Carbohydrate deficient serum transferrin in a new systemic hereditary syndrome

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Abstract
Four patients with a new, inherited, complex developmental deficiency syndrome were studied. The syndrome affects the central and peripheral nervous system, and also the retina, liver, bone, adipose tissue, and genital organs. Abnormalities of glycoproteins, glycocopeptide hormones, and lipids have been found in serum from these patients, the most pronounced being increased cathodal forms of transferrin. Isoforms of serum transferrin were therefore analysed qualitatively and quantitatively by isoelectric focusing and isocratic anion exchange chromatography, and the carbohydrate composition was determined in transferrin isolated by immune affinity chromatography. All the patients had about tenfold raised serum concentrations of isotransferrins with higher isoelectric points than normal. Similar findings, though less pronounced, were made in all the fathers and in one of the mothers. Half the transferrin in the patients was constantly present in two principal abnormal cathodal forms in approximately equal amounts. Carbohydrate determinations in purified transferrin showed quantitatively similar deficiencies of sialic acid, galactose, and N-acetylglucosamine, the mannose content being normal. The results suggest that either two or all of the normally four terminal trisaccharides in transferrin may be missing. A defect in the synthesis or catabolism, or both, of this trisaccharide, which is common to many secretory glycoconjugates, is likely. Apart from providing a quantitative diagnostic method, the present findings may serve as a basis for further studies of the metabolic deficiency in this syndrome.

A new hereditary disorder has recently been identified in four girls with severe neurological symptoms associated with complex biochemical abnormalities. The neurological syndrome includes psychomotor retardation, pronounced cerebellar hypoplasia with corresponding cerebellar dysfunction, alternating internal strabismus, and peripheral sensorimotor neuropathy. Convulsions and stroke like episodes have occurred in some patients. Other organs are variably affected, including retinitis pigmentosa, skeletal abnormalities, lipodystrophy and fat accumulations on the buttocks, hypertrophy of the labia majora, and retracted nipples. In all four patients there was variable hepatomegaly with distension of the endo-plasmic reticulum and Golgi apparatus, and lipid and membrane like inclusions in the lysosomes of hepatocytes.

A number of abnormalities of serum glycoproteins, lipids, hormones, and enzymes have been found in these patients—for example, low concentrations of albumin, haptoglobin, apoprotein B, thyroxine binding globulin, transcortin, and cholesterol in the serum, and fluctuating concentrations of prolactin, growth hormone, and follicle stimulating hormone. Some lysosomal enzyme activities in serum are raised, (aryl sulphatase A, beta-galactosidase and N-acetylgalcosaminidase), and others are normal (sialidase and alpha-mannosidase). The microheterogeneity of transferrin from serum and cerebrospinal fluid was abnormal in all the patients, suggesting a deficiency in the negatively charged carbohydrate sialic acid. A similar abnormality was also found in other serum glycoproteins, but to a lesser extent.

Studies of the carbohydrate content of total serum glycoproteins indicated that the carbohydrate deficiency was complex and included a reduction of about 40% not only of sialic acid, but also of galactose and N-acetylglucosamine in the patients and to a lesser degree in their fathers as well. The carbohydrate content of total serum glycoproteins is, however, affected by the concentrations of the various glycoproteins, which also differ in their individual carbohydrate content and composition. In order to obtain more detailed information on this carbohydrate deficiency in a single quantitatively important glycoprotein, serum transferrin isoforms were studied qualitatively and quantitatively, and the carbohydrate content was determined in the isolated glycoprotein.

Subjects and methods
For analysis of carbohydrate concentrations in transferrin, serum was prepared from venous blood from the four female patients aged 2–5–12 years, and from four age matched controls. The patients, including monozygous twins, came from three unrelated families (table 1). For qualitative and quantitative analyses of isoforms of serum transferrin, samples were collected from six control children aged 2–14 years, from two clinically unaffected siblings aged 3–5–4 years, and from the three clinically healthy fathers and mothers. There was no history of alcohol abuse in the parents, and they had agreed not to use any alcohol for the two weeks preceding sampling. Serum samples were immediately frozen and...
Table 1  Summary of principal clinical symptoms shown by the patients

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Description</th>
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<tbody>
<tr>
<td>Growth</td>
<td>Mostly at or below the third centile</td>
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<tr>
<td>Psychomotor retardation without regression</td>
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<tr>
<td>Alteration of internal strabismus</td>
<td></td>
</tr>
<tr>
<td>Hypotonia, trunk ataxia, hyporeflexia</td>
<td></td>
</tr>
<tr>
<td>Localised fat accumulations, lipodystrophy, obesity</td>
<td></td>
</tr>
<tr>
<td>Prominent thorax, scoliosis</td>
<td></td>
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<tr>
<td>Variable hepatomegaly</td>
<td></td>
</tr>
<tr>
<td>Variable retinitis pigmentosa</td>
<td></td>
</tr>
<tr>
<td>Unexplained fits with coma, convulsions, and hemiplegia</td>
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</tr>
</tbody>
</table>

stored at -80°C until analysed. The samples were transported without thawing in dry ice from Belgium to Sweden within 24 hours.

ISOELECTRIC FOCUSING
A 40 µl sample of whole serum diluted 1:75 in distilled water was analysed from each subject in thin layer polyacrylamide gel in a pH gradient ranging from 2.2 to 11.4 and stained by silver staining.9 Transferrin components were identified by subsequent immunofixation of serum samples containing a constant amount of transferrin (0.5 µg) using antibodies against human serum transferrin (DAKO).6 Samples containing 25 µg of iron saturated purified transferrin from each subject were examined and stained with Coomassie brilliant blue. Densitometry was then carried out and the relative amount of each component was calculated as a percentage of the total.7 Genetic typing of serum transferrin was carried out in the patients after neuraminidase treatment of serum, isoelectric focusing, and immunofixation using a sample with a known phenotype of C 1–2 as reference.8 Total transferrin concentration in the serum and in the samples with purified transferrin were determined by single radial immunodiffusion on NOR-Partigen plates (Behring).

DETERMINATION OF CARBOHYDRATE DEFICIENT TRANSFERRIN (CDT) IN SERUM
After iron saturation and dilution, duplicate samples of 100 µl of serum were subjected to isocratic microanion exchange chromatography at pH 5-65 after which a transferrin radio-immune assay of the eluate was carried out as previously described.9 This method, initially developed for diagnosing alcohol abuse, measures the concentration of cathodal isotransferrins in serum (isoelectric point ≥5-7) while the normal main components (isoelectric point <5-7) are retained by the anion exchanger.3 Except when rare genetic D variants of transferrin are present,9 increased concentrations of carbohydrate deficient transferrin (>74 mg/l) indicate a reduction in the content of the charged carbohydrate sialic acid in transferrin.9 Variations in the content of neutral carbohydrate in transferrin (galactose, N-acetylglucosamine, and mannos) can probably not be detected by this method.10 The specificity of this assay for detecting alcohol abuse is 99% in large clinical studies in adults.9 11 12

PURIFICATION AND CARBOHYDRATE DETERMINATIONS OF SERUM TRANSFERRIN
Samples of 2-5 ml of serum from patients and controls were diluted 1:1 in 0.05 M sodium orthophosphate and 0.02 M sodium citrate at pH 7.2, and applied to 1-2 ml columns of antitransferrin-Sepharose 4B. Chromatography was carried out as previously described.13 14 Two columns were used for each individual. The eluted transferrin from each subject was pooled, neutralised, dialysed, and concentrated to a final transferrin concentration of 1.56–3.34 g/l as determined by single radial immunodiffusion.14 Aliquots were saturated with iron and subjected to isoelectric focusing while the remainder of the concentrated eluates was used for carbohydrate analyses.14

The amounts of sialic acid, galactose, N-acetylgalactosamine, and mannos were determined after acid hydrolysis by colorimetric15 16 or enzymatic17 methods previously described,14 with the exception that the galactose content was analysed after hydrolysis in 0.5 M sulphuric acid at 100°C for four hours, which had been found to improve reproducibility (H Stibler, unpublished observations). All samples were analysed for the same carbohydrate simultaneously on the same day and at least two specimens were examined. The variation of these methods (SD), varied between 0.07 and 4.7 nmol.14

Results
In the type of pH gradient used for isoelectric focusing, normal, iron saturated serum transferrin separates into six components within the pH range 5-2–5-7.4 The normal main component has an isoelectric point of 5-4 and corresponds to transferrin containing four sialic acid residues in its two carbohydrate chains (tetrasiotransferrin).4 10 14 18 Anodal and cathodal isotransferrins are normally minor bands: higher glycosylated forms with isoelectric points of 5-2 and 5-3, and trisialotransferrin and disialotransferrin with isoelectric points of 5-6 and 5-7, respectively.4 10 14 18 In all the patients there was a pronounced abnormality of the microheterogeneity of transferrin in native serum as well as in transferrin purified by affinity chromatography (figs 1 and 2). There was a pronounced increase (see below) of components with isoelectric points of 5-7 and 5-9 (disialotransferrin and asialotransferrin). Only a minor band at isoelectric point 5-8 (monosialotransferrin) was present, while components corresponding to trisialotransferrin, tetrasiotransferrin, and pentasialotransferrin (isoelectric points 5-6, 5-4, and 5-3, respectively) were less marked than normal. Moderate increases of disialotransferrin and asialotransferrin were observed in serum from all the fathers (fig 1). Neuraminidase treatment completely abolished the abnormal transferrin heterogeneity, and transferrin phenotypes were the common C1 in three patients, and C1–2 in one patient. Quantitative determination of carbohydrate deficient transferrin (isoelectric point >5-65) showed approximately a tenfold rise in cathodal isotransferrins in all the patients compared with normal values 3 9 12. The carbohydrate deficient transferrin values were on average twice the normal in all three fathers, and slightly higher than normal in one of the mothers (86 mg/l).
Figure 1 Isoelectric focusing of native serum proteins with silver staining within the pH range 2.2-11. The isoelectric points of transferrin are indicated at the right. A = mother of C and D. B = father of C and D. C and D = patients. E = mother of G. F = father of G. G = patient. H = mother of J. I = father of J. J = patient. Note the increase of disialotransferrin and asialotransferrin (isoelectric point 5.7 and 5.9) and the low amount of trisialotransferrin and monosialotransferrin (isoelectric point 5.6 and 5.8) in the patients. Note also the slight increase of disialotransferrin in the fathers. The transferrin pattern seems normal in the mothers.

The other two mothers and the siblings had normal carbohydrate deficient transferrin concentrations (table 2). For the sake of comparison, carbohydrate deficient transferrin values from alcoholic patients are included in table 2. Total transferrin concentration in serum was reduced (1.40 and 1.56 g/l) in two of the patients, but normal in the other two cases (2.05 and 2.94 g/l) (normal range 2.02-3.90 g/l).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Mean (SD) carbohydrate deficient transferrin concentration (mg/l)</th>
</tr>
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<tbody>
<tr>
<td>Patients (n=4)</td>
<td>591 (77)</td>
</tr>
<tr>
<td>Siblings (n=2)</td>
<td>63 (2)</td>
</tr>
<tr>
<td>Child controls (n=6)</td>
<td>62 (18)</td>
</tr>
<tr>
<td>Fathers (n=3)</td>
<td>125 (50)</td>
</tr>
<tr>
<td>Mothers (n=5)</td>
<td>64 (15)</td>
</tr>
<tr>
<td>Healthy adult controls (n=155)</td>
<td>53 (11)</td>
</tr>
<tr>
<td>Alcoholic patients (n=100)</td>
<td>133 (57)</td>
</tr>
</tbody>
</table>

Table 3 Carbohydrate concentrations in purified whole serum transferrin from patients and controls

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Patients</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>Sialic acid:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin (µmol/g)</td>
<td>37.40 (2.07)</td>
<td>54.59 (2.27)</td>
</tr>
<tr>
<td>Transferrin (mol/mol)</td>
<td>2.8 (0.2)</td>
<td>4.4 (0.2)</td>
</tr>
<tr>
<td>Galactose:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin (µmol/g)</td>
<td>38.72 (2.58)</td>
<td>54.71 (3.38)</td>
</tr>
<tr>
<td>Transferrin (mol/mol)</td>
<td>3.0 (0.2)</td>
<td>4.2 (0.1)</td>
</tr>
<tr>
<td>N-acetylgalactosamine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin (µmol/g)</td>
<td>83.38 (4.36)</td>
<td>118.33 (5.06)</td>
</tr>
<tr>
<td>Transferrin (mol/mol)</td>
<td>6.4 (0.3)</td>
<td>9.1 (0.4)</td>
</tr>
<tr>
<td>Mannose:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin (µmol/g)</td>
<td>76.05 (1.82)</td>
<td>78.85 (5.43)</td>
</tr>
<tr>
<td>Transferrin (mol/mol)</td>
<td>5.6 (0.1)</td>
<td>6.1 (0.4)</td>
</tr>
</tbody>
</table>

The molar ratios were calculated from a molecular weight of 77,000 for transferrin.
glycoprotein. The average reduction of the three saccharides may therefore amount to 66% in the abnormal fractions. As these mainly consisted of disialotransferrin (29%) and asialo-
transferrin (24%), and the three carbohydrates were reduced to the same extent, it may be suggested that the disialotransferrin component lacks two, and the asialotransferrin four, sialic
acid, galactose, and N-acetylglucosamine residues peripheral to mannose (fig 3). Based on the relative amounts of normal and abnormal iso-
transferrins, this type of proposed carbohydrate defect would result in expected total carbo-
hydrate contents of 2.6 for sialic acid and galactose, 6.9 for N-acetylglucosamine, and 6.1 for mannose (in mol/mol of transferrin), which are close to the total values obtained in the patients (table 3).

Discussion

The extreme alteration of transferrin microhe-
terogeneity on isoelectric focusing, with more than half of this glycoprotein being abnormal in our patients, seems to be almost specific for this disease. An abnormality of this kind has also
been described recently in seven Swedish chil-
dren (four girls and three boys from four fami-
lies) with a constellation of complex clinical symptoms similar to those in our cases.19 Based
on the transferrin pattern on isoelectric focus-
ing, these authors called the disease 'di-
sialotransferrin developmental deficiency syndrome'.19 From their first figure it is,
however, clear that both the disialotransferrin and asialotransferrin components were greatly
increased in serum as in our patients. The clini-
cal picture, together with the most unusual appearance of the transferrin, suggests that these patients suffer from the same or a closely related metabolic disorder. In several thousands of serum samples examined by isoelectric focusing by us and others39 20 a cathodal distribu-
tion of transferrin of this type, though less pronounced, has only been found in alcohol abusers. The same result has been obtained with the more rapid and simple quantitative carbo-
hydrate deficient transferrin assay.3 9 11 12 21 Slightly increased carbohydrate deficient trans-
 ferrin concentrations may occur in some patients with primary biliary cirrhosis, and in patients with rare genetic D-variants of trans-
ferrin.9 11 12 Carbohydrate deficient transferrin concentrations of the magnitude of those in the present cases, however, have only occasionally been found in alcoholics. With these exceptions in mind, the carbohydrate deficient transferrin assay seems to be highly specific for this syndrome.

The results of carbohydrate analyses in total serum glycoproteins1 2, and now specifically in isoelectric transferrin, clearly show that the carbohydrate deficiency includes not only the charged sialic acid but also the neutral galactose and N-acetylglucosamine to almost exactly the same extent, which together constitute the terminal trisaccharides in transferrin (fig 3) and in many other secretory glycoproteins.32 The normal mannose content and the degree of reduction of N-acetylglucosamine suggest that the oligosaccharide core is probably intact. The carbohydrate defect previously shown in total serum glycoproteins 1 2 cannot be quantitatively explained by this abnormality in transferrin alone. Other serum glycoproteins can therefore be expected to be similarly affected, and this supposition is also indirectly supported by preliminary isoelectric focusing analyses.2 One of the reasons why this change is more readily observed in transferrin may be its relative independence of the carbohydrate units for secretion and survival in circulation in contrast to many other serum glycoproteins.23 24

The fact that the transferrin abnormality con-
ists of increases of disialotransferrin and asialotransferrin, and only to a small extent of monosialotransferrin together with the reduced trisialo-component, argues against a simple cata-
bolic effect of glycosidases. Such an action would be expected to cause a successive or random increase in cathodal isotransferrin.4 18

provided that the recognition and elimination of transferrin with two or four defective oligosaccharide antennas is not selectively impaired.22 The normal sialidase activity and normal concentrations of free sialic acid, galactose, and N-acetylglucosamine in serum are also evidence against a glycolytic mechanism.2

In view of the morphological pathology of the endoplasmic reticulum and Golgi apparatus, a synthesis defect has to be considered.2 After en bloc incorporation of the core oligosaccharide in the rough endoplasmic reticulum, followed by mannose trimming, the carbohydrates of the terminal trisaccharides in serum glycoproteins are successively added in the Golgi complex.23 In the case of transferrin at least five glycosyl-
transferases are needed for completion of the chain.24 Considering the transferrin pattern and its carbohydrate contents in the patients, a partial deficiency of N-acetylglucosaminyl-
transferase I or II can be hypothesised (fig 3). Preliminary results have indicated a 40% reduction of non-specified N-acetylglucosa-
miminyltransferase but also of sialyltransferase activity in serum.2 To elucidate the basic defect, studies of the intracellular enzymes with specific isozyme analyses are in progress. It may be of interest that in alcoholic patients with a carbohydrate deficiency in transferrin similar to that shown by the patients with this syn-
drome,18 several glycosyltransferases have low activities, apparently as result of an inhibitory action of acetaldehyde,26 which also causes morphological changes of the Golgi apparatus.27

The essential similarity in symptoms, particu-
larly those of the nervous system, between this inherited syndrome and the acquired complica-
tions of chronic alcoholism in adults is notable. These two conditions may therefore offer ways to gain greater insight into the role of the carbo-
hydrates in both secretory glycoproteins for the development and maintenance of the human nervous system.

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