

Primary structure of glycans isolated from human leucocyte lactotransferrin

Absence of fucose residues questions the proposed mechanism of hyposideraemia

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Lactotransferrin was highly purified from lysates of human neutrophilic leucocytes by immuno-affinity chromatography. A comparative analysis of the molar carbohydrate compositions of human leucocyte lactotransferrin and human milk lactotransferrin reveals that the glycans of leucocyte lactotransferrin differ essentially by the absence of fucose residues. Structural analysis combining methylation-mass spectrometry and 400 MHz ¹H-n.m.r. spectrometry of oligosaccharide alditols released from human leucocyte lactotransferrin shows the presence of two disialylated and non-fucosylated biantennary glycans of the *N*-acetyl-lactosaminic type. These results question a previously proposed mechanism for hyposideraemia in which the leucocyte lactotransferrin was involved and in which the fucose residues played a key role.

INTRODUCTION

Lactotransferrin (also called lactoferrin) was first described in neutrophilic polymorphonuclear leucocytes by Masson *et al.* (1969) and has been supposed to play an important role in the hyposideraemia commonly found during inflammation (Van Snick *et al.*, 1974). In particular, the lactotransferrin released by leucocytes after stimulation by interleukin-1 (Heylar & Sherman, 1987) is supposed to bind iron from the serum transferrin and then to be taken up by the liver. Studies concerning the interaction of human and mouse lactotransferrins with rat hepatic receptors (Prieels *et al.*, 1978) led to the conclusion that these receptors are lectinic and recognize α -1,3-linked fucose residues.

Human milk lactotransferrin (Montreuil & Mullet, 1960; Montreuil *et al.*, 1960) is a well-known glycoprotein whose complete amino acid sequence has been reported (Metz-Boutigue *et al.*, 1984), as well as the structure of the two glycans linked to the single polypeptide chain (Spik *et al.*, 1982). All of these glycans are of the sialyl-*N*-acetyl-lactosaminic type and are α -1,6-fucosylated at the *N*-acetylglucosamine residue linked to asparagine. They are highly heterogeneous owing to a variable number of sialic acid residues (one or two) and to the presence or the absence of a fucose residue α -1,3-linked to the external *N*-acetylglucosamine residue. A recent comparative study (Moguilevsky *et al.*, 1984) did not detect any significant differences between milk and leucocyte lactotransferrins with respect to molecular mass, isoelectric point, stability of iron complex and uptake by the liver. However, no results have been reported concerning either the composition or the structure of the glycan moiety of leucocyte lactotransferrin.

As the role of the glycans of leucocyte lactotransferrin is considered to be of fundamental importance with respect to its biological activity, we have undertaken to determine their structure. In the present paper we report the complete structure of the two glycans of leucocyte lactotransferrin resolved on the basis of methylation-mass spectrometry and 400 MHz ¹H-n.m.r. spectroscopy.

MATERIALS AND METHODS

Materials

Equipment for electrophoresis, molecular-mass markers and Sephadex G-25 prepacked columns were from Pharmacia Fine Chemicals (Uppsala, Sweden), Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.), ²H₂O (99.95 atom % ²H) was from the Commissariat à l'Energie Atomique (Saclay, France), phenylmethane sulphonyl fluoride, aprotinin, dithiothreitol and agarose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Na¹²⁵I (carrier-free, 15.6 mCi/mg) was from Amersham International (Amersham, Bucks., U.K.) and Star Tubes were from Nunc (Kamstrup, Denmark). All other reagents were of the highest purity.

Isolation of polymorphonuclear cells

Human heparinized blood samples obtained from healthy volunteers were pooled and centrifuged at 800 *g* for 10 min, and the buffy coat was removed with a pipette. The remaining erythrocytes were haemolysed by resuspension of the buffy coat in 10 ml of 10 mM-KHCO₃/0.154 M-Tris/HCl buffer, pH 7.4, containing 0.1 mM-EDTA and incubation for 30 min at 4 °C. The leucocyte pellet was then washed three times with phosphate-buffered saline (0.143 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4).

Affinity chromatography of lactotransferrin from leucocytes

Antibodies against human milk lactotransferrin were raised by intradermal injections of iron-saturated lactotransferrin with complete Freund's adjuvant (Vaitukaitis *et al.*, 1971). IgGs specific for human lactotransferrin were purified from rabbit serum by affinity chromatography on lactotransferrin immobilized on Sepharose 4B by the CNBr procedure (March *et al.*, 1974) at 5 mg of lactotransferrin/ml of bead. Briefly, 50 ml of antiserum was passed through the column at a flow rate of 60 ml/h. The column was washed by 100 ml of Tris-buffered saline (150 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0),

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followed by 100 ml of the same buffer containing 1 M-NaCl (final concn.) and eluted by 0.2 M-glycine/HCl buffer, pH 2.8. Each fraction was immediately adjusted to pH 7.5 by adding 1 M-Tris solution. Affinity-purified IgGs were dialysed against 0.2 M-sodium bicarbonate buffer, pH 8.5, for 48 h and immobilized on Sepharose 4B at 5 mg of IgG/ml of bead.

The leucocytes, resuspended in phosphate-buffered saline (20 ml) containing the proteinase inhibitors (each 1 mM) aprotinin, phenylmethanesulphonyl fluoride, MgCl_2 , dithiothreitol and *o*-phenanthroline, were disrupted by sonication. Cell debris was removed by centrifugation. Protein was assayed in cell lysates as described by Peterson (1977). The cell lysates were loaded on to a specific anti-(human lactotransferrin) IgG-Sepharose 4B column (1.15 cm $\times$ 9 cm). The column was washed and the leucocyte lactotransferrin was eluted from the affinity column in the conditions described above for the elution of specific human lactotransferrin IgG. Then the eluted fractions containing leucocyte lactotransferrin were pooled, dialysed against distilled water and freeze-dried.

Radio-immunoassay

The amount of leucocyte lactotransferrin in cell lysates was determined by radio-immunoassay (Johnstone & Thorpe, 1982) with Star Tubes, with high binding capacity and ^{125}I -labelled human milk lactotransferrin as a competitive binding antigen.

The iodination of human lactotransferrin (0.25 mg) was performed as described previously (Fraker & Speck, 1978; Salicinsky *et al.*, 1981) with the Iodogen reagent and Na^{125}I (0.2 mCi) in 50 mM-sodium phosphate buffer, pH 7.4, for 10 min on ice. The excess reagent was removed by gel filtration on a Sephadex G-25 column.

Immunological and electrophoretic techniques

The purity of human lactotransferrin isolated from leucocytes was checked by SDS/PAGE on a 10% polyacrylamide gel in accordance with Laemmli (1970). Electrophoresis was performed on a 1.5 mm-thick slab. A mixture of molecular-mass markers (14–94 kDa) was run in parallel and stained with Coomassie Blue.

The presence of identical epitopes in human lactotransferrins isolated from milk and from leucocytes was analysed by the radial immunodiffusion technique (Ouchterlony, 1949).

Release by hydrazinolysis of oligosaccharide alditols from human leucocyte lactotransferrin

Oligosaccharide alditols were prepared from human leucocyte lactotransferrin by hydrazinolysis, *N*-reacetylation and reduction (Reading *et al.*, 1978; Bendiak & Cumming, 1986). Hydrazinolysis was performed in a sealed glass tube for 16 h at 100 °C. Hydrazine was removed under a flow of N_2 and then *in vacuo* in the presence of conc. H_2SO_4 . The residue was dissolved in saturated NaHCO_3 (1 ml), and acetic anhydride was added every 5 min, in 10 μl portions, during 20 min. After reaction time of 1 h, the products were deionized by the addition of Dowex 50-X2 (H^+ form; 200–400 mesh) cation-exchange resin. Reduction was performed with NaBH_4 and the mixture was kept overnight at room temperature. The products were then passed through a Bio-Gel P-2 column (0.2 cm $\times$ 40 cm) and eluted with distilled water. The oligosaccharide alditols were monitored by u.v. absorbance at 206 nm.

Carbohydrate composition

The monosaccharide molar proportions present in the glycan moiety of the leucocyte lactotransferrin and in the liberated oligosaccharide alditols were determined after methanolysis and g.l.c. of the trimethylsilylated glycosides on a capillary CP-Sil

5 CB column (0.25 mm $\times$ 25 m) (Kamerling *et al.*, 1975). The reduced-end residues were characterized as alditols.

Methylation analysis of the oligosaccharide alditols

The oligosaccharide alditols were permethylated by use of the lithium methylsulphonyl carbanion reagent (Hakomori, 1964; Paz-Parente *et al.*, 1985). The permethylated oligosaccharide alditols were subjected to methanolysis, and the partially methylated methyl glycosides were peracetylated by use of a pyridine/acetic anhydride (1:5, v/v) solution at 37 °C for 18 h. The peracetylated and permethylated methyl glycosides were then separated by g.l.c. on a capillary column (0.3 mm $\times$ 25 m) coated with OV-101 (Fournet *et al.*, 1981). The monosaccharide derivatives were also identified by g.l.c.–m.s. with the use of a Ribermag R 10.10 mass spectrometer (Riber, Rueil-Malmaison, France), coupled to the data system Sydar 121.

^1H -n.m.r. spectroscopy

The oligosaccharide alditols were repeatedly dissolved in $^2\text{H}_2\text{O}$ at room temperature and at pH 7 with intermediate freeze-drying (Vliegthart *et al.*, 1983). The deuterium-exchanged oligosaccharide alditols were submitted to ^1H -n.m.r. spectroscopy at 400-MHz on a Bruker AM 400 WB spectrometer operating in the pulsed Fourier-transform mode and equipped with Bruker Aspect 3000 computer at a probe temperature of 300 K (Centre Commun de Mesures, Université des Sciences et Techniques de Lille Flandres-Artois). Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone in $^2\text{H}_2\text{O}$ (δ 2.225 p.p.m. with an accuracy of 0.002 p.p.m.).

RESULTS

Purification of human leucocyte lactotransferrin

In a typical experiment the leucocyte extract obtained from 400 ml of heparinized blood (10^8 neutrophils) contained 105 mg of protein and 3.66 mg of leucocyte lactotransferrin as assayed by radio-immunology.

The radio-immunoassay of lactotransferrin performed on human neutrophilic leucocyte lysates provided reproducible results that allowed us to fix the lactotransferrin content at $3.66 \pm 0.2 \mu\text{g}/10^6$ cells. This result is similar to that obtained by Leffel & Spitznagel (1975), namely $3.4 \pm 0.34 \mu\text{g}/10^6$ cells, but quite different from the value of 10–20 μg of lactotransferrin/ 10^6 leucocytes reported by Bennet & Kokocinsky (1978).

After the one-step purification by immuno-affinity the recovery was about 95% (3.5 mg). The stained polyacrylamide gel revealed that human leucocyte lactotransferrin was obtained at high purity, only one band being observed. When human milk lactotransferrin was run in parallel with human leucocyte lactotransferrin, both proteins have the same migration in SDS/PAGE, suggesting the molecular mass of leucocyte lactotransferrin, as found by Moguilevsky *et al.*, (1984), is similar to that calculated (81442 Da) for human milk lactotransferrin (Metz-Boutigue *et al.*, 1984).

Milk and leucocyte human lactotransferrins fully cross-reacted (Fig. 1) with antibodies raised against human milk lactotransferrin. The result indicates that the polypeptide chains of the two human lactotransferrins possess identical or similar antigenic determinants.

Glycan structure determination

Comparison of carbohydrate compositions of human milk and leucocyte lactotransferrins. Native leucocyte lactotransferrin was examined for its percentage carbohydrate composition, and a

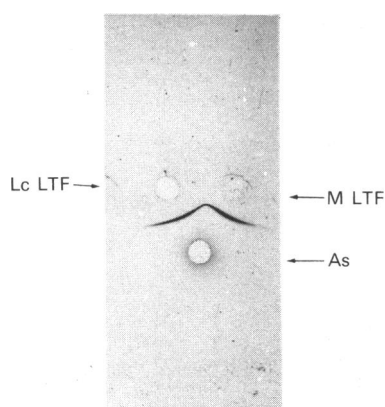


Fig. 1. Radial immunodiffusion of human milk lactotransferrin and human leucocyte lactotransferrin against rabbit anti-(human milk lactotransferrin) serum

Key: M LTF, milk lactotransferrin; Lc LTF, leucocyte lactotransferrin; As, antiserum against human milk lactotransferrin.

mean value of 5.9 % was obtained. This result is very similar to that reported for milk lactotransferrin (Spik *et al.*, 1982). The carbohydrate composition (2.27 % neutral monosaccharides, 2.13 % hexosamines and 1.38 % sialic acids) and the carbohydrate molar proportions of leucocyte lactotransferrin (Table 1) indicate that this glycoprotein of 80 kDa molecular mass contains, like milk lactotransferrin, **two glycans of the biantennary *N*-acetyl-lactosaminic type**. That the molar proportions of the monosaccharides are close to whole numbers suggests homogeneity of the glycan structure. The major difference that was observed on comparing human milk and leucocyte lactotransferrins carbohydrate compositions resides in the absence of fucose residues from human leucocyte lactotransferrin.

Structure of oligosaccharide alditols released by hydrazinolysis from leucocyte lactotransferrin. The oligosaccharide alditols from the human leucocyte lactotransferrin were obtained in good yield after gel filtration on a Bio-Gel P-2 column (70 %) and fully re-*N*-acetylated. Chemical analysis provided the same results as those obtained for human serotransferrin (Spik *et al.*, 1975). Determination of the molar carbohydrate composition (Table 1) shows that the proportion of the different monosaccharides may be interpreted as indicating the **presence of glycan possessing a biantennary structure of the disialylated *N*-acetyl-lactosaminic type**.

Table 1. Molar carbohydrate compositions of human milk lactotransferrin and human leucocyte lactotransferrin

The molar proportions were calculated on the basis of 3 mol of mannose residue/mol of glycan

	Molar carbohydrate composition					
	Fuc	Gal	Man	GlcNAc	GlcNAc-ol	NeuAc
Milk lactotransferrin*	1.4	2.4	3	4.5	0	1.3
Leucocyte lactotransferrin	0	2.1	3	3.9	0	1.8
Oligosaccharide alditol†	0	2.1	3	2.8	0.4	1.7

* According to Spik *et al.* (1982).

† Released from leucocyte lactotransferrin by hydrazinolysis.

Table 2. Molar proportions of monosaccharide methyl ethers present in the methanolysate of permethylated oligosaccharide alditols released by hydrazinolysis from human leucocyte lactotransferrin

Molar proportions were calculated on the basis of 1 mol of 2,4-di-*O*-methylmannose residue/mol of oligosaccharide alditol. The numbers in parentheses correspond to the theoretical values.

Monosaccharide methyl ether	Molar proportion
2,3,4-Tri-OMe-Gal	1.9 (2)
2,4-Di-OMe-Man	1 (1)
3,4,6-Tri-OMe-Man	1.5 (2)
3,6-Di-OMe-GlcNAcNMe	3 (3)
1,3,5,6-Tetra-OMe-GlcNAcNMe-ol	0.1 (1)
4,7,8,9-Tetra-OMe-NeuAc	1.9 (2)

In addition, the molar proportions of the monosaccharide methyl ethers (Table 2) indicate that the **two galactose residues are substituted at the C-6 position, suggesting that they are α -2,6-sialylated**. The results obtained by methylation confirm the homogeneity of the released oligosaccharide alditols and underline a difference from milk lactotransferrin, in which the three following types of biantennary *N*-acetyl-lactosaminic glycan structures have been characterized: disialylated monofucosylated, monosialylated monofucosylated and monosialylated difucosylated (Spik *et al.*, 1982).

The 400 MHz ^1H -n.m.r. parameters of the oligosaccharide alditol obtained from human leucocyte lactotransferrin are compiled in Table 3 and the n.m.r. spectrum is depicted in Fig. 2.

The chemical-shift values of the H-1 and H-2 atoms of the Man-3, Man-4 and Man-4' residues (Table 3) are exactly identical with those of a classical biantennary α -2,6-disialylated glycan structure (Fig. 3), such as that found for human serum transferrin (Dorland *et al.*, 1977), and for oligosaccharide isolated from the

Table 3. 400 MHz ^1H -n.m.r. chemical shifts of structural reporter groups of constituent monosaccharides for the oligosaccharide alditol derived from human leucocyte lactotransferrin

For numbering of monosaccharide residues and complete structure see Fig. 3. Chemical shifts are given for neutral solution at 27 °C in p.p.m. downfield from internal acetone in $^2\text{H}_2\text{O}$.

Reporter group	Residue	Chemical shift (p.p.m.)
H-1	GlcNAc-2	4.642
	Man-3	4.774
	Man-4	5.133
	Man-4'	4.947
	GlcNAc-5	4.605
	GlcNAc-5'	4.605
H-2	Gal-6	4.444
	Gal-6'	4.444
	Man-3	4.255
	Man-4	4.196
H-3 _{ax}	NeuAc-6	1.718
	NeuAc-6'	1.718
H-3 _{eq}	NeuAc-6	2.670
	NeuAc-6'	2.673
NAc	GlcNAc-1o1	2.056
	GlcNAc-2	2.082
	GlcNAc-5	2.070
	GlcNAc-5'	2.065
	NeuAc-6,6'	2.030

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