Bi- and Tri-Antennary Human Transferrin Glycopeptides and their Affinities for the Hepatic Lectin Specific for Asialo-Glycoproteins

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Glycopeptides were isolated from a proteolytic digest of human transferrin. After mild acid hydrolysis the desialylated glycopeptides were labelled by the galactose oxidase/ NaB^3H_4 procedure and then fractionated by Sephadex-gel filtration or by anion-exchange chromatography. Either technique allowed separation of the two heterosaccharide chains (designated glycan I and glycan II) previously described for this protein by Spik, Vandersyppe, Fournet, Bayard, Charet, Bouquelet, Strecker & Montreuil (1974) (in *Actes du Colloque Internationale No. 221* vol. 1, pp. 483–499). Subsequent chromatography on Sepharose–concanavalin A separated fractions containing different quantities of carbohydrates for each glycan, as indicated by analyses. The isolated glycan fractions were then tested for their abilities to bind to the immobilized rabbit hepatic lectin. Our studies suggest that either glycan can have a bi- or tri-antennary structure. Desialylated biantennary glycans I and II did not bind to the hepatic lectin. Desialylated triantennary glycan I was slightly retarded by the hepatic lectin, whereas the triantennary glycan II consisted of equal quantities of a retarded and a bound type. Desialylated triantennary glycan II was totally displaced from the hepatic lectin by using a buffer containing 0.05M-EDTA. The results suggest that greater structural heterogeneity exists in the carbohydrate moiety of human transferrin than was previously envisaged. Such heterogeneity could be reflected in several molecular forms of human transferrin, which, after desialylation, differ significantly in their affinities for the hepatic lectin.

According to Jamieson *et al.* (1971) and Spik *et al.* (1974), human transferrin contains two biantennary (i.e. two-forked) heteroglycans of identical structure. Glycan I and glycan II are linked by *N*-glycosidic bonds to the peptide sequences Asn-Lys-Ser and Gly-Ser-Asn-Val-Thr respectively (Spik *et al.*, 1974; Graham & Williams, 1975). Later Montreuil & Spik (1975) also found a small quantity of a triantennary (i.e. three-forked) glycan in transferrin, which was linked only to the Asn-Val-Thr sequon (Marshall, 1974). No primary structure could be assigned to this particular glycopeptide.

We have shown that human transferrin phenotype C consists of three molecular species, which can be separated by anion-exchange chromatography (Regoeczi *et al.*, 1977). The predominant molecular species [designated 2Fe-TfC-5-6 by Wong *et al.* (1978)] was quantitatively retained by Sepharose–concanavalin A, whereas the more anodic transferrin species (2Fe-TfC-6-6) was not. On the basis of the work by Krusius *et al.* (1976), we have tentatively

concluded from this finding that those transferrin molecules that possess a combination of one bi-antennary and one triantennary heteroglycan are contained in transferrin fraction 2Fe-TfC-6-6. However, after desialylation and intravenous administration to rats (Regoeczi *et al.*, 1978), partition of this transferrin fraction between liver and plasma markedly deviated from expected values (Wong *et al.*, 1978). This poses the question of further heterogeneity in the carbohydrate composition of human transferrin and prompted the work described here.

Materials and Methods

Materials

Human transferrin was obtained from Behringwerke A. G. (Marburg, German Federal Republic) and its purity established before (Regoeczi *et al.*, 1977). Galactose oxidase (EC 1.1.3.9) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) and purified by an affinity method (Hatton & Regoeczi, 1976); DEAE-cellulose (DE-52) was from Reeve Angel (Clifton, NJ, U.S.A.); a low-specificity proteinase (ex *Streptomyces griseus*) was

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from Sigma Chemical Co. (St. Louis, MO, U.S.A.; catalogue no. P-5130); trifluoroacetic anhydride was from BDH, (Toronto, Ont., Canada); Tabsorb was from Regis Chemical Co. (Morton Grove, IL, U.S.A.); NaB^3H_4 was from New England Nuclear (Lachine, Que., Canada); Sephadex-gel-filtration media and Sepharose-concanavalin A were from Pharmacia (Montreal, Que., Canada).

Column chromatography

All systems were at room temperature unless indicated otherwise. Columns (42cm $\times$ 2.2cm) of Sephadex G-50 were equilibrated with 0.1 M-pyridine buffered to pH 5.5 with acetic acid. Sephadex G-25 (coarse or superfine; column, 50–51 cm $\times$ 2.0cm) was equilibrated with deionized water to which toluene (1ml/litre) was added.

DEAE-cellulose columns (30 cm $\times$ 1cm) were equilibrated with 0.01 M-Tris/HCl, pH8.0. After 1–5 mg of asialo-glycopeptides had been loaded, the column was eluted with 100ml of 0.01 M-Tris/HCl, pH8.0, and then 0.2 M-Tris/HCl, pH8.0.

Sepharose-concanavalin A columns (10cm $\times$ 1cm) were used at 4°C with the elution system described by Krusius *et al.* (1976). Column loads of 1–5 mg of desalted asialo-glycopeptides were used.

The lectin specific for asialo-glycoproteins was prepared from rabbit liver by the procedure of Stockert *et al.* (1974) and conjugated to Sepharose 6B as described before (Wong *et al.*, 1978). Sepharose-lectin columns (8–10cm $\times$ 1cm) were equilibrated with 0.05 M-Tris/HCl, pH7.8, containing 0.05 M- CaCl_2 and 0.15 M-NaCl. After loading (5–20 μg of [^3H]asialo-glycopeptides), the column was washed with 50ml of the Tris/ Ca^{2+} buffer. Any bound asialo-glycopeptide was displaced by 0.05 M-EDTA (disodium salt) in 0.15 M-sodium acetate, pH6.2, containing 0.3 M-NaCl. From a fraction volume of 1.6 ml, 1.0 ml was taken for liquid-scintillation counting in aqueous counting scintillant (ACS; Amersham Corp., Arlington Heights, IL, U.S.A.) in a Beckman LS220 counter.

Preparation of transferrin glycopeptides

Apo-transferrin was dissolved to a concentration of 20 mg/ml in 0.01 M-Tris/HCl, pH8.0 (freshly prepared from sterile water). Proteinase was added (1 mg of enzyme/100 mg of protein) at 24 h intervals during incubation at 37°C for 4 days. Before each addition of enzyme, the pH was adjusted to 8.0 with 1 M-Tris as necessary. Toluene (approx. 10 μl /day) was added to inhibit bacterial growth. The glycopeptide fraction was separated from the other constituents by Sephadex G-50 chromatography in 0.1 M-pyridine/acetic acid. The fractions were analysed for sugars by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956), pooled and freeze-dried.

Desialylation and ^3H -labelling of the glycopeptides

Glycopeptide preparations were desialylated by mild acid hydrolysis. Approx. 3–5 mg of glycopeptide was dissolved in 2 ml of 0.05 M- H_2SO_4 and heated at 76–78°C for 1 h. After cooling, the hydrolysate was neutralized by the addition of 4 M-NaOH and the products were chromatographed on Sephadex G-25.

The ^3H -labelling procedure was based on the galactose oxidase/ NaB^3H_4 method of Morell *et al.* (1966) and has been described in detail elsewhere (Hatton *et al.*, 1978).

Amino acid analysis

Glycopeptides (approx. 100 μg) were hydrolysed with 2 ml of redistilled constant-boiling 6.09 M-HCl at 105°C for 20–22 h. HCl was removed by rotary evaporation. For most glycopeptides, analysis was undertaken by the g.l.c. method of Kaiser *et al.* (1974). Before derivative formation, ornithine (100 nmol) was added to each hydrolysate as an internal standard. A standard amino acid mixture (Beckman Instruments, Palo Alto, CA, U.S.A.), to which ornithine was also added, was used as a reference. In addition to this procedure, conventional amino acid analysis using a Beckman 120C analyser was used for some samples.

Carbohydrate analysis

Sialic acid was determined by the method of Warren (1959) after mild acid hydrolysis. Neutral sugars were measured as their alditol acetates after hydrolysis of the glycopeptide by using Dowex 50 in the H^+ form as described by Lehnhardt & Winzler (1968). Hexosamines were measured after hydrolysis in 4 M-HCl for 6 h at 100°C by the procedure for amino acid analysis in a Beckman 120C analyser.

Results

Separation of glycans I and II

This was accomplished by chromatography of the asialo-glycopeptides either on superfine Sephadex G-25 or on DEAE-cellulose.

The result of chromatographing a salt-free preparation of asialo-glycopeptides on superfine Sephadex G-25 is shown in Fig. 1. From absorbance measurements (215 nm) and the hexose determinations, two partially separated peaks emerged. On analysis (Table 1), peak 1 contained the amino acids aspartic acid, serine, glycine, valine, threonine and a small amount of glutamic acid, whereas peak 2 contained aspartic acid and lysine with trace amounts of four other amino acids, including serine. Chromatography of [^3H]asialo-glycopeptides resulted in a similar elution profile.

DEAE-cellulose resolved the asialo-glycopeptides into three peaks, designated *a*, *b* and *c*, as shown by

the absorbance (215nm) and hexose content of the effluent (Fig. 2). Amino acid analyses (Table 1) showed that peak *a* contained only aspartic acid and lysine, peak *b* largely aspartic acid and serine, and peak *c* aspartic acid, serine and valine.

Applying the peptide-sequence data of Spik *et al.* (1974) to the results in Table 1, the amino acid analyses of peaks 1, *b* and *c* are consistent with a glycopeptide mixture from the glycan-II sequence, Gly-Ser-Asn-Val-Thr, and peaks 2 and *a* probably consist of Asn-Lys with trace amounts of Asn-Lys-Ser, which is consistent with the sequon for glycan I.

The relationship between respective resolutions obtained by gel-filtration and anion-exchange chromatography was confirmed as follows. Peak 1 from superfine Sephadex G-25 yielded a small peak *a* (approx. 10% of the load), a peak *b* (50%) and a peak *c* (40%) on DEAE-cellulose; in contrast, peak 2 chromatographed entirely in the peak *a* position.

Chromatography of asialo-glycopeptides on Sepharose-concanavalin A

This medium fractionated the asialo-glycopeptides

Fig. 1. Chromatography of human transferrin asialo-glycopeptides (approx. 4.3 mg) on Sephadex G-25 superfine with distilled water as the eluent
Peaks 1 and 2 were eluted. ○, *A*₂₁₅; ■, hexose analysis (490nm); ▲, elution profile of [³H]asialo-glycopeptides. Fraction volume was 2.0ml.

Fig. 2. Separation of human transferrin asialo-glycopeptides on DEAE-cellulose on elution with 0.01 M-Tris/HCl, pH 8.0.
The arrow indicates the start of elution with 0.2 M-Tris/HCl, pH 8.0. Fraction volume was 2.0ml. ●, *A*₂₁₅; ■, hexose analysis (490nm). The fractions enclosed by brackets were pooled for further study of peaks *a*, *b* and *c*. No analyses were made on the small peak displaced by 0.2 M-Tris/HCl buffer, pH 8.0.

Table 1. Amino acid analyses of the peaks obtained by chromatography of human transferrin asialo-glycopeptides either on Sephadex G-25 superfine or DEAE-cellulose

The results are expressed as molar ratios, aspartic acid being taken as 1.00; Tr indicates <0.1. Each value represents the mean of the least two analyses.

Amino acid analyses of asialo-glycopeptides						
Amino acid	Fractionation on ...	Sephadex G-25		DEAE-cellulose		
		Peak 1	Peak 2	Peak <i>a</i>	Peak <i>b</i>	Peak <i>c</i>
Aspartic acid		1.0	1.0	1.0	1.0	1.0
Threonine		0.2	Tr	0	Tr	Tr
Serine		0.9	Tr	Tr	1.1	1.0
Glutamic acid		0.1	Tr	Tr	0.1	0.3
Glycine		0.6	Tr	Tr	0.3	Tr
Alanine		Tr	0	0	0	Tr
Valine		0.3	0	Tr	0.1	0.4
Lysine		Tr	1.1	0.9	Tr	Tr

Table 2. Analyses of peaks *A* and *B* obtained by chromatography of human transferrin glycopeptides on Sepharose-concanavalin *A*

Amino acids are expressed as their molar ratios relative to aspartic acid (1.0); monosaccharides are related to mannose (3.0). 'Tr' indicates < 0.1. Each value represents the mean of at least two analyses.

	Peak <i>A</i>	Peak <i>B</i>
Amino acids		
Aspartic acid	1.0	1.0
Threonine	0.3	Tr
Serine	0.6	0.7
Glutamic acid	0.4	Tr
Glycine	0.4	0.4
Alanine	Tr	0
Valine	0.2	0.3
Lysine	0.5	0.4
Glucosamine*	2.7	3.3
Monosaccharides		
<i>N</i> -Acetylneuraminic acid	2.9	1.6
Galactose	2.9	2.1
Mannose	3.0	3.0
Glucosamine†	5.0	3.3

* Glucosamine values obtained from amino analysis (22h hydrolysis at 106°C in 6M-HCl).

† Glucosamine values obtained from 6h hydrolysates (4M-HCl at 100°C).

Table 3. Carbohydrate analyses of peaks *aA*, *aB*, *bA* and *bB* obtained by chromatography of peaks *a* and *b* (from DEAE-cellulose) on Sepharose-concanavalin *A*

Each value is expressed as the molar ratio relative to aspartic acid (1.0). See Table 1 for details of the amino acid analyses of peaks *a* and *b*.

Monosaccharides	<i>aA</i>	<i>aB</i>	<i>bA</i>	<i>bB</i>
Galactose	3.0	1.8	2.8	2.3
Mannose	2.8	3.1	2.9	2.8
Glucosamine	5.4	3.8	5.0	4.1

into two peaks. A small fraction (designated peak *A*), equivalent to 15–20% of the carbohydrate load, passed through the column, whereas the remainder of the load was retained. The bound fraction, peak *B*, was eluted by 0.015M- α -methyl glucoside. Similar separation was obtained with samples of human transferrin glycopeptides that had not been desialylated.

Amino acid analyses were made on samples of peaks *A* and *B*. Each peak contained aspartic acid, serine, valine, lysine and glutamic acid (Table 2). However, significant differences between *A* and *B* were found from carbohydrate analyses. Peak *A* contained greater amounts of galactose, *N*-acetylglucosamine and, for the sialylated glycopeptides, *N*-acetylneuraminic acid than did peak *B*. Taking the

mannose content as being 3 residues/molecule, the quantities of the other sugar types in peak *A* were equivalent to a triantennary glycan, whereas the values for peak *B* were consistent with a biantennary glycan.

Chromatography of the [³H]asialo-glycopeptides on Sepharose-concanavalin *A* after their pre-fractionation into peaks 1 and 2 on superfine Sephadex G-25 led to the following observations. Peak 1 separated into a non-retained fraction (designated fraction 1*A*, corresponding to 14–15% of the radioactivity load) and a retained fraction (fraction 1*B*; 82–84%), which was eluted by α -methyl glucoside. Of peak 2, 9–11% of the radioactivity was not bound by Sepharose-concanavalin *A* (fraction 2*A*). The bound fraction (fraction 2*B*; 86–88%) was displaced by α -methyl glucoside.

Chromatography of peak *a* from DEAE-cellulose (Fig. 2) on Sepharose-concanavalin *A* yielded an unbound (peak *aA*) and a bound (*aB*) fraction. Similarly, peak *b* from DEAE-cellulose, when chromatographed on Sepharose-concanavalin *A*, produced an unbound (peak *bA*) and a bound (*bB*) fraction. The results of amino acid and carbohydrate analyses made on these fractions are given in Table 3. From the molar ratios of galactose and glucosamine relative to mannose, the fractions derived from peak *a* (glycan 1) are consistent with a triantennary (peak

Fig. 3. Affinity chromatography of [³H]asialo-glycopeptide fractions 1*A*, 1*B* and 2*B* on the immobilized rabbit hepatic lectin

The column was eluted with 0.05M-EDTA (disodium salt) at fraction 15 (arrowed). Fraction volume was 1.6ml. ●, Fraction 2*B*; ○, fraction 2*A*; ▲, fraction 1*A*. Fraction 1*B* chromatographed in a similar manner to fraction 2*B* and has not been included.

aA) and a biantennary (peak *aB*) structure located on the sequon Asn-Lys-Ser. Also, the fractions from peak *b* (glycan 2) are consistent with a triantennary (peak *bA*) and a biantennary (peak *bB*) structure attached to the sequon Asn-Val-Thr. From the analyses, peaks *aA* and *bA* amounted to 12 and 8% (by wt.) of peaks *a* and *b* respectively.

Studies with the immobilized rabbit hepatic lectin

The asialo-glycopeptide preparations, designated 1*A*, 1*B*, 2*A* and 2*B* (see above), were further analysed for their ability to bind to this galactose-specific lectin. Because the capacity of the hepatic lectin columns was very small, approx. 10 µg of asialo-glycopeptide was the maximum load for a 10 ml column. Consequently, only experiments with [³H]-asialo-glycopeptide fractions are shown in Fig. 3. Asialo-glycopeptide 1*A* was separated into two portions by the lectin: approx. 50% of the load was retarded and the remainder was bound. The bound fraction was displaced by elution with 0.05 M-EDTA (disodium salt). Rechromatography of the retarded peak on the lectin did not change the elution pattern. Furthermore varying the load (0.6–6 µg) did not influence the position of the retarded peak. In contrast, fraction 2*A* was slightly retarded by the column and only 3–4% of the load was bound. Both fractions 1*B* and 2*B* passed unretarded through the column.

Discussion

Data presented in Tables 2 and 3 give support to the claim of Montreuil & Spik (1975) that some of the human transferrin molecules bear a combination of one biantennary and one triantennary glycan. However, in contrast with their suggestion that the triantennary heterosaccharide chain is solely confined to the Asn-Val-Thr sequon of glycan II, our studies indicate that such a structure may also occur as glycan I. From these results, we infer that variations in the carbohydrate composition of human transferrin may be more extensive, at least in a qualitative sense, than hitherto realized.

Demonstration of the additional heterogeneity relied on subjecting transferrin glycopeptide preparations to at least two successive chromatographic procedures, one of which achieved separation of the glycans according to their domain of attachment and another according to the number of oligosaccharide branches. Separation of glycan types I and II by superfine Sephadex G-25 was an unexpected finding and the principle involved is not entirely clear. Nevertheless, the amino acid compositions of glycopeptide peaks 1 and 2 obtained by this method agreed satisfactorily with the values obtained independently by anion-exchange chromatography. In retrospect, DEAE-cellulose is probably the preferred choice because of the lesser risk of cross-contamin-

ation, which necessitates the sacrifice of portions of the peptide peaks in the case of the gel-filtration method. The ability of immobilized concanavalin A to distinguish between two- and three-forked glycans was reported by Krusius *et al.* (1976). If triantennary glycopeptides in human transferrin only occurred as glycan II, all glycopeptides from peak 2 of the Sephadex G-25 column (containing the Asn-Lys-Ser sequon) would have been retained by Sepharose-concanavalin A, which was clearly not the case.

Additional evidence for a three-forked heterosaccharide on glycans I and II comes from the chromatographic studies of the DEAE-cellulose peaks *a* and *b* on Sepharose-concanavalin A. From the analyses of the fractions *aA*, *aB*, *bA* and *bB* (Tables 1 and 3), the carbohydrate contents of *aA* and *bA* resemble that of the triantennary glycan proposed by Spik *et al.* (1974), whereas those of *aB* and *bB* relate more closely to the values obtained for a biantennary glycan.

Binding of human asialo-transferrin by the hepatic lectin in the intact rat is now established (Regoecci *et al.*, 1978), but the galactose requirement for this interaction is not known at a glycan level. Therefore it was of a particular interest to compare the behaviours of asialo-glycopeptide preparations 1*A*, 1*B*, 2*A* and 2*B* on columns of the immobilized lectin from rabbit liver. In these studies, biantennary glycans (preparations 1*B* and 2*B*) exhibited no detectable binding, which is in agreement with our earlier observations *in vivo* on the two-forked glycan from bovine transferrin (Hatton *et al.*, 1978). However, human asialo-transferrin is retarded by the lectin column (Wong *et al.*, 1978), suggesting that two biantennary glycans can co-operatively potentiate the interaction of an asialo-glycoprotein with the hepatocyte plasma membrane. The triantennary glycan II (preparation 1*A*) contained approximately equal quantities of a fraction that was bound and a fraction that was retarded by the liver lectin. In contrast, the overwhelming portion (approx. 96%) of the triantennary glycan I (preparation 2*A*) was only slightly retarded by the lectin column (a minor portion that was bound probably represented contamination with triantennary glycan II from the chromatography on Sephadex G-25).

These results suggest that, with regard to affinity for the hepatic lectin, there exist at least two classes of triantennary glycans in human transferrin. Future clarification of the underlying structural differences seems highly desirable. Regrettably, technical and economical problems have so far prevented the assembly of hepatic lectin columns of preparative capacity large enough for this purpose and our attempts to achieve the same kind of separation by a more readily available galactose-specific lectin, RCA II from *Ricinus communis*, have been unsuccessful.

From our present results, we can predict the existence of at least three types of human transferrin with respect to carbohydrate content: (1) molecules possessing two biantennary glycans; (2) molecules possessing a biantennary glycan I and a triantennary glycan II; (3) molecules possessing a triantennary glycan I and a biantennary glycan II. From the studies with [^3H]asialo-glycopeptides approx. 10 % of glycan II and 6 % of glycan I may be of a triantennary type, but this calculation should be regarded with caution because it assumes that each galactosyl group has an equal probability of becoming tritiated in the galatose oxidase/ NaB^3H_4 procedure. In agreement with the above prediction, we have recently succeeded in isolating three populations of human asialo-transferrin molecules with distinctly different affinities for the hepatic lectin of the rat (Regoeczi *et al.*, 1979).

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