

Carbohydrate-deficient glycoprotein syndromes: inborn errors of protein glycosylation

G Keir¹, B G Winchester² and P Clayton³

From the ¹Department of Neuroimmunology, Institute of Neurology, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, the ²Division of Biochemistry and Genetics, Institute of Child Health, Great Ormond Street Hospital, London, and the ³Institute of Child Health, Great Ormond Street Hospital, London, UK

SUMMARY. The carbohydrate-deficient glycoprotein (CDG) syndromes (CDGS) are a series of autosomal recessive enzyme deficiencies which result in incomplete glycosylation of plasma proteins. CDGS types Ia and Ib have been related to deficiencies of phosphomannomutase and phosphomannose isomerase, respectively, while CDGS type II results from a deficiency of N-acetylglucosaminyltransferase II. Secondary CDG syndromes are associated with galactosaemia and hereditary fructose intolerance. The diagnosis of CDGS is most easily made by studying the glycoforms of suitable marker proteins using either electrophoresis or isoelectric focusing. This paper reviews the structure of the glycan chains of proteins and structural alterations in CDGS. It also outlines analytical techniques which are useful in the laboratory study of protein glycoforms and the diagnosis of CDGS.

Additional key phrases: phosphomannomutase, phosphomannose isomerase, N-acetylglucosaminyltransferase II, galactosaemia

The majority of plasma proteins are glycoconjugates in which one or more carbohydrate chains, or glycans (each consisting of several linked monosaccharides) are covalently linked by glycosidic links to amino acid side chains of the polypeptide. Two types of glycosidic linkages occur between glycans and proteins. *N*-glycans are linked to the protein through the amide nitrogen atom of an asparagine (Asn) residue, whilst *O*-glycans are usually connected to the oxygen atom of either a serine (Ser) or a threonine (Thr) residue. Occasionally, *O*-glycosylation occurs through hydroxylysine or hydroxyproline residues. Many glycoproteins contain only a single type of glycan link but some, including procollagen, glyophorin, and human chorionic gonadotrophin have both types of linkages and are classed as *N*-, *O*-glycoproteins.

It is important to distinguish between *glycosylation*, in which the oligosaccharyl chains are linked to proteins by a glycosidic link formed by an enzyme-catalysed stereo-specific reaction, and *glycation* in which carbohydrates react non-enzymatically with primary amine groups to form a Schiff base which then rearranges to give a stable adduct. Glycated proteins represent the chemical equilibrium between the sugar and the protein and examples include HbA_{1c} and glycated albumin. The phenomenon of protein glycation will not be considered further here.

It has been recognized for some time that the glycosylation of proteins has great potential for carrying biological information. Although the precise physiological role of the glycan chains is known for only a few glycoproteins, it is clear that the physiochemical properties of a glycoprotein depend upon both the protein and carbohydrate components, and that synthesis of specific carbohydrate side chains at defined sites is essential for biological functions. Since cells are capable of producing a wide variety of oligosaccharyl structures, sophisticated mechanisms exist to ensure that the correct structures

This article was prepared at the invitation of the Clinical Laboratory Investigations Standing Committee of the Association of Clinical Biochemists, but does not necessarily reflect its views.

Correspondence: Dr G Keir.
E-mail: gkeir@ion.ucl.ac.uk

are added to the numerous glycoproteins that are assembled.¹⁻³

Alterations in the glycosylation of proteins occur in association with certain diseases, one example being the abnormal fucosylation seen in malignancy.⁴ Such changes in protein-bound carbohydrates are secondary to the underlying pathology. There are also examples in which defective glycosylation results from changes in the sequences of amino acids of an individual protein, e.g., α_1 -antitrypsin Z.

Primary disorders of *N*-glycosylation have been identified in only five conditions. These are leukocyte adhesion deficiency type II,^{5,6} inclusion body cell disease (I-cell disease),⁷ congenital dyserythropoietic anaemia type II⁸ (previously known as hereditary erythroblastic anaemia with multinuclearity and a positive acid serum lysis test, or HEMPAS), paroxysmal nocturnal haemoglobinuria⁹ and carbohydrate-deficient glycoprotein syndromes (CDGS).¹⁰

The CDGS and the secondary carbohydrate-deficient glycoprotein disorders galactosaemia and hereditary fructose intolerance form the subject of this review.

GLYCOSYLATION OF PROTEINS

The co-translational transfer of a common precursor oligosaccharide, $\text{Glc}_3\text{Man}_{5,9}\text{GlcNAc}_2$ (where Glc = glucose, Man = mannose, GlcNAc = *N*-acetylglucosamine), from a dolichol pyrophosphate lipid carrier to the nascent polypeptide chain initiates the assembly of *N*-linked glycans (Fig. 1).¹¹⁻¹³ This step is catalysed by an oligosaccharyltransferase in the lumen of the rough endoplasmic reticulum,¹⁴ which transfers the precursor glycan *en bloc* to an asparagine residue within the recognition tripeptide Asn-X-(Ser/Thr/Cys), where X = any amino acid other than proline or aspartic acid and Cys = cysteine. Not all of the consensus sequences in glycoproteins are necessarily glycosylated. Since all newly synthesized glycoproteins pass through the same assembly pathway and are thus exposed to the same transferases, it is probable that the conformation of the growing polypeptide chain determines whether a particular site is glycosylated.

Once the precursor glycan has been attached to the polypeptide chain it is then subjected to trimming by several glycosidases, each removing a specific monosaccharyl residue. Middle-stage processing then takes place, involving elongation of the processed oligosaccharides by the addi-

tion of new glycoside residues. Finally, late-stage processing involves further elongation of the glycan chains, sometimes with additional modifications such as sulphation or phosphorylation.

As a consequence of the processing pathways the *N*-glycans of mature glycoproteins all have a common invariant core which is composed of three D-mannose and two *N*-acetyl-galactosamine residues. Additional monosaccharide units are attached to this core and allow the glycan chains to be grouped into high mannose, complex, hybrid and poly-*N*-acetylglucosamine types (Fig. 2).

It should be noted that the glycosylation pathway is not template-based, i.e., geared towards the mass production of invariant structures. The glycosylation 'fingerprint' or glycoform of an individual cell depends upon the concentrations and substrate specificities of the glycosidases and glycosyltransferases that are expressed in that individual cell. The expression of these processing enzymes can vary with the physiological or pathological state of the cells, and vary between individual cells in the same state. Furthermore, the nature of the glycan structure at a particular glycosylation site will depend upon which processing enzymes recognize it as a substrate. This can vary from site to site within an individual polypeptide as well as between the potential glycosylation sites on different polypeptides. Finally, the glycan structures at any given glycosylation site may show microheterogeneity as a consequence of subtle conformational difference existing within the individual monosaccharide units which make up the glycans.

Any individual glycoprotein molecule therefore has a glycan structure that is only one of a range of possible glycan structures associated with that specific protein. The overall spectrum of glycoforms found for a particular glycoprotein is often species- and tissue-specific. Furthermore, the range of glycoforms produced for a particular glycoprotein may change during the normal development of tissues, as well as varying as a consequence of pathological processes.

GLYCAN STRUCTURE OF SERUM TRANSFERRIN

Transferrin is a useful model glycoprotein, and one that is pivotal to the diagnosis of the carbohydrate-deficient glycoprotein syndromes.

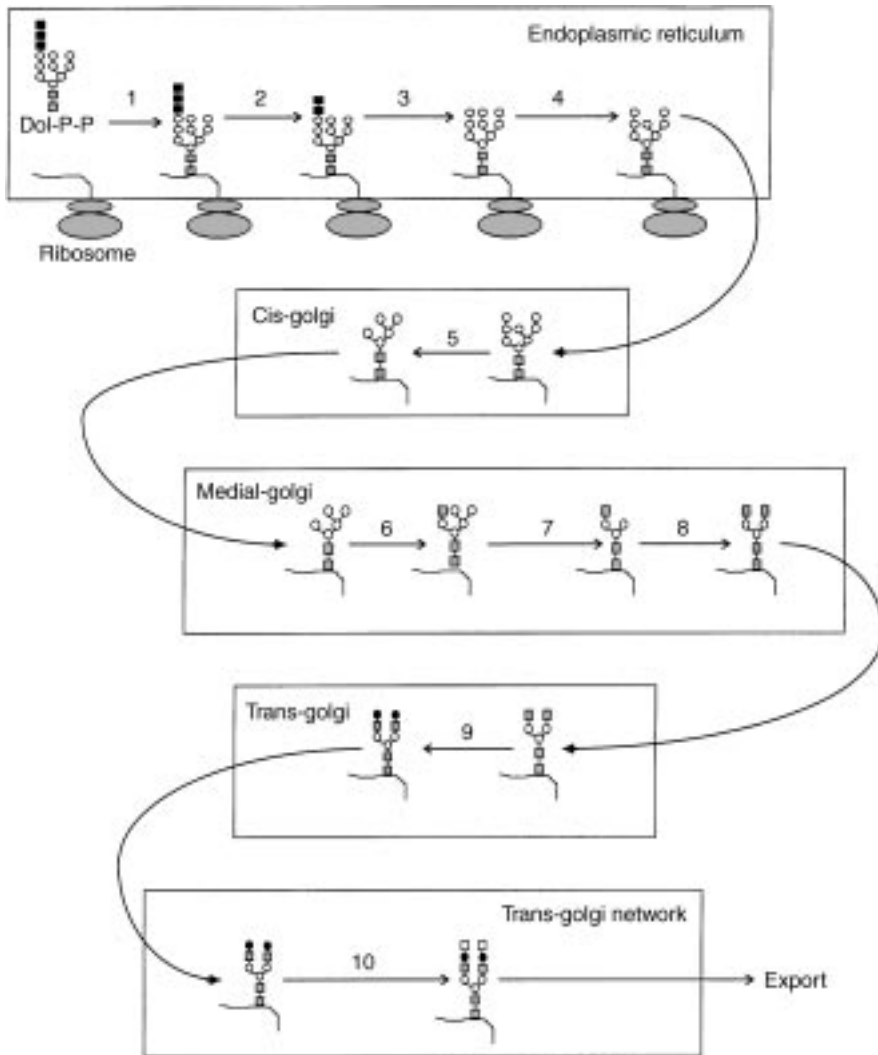


FIGURE 1. A representative pathway for the formation of a bi-antennary glycan chain. The enzymatic steps are: 1, oligosaccharyltransferase transfers the glycan chain precursor from dolichol pyrophosphate (Dol-P-P) to the nascent protein; 2, 3, α -glucosidases remove the glucose units from the glycan; 4, α -(1,2)-mannosidase removes a specific α 1-2-linked mannose; 5, α -mannosidase I then completes the removal of the α 1-2-linked mannoses, which allows 6, N-acetylglucosaminyltransferase I to add the first N-acetylglucosamine (GlcNAc) unit to the core using uridine diphosphate-GlcNAc (UDP-GlcNAc) as a donor; 7, α -mannosidase II then clips further mannose units from glycan, which allows 8, N-acetylglucosaminyltransferase II to begin extending the second antenna again using UDP-GlcNAc as a donor; 9, β (1,4)galactosyltransferase then adds the penultimate galactosyl residues, donated from UDP-galactose, and finally 10, α (2,3)sialyltransferase completes each antenna by transferring sialic acid from CMP-NAcNeu. Intermediary structures formed within this pathway are used as substrates for the production of alternative glycan structures, e.g., triantennary chains. ■ = glucose; ○ = mannose; ■ = N-acetylglucosamine; ● = galactose; □ = N-acetylneuraminic acid.

Human transferrin consists of a single polypeptide chain of 679 amino acids with two N-linked glycan chains attached to Asn residues 413 and 611.¹⁵ The glycan chains are both of the

complex type, with bi- and tri-antennary chain forms being most commonly represented.¹⁶ Each antenna of the glycan terminates in a sialic acid (N-acetylneuraminic acid, NAcNeu) residue,

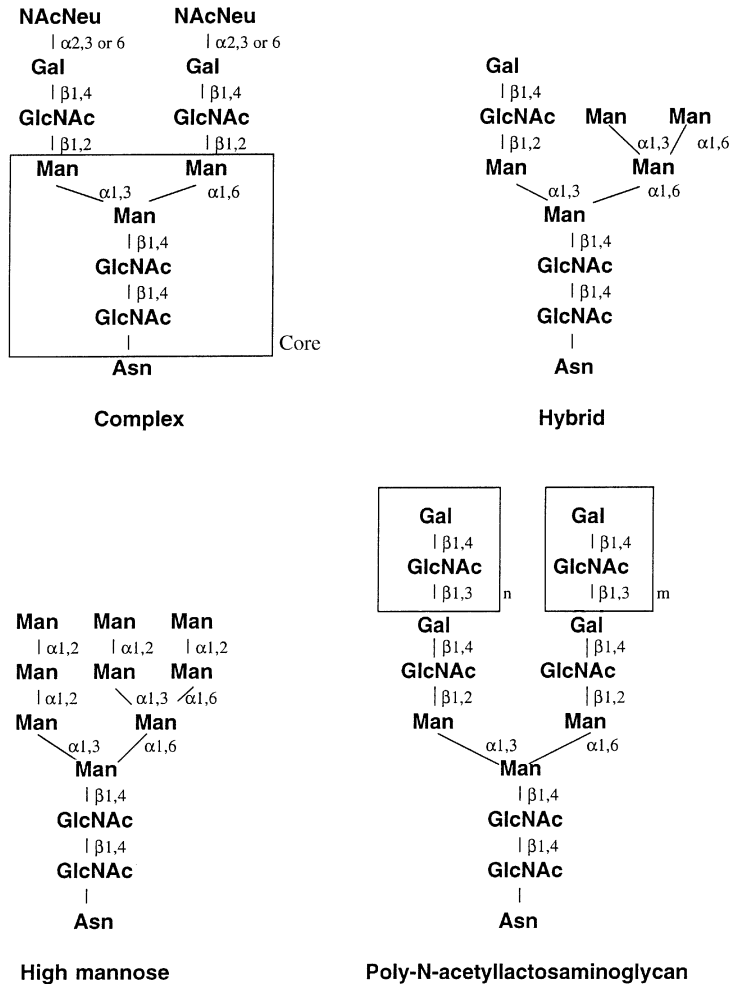


FIGURE 2. The structure of the four major N-glycan structures found on proteins. The core structure of $Man_3-GlcNAc_2$ is repeated throughout all four glycan groups. In the poly-N-acetyllactosaminoglycan group n and m are repeating structures. NAcNeu = N-acetyl neuraminic acid; Gal = galactose; GlcNAc = N-acetylglucosamine; Man = mannose; Asn = asparagine.

which, since it carries a negative charge, contributes to the overall charge on the glycoprotein (Fig. 3). The degree of sialylation thus influences the electrophoretic behaviour of transferrin and it is possible to separate the component glycoforms using a suitable technique.¹⁷

The antennary form of the glycan occupying a particular glycosylation site on transferrin varies, and the permutations explain much of the observed heterogeneity of serum transferrin.

Approximately 85% of the transferrin in normal serum is in the tetra-sialyl form, with four sialic acid residues probably present as two bi-antennary structures. Penta-sialyl and tri-sialyl forms make up most of the remainder. Only trivial amounts of transferrin containing fewer than three sialic acid residues are seen in normal serum and this can be explained by the removal of underglycosylated transferrin from the circulation by the asialoglycoprotein receptor.^{18,19}

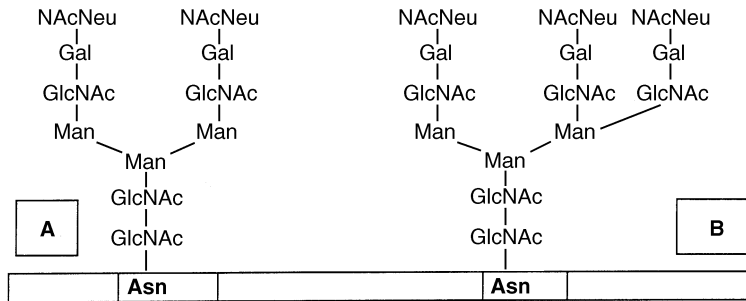


FIGURE 3. The most common structures of the N-glycan chains of transferrin. The polypeptide chain has 2 potential glycosylation sites and each can be occupied by either a bi- or a tri-antennary glycan structure. The most common combinations give rise to transferrin having 3, 4, 5 or 6 sialic acids. NacNeu = N-acetyl neuraminic acid; Gal = galactose; GlcNAc = N-acetylglucosamine; Man = mannose; Asn = asparagine.

PRIMARY CARBOHYDRATE-DEFICIENT GLYCOPROTEIN SYNDROMES

In the late 1970s a pair of monozygotic twins were reported as having developmental delay in association with endocrine and biochemical abnormalities.^{20,21} These children were later found to have unusual patterns of serum transferrin on isoelectric focusing, and partial deficiencies of protein-bound sialic acid, galactose and N-acetylglucosamine were identified.²² As further cases were investigated it became apparent that this hypoglycosylation affected many, possibly all, plasma glycoproteins.^{23,24} It seemed likely, therefore, that these patients had a disorder primarily affecting protein glycosylation.^{25–28} As a result of the characteristic partial deficiency of several carbohydrates from glycoproteins, the term carbohydrate-deficient glycoprotein syndrome is used to describe these conditions.

On the basis of clinical and laboratory findings, five types of CDGS have so far been reported. It is also recognized that other inborn errors of carbohydrate metabolism, such as galactosaemia and hereditary fructose intolerance, also give rise to carbohydrate-deficient glycoproteins. Collectively, the conditions offer important insights into the metabolic control of the glycosylation pathway.

For historical reasons the classification of CDGS is based upon the glycoform pattern observed for transferrin. CDGS type Ia and type Ib give transferrin glycoform patterns which are indistinguishable. The subtypes differ in the nature of the enzyme deficiency; type Ia is due to a deficiency of the enzyme phosphomanno-

mutase (PMM), while type Ib is associated with a deficiency of phosphomannose isomerase (PMI). These two subtypes may also differ in clinical presentation, although at the time of writing only one patient with type Ia had been described in the literature, with a report on a further four cases awaited.

CDGS TYPE I

Clinical aspects

Infants with CDGS type Ia have subtle abnormalities at birth.^{29–31} These include an antimongoloid slant to the eyes; abnormal fat pads, particularly on the upper outer aspect of the buttocks; *peau d'orange* skin on the legs; long fingers and toes; and limited movement of the hips and knees. They fail to thrive from birth and by 6 weeks roving eye movements and marked head lag on pulling to sit are often evident. Ascites may appear in the neonatal period; the liver size is normal or modestly increased. The infants are prone to accumulate fluid in the pericardial and pleural cavities and may present with tamponade. Cardiac failure can also be due to cardiomyopathy.^{32,33} Proteinuria is common, although the nephrotic syndrome is unusual.^{34,35} The kidneys are large and cysts may be demonstrated by ultrasound. Affected infants are also prone to stroke-like episodes and periods of stupor. Bruising and intracranial haemorrhage have been described. Opportunistic infections have been documented in a few infants. There is a high mortality in infancy amongst the most severely affected patients.

In the survivors, developmental delay becomes apparent during the first year of life. The truncal hypotonia is associated with ataxia, which makes walking very difficult for these children. Some patients have acquired microcephaly. An alternating convergent squint is common. Neurological imaging shows marked atrophy of the cerebellum (and sometimes of the brain stem) and there may be a substantial collection of fluid in the posterior fossa, suggestive of a Dandy-Walker cyst.^{31,36-39}

During childhood the lower limb tendon reflexes are lost and a neuropathy can be detected on electromyoneurography. Stroke-like episodes can also occur. A pigmentary retinopathy can often be detected, often associated with abnormalities on electroretinography,⁴⁰⁻⁴² although none of the patients has developed blindness as a result. Kyphoscoliosis and pectus carinatum become apparent and this, together with incomplete extension at the hips and knees, give the children a very hunched appearance. Linear growth is retarded.⁴³

During teenage years the children continue to make developmental progress, albeit remaining significantly delayed (IQ approximately 50-60). They are extrovert and their understanding is better than their gross motor and expressive skills. Most manage to communicate reasonably well despite dysarthria. Motor neuron weakness in lower limbs progresses and is associated with atrophy of the lower legs. Female patients generally fail to develop secondary sexual characteristics due to primary ovarian failure,⁴⁴ although pubertal development has been observed.⁴⁵ Males do undergo puberty with the development of secondary sexual signs, but many have a low serum testosterone concentration with either normal or only slightly raised gonadotrophin concentrations as well as testicular atrophy. In adult life, signs of premature aging can become obvious.

Systematic prevalence studies of CDGS type I have not yet been published, but preliminary estimates of prevalence are in the region of 1/40 000 to 1/60 000. Overall mortality is around 15%, with a median age at death of 2.2 years (range 1 week to 11 years).

Molecular biology of CDGS I

Structural studies using nuclear magnetic resonance and mass spectroscopy reveal that the carbohydrate deficiency in transferrin is not restricted to the sialic acid residues and that some transferrin molecules are missing complete

glycan chains.^{46,47} CDGS type I is therefore a deficiency in glycosylation site occupancy. Since up to 50% of the transferrin in affected individuals is glycosylated normally, the condition is one of a relative deficiency and not a total lack of glycosylation ability (Fig. 4), which points to a defect in the early steps of glycosylation (probably resulting from a lack

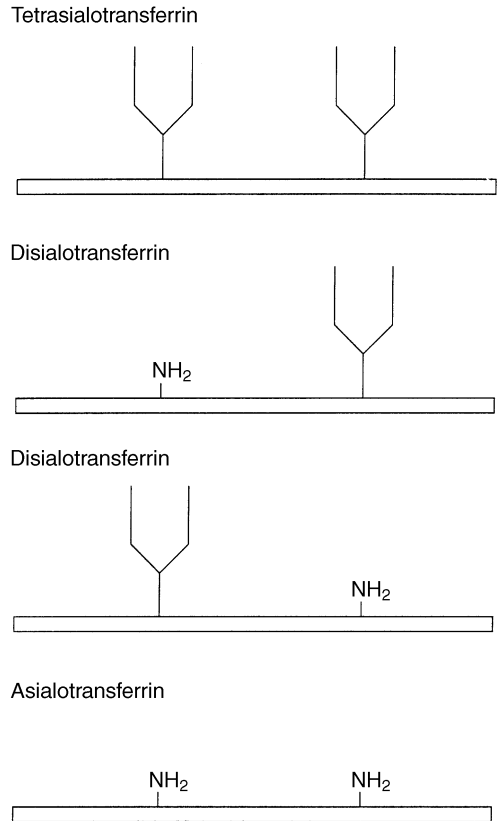


FIGURE 4. The various transferrin glycoforms found in carbohydrate-deficient glycoprotein syndromes (CDGS) Type I. Using isoelectric focusing, the abnormal transferrin bands observed correspond to the disialo- and asialoglycoforms. Using electrophoresis, the two disialoglycoforms are indistinguishable; the barbitone buffer anion also binds to the free amino site, giving it a net negative charge. This means that, on electrophoresis, the abnormal bands migrate as if they were trisialo- and disialotransferrin. This phenomenon is only seen in CDGS transferrin because the asparagine residue is unoccupied. In normal cerebrospinal fluid in which the asialo-transferrin is due to a lack of sialic acid only and both glycosylation sites are occupied, the glycoform pattern is the same by both electrophoresis and isoelectric focusing.

of the dolichopyrophosphate oligosaccharide precursor or in its transfer on to the nascent peptide).⁴⁸ A supply problem is most likely as there is decreased incorporation of [³H]mannose into both *N*-linked oligosaccharides and their lipid precursors in the fibroblasts of patients with CDGS type I,⁴⁹ while the activity of the transfer enzyme, *N*-oligosaccharyltransferase, is normal in fibroblasts.⁵⁰

It has been shown that there is marked reduction in the activity of PMM in the fibroblasts of CDGS type Ia patients.⁵¹ PMM catalyses the interconversion of mannose-6-phosphate and mannose-1-phosphate (Fig. 5). A deficiency would be expected to reduce availability of mannose-1-phosphate and, thus, the GDP-mannose needed for the synthesis of dolichopyrophosphate-oligosaccharides. Supplying mannose, but not glucose, to CDGS type Ia fibroblasts in culture in part corrects the altered glycosylation.⁵² This is somewhat surprising, since the bulk of the endogenous mannose required for glycoprotein synthesis is assumed to be derived from glucose by the action of phosphomannose isomerase, and the activities of this enzyme, as well as that of hexokinase and phosphoglucose isomerase, have been shown to be normal in CDGS type Ia.⁵³ This implies that glucose is not the predominant source of mannose for glycoprotein synthesis, but rather that mannose is more directly required.

Although supplying mannose to CDGS cells in culture improves their glycoprotein synthesis, and oral ingestion of mannose increases its blood levels,⁵⁴ it is unlikely that this will prove

a suitable therapeutic approach for CDGS type Ia.

Alternative explanations for the CDGS type I phenotype include a deficiency of dehydrodolichol reductase activity.⁵⁵ This argument is based upon the observation that patients with CDGS type Ia retain between 10 and 45% of their PMM activity, which should be sufficient to cope with the demands for mannose-1-phosphate made by the cell; furthermore, PMM-deficient cells should accumulate (GlcNAc)₂-pyrophosphate-dolichol, yet this is not found. Phosphoglucomutase is capable of catalysing the conversion of mannose-6-phosphate to mannose-1-phosphate *in vitro* and, while the importance of this reaction *in vivo* is not known, the possibility of phosphoglucomutase acting as some form of salvage pathway should not be discounted. It has also been shown that alkaline phosphatase in the serum of patients with CDGS I lacks the glycan chain derived from the glycosylphosphatidylinositol (GPI) anchor.^{56,57} This may reflect a deficiency of dolichylphosphomannose, a substrate used for glycan formation of the GPI anchor.

Genetics of CDGS type Ia

CDGS type Ia is inherited as an autosomal recessive trait.⁵⁸ The incidence of the disease is not yet known, but more than 200 patients have been identified from more than 14 countries.

Phosphomannomutase genes are present on two chromosomes. The *PMM1* gene is on chromosome 22q13,^{59,60} with a second PMM gene (*PMM2*) located on chromosome 16p13.

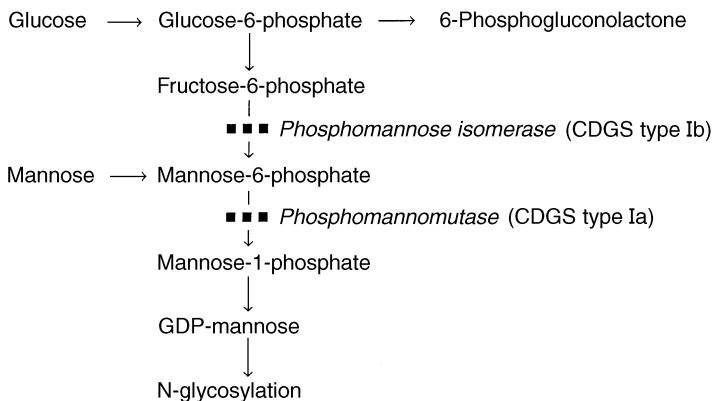


FIGURE 5. The early stages in the metabolism of monosaccharides showing the reaction catalysed by phosphomannose isomerase and phosphomannomutase, the two principal enzymes so far associated with carbohydrate-deficient glycoprotein syndrome (CDGS) type I. GDP = guanosine diphosphate.

The *PMM2* gene encodes a protein which is 66% homologous to that of *PMM1*.⁶¹ Eleven out of sixteen cases of CDGS I studied were found to have mis-sense mutations in the *PMM2* gene, all of which had a reduced PMM enzyme activity.

Linkage analysis maps the CDGS gene to chromosome region 16p13.3–p13.12.^{62–64} Of 17 families sharing a particular haplotype, 15 came from the same region of southwest Sweden, southern Norway and eastern Denmark, suggesting that they share a common ancestral CDGS I mutation. By contrast, studies in other non-Scandinavian families suggest multiple founder effects, with various spontaneous mutations occurring in separate geographical locations.

Laboratory aspects

The clinical biochemistry is dominated by abnormalities of *N*-glycosylation of numerous plasma glycoproteins,^{23,24} including transferrin. An additional common finding is hypoalbuminaemia. While proteinuria is a common feature, nephrotic syndrome and renal failure are not typical and it is unlikely that the observed hypoalbuminaemia can totally be explained by circulatory loss.

The concentrations of a wide range of clotting factors and their inhibitors are decreased in blood, particularly factors II, V, X, XI, antithrombin and protein C, while levels of fibrinogen D-dimer are frequently increased.⁶⁵ Either of these effects could give rise to coagulopathies which may contribute to the stroke-like episodes that are observed in cases of CDGS. Coagulation screening assays, such as the activated partial thromboplastin time (APTT) and prothrombin time (PT) are often normal, so analysis of individual coagulation factors is recommended,⁶⁶ particularly when there are clinical symptoms or if liver biopsy or elective surgery is being considered.

Thyroxine-binding globulin (TBG) concentrations are low in about 75% of cases. In many cases of CDGS total plasma thyroxine (T_4), triiodothyronine (T_3) and reverse T_3 (rT_3) concentrations are subnormal, often disproportionate to the TBG level.⁶⁷ The finding of unexplained neonatal hypothyroidism or a biochemically euthyroid state with low TBG should prompt investigation for CDGS.

Cases of CDGS have also been described with other biochemical findings, including non-ketotic hyperglycaemia,⁶⁸ and with isolated, unexplained increases in blood lysosomal enzymes, particularly β -hexosaminidase.

CDGS TYPE IB

About 20% of patients with transferrin glycoform patterns typical of CDGS type I have no reduction in fibroblast PMM activity.⁶⁹ A deficiency in PMI⁷⁰ may explain some of these cases. CDGS type I associated with a deficiency in PMI has been classed as CDGS type Ib.

Molecular biology of CDGS type Ib

In the one case so far reported CDGS type Ib is caused by a genetic defect in the *PMII* gene. PMI deficiency reduces the production of mannose-6-phosphate from fructose-6-phosphate (Fig. 5). The transferrin glycoform patterns of CDGS types Ia and Ib are identical, and enzyme studies are necessary to distinguish the two conditions. The use of oral mannose in CDGS type Ib is more successful and leads to a considerable improvement in the patterns of protein glycoforms. Care has to be taken, however, to prevent mannose toxicity.⁷¹

The reasons for the difference in clinical phenotype between CDGS types Ia and Ib are not yet clear, but could be related to the fact that mannose uptake from both mannose and glucose are blocked in PMM deficiency, whereas the uptake of exogenous mannose is unaffected in PMI deficiency (Fig. 5). This could also explain the difference in response of these two conditions to oral mannose.

CDGS TYPE II

Clinical aspects

This condition has so far been described in two children from unrelated families. The first was a 3-year-old Iranian girl born of consanguineous parents,⁷² the other a Belgian boy.⁷³ In contrast to CDGS I, the patients had more severe psychomotor retardation but a normal cerebellum on magnetic resonance imaging (MRI) scan and no peripheral neuropathy. Some dysmorphic features were present, including large, dysplastic, posteriorly rotated ears, thoracic deformity, widely spaced nipples, dystrophic limbs and proximally implanted thumbs. Both cases had stereotypic handwashing movements and ventricular septal defects, osteopenia and hypogonadism.

Molecular biology

Nuclear magnetic resonance analysis and electrospray mass spectrometry of purified CDGS type II transferrin show that its glycan chains consist primarily of truncated, mono-antennary, monosialylated *N*-glycans (Fig. 6). The difference in

molecular weights between normal transferrin and the disialotransferrin of the patients indicate two moles of the antennary chain α -NacNeu (2 \rightarrow 6) β -D-Gal(1 \rightarrow 4) β -D-GlcNAc (where Gal = galactose) to be missing, consistent with a deficiency in N-acetylglucosaminyltransferase II (GlcNAcT-II). The attachment of antennae to the common (Man)₃(GlcNAc)₂-Asn core structure is initiated by the sequential action of six N-acetylglucosaminyltransferases (numbered GlcNAcT-I to GlcNAcT-VI). Prior action of GlcNAcT-I is essential for the actions of GlcNAcT-II, -III and -IV, while action of GlcNAcT-II is required prior to that of GlcNAcT-V. Synthesis of complex N-glycans cannot occur until after both GlcNAcT-I and GlcNAcT-II have acted. Patients lacking GlcNAcT-II activity are therefore unable to attach GlcNAc residues in the α 1 \rightarrow 6 position of the core, resulting in incomplete structures.

A deficiency of GlcNAcT-II was confirmed in fibroblasts which showed a marked reduction in activity to approximately 1% that of normal.⁷⁴ Adding GlcNAcT-II from normal fibroblasts to extracts of patients' fibroblasts replaced the missing activity and indicated that the deficient activity in the patient was not due to the presence of an enzyme inhibitor. Blood relatives of patients have been shown to have GlcNAcT-II activities between 32 and 67% of normal, consistent with an autosomal recessive disease.⁷⁵

Laboratory aspects

Proteinuria was absent, with normal serum albumin and glutamic pyruvate transaminase activity. Coagulopathies due to deficiencies of clotting factors IX, XI, XII, antithrombin III, protein C, protein S and heparin cofactor II

were found. The serum activity of β -glucuronidase was reduced. Again, the most characteristic feature was the transferrin glycoform pattern. The normal tetra-sialo transferrin band was completely absent and was substituted by a heavy band corresponding to disialo-transferrin. Total serum transferrin concentrations were normal.

CDGS TYPE III

Clinical aspects

This has been described in two children of unrelated families.⁷⁶ One girl was a 9-year-old from Sweden, the other a 2-year-old from Germany. Both patients displayed perinatal floppiness, a slightly dystrophic appearance, severe psychomotor retardation, tetraparesis, infantile spasms, optic atrophy, depigmented skin and hepatomegaly. In early infancy the hypotonia was associated with brisk tendon reflexes, but later the flaccid tetraparesis was associated with absent reflexes. Central and cortical atrophy and generalized dysmyelination were apparent on computed tomography and MRI. In one case hypoplasia of the corpus callosum and a Dandy-Walker cyst were present. Cortical evoked potentials were severely abnormal from early infancy.

Laboratory aspects

Intermittent elevation of liver enzyme concentration was detected. Cerebrospinal fluid (CSF) protein concentrations were not elevated, and the levels of albumin and other proteins in the plasma were normal. Although increased levels of carbohydrate-deficient transferrin were found using chromatographic methods, isoelectric focusing patterns revealed only a slight increase

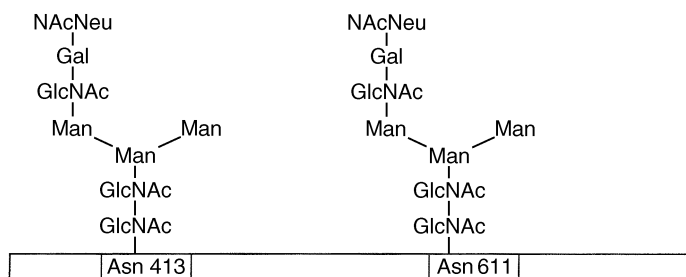


FIGURE 6. The proposed glycan structure found in transferrin in carbohydrate-deficient glycoprotein syndrome type II. The lack of N-acetylglucosaminyl transferase results in a deficiency of the antennary arm connected through the Man (α 1 \rightarrow 3) linkage. NacNeu = N-acetyl neuraminic acid; Gal = galactose; GlcNAc = N-acetylglucosamine; Man = mannose; Asn = asparagine.

in the 0, 1, 2 and 3 sialo-transferrin fractions. Transferrin-bound sialic acid, galactose and *N*-acetylglucosamine were decreased by an average of 15%. The nature of the structural difference and any associated enzyme abnormalities are not yet known.

CDGS TYPE IV

Clinical aspects

The only two cases so far reported⁷⁷ are a girl of Turkish descent and a German boy. Both showed microcephaly with severe epilepsy and absent psychomotor development. Dysmorphic features included adducted thumbs, a high arched palate and posteriorly rotated, dysplastic ears. One of the infants had multiple contractures, the other had optic atrophy and a coloboma of the iris. Muscle tone was strikingly abnormal but varied from hypotonicity to hypertonicity. In one patient MRI showed atrophy of the cerebrum and corpus callosum; in the other the cerebellum was atrophied more than the cerebrum. Generalized clonic-tonic seizures and myoclonic fits were observed and in both cases the electro-encephalograph (EEG) showed hypsarrhythmia.

Laboratory aspects

Routine biochemistry of blood and CSF was generally unremarkable, with only slight reductions in the plasma concentrations of anti-thrombin III and apolipoprotein B. TBG and albumin concentrations were both within the normal range. Quantitative determination of serum carbohydrate-deficient transferrin shows moderately elevated values. Isoelectric focusing revealed the absence of the asialo-transferrin band typically seen in CDGS type I, with an increase in disialo-transferrin, which is less than that found in CDGS type I and different also from that of types II and III.

LABORATORY METHODS IN DETECTION OF CDGS

The laboratory diagnosis of CDGS rests on two observations:

- 1 the detection of charge heterogeneity within individual glycoproteins, and
- 2 the demonstration that this charge heterogeneity is a function of glycan side-chain structure.

One corollary of these observations is that the charge heterogeneity cannot be due to difference

in the primary sequence of amino acids of the protein, i.e., a sequence polymorphism. The critical step, therefore, is to exclude amino acid substitutions as an explanation of charge heterogeneity. Once the nature of the glycoprotein abnormality has been established, appropriate enzyme activities can then be measured.

Detecting charge heterogeneity

Detecting charge isoforms of proteins is straightforward and glycoforms of transferrin have been resolved using techniques including isoelectric focusing,⁷⁸ acrylamide gel electrophoresis,⁷⁹ two-dimensional electrophoresis,⁸⁰ chromatography,⁸¹ capillary electrophoresis⁸² and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).⁸³

One convenient approach involves agarose electrophoresis with nitrocellulose immunoblotting (Figs 7 and 8). The common C1 and C2 phenotypes are not resolved by this technique, but many other phenotypic variants are. Double, slow-moving or absent transferrin bands which are found following serum electrophoresis should always be investigated for glycan abnormalities, although an apparently normal transferrin band does not exclude CDGS. Quantitative measurement is also unreliable, as the total concentration of transferrin is often normal.

CSF is an ideal reference material for use in any method since it contains a natural mixture of transferrin glycoforms having from zero to six sialic acids (inclusive). Serum, by contrast, contains only trivial amounts of transferrins having fewer than three sialic acid residues. We routinely include normal CSF and serum on each electrophoresis run.

Proving glycan structure

The cornerstone of diagnosing CDGS is to show that any charge heterogeneity observed results from alterations in glycan chain structure. This is easily demonstrated by using electrophoresis to detect the change in the overall charge of the transferrin that occurs when all the sialic acid residues are enzymatically removed (Fig. 9). As sialic acid is the only monosaccharide that contributes to the overall charge of the molecule, when all sialic acids are removed the only charge left is that intrinsic to the polypeptide chain. Removing the sialic acid residues therefore reduces all glycoforms to the asialo-protein. If the asialo-protein retains heterogeneity, then this can only result from amino acid substitutions in

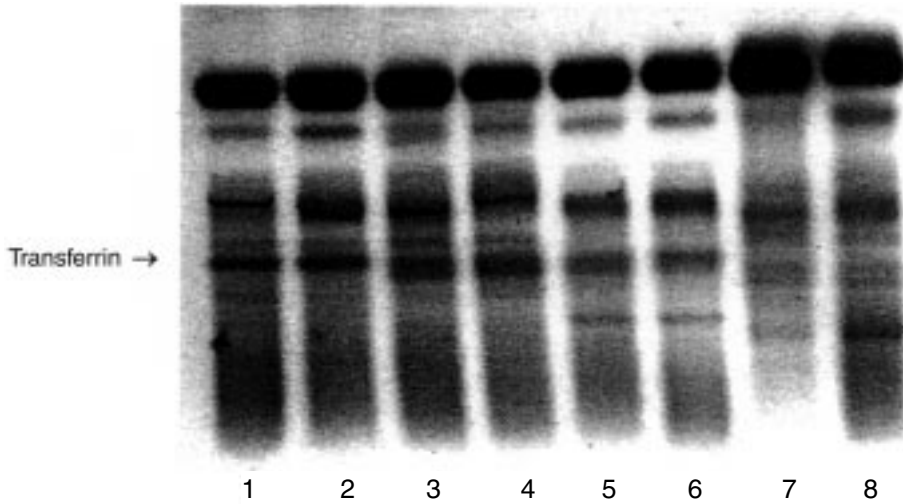


FIGURE 7. Serum agarose electrophoresis patterns in carbohydrate-deficient glycoprotein syndromes (CDGS). Lanes 1, 2 and 6 are normal serum. Lanes 3, 4 and 5 show double transferrin bands due to phenotypic variants. Lanes 7 and 8 show carbohydrate-deficient transferrin in two cases of CDGS.

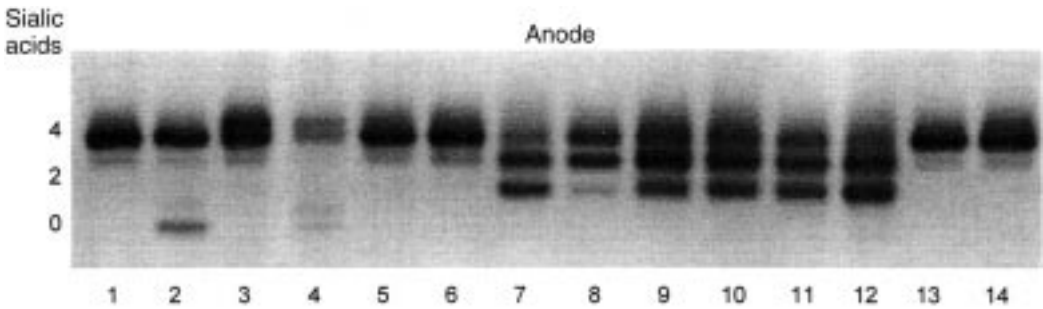


FIGURE 8. Transferrin glycoforms detected by nitrocellulose immunoblotting after agarose electrophoresis. Lanes: 1, control 'normal' serum; 2, control 'normal' cerebrospinal fluid (CSF); 3, phenotypic (fast) variant serum; 4, phenotypic variant CSF; 5, purified control serum transferrin; 6, purified control serum transferrin; 7, 9, 10, 11, 12, carbohydrate-deficient glycoprotein syndrome (CDGS) type I; 8, phenotypic (slow) variant; 12, CDGS type I; 13, mother of case 12; 14, father of case 12. Note the double asialotransferrin band in the CSF of lane 4.

the polypeptide chain. Neuraminidase digestion of phenotypic variants results in two asialo-protein bands in the heterozygous state, or only one band in the homozygous state.

Prenatal diagnosis of CDGS

Prenatal diagnosis of CDGS by analysis of transferrin glycoforms in foetal blood is not possible.^{84,85} Although the foetal liver is capable of synthesizing transferrin by 10 weeks of gestation, it seems that nearly all of the transferrin in the foetal circulation is derived from the maternal blood, so the glycoform pattern is misleading. After birth, maternal transferrin

decays from neonatal blood with a half-life of about 8 days,⁸⁶ during which time it is replaced by the neonate's transferrin. Although abnormalities are detectable earlier, we recommend allowing 3 weeks after the birth before analysis of neonatal blood transferrin, to minimize the risk of false negative results. It is possible to analyse blood spots for transferrin⁸⁷ which offers great convenience when samples need to be referred to distant laboratories for analysis, although in our hands this has not proved totally reliable. Amniotic fluid α -fetoprotein glycoforms may be possible markers for diagnosis *in utero*.⁸⁸ Elevated levels of the lysosomal enzymes β -hexosaminidase and

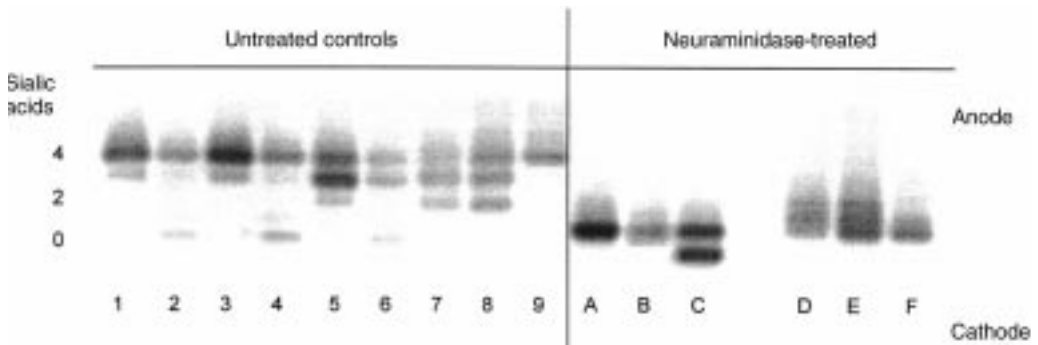


FIGURE 9. Effects of neuraminidase on transferrin glycoforms. Lanes: 1, normal serum; 2, normal cerebrospinal fluid (CSF); 3, normal serum; 4, normal CSF; 5, phenotypic variant serum; 6, phenotypic variant CSF; 7, carbohydrate-deficient glycoprotein syndromes (CDGS) type I serum; 8, CDGS type I serum; 9, normal serum; A, normal serum; B, normal CSF; C, phenotypic variant; serum (note double band); D, CDGS type I; E, CDGS type I; F, normal serum.

β -glucuronidase in amniotic fluid are additional features useful to support a diagnosis.⁸⁹ One promising approach is to measure PMM activity in either cultured amniocytes or chorionic villus cells,⁸⁹ although this is applicable only to foetuses which are PMM-deficient. Owing to residual PMM activity it is essential that enzyme studies be supported by genetic linkage analysis to confirm involvement of the *PMM2* locus. In due course, diagnosis will be possible by detection of specific mutations in the *PMM2* gene.⁶¹

SECONDARY CDGS

The transferrin glycoform pattern characteristic of CDGS type I is not restricted to this condition. Other inborn errors of carbohydrate metabolism give rise to similar abnormalities and need to be excluded from the different diagnosis.

Galactosaemia

Classical galactosaemia is caused by a deficiency of galactose-1-phosphate uridylyltransferase. The disorder usually presents in the first weeks of life with liver dysfunction, sepsis, Fanconi syndrome and cerebral oedema, and is life threatening if left untreated. Starting a galactose-restricted diet resolves the acute disorder in most cases. Many of the clinical manifestations of the condition have been explained by the accumulation of galactose-1-phosphate, although this is probably not the only factor involved.

Galactosaemia also gives rise to a pattern of serum transferrin glycoforms that is indistinguishable from that seen in CDGS type I, and

galactosaemia should be excluded before a diagnosis of CDGS is made. The glycosylation defect in galactosaemia is a secondary feature, and within 1–2 weeks of starting a galactose-free diet the transferrin glycoform pattern reverts to normal (Figs 10 and 11).

From the accumulated experience of over 20 years of neonatal screening for galactosaemia it has become apparent that even the earliest and most careful dietary restriction does not prevent all chronic complications of this disorder. Even well-managed galactosaemic patients develop a long-term encephalopathic syndrome characterized by delayed development, declining IQ with age, speech deficiency and personality disorders.^{90,91} Physical findings include delayed puberty, with females showing primary amenorrhoea associated with hypergonadotrophic hypogonadism. *Post mortem* studies have shown

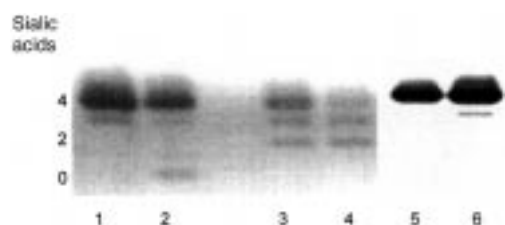


FIGURE 10. Transferrin glycoforms in galactosaemia. Lanes: 1, control serum; 2, control cerebrospinal fluid; 3, galactosaemia (untreated); 4, galactosaemia (untreated); 5, galactosaemia (case 3, 4 weeks after starting diet); 6, galactosaemia (case 4, 6 weeks after starting diet).

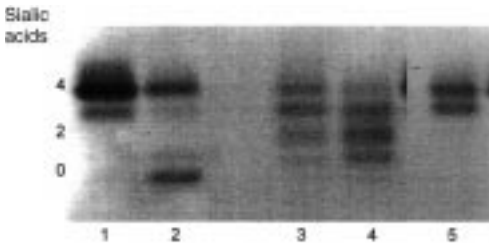


FIGURE 11. *Transferrin glycoforms in galactosaemia. Lanes: 1, control serum; 2, control cerebrospinal fluid; 3,4, galactosaemia, untreated (same patient samples on 2 separate days); 5, patient after 4 weeks diet. Note: this patient has a slow phenotypic variant as well as galactosaemia! If the pre-treatment pattern on this patient is compared with those in Fig. 9, it can be seen that this patient shows four glycoform bands, while the previous patients show only three. The additional band is a reflection of variant transferrin.*

cortical neuronal degeneration and atrophy of the cerebellum and basal ganglia.

Most patients with galactosaemia, even those with good dietary control, display persistent increases in red cell galactose-1-phosphate concentration and urinary galactitol excretion. This has traditionally been explained either by dietary non-compliance or by cryptic sources of galactose, such as fruit and vegetables. However, it would appear that both normal adults and adults with galactosaemia are capable of synthesizing more than 1g of galactose per day.⁹² In the face of this amount of endogenous galactose production, dietary manipulation is likely to have only a small influence and the implications of this on long-term complications need to be investigated. Although the serum protein glycoform pattern appears to normalize with dietary treatment, the possibility of subtle glycosylation problems remains. For example, there is evidence for a reduction in glycolipids containing galactose, with a concomitant increase of their precursors in the brain.⁹³

The nature of the metabolic block in protein glycosylation seen in galactosaemia is uncertain; however, structural studies have shown that the glycan side chain of transferrin lacks both sialic acid and galactose.⁹⁴ Since galactose is the penultimate monosaccharide on the glycan chain, it is tempting to suggest that the accumulation of galactose-1-phosphate either prevents the incorporation of galactose into the structure, as this normally involves a β 1, 4 linkage, or that there is a deficiency of the donor molecule uridine diphosphate (UDP)-galactose.

In either case the absence of a galactose residue means that there is no substrate for the terminal sialic acid to attach to. As with CDGS type I, about half of the serum transferrin is glycosylated normally, suggesting that some UDP-galactose is available, probably through epimerization of UDP-glucose. There are nevertheless differences between the glycan patterns for lysosomal enzymes seen in galactosaemia compared with CDGS I, which may indicate that the metabolic block is different in the two conditions.

Hereditary fructose intolerance

Hereditary fructose intolerance (HFI) is an autosomal recessive disorder resulting from a deficiency of fructose-1-phosphate aldolase. Untreated cases of HFI show patterns of serum transferrin glycoforms which are indistinguishable from those of CDGS type II.⁹⁵ HFI results in an accumulation of fructose-1-phosphate and this has been shown to inhibit PMM.⁹⁶ It is therefore likely that in HFI there is an inhibition of an early step in the *N*-glycosylation pathway, causing the same or similar underglycosylation of proteins as is seen in CDGS type I. A major difference from CDGS type I is that in HFI the error in the glycosylation pathway may prove to be limited to those tissues possessing the fructose pathway, principally the liver, kidney and small intestine.

Acknowledgements

The authors would like to thank their colleagues from the Department of Neuroimmunology (Institute of Neurology) and the Division of Biochemistry and Genetics (Institute of Child Health). Particular appreciation is due to Professor E J Thompson and Mr M Chowhan (Institute of Neurology) and Drs N Mian, A Johnson, E Di Tomasi and J Charleston (all Institute of Child Health), as well as to the physicians and clinical scientists who provided clinical information and material.

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Accepted for publication 14 July 1998