

CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II: MOLECULAR BASIS AND CLINICAL ASPECTS

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ABSTRACT

Congenital dyserythropoietic anemia of type II (CDA II) is a rare disorder, usually present in childhood, with a clinical picture of chronic anemia of mild to moderate degree, splenomegaly and intermittent or persistent jaundice. It is transmitted by autosomal recessive inheritance and is characterized by the presence of a large number of multinucleate and binucleate erythroblasts in the bone marrow and typical morphological abnormalities of the membrane of circulating erythrocytes. SDS-PAGE of red blood cell membrane proteins shows a narrower aspect and a faster migration of band 3 (anion exchange transporter). This aspect is consistent with decreased glycosylation of this protein.

The genetic mutations responsible for the glycosylation defect in CDA II have not yet been identified. Analysis of carbohydrate structures and biochemical data indicate that the activity of either GnT II or α -Man II is reduced in different families, suggesting that the disease is genetically heterogeneous. Molecular cloning of the GnT II and α -Man II DNA sequences has allowed direct investigation of the genetic mutations underlying the glycosylation defect in CDA II patients to begin.

Key words: congenital dyserythropoietic anemia type II, CDA II, dyserythropoiesis, anion exchange transporter, glycosylation, bone marrow, HEMPAS

Dyserythropoiesis is the term used to describe any alteration of the normal differentiation-proliferation pathway of the erythroid lineage. This alteration is manifested by morphologically abnormal erythroblasts, mainly bi-nuclearity and gigantism, and by ineffective erythropoiesis resulting from a discrepancy between erythroid output from marrow to circulation and erythroid marrow content, i.e. everything responsible for quantitative and qualitative decreases in erythropoiesis.

In the normal marrow approximately 3-5 cells out of 1000 erythroblasts are abnormal; however, normal individuals do not display binucleates or pluripolar mitosis.

Dyserythropoiesis may represent a physiologi-

cal condition or a disease. The latter could be a principal (CDA) or a secondary characteristic.

Multinucleated erythroblasts and karyorrhexis are seen occasionally in nutritional anemias (megaloblastic anemia, iron deficiency, folic deficiency, vit. B6 and vit. E deficiencies), leukemia and hemolytic anemia, and are indicative of bone marrow stress. Furthermore, dyserythropoietic erythroblasts may be observed in aplastic anemia, sideroblastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and during the recovery from bone marrow transplantation.

Physiologic dyserythropoiesis is present in those conditions in which a maturation blockage results from passage from a condition of rel-

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ative hypoxia to normal tissue oxygenation: erythropoiesis during the neonatal period, congenital cardiopathies with cyanosis, following surgery, or reduction of erythrocytosis due to hypobarism.

This review will deal with congenital dyserythropoietic anemia type II (CDA II), which represents the most frequent form of CDA. This type was established by Heimpel and Wendt on morphological criteria in 1967;¹ later Crookston *et al.* described two distinctive laboratory findings: positivity of acidified serum hemolysis and anti-i agglutination.² This latter author named this condition HEMPAS (*hereditary erythroblastic multinuclearity with positive acidified serum*).

For some years the main distinctive feature between the different forms of CDA was bone marrow morphology. Type I is characterized by megaloblastoid and binucleated (2-5%) erythroblasts with chromatin bridges; type II is characterized by bi- and multinucleated (10-40%) erythroblasts with karyorrhexis. In contrast, type III shows multi-nucleated cells (gigantoblasts: 10-40%).³ The pathogenesis of these diseases is still unknown; the functional defects could be due to a discrepancy between proliferation and maturation. This leads to enhanced ineffective erythropoiesis with a probable defect in the correct mitosis pathway.

From this point of view, the disease could be considered an effect of a cell cycle anomaly. This induces multinuclearity and a maturative discrepancy between the nucleus and cytoplasm, which in turn could cause enhanced intramedullary death and morphological abnormalities (multinuclearity).^{4,5}

Knowledge about CDA II has undergone two fundamental steps. The first was the discovery of the red cell membrane protein abnormality, which could have been hypothesized by the increased agglutinability and Ham test positivity. The second was the discovery of the protein glycosylation defect.⁷ Recently two putative genes, which appear to be involved in the glycosylation deficiency, have been cloned (see also later).

Type II CDA has been reported in more than 120 patients. Anemia is noted between infancy and adulthood and varies from mild to requiring regular transfusions.

The exact prevalence of this disease as well as the frequency of the gene are still unknown; this is due to the heterogeneity of clinical manifestations and to the fact that the majority of cases were probably undiagnosed. It is noteworthy that the highest frequency of clinical descriptions and diagnoses are in Italy. This might suggest that Italian hematologists are more aware of this kind of pathology, but it is possible to hypothesize a greater prevalence of this gene in the Italian (and Mediterranean) territory.

This review will concentrate on our current knowledge of the clinical, biochemical, genetical and pathophysiological aspects of CDA II. Furthermore, we will provide more recent information regarding diagnostic protocols for hematologists; finally, we will speculate about the future scenario of this interesting anemia.

Red blood cell membrane

This section will deal with the normal membrane structure, emphasizing the aspects that are most involved in the genesis of membrane defects in CDA II. The red cell membrane is made up of a phospholipid bilayer and a proteinic structure. The latter is composed of integral membrane proteins which penetrate or transverse the lipid bilayer and interact with the hydrophobic lipid core. They include transport proteins such as band 3, the anion exchange channel, and the glycoporphins, which carry membrane receptors and antigens. The peripheral proteins (known also as cytoskeleton) are a protein network that laminates the inner surface of the red cell membrane.⁸

The cytoskeleton contains 4 major proteins and hundreds of minor ones. Its major proteins are spectrin, ankyrin, actin and protein 4.1. Spectrin is the major skeletal component; it is a heterodimer of two polypeptide chains aligned side by side in an antiparallel arrangement. Heterodimers self-associate in head-head contact, whereas at their opposite ends spectrin dimers bind to actin short filaments, an interaction that is greatly enhanced by protein 4.1. The spectrin/actin/4.1 complex (also known as the junctional complex) interacts with the membrane-spanning glycoproteins, particularly gly-

cophorin C, and with several other modulating proteins such as demantin (protein 4.9), adducin and tropomyosin.⁹ Furthermore, ankyrin binds to spectrin at a site near the end of the molecule involved in dimer-tetramer association; ankyrin in turn binds the band 3 intra-cytoplasmic domain (protein 4.2 regulates and stabilizes this association).^{8,9}

Band 3 is the conduit for the physiologically important chloride-bicarbonate exchange. It is also a major binding site for a variety of enzymes and cytoplasmic membrane components. These two functions (transport and binding) are relegated to two structurally independent domains of the molecule.¹⁰ A 52 kd membrane domain contains the anion exchange channel; it is composed of 14 transmembrane helices connected by hydrophilic segments that must form the transport channel, but how they do this is unknown.

Evidence suggests that band 3 is a mixture of dimers and tetramers in the membrane. Normally 70% is in the dimer form and 30% in tetramers and oligomers. Almost all of the latter form is associated with membrane skeleton, which fits with other evidence that ankyrin binds preferentially to band 3 tetramers.¹¹ Band 3 contains a single fatty acid esterified to Cys 843 and a single complex carbohydrate attached to Asn 642. It is a complex lactosaminoglycan which is variable in length and contains Ii blood group antigens. In fetal cells it is unbranched and has i specificity, whereas in adults a branched structure with I reactivity is present.¹⁰

Defects of band 3 have been identified in four different red blood cell diseases: hereditary spherocytosis (decreased synthesis or aa. substitution); Southeast Asian ovalocytosis (deletion of aa. 400-408); acanthocytosis (variants associated with increased anion transport).¹² In the remaining defect, CDA II, a lactosaminoglycan side chain of band 3 is improperly constructed due to a lack of the relevant glycosyltransferases (see also next sections).

Clinical findings

CDA II is the most common of the congenital dyserythropoietic anemias. The geographic dis-

tribution of affected patients suggests a higher frequency of the gene in northwest Europe, Italy and North Africa.^{2,13} Like other patients with rare recessive genetic diseases, these subjects appear more often in a restricted area of the world, where people have a higher chance of intermarriage with relatives. Both sexes are equally affected and heterozygotes of CDA II, parents and some children, are hematologically normal. CDA II patients suffer from life-long anemia. It results from a combination of the death of erythroblasts in the bone marrow (ineffective erythropoiesis) and an increased breakdown of released red cells (peripheral hemolysis). The red cell glycosylation defects are responsible for both mechanisms. Erythrokinetic studies with ⁵⁹Fe and ⁵¹Cr showed that the degree of ineffective erythropoiesis and of peripheral hemolysis varies considerably among CDA II patients.^{14,15} Direct measurement of these factors, together with determination of sites of red cell destruction, is an essential procedure for having quantitative information about the mechanism of anemia.¹⁶ In fact, the spleen often appears to be the principal site of red cell destruction in cases with marked hemolysis.¹⁷

Anemia is often first noted in infancy or childhood and it shows great range from mild to severe. In some cases regular transfusions are required but it is rare that severe anemia is present from birth. White blood cells and platelets are normal; nevertheless, some cases of CDA II have displayed ultrastructural abnormalities not only in erythroid cells, but also in granulocytes, macrophages and platelets.^{18,19} In severe cases, mental and sensory abnormalities have been reported. Jaundice from an increase of indirect bilirubin, hepatosplenomegaly and gallstones are common. The plasma ferritin concentration may be normal²⁰ but its increase is independent of the degree of anemia, while it is related to patient age, sex and the increase in total erythroid activity.²¹

Hemosiderosis is the most important long-term complication except in those patients protected by ongoing iron loss such as menstruation, pregnancy or hemosiderinuria.^{22,23} Iron accumulation does occur, both from transfusions and from increased intestinal absorption,

even in untransfused patients, and death may result from intractable heart failure secondary to iron overload. Moreover, excessive iron deposition could result in diabetes mellitus, hypogonadotropic hypogonadism and liver cirrhosis.²⁴ For these reasons blood transfusions should be minimized and iron therapy is always contraindicated. No clinical trial to prevent tissue siderosis has been performed, yet the use of prophylactic phlebotomy or the administration of iron chelating agents such as desferoxamine must be considered in the management of CDA II patients.

No satisfactory treatment is available, but partial benefit has been reported with splenectomy in patients with severe anemia because of the increase in red cell life span and abrogation of the need for transfusions. In any case, prior to undertaking surgery, erythrokinetic studies can be performed to quantify the extent of peripheral red blood cell destruction.^{15,17} The spleen-liver ratio determined by ⁵¹Cr surface counting may be a sufficiently valid index to guide a clinical decision about splenectomy.

Most patients do not require regular transfusion therapy; however, blood transfusion may become necessary in certain instances. For example, these patients are susceptible to aplastic crises from infection with parvovirus or other pathogens.²⁵

It appears that patients with CDA II are predisposed to liver cirrhosis irrespective of secondary tissue siderosis. It is likely that the primary gene defect of the glycosylation enzyme affects liver cells. In fact, incompletely processed N-glycans have been detected in serum glycoproteins such as transferrin in CDA II patients. These aberrant glycoproteins may be recognized by oversaturated receptors in hepatocytes and in Kupffer cells. An enormous amount of serum glycoproteins is cleared from circulation into the liver and the reticuloendothelial system, exceeding the clearance capacity. Thus it seems unavoidable that these patients eventually develop cirrhosis. Besides, the abnormal transferrin must be subsequently digested by lysosomal proteases and free iron could be precipitated. This mechanism could be one of the causes of hemosiderosis.²⁶ Diabetes and gallstones may

also be caused by a primary gene defect of the glycosylation enzymes.⁴

Many tissues are apparently unaffected by CDA II. In these cases the possible existence of tissue-specific isozymes should be considered. The persistent erythropoietic stimulation associated with chronic anemia can produce asymptomatic extramedullary hematopoiesis that may mimic tumors of the mediastinum, abdomen and vertebral column. Interestingly, two CDA II siblings improved their hemoglobin levels after they developed large mediastinal masses, which became foci of effective hemopoiesis.²⁷ In these masses an embryonic glycosylation isozyme could be expressed that corrects the anemia.²⁸

When a case of CDA II is diagnosed, it is important to identify siblings who may also be affected. Even if they are asymptomatic, monitoring for iron overload may be beneficial.

Diagnosis

Congenital dyserythropoietic anemia type II is a rare disorder, usually present in childhood, with a clinical picture of chronic anemia of mild to moderate degree, splenomegaly and intermittent or persistent jaundice.² It is transmitted by autosomal recessive inheritance and is characterized by the presence of a large number of multinucleate and binucleate erythroblasts in the bone marrow (Figure 1) and typical morphological abnormalities of the membrane of circulating erythrocytes.²⁹ Bone marrow examination demonstrates five to ten times more erythroblasts than normal (erythroid hyperplasia); early erythroblasts are relatively normal, but about 10%-40% of more mature erythroblasts (late polychromatophilic and oxyphilic erythroblasts) are bi- or multinucleated. Binuclearity is also evident at the basophilic erythroblast level in the marrow of severe cases (*P. Izzo, unpublished results*). Multinuclearity is believed to result from a failure of cell division, while nuclear division occurs normally.

Extensive morphological anomalies have been observed at electron microscopy of CDA II erythroblastic cells, the most peculiar being the presence of the so-called double membrane, i.e. peripheral cisternae running parallel to and

beneath the plasma membrane.^{30,31} The same alteration can also be seen in CDA II erythrocytes; studies with immunogold electron microscopy revealed that the second membrane of the red cell double membrane lacks plasma membrane components. These results suggest that the second membrane may be the extension of the endoplasmic reticulum rather than a duplication of the plasma membrane.³²

CDA II red cells display enhanced agglutination with anti-i antibodies and are lysed when incubated in acidified serum by an alloantibody present in many normal sera. An important test classically utilized for CDA II diagnosis, Ham's test, is based upon this characteristic. In only one other syndrome have red blood cells been shown to be lysed in acidified serum: paroxysmal nocturnal hemoglobinuria (PNH).^{6,33} This syndrome is clearly different from CDA II and there is no difficulty in distinguishing the two. Furthermore, the serum of patients with CDA II always lacks the antibody and thus, unlike the cases with PNH, lysis does not occur in autologous serum.^{2,6,29,33} Compelling evidence indicates that hemolysis of CDA II cells in acidified serum is dependent on the presence of a naturally occurring IgM antibody. This antibody, present in the plasma of approximately 30% of the population, presumably recognizes a particular antigen that arises as a consequence of the abnormal glycosylation of erythrocyte membrane constituents. Recently some experimental data demonstrated that aberrant regulation of complement also contributes to the susceptibility of CDA II erythrocytes to acidified serum lysis.³⁴

Three types of CDA have been described (CDA I-III), as well as a number of variants. All are characterized by variable degrees of anemia with insufficient reticulocytosis and ineffective erythropoiesis. Reticulocyte counts are normal or more often inadequately elevated, ranging from 60 to 180×10⁹/L. CDA II red cells are usually normochromic and normocytic; smears reveal anisocytosis and poikilocytosis, and frequently a discrete number of spherocytes are also present (Figure 1). Shortened half-life of plasma iron clearance, increased plasma iron turnover and, frequently, iron overload are observed. Typically, elevation of serum lactate dehydrogenase and

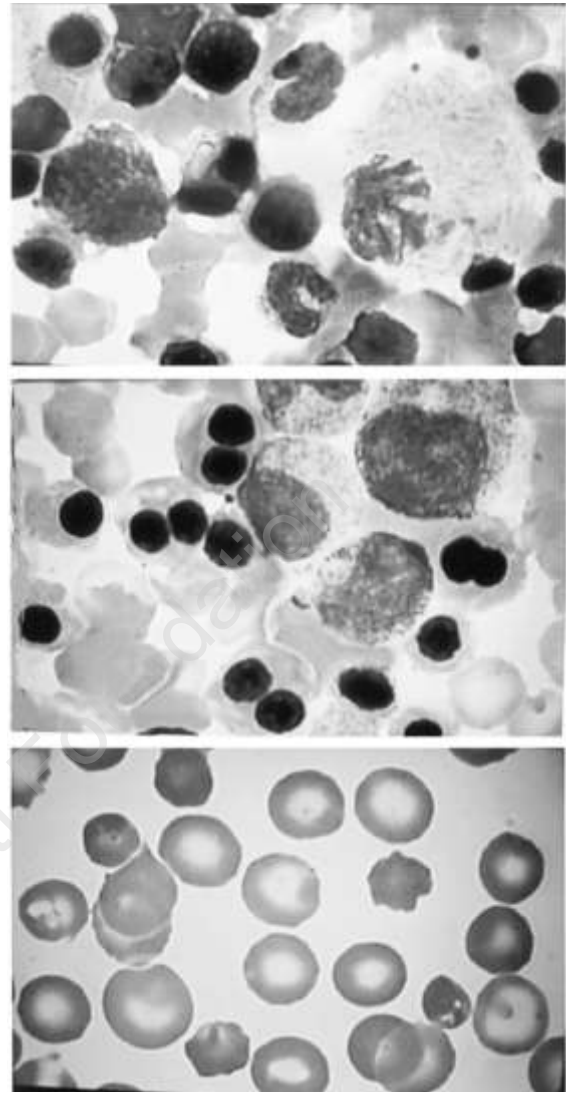


Figure 1. Morphological aspects of peripheral RBC and bone marrow in CDA II. Top: peripheral blood smear of a splenectomized CDA II patient. Presence of a double population: one macrocytic and leptocytic and the second spherocytic. Middle: bone marrow of a CDA II patient. Presence of polychromatophilic and orthochromatic binuclear erythroblasts. Bottom: bone marrow of a CDA II patient. Gaucher-like cells with needle-like cytoplasmic inclusions.

depression of serum haptoglobin are noted. Red cell osmotic fragility appears to be increased in a high number of patients. Differential diagnosis between CDA II and the other CDAs is initially based on red cell size (CDA II erythrocytes are generally normocytic, unlike macrocytic CDA I red cells) and on the inheritance pattern (CDA II shows a recessive pattern of inheritance, unlike

the dominantly inherited CDA III). Once the presence of CDA II is suspected, a definitive diagnosis is made by demonstrating the previously mentioned characteristic light and electron microscopic findings as well as positivity for Ham's test.

Differential diagnosis between CDA II and non dominant hereditary spherocytosis (HS) seems to be, in our experience, an intriguing and little-known problem up to now. Alteration of the normally utilized indices of hemolysis, i.e. indirect bilirubin, hemopessin and haptoglobin, associated with the presence of spherocytes on the blood smear and with the frequent observation of increased osmotic fragility could erroneously direct some CDA II patients toward a diagnosis of HS. This is especially possible in CDA II subjects showing reticulocyte numbers ranging from 120 to $180 \times 10^9/L$. Careful evaluation of the ratio between reticulocyte count and hemoglobin level and often electrophoretic studies of the red cell membrane (see next section) are helpful in arriving at an accurate diagnosis.

New diagnostic criteria

Acrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) reveals numerous biochemical changes in circulating CDA II erythrocytes. Band 3 was repeatedly found to be narrower and migrate faster (Figure 2).^{7,35-38} This aspect was related to insufficient incorporation of lactosaminoglycans, as suggested by red cell labelling experiments with the galactose oxidase/NaB (^{-3}H)₄ method.³⁹ As a matter of fact, the slightly decreased molecular weight of band 3 in CDA II erythrocytes is consistent with decreased glycosylation of this protein. The defect in glycosylation is not an exclusive finding of band 3 but concerns the major membrane glycoproteins of CDA II erythrocytes.³⁸ By contrast, glycolipids appear to be overglycosylated.⁴⁰

The molecular basis for this alteration, which in turn could involve pathologies of enzymes catalyzing essential steps in the biosynthetic pathway leading to membrane protein glycosylation, will be discussed in the next section.

Narrower and more anodic migration at acrylamide gel electrophoresis of a quantitatively normal band 3 represents a typical feature of CDA II erythrocytes. In CDA I for example, band 3 is qualitatively and quantitatively normal, and in HS with reduced band 3 this protein is diminished by 20 to 40% but retains normal width and position at acrylamide gel electrophoresis.⁴¹ Recently, we showed the presence of three minor proteins with apparent molecular weights of 74, 59 and 58 Kd, respectively, in the red cell membrane of CDA II patients. These bands were lacking in normal controls, in CDA II obligate carriers, in patients presenting with CDA I, HS (with or without band 3 deficiency), and in various cases of HE.⁴² Amino acid sequencing revealed that the 74 Kd protein was the glucose-regulated protein referred to as GRP 78; the 59 Kd protein was the protein disulfide isomerase (PDI) and the 58 Kd protein was calreticulin, a high affinity Ca^{2+} binding protein.^{43,44} All these proteins are major constitutive proteins of the endoplasmic reticulum lumen. Their presence strongly supports the hypothesis that the second membrane of the well-known double membrane system present in CDA II erythrocytes must arise from the endoplasmic reticulum. Antibodies against these three proteins are available; detection of GRP 78, calreticulin and

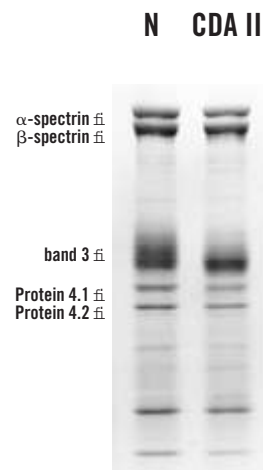


Figure 2. Analysis of RBC membrane proteins by means of SDS-PAGE. The electrophoretic pattern of a CDA II patient shows a band 3 that is narrower and migrates faster.

PDI in CDA II red blood cells by immunofluorescence microscopy techniques has been suggested as a novel, non invasive and straightforward diagnostic tool.⁴²

Biochemical and molecular findings

Carbohydrate structure analysis of membrane and soluble glycoproteins and biochemical data indicate that CDA II is associated with defects in the biosynthesis of complex N-linked oligosaccharides.^{4,5} Erythrocyte membrane glycoproteins of CDA II patients lack poly lactosamine structures. The lack of this large carbohydrate moiety may increase hydrophobicity and induce clustering of membrane glycoproteins.⁴⁵ The altered distribution of membrane glycoproteins may result in membrane abnormalities which are morphologically visible¹⁶ and could interfere with functions required for intracellular transport and compartmentalization, cell division and differentiation.

Defects in the activity of glycosylation enzymes have been implicated in a few very rare human diseases, including CDA II,⁴ I-cell disease⁴⁶ and carbohydrate deficient glycoprotein syndrome type II,^{47,48} indicating that the glycosylation enzymes play a fundamental role in the normal development and function of higher organisms. Consistent with this notion, *in vivo* disruption of the biosynthesis of the N-linked oligosaccharides by gene-targeting^{49,50} causes arrest of the development of mouse embryos at an early post-implantation stage. Moreover, *in vivo* alteration of the pattern of terminal glycosylation by ectopic expression of a viral sialidase blocks the development of transgenic mouse embryos at the very early stage of morula compaction or interferes with tissue organization,⁵¹ further supporting the model that cell surface carbohydrates play a key role in the transfer of positional information during mammalian development.

Biosynthesis of complex N-linked oligosaccharides

The oligosaccharide moieties of glycoproteins are usually bonded to the polypeptide backbone through a glycosidic linkage to the amino group of asparagine (Asn) residues and/or to the

hydroxyl group of serine (Ser) and threonine (Thr) residues. These structures are referred to as N-linked or O-linked oligosaccharides.

The N-linked oligosaccharides of membrane or soluble glycoproteins can be grouped into three major types: high mannose, hybrid, and complex oligosaccharides. Typical examples of these oligosaccharides are illustrated in Figure 3. The three types all share the structure $\text{Man}\alpha 1,3(\text{Man}\alpha 1,6)\text{Man}\beta 1,4\text{GlcNac}\beta 1-4\text{GlcNac}$, termed *the core pentasaccharide* (indicated by the dashed box in Figure 3). As suggested by the presence of the common core structure, all three types of N-linked oligosaccharides derive from the same precursor, which is differently processed and elongated.

The synthesis of N-linked oligosaccharides may be divided in two distinct stages. Stage 1 involves the synthesis of a large branched oligosaccharide linked to the polyisoprenoid alcohol dolichol.⁵² The lipid-linked oligosaccharide is transferred *en bloc* from the dolichol pyrophosphate donor to asparagine residues while the polypeptide chains are being translocated across the membrane of the rough endoplasmic reticulum (RER).⁵³

Stage 2 of N-linked oligosaccharide synthesis occurs as the glycoprotein is transported from the RER through the Golgi membranes to the cell surface, where it is either secreted or integrated into the plasma membrane. During transport through the RER and Golgi membranes the precursor oligosaccharide is processed by specific glucosidases and mannosidases. Removal of the glucoses seems to act as a sorting signal for the transport of properly folded glycoproteins from the ER to the Golgi membranes. Incompletely or misfolded glycoproteins are recognized and reglucosylated by a specific UDP-glucose: glycoprotein glucosyltransferase.⁵⁴ Current evidence indicates that monoglucosylated glycoproteins are retained in the ER by interaction with calnexin^{55,56} and calreticulin,^{57, 58} two ER proteins which share blocks of sequence similarity and lectin-like properties. As a result of the removal of four $\alpha 2$ -linked mannose residues, the $\alpha 3$ -mannose of the core becomes exposed. Biochemical data indicate that this conversion can be catalyzed by several $\alpha 1,2$ -

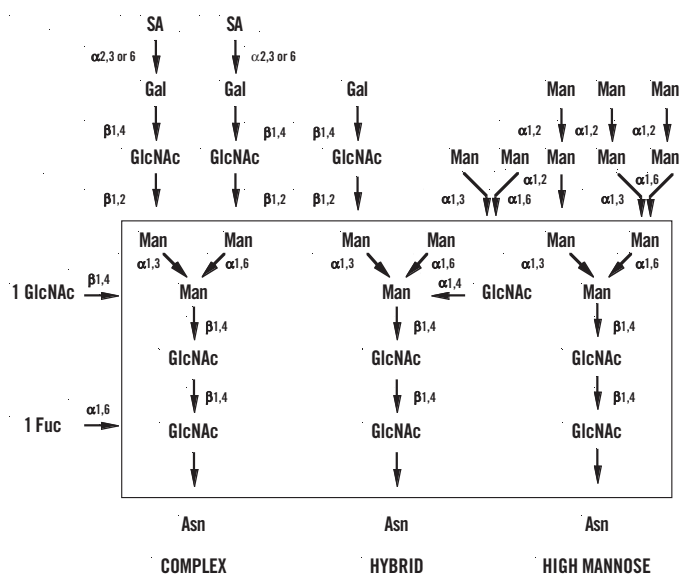


Figure 3. Structures of N-linked oligosaccharides. The dashed box encloses the Man₃GlcNAc₂ core penta-saccharide. High mannose-type oligosaccharides have only additional mannose residues linked to both α -mannoses of the core. Complex oligosaccharides may contain two (as shown in the figure), three, four or five antennae composed of fucosylated and sialylated lactosaminyl (Galb1,4-GlcNAcb1-) structures branching from the two α -mannoses of the core. In hybrid-type N-linked oligosaccharides, mannose residues are linked to the α 6-mannose of the core, whereas the α 3-mannose is elongated with a lactosaminyl (Galb1,4-GlcNAcb1-) structure. N-linked oligosaccharides carrying a GlcNAc residue in β 1,4 linkage to the β -mannose of the core are referred to as bisected structures. Most hybrid-type oligosaccharides are isolated as bisected structures.

mannosidases that show distinct properties, subcellular localization, and specificities. However, the role of distinct α 1,2-mannosidases in the processing steps of the biosynthetic pathway of N-linked oligosaccharides remains to be elucidated.^{59,60}

The processing and elongation reactions occurring in late stage 2 of the biosynthetic pathway are summarized in Figure 4. Depending upon the extent to which the mannosyl residues are removed, the precursor oligosaccharide can be elongated in distinct compartments of the Golgi membranes by the sequential action of specific glycosyltransferases to become terminally glycosylated. The glycosyltransferases catalyze the transfer of monosaccharide residues from an activated donor substrate, usually a nucleotide-sugar, to the non-reducing termini of the acceptor oligosaccharide. These membrane-bound enzymes are typically grouped into families based on the type of monosaccharide they transfer, and all share a similar domain architecture and type II transmembrane topology.⁶¹ The branching pattern of complex N-linked oligosaccharides is specified by the action of distinct N-acetylglucosaminyltransferases (GnT), each one of which catalyzes the transfer in a specific glycosidic linkage of a GlcNAc residue from the donor substrate UDP-GlcNAc to a mannose residue of the trimannosyl core.⁵³ The processing interme-

diate Man₅GlcNAc₂ (indicated by an asterisk in Figure 4) constitutes the substrate for GnT I, which catalyzes the transfer in a β 1,2 linkage of a GlcNAc residue from the donor substrate to the α 3-mannose of the core. In mutant cells lacking GnT I activity, further processing and elongation of the oligosaccharide is halted.⁶² Following the addition of a GlcNAc residue to the α 3-mannose, the oligosaccharide becomes a substrate for α -mannosidase II (α -Man II), GnT III, GnT IV, and β 1,4-galactosyltransferase.⁶³ The action of α -Man II generates the acceptor substrate for GnT II, which catalyzes the transfer of a GlcNAc residue in a β 1,2 linkage from the donor substrate to the α 6-mannose of the core.⁶⁴ This reaction initiates the formation of biantennal N-linked oligosaccharides and constitutes an essential step in the biosynthetic pathway leading from hybrid-type to complex N-linked oligosaccharides. The formation of triantennal N-linked oligosaccharides is initiated by the action of GnT IV, which catalyzes the transfer of a second GlcNAc residue in a β 1,4 linkage to the α 3-mannose of the core. The action of GnT V and GnT VI initiates formation of the antennae linked β 1,6 and β 1,4, respectively, to the α 6-mannose of the core. *In vitro* studies indicate that the activities of GnT V and GnT VI require the prior action of GnT II.^{53,63}

The antennae branching from the α -man-

The level of poly lactosamine structures in complex N-linked oligosaccharides appears to be controlled by the expression of GnT V.⁷³⁻⁷⁵ The patterns of terminal glycosylation of complex N-linked oligosaccharides are determined by the action of distinct fucosyl- and sialyl-transferases. Members of these two glycosyl-transferase families catalyze the transfer in a variety of glycosidic linkages of a fucose or sialic acid residue from their donor substrate (GDP-fucose or CMP-sialic acid) to lactosaminyl structures. The differential modification of these structures generates a vast array of complex N-linked oligosaccharides, many of which show a cell type- or tissue-specific pattern of expression and are developmentally regulated.⁷²

Altered N-linked carbohydrate biosynthesis in HEMPAS

Band 3 poly lactosaminoglycans isolated from normal adult human erythrocytes are composed of a biantennal tri-mannosyl core structure elongated with branched poly lactosaminyl [3Gal β 1,4GlcNAc β 1,-]n repeats in the antennae linked β 1,2 to the α -mannoses of the core^{67,68,70} (Figure 5, structure a). Fast atom bombardment mass spectrometry (FAB-MS) analysis of band 3 poly lactosaminoglycans isolated from the erythrocyte membranes of several CDA II patients revealed in one case the presence of incompletely processed tri- and penta-mannosyl hybrid-type structures (Figure 5, structures b and c) and in three cases the presence of these abnormal structures in addition to complex N-linked oligosaccharides (Figure 5, structure e).⁷⁶ Structural analysis of erythrocyte membrane glycopeptides from another CDA II patient showed the accumulation of penta-mannosyl hybrid-type oligosaccharides (Figure 5, structure b), in addition to high-mannose (Figure 5, structure d) and complex oligosaccharides (Figure 5, structure d).⁴ Finally, in a patient affected by a variant form of CDA II, FAB-MS analysis of erythrocyte membrane glycopeptides revealed the presence of high mannose-type oligosaccharides, with the penta-mannosyl moiety (Figure 5, structure d) being the most abundant structure.⁷⁷

The characteristic presence in CDA II patients

of tri-mannosyl and penta-mannosyl hybrid-type oligosaccharides (Figure 5, structures b and c) has led to the suggestion that the glycosylation defect is the result of a deficiency of either GnT II or α -Man II activity. A deficiency of either one of these enzymes would partially preclude initiation of the antenna linked β 1,2 to the α 6-mannose of the core, causing accumulation of incompletely processed hybrid-type oligosaccharides and reducing the overall synthesis of complex oligosaccharides elongated with poly lactosamines.

Enzyme deficiencies that could account at least in part for these structural findings have been demonstrated by *in vitro* enzymatic assays in only a few patients. In many cases of CDA II, the enzyme deficiency underlying the biochemical defect has not been identified. A specific marked reduction of GnT II activity, although to different extents, was observed in the microsomal fraction of peripheral blood lymphocytes (PBL) isolated from two CDA II patients. In these two patients an analysis of the carbohydrate moieties of erythrocyte membrane band 3 and serum transferrin revealed the presence of tri- and penta-mannosyl hybrid-type N-linked oligosaccharides.^{76,78} Conversely, a deficiency of α -Man II activity was noted in the microsomal fraction of PBL isolated from another CDA II patient, as suggested by FAB-MS analysis of erythrocyte membrane glycopeptides showing an accumulation of penta-mannosyl hybrid-type structures.⁴ In four patients, the level of either GnT II or α -Man II activity relative to the degree of GnT I activity was reported to be almost normal,⁴⁸ suggesting that the lack of band 3 poly lactosamine structures in these subjects could be the result of a deficiency of other enzyme(s) involved in the pathway. Alternatively, the glycosylation defect could be due to either an imbalance or an overall reduction in the activities of GnT I, GnT II and α -Man II. These enzymes all catalyze key steps of the pathway leading from hybrid-type to complex oligosaccharides, and their expression is coordinately regulated within the cell to ensure the proper branching pattern of N-linked oligosaccharides. The interdigitations between the activities of GnT I, GnT II and α -Man II could amplify the effect on the routing of the

pathway of even a slight imbalance or reduction in their activities. The physiological substrate for GnT II is generated by the action of α -Man II, which is dependent on the prior action of GnT I.

Cloning and chromosomal mapping of the GnT II and α -Man II genes

The genetic mutations responsible for the glycosylation defect in CDA II have not yet been identified. An analysis of carbohydrate structures and biochemical data indicate that the activity of either GnT II or α -Man II is reduced in different families, suggesting that the disease is genetically heterogeneous.

Although a deficiency of either GnT II or α -Man II could determine a very similar phenotype, definition of the genetic basis of CDA II must await the results of genetic linkage analysis and elucidation of the mechanisms that regulate the expression of the GnT II and α -Man II genes. In principle, several types of mutations could cause a deficiency of GnT II and/or α -Man II in CDA II patients. First, structural mutations of the GnT II and α -Man II genes could alter the functional properties and specificities or modify the subcellular localization of the encoded enzymes. Second, mutations in cis-acting regulatory regions may disrupt transcriptional or post transcriptional control of the expression of the GnT II and α -Man II genes. Molecular cloning of the GnT II⁷⁹⁻⁸¹ and α -Man II^{82,83} DNA sequences has allowed us to initiate a direct investigation of the genetic mutations underlying the glycosylation defect in CDA II patients.

The DNA sequences coding for GnT II are organized in a single exon uninterrupted by introns in both the rat⁷⁹ and human⁸¹ genome. The human gene for GnT II has been mapped to the long arm of chromosome 14 (14q21) by fluorescent *in situ* hybridization (Figure 6).⁸¹ Multiple polyadenylation signals with the consensus sequence AATAAA are found in the 3' untranslated region of either the human (Figure 7) or the rat GnT II gene.^{79,80} The 5' untranslated region (UTR) of the GnT II gene is GC rich (76%) and contains multiple sequence motifs which could be recognized by tissue-specific (Sp1 and AP2) transcription factors. The rat

and human GnT II DNA sequences are highly similar. The sequence similarity between rat and human GnT II DNA is 89% in the coding region and 86% in the 3' UTR.⁸⁰ The conserved sequence similarity of the GnT II 3' UTR across mammalian species suggests a conserved function of this region.

Ribonuclease protection assays and Northern blot analysis indicate that transcription of the human GnT II gene starts ~450 bp upstream from the translation initiation codon, generating two mature transcripts of 2.8 and 2.0 kb. The two GnT II transcripts differ at their 3' end, terminating 16 nucleotides downstream from two distinct polyadenylation signals, as indicated by sequence analysis of human liver GnT II cDNA clones (Figure 7; D'Agostaro *et al.*, unpublished data). The total and relative abundance of the 2.0 and 2.8 kb GnT II transcripts varies in different human organs and tissues (ref. #81 and D'Agostaro *et al.*, unpublished data). The variation in the overall level of GnT

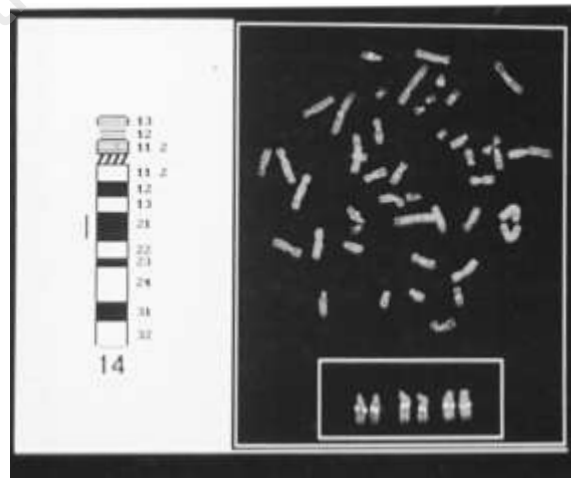


Figure 6. Mapping of the human GnTII gene by fluorescent *in situ* hybridization. Human metaphase spreads were obtained from PHA-stimulated peripheral blood lymphocytes isolated from a healthy adult donor. Chromosome preparations were hybridized *in situ* with DNA of phage clone HG30 labeled with biotin by nick translation. Biotin-labeled DNA was detected with FITC-conjugated avidin. Chromosomes were identified by simultaneous DAPI staining, which produces a Q-banding pattern. Digital images were obtained using a Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics). FITC and DAPI fluorescence were detected using Pinkel #1 specific filter set combinations (Chroma Technonology), captured without any image shifting, and recovered separately as gray scale images. Pseudocoloring and merging of images were performed using Adobe Photoshop software.

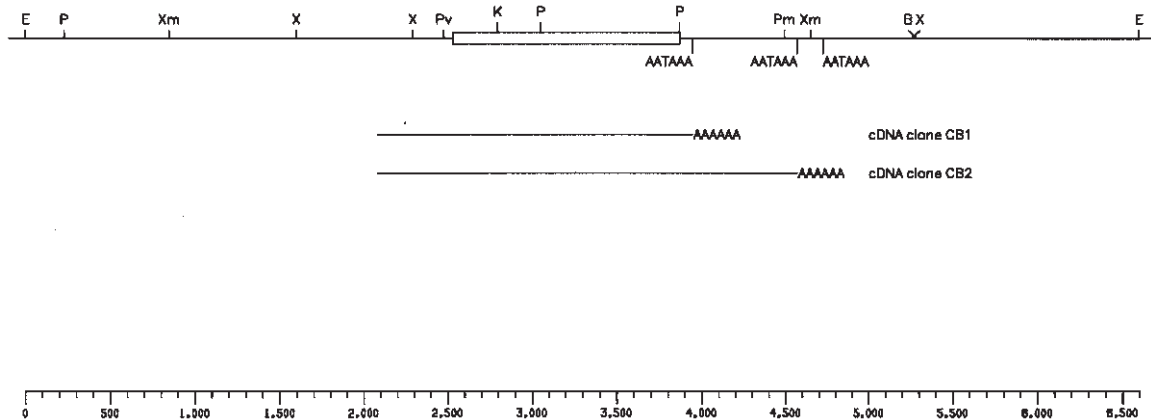


Figure 7. Restriction Map of the GnT II gene. Partial restriction map of the 6.6 Kbp EcoRI fragment of γ -phage clone HG30 containing the human GnT II gene. The sequence corresponding to the coding region is indicated by the open box. The 2.0 and the 2.8 Kb transcripts of the GnT II gene are illustrated below the restriction map. Only restriction sites relevant to the sequence analysis are indicated: E, EcoRI; K, KpnI; P, PstI; Pm, PmeI; X, XhoI; XmnI; Pv, Pvu II.

II mRNAs could be the result of transcriptional control mediated by tissue-specific transcription factors that specifically recognize and bind to sequence motifs present in the 5' UTR of the human GnT II gene.

The human α -Man II gene displays complex exon-intron organization (K. Moremen, personal communication) and has been mapped to chromosome 5 (5q2.1-2.2) by screening a panel of somatic cell hybrids⁸² and by radioactive *in situ* hybridization.⁸³ Northern blot analysis has shown that transcription of the human α -Man II gene generates multiple mRNA species. Two α -Man II transcripts of similar size (~7.6 kb) were detected by Fukuda *et al.*⁴ in polyA⁺-enriched RNA preparations of EVB-transformed cell lines derived from the PBL of three healthy adult donors. We found two major α -Man II transcripts of 7.2 and 7.0 kb in length and two minor α -Man II transcripts 4.4 and 4.2 kb in length (Figure 8) in polyA⁺-enriched RNA preparations of EVB-transformed cell lines derived from the PBL of several normal adult donors, using the same fragment of human α -Man II cDNA as hybridization probe. Four α -Man II transcripts of 7.5, 6.1, 4.3, and 4.0 kb have also been detected in various mouse organs.⁸² The origin and functional significance of the multiple α -Man II transcripts remains to be elucidated. These tran-

scripts could be generated by transcription events starting at distinct initiation sites, by differential splicing of a primary transcript, or by transcription of a similar but distinct gene(s) or pseudo α -Man II gene. In the absence of information about the transcriptional unit encoded by the human α -Man II gene, it is intriguing to speculate that all or some of the α -Man II transcripts could represent the products of differential utilization of distinct polyadenylation sig-

DS AV TO DM

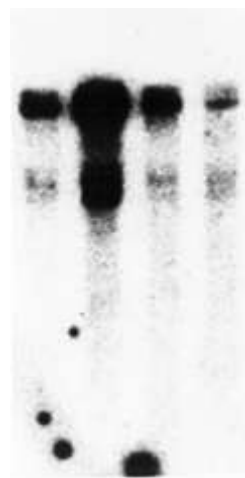


Figure 8. Northern blot analysis. Five μ g aliquots of poly(A)⁺-enriched RNA from lymphoblastoid cells derived from healthy adult donors by transformation with EBV were fractionated by electrophoresis through denaturing 0.9% agarose gel.

A fragment of human α -Man II cDNA (Gen Bank accession number U31520), extending from nucleotide 1 to 1819, was used as a probe. The migration of the two ribosomal RNAs is indicated by the arrows on the left side. RNAs of a defined length were used as mol-

nals during maturation of the primary transcript of the α -Man II gene. As a matter of fact, three types of α -Man II cDNA clones terminating within 20 nucleotides downstream from three distinct polyadenylation signals have been isolated from a mouse cDNA library.⁸²

Defective expression of GnT II and α -Man II genes in CDA II: a hypothesis

In order to establish whether regulatory or structural mutations of the genes encoding the glycosylation enzymes occur in CDA II patients, polyA+-enriched RNA preparations from EBV-transformed cell lines derived from affected and healthy individuals were subjected to Northern blot analysis. A marked reduction in the two α -Man II transcripts, migrating as a close doublet of ~7.6 kb, was observed in one patient,⁴ in accordance with carbohydrate structure analysis and biochemical data demonstrating a severe deficiency of α -Man II activity in this patient.

Molecular cloning of the GnT II gene has enabled us to extend molecular analysis to CDA II patients showing a defect of GnT II activity and to monitor the transcription products of both the GnT II and α -Man II genes. RNA preparations isolated from cell lines of four patients and eight related and unrelated healthy individuals were subjected to Northern blot analysis using GnT II and α -Man II cDNA fragments as hybridization probes. In all patients the levels of the 2.0 kb GnT II transcript and of the α -Man II transcripts of 7.0, 4.4 and 4.2 kb were consistently and significantly reduced. Conversely, the amount of the 2.8 kb GnT II transcript and of the 7.2 kb α -Man II transcript showed no significant variation between CDA II subjects and unaffected individuals. These results may suggest that the glycosylation defect observed in HEMPAS patients is caused by regulatory mutation(s) disrupting the mechanisms that control the maturation of the primary transcripts of both the GnT II and α -Man II genes. Since the 2.8 and 2.0 kb GnT II transcripts are presumably generated by utilization of distinct polyadenylation signals during maturation of the primary transcript of the GnT II gene, it is assumed that this process also takes place during maturation of the primary α -Man II transcript,

as suggested by sequence analysis of murine cDNA clones for α -Man II. The hypothesis can be extended to suggest that the genetic mutation(s) occurring in HEMPAS patients are not linked to either the GnT II or α -Man II gene, and may alter the properties of a tissue-specific cellular factor which specifies the utilization of distinct polyadenylation signals within the 3'UTR of mRNAs to achieve a unique expression profile of mature transcripts.

According to our current knowledge of the function of the 3' UTR of eukaryotic mRNAs^{84,85} the stability and/or translational proficiency of individual mature transcripts for GnT II and α -Man II could, in turn, be under controls mediated by 3'UTR-sequences acting in concert with tissue-specific regulatory factors.

The expression of other glycosylation enzymes could also be regulated by mechanisms of post-transcriptional and translational control and could be defective in CDA II patients.

The GnT II and α -Man II genes are ubiquitously expressed, yet many tissues and organs of CDA II patients are apparently unaffected by the glycosylation defect. Mutation(s) disrupting tissue-specific regulatory mechanisms of expression of glycosylation enzymes could provide an explanation for the appearance in these patients of severe pathologic effects, mainly in hepatocytes and cells of the erythroid lineage. Structural characterization of the GnT II and α -Man II genes in CDA II patients and genetic linkage studies are in progress. The knowledge to be gained from a study of the mutations that cause glycosylation defects in these patients should aid in elucidating the role of defined carbohydrate structures in the differentiation and maturation of specific cell lineages. Moreover, these studies will give us insight into the link between a primary gene defect and phenotype in different cell types, and may aid in clarifying the molecular mechanisms by which differentiated phenotypes are induced during development or altered in human pathologies.

Conclusions

CDA II is a hereditary anemia whose prevalence is unknown. One reason for this is certain-

ly due to the heterogeneity of the clinical picture; in fact, it is possible that in the majority of cases this condition could be symptomless and could lead to siderosis in adulthood. Also in this respect, the clinical course of CDAII resembles that of thalassemia intermedia.⁸⁶

For many years this condition was diagnosed by means of bone marrow biopsy and observation of characteristics of the erythroblasts. It is evident that this diagnostic procedure was reserved for the most severe clinical forms.

The finding of peculiar characteristics at the red blood cell membrane level and the possibility of revealing them by means of SDS-PAGE led to a painless diagnosis for patients. Unfortunately, SDS-PAGE is still not a widespread diagnostic tool. More recently, the availability of antibodies against calreticulin and the demonstration that the presence of this protein on the surface of the red cell is a new characteristic of CDA II have also made diagnosis possible in transfused patients in a way that is non invasive and very easy to perform.

The cloning of two candidate genes opens new perspectives at the diagnostic level: the possibility of diagnosing the heterozygous state. It will be possible in the near future to characterize the molecular defects in CDA II patients; in this way it will be clear whether the various clinical pictures are due to different molecular defects or to a characteristic defect worsened by different factors. This knowledge will lead to new insights into the field of relationships between differentiation and proliferation of erythroid progenitors.

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