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Diseases of aberrant glycosylation

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Recently a defective glycosylation of glycoconjugates has been implicated in the pathogenesis of a number of heritable or acquired diseases of humans. Herein I discuss them under the name of diseases of aberrant glycosylation. These are: congenital dyserythropoietic anemia type II, carbohydrate-deficient glycoprotein syndrome, I-cell disease, galactosemia in subjects on galactose-free diet, variants of leukocyte adhesion deficiency, and of Ehlers-Danlos syndrome, paroxysmal nocturnal hemoglobinuria, and Tn syndrome. Regarding the present views on the function of glycoconjugates it is probably significant that in most instances defective or missing glycoproteins (or proteoglycans,) but not glycosphingolipids, are probably involved in the pathogenesis of these diseases.

Lysosomal storage diseases, most of which are caused by impaired catabolism of glycoconjugates, have been known for a long time. Recently biosynthetic rather than catabolic defects of glycoconjugate metabolism have been implicated in the pathogenesis of several diseases. I propose to bring them together under the name of diseases of aberrant glycosylation. These are: congenital dyserythropoietic anemia type II, carbohydrate-deficient glycoprotein syndrome, I-cell disease, galactosemia in subjects on galactose-free diet, variants of leukocyte adhesion deficiency and of Ehlers-Danlos syndrome, paroxysmal nocturnal hemoglobinuria, and Tn syndrome. It should be emphasized that by diseases of aberrant glycosylation I mean only those conditions in which aberrant glycosylation is a probable cause of the disease.

Congenital dyserythropoietic anemia type II. The illness has been classified into three types I, II and III [1]. Documented aberrant glycosylation occurs in type II. Main laboratory findings are anemia, multinuclearity of late erythroblasts, and ineffective erythropoiesis [2]. Multinucleated cells are presumably destroyed within the bone marrow and thus ac-

[1] Abbreviations: CDA II, congenital dyserythropoietic anemia type II; CDG syndrome, carbohydrate-deficient glycoprotein syndrome; PNH, paroxysmal nocturnal hemoglobinuria; Dol, dolichol; PI, phosphatidylinositol, GPI, glycosylphosphatidylinositol. Enzymes: Gal4transferase, glycoprotein 4-β-galactosyl--transferase (EC 2.4.1.38); Gal3transferase, glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase (EC 2.4.1.122); Galtransferase I, xylosylprotein 4-β-galactosyltransferase (galactosyltransferase I), (EC 2.4.1.133); Gntransferase II, *N*-acetylglucosaminyltransferase II (EC 2.4.1.143); GlcNAc3transferase, *N*-acetyllactosaminide β-1,3-*N*-acetylglucosaminyltransferase (EC 2.4.1.149); IGntransferase, β-6-*N*-acetylglucosaminyltransferase, UDP-GlcNAc:GlcNAcβ1-3Gal(-R) β1-6(GlcNAc to Gal).

count for ineffective erythropoiesis. CDA II[1] erythrocytes exhibit a new antigen recognized by a hemolytic antibody present in sera of about 30% of the population. Tests for the detection of the antibody are performed in acidified serum and hence another name for the disease is HEMPAS (Hereditary Erythroblast Multinuclearity with Positive Acidified Serum test [3]). Another serological abnormality of CDA II erythrocytes is a simultaneous expression of "I" and "i" antigens. In healthy adult subjects "I" but not "i" antigen is present in erythrocytes whereas in cord erythrocytes the expression is reversed [4].

Aberrant glycosylation in CDA II is manifested in glycosphingolipids [5] and glycoproteins [6] of the erythrocyte membrane. Glycoproteins are underglycosylated whereas ceramides overglycosylated both with polyglycosyl chains [7, 8]. These chains, initially discovered and characterised in erythrocytes as carbohydrate portions of complex glycosphingolipids with ABH and "Ii" antigenic activities (polyglycosylceramides), [9–11], are now commonly designated lactosaminoglycans. The repeating core structure of lactosaminoglycans from erythrocytes of adults is shown in Fig. 1.

In cord erythrocytes and in those of a rare heritable "i" phenotype the amount of branches originating from carbon atom 6 of galactose residues is much reduced and the chains are usually linear [12]. Highly branched lactosaminoglycans bind anti-I antibodies whereas linear lactosaminoglycans bind those with anti-i specificity (for refs. see [13]). Thus, the "I" type develops from "i" through branching of lactosaminoglycans [10, 14, 15]. The branching enzyme is β-6-*N*-acetylglucosaminyltransferase (IGntransferase, for refs. see [16]).

Glycosphingolipids bearing lactosaminoglycans contain on the average 3 repeating units, i.e. about 22–24 sugar residues. Lactosaminoglycans are also carbohydrate components of band 3 and band 4.5 erythrocyte glycoproteins (for refs. see [17]). Those of band 3 protein (anion transporter of erythrocytes) are linked as a biantennary structure to a typical mannose core of complex type glycoproteins (see Fig. 2). Both antennae contain several repeating units

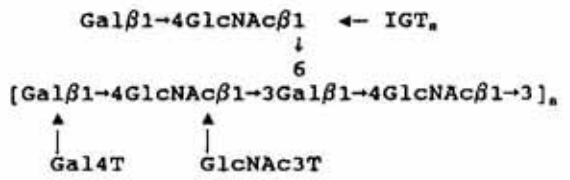

Fig. 1. Structure and biosynthesis of the repeating unit of erythrocyte lactosaminoglycans [9, 11, 18]. Terminal β-Gal residues are, depending on ABO genotype, substituted with carbohydrate structures that define A, B, H blood group specificity. Some side chains of the core structure may be longer than one lactosaminyl unit [9, 11]. Large arrows indicate transfer reactions catalysed by respective transferases.

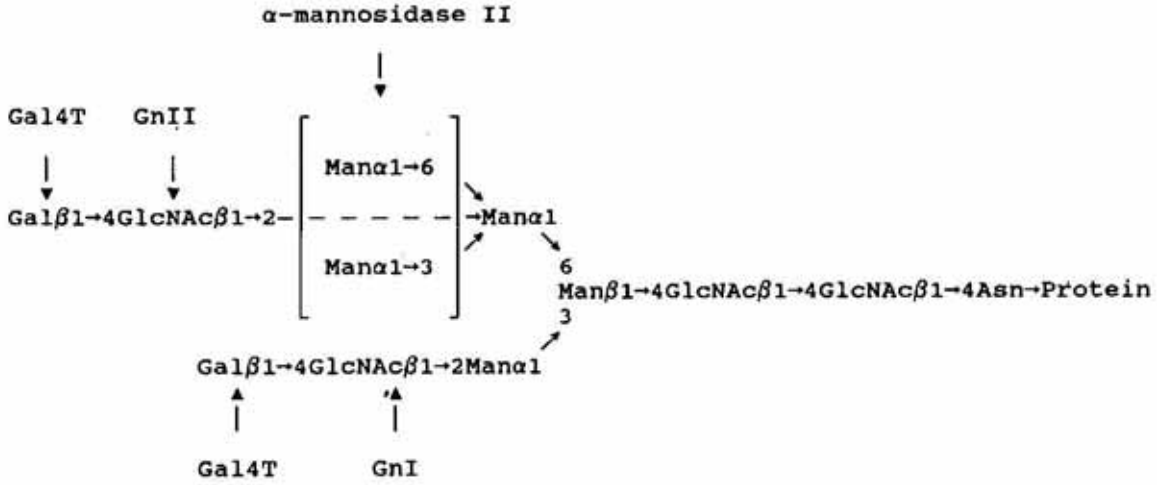

Fig. 2. Structure and biosynthesis of the outer portion of the core of complex glycoproteins. Transfer of GlcNAc to Manα1→3Man arm of the core by Gntransferase I (GnI) (β-1,2-*N*-acetylglucosaminyltransferase, EC 2.4.1.101) is a signal for α-mannosidase II to cleave two terminal mannose residues (in brackets) from the Manα1→6Man arm; subsequently, this arm is substituted with GlcNAc residue through the action of Gntransferase II (GnII). Both arms are now ready for the biosynthesis of lactosaminoglycan antennae through the action of Gal4transferase (Gal4T), GlcNAc3transferase (GlcNAc3T) and IGntransferase (IGnT). Transfer reactions are marked as in Fig. 1.

of lactosaminoglycans but that on the Manα1-6Manβ1-4GlcNAc arm of the core is longer [18]. Underglycosylation of band 3 protein in CDA II erythrocytes is brought about by a deficiency of one of three enzymes: Gntransferase II [19], or α-mannosidase II (3.2.1.114) [20], or membrane-bound Gal4transferase [21]. As a result of the deficiency of either Gntransferase II or α-mannosidase II the first GlcNAc residue cannot be attached to the Manα1→6Manβ1→4GlcNAc arm of the core and consequently of the whole lactosaminoglycan antenna (see Fig. 2). Under these conditions enzymes that normally biosynthesize lactosaminoglycans (Gal4transferase, GlcNAc3transferase, and IGntransferase, see Fig. 1) are presumably idle and overglycosylate ceramides. Polyglycosylceramides from CDA II erythrocytes though much increased in amount are nevertheless less branched and, like the erythrocytes they were isolated from, exhibit both "I" and "i" antigenic activity [8]. Deficiency of Gal4transferase abolishes the biosynthesis of lactosaminoglycans themselves [21]. Thus lactosaminoglycans are missing from both glycoproteins and glycolipids. It should be pointed out, however, that the structure of a glycopeptide isolated from pronase digests of erythrocyte membranes from a patient with putative Gal4transferase deficiency does not meet expectation. The glycopeptide contains GlcNAc-unsubstituted terminal mannose residues on both arms of the mannose core, including two mannose residues on the upper arm that should have been cleaved by mannosidase II. In contrast, structures of glycopeptides from erythrocyte membranes of patients with Gntransferase II and mannosidase II deficiencies fully support the proposed enzyme defects [19, 20]. Deficiency of α-mannosidase II seems to be generalized and serum glycoproteins are also affected [22].

Band 3 protein accounts for about 30% of total membrane proteins. When underglycosylated with lactosaminoglycans it is poorly soluble in water [22, 23]. Electron microscopy reveals that in CDA II band 3 protein is unevenly distributed in erythrocyte membranes and forms clusters. It is likely that in late erythroblasts — when biosynthesis of band 3 is accelerated [17] — the underglycosylated band 3 glycoprotein may precipitate in the Golgi apparatus and in the plasma membrane, thus deranging cell division but not necessarily nuclear division [23]. Indeed Dr. M.N. Fukuda has recently produced evidence that normal human erythroblasts cultured in the presence of swainsonine, a mannosidase II inhibitor, became multinucleated [23]. This elegant experiment has proved beyond doubt that CDA II is a disease of aberrant glycosylation. The nature of HEMPAS antigen in CDA II erythrocytes has not been so far resolved.

Carbohydrate-deficient glycoprotein syndrome. This heritable, multisystem disease was discovered in 1980 (for refs. see [24, 25]). It is usually diagnosed at infancy or early childhood. Clinical symptoms are among others: mental and motor retardation, cerebellar ataxia, liver insufficiency, and some anatomical abnormalities. In adult patients premature ageing is evident. The disease has been classified into three types [26].

Aberrant glycosylation is seen in serum proteins. In fact, the diagnosis of CDG is confirmed by electrofocusing of serum transferrin. In CDG patients this protein exhibits substantial microheterogeneity with several isoforms showing pI above 5.7 [27]. Other serum glycoproteins also show a cathodal shift when examined by isoelectric focusing or high resolution two-dimensional polyacrylamide gel electrophoresis [28]. Evidence was presented that the abnormal isoforms of transferrin resulted from deficient glycosylation with sialic acid, galactose and *N*-acetylglucosamine of the non-reducing termini of two N-glycans that are present in the glycoprotein. Mannose residues in carbohydrate-deficient transferrin were at the normal concentration [27] suggesting that deficient glycosylation may occur in the Golgi apparatus. Recently this evidence has been challenged by demonstration that carbohydrate-deficient transferrin lacks entire glycans: isoforms were described which contained two glycans, one glycan and no glycans [29, 30]. These findings imply that the biochemical defect in CDG syndrome occurs at a very early stage of N-glycan biosynthesis, presumably at the lipid intermediate level or translocation across endothelial membrane. The contents of dolichyl phosphate, and UDP-GlcNAc:dolichyl phosphate Glc-NAc1-phosphate transferase [EC 2.7.8.15] in CDG fibroblasts were, however, normal [31]. Also normal was the activity of N-oligosaccharyltransferase [EC 2.4.1.119] assayed with Dol-P-P-$(GlcNAc)_2$ as

carbohydrate donor and hexapeptide as acceptor substrate [32]. Thus, the pathogenesis of CDG syndrome remains to be elucidated. It should be emphasized that, whereas some functional disturbances in CDG syndrome like those involving the coagulation or complement systems [25] may be explained by deficient glycosylation of the respective factors, some other biochemical findings are explained less readily. Hypoalbuminaemia in type I patients and increased serum growth hormone and insulin levels belong to the latter category [25]. The endocrinological abnormalities may result from deficient glycosylation of hormonal receptors. Interestingly, no evidence of aberrant glycosylation was found in one 19 week-old CDG fetus [33]. Carbohydrate-deficient isoforms of transferrin, α_1-antitrypsin, and β-hexosaminidase appeared only in the 2nd and 3rd week of post-natal life though clinical symptoms of the disease were clearly present already at birth.

I-cell disease. The illness is usually classified as a storage disease [34] but in fact it is caused by the absence of mannose 6-phosphate recognition marker on lysosomal enzymes [34, 35]. Enzymes deficient in mannose 6-phosphate are not recognized by the specific receptor in transport vesicles [36] and are exported rather than directed to their proper lysosomal destination. The deficient enzyme is GlcNAc-phosphotransferase (EC 2.7.8.17) that links GlcNAc-1-P (from UDP-GlcNAc) to carbon atom 6 of mannose. Subsequently GlcNAc residues are cleaved.

Galactosemia. Classic galactosemia is an inborn error of metabolism secondary to the deficiency of galactose-1-phosphate uridyltransferase (EC 2.7.7.9). This causes accumulation in the body of galactose and galactose metabolites (especially of galactose-1-phosphate) that are toxic at high concentrations (for refs. see [37]). The disease may be fatal unless infants are put on a galactose-free diet. Recent evidence suggests, however, that galactose-free diet even commenced right after birth does not prevent gradual decline of intelligence and neurological complications [38–40]. The symptoms may be at least partly due to a defective galactosylation of glycoconjugates caused by a low concentration of UDP-Gal in the cells of patients with galactosemia [41, 42]. Recently, deficient galactosylation of glycoconjugates in galactosemic patients involving both glycoproteins [43, 44] and glycosphingolipids [44, 45] has been reported.

Leukocyte adhesion deficiency. Only two such cases have been so far reported [46]. They were unrelated boys with mental retardation, short stature, and a distinctive facial appearance. Both suffered from recurrent bacterial infections. The boys lacked H and Lewis blood group antigens in secretions and red cells. Neutrophils exhibited a decreased motility and were unable to adhere to endothelial cells before and after induction of the expression of E-selectin. Absence of the ligand (sialyl-Lewis X) for E and P selectins in neutrophils was reported. Other variants of leukocyte adhesion deficiency are known but presumably without the involvement of carbohydrates.

Ehlers-Danlos syndrome and other diseases of connective tissue. Ehlers-Danlos syndrome describes a heritable, heterogeneous group of disorders of the connective tissue characterized by hypermobile joints and often hyperextensibility as well as thinness of skin [47, 48]. About 10 types of the syndrome have been differentiated. Deficiency of galactosyltransferase I was reported in skin fibroblasts from one prematurely-aged patient [49]. The enzyme catalyses the second glycosyl transfer step in the biosynthesis of dermatan sulfate proteoglycan II (decorin) and of a number of other proteoglycans. Fibroblasts of the patient released into culture medium incomplete proteoglycan molecules possessing unsubstituted xylose residues attached to the core protein. Surprisingly, a deficiency of the third glycosyl transfer reaction catalysed by galactosyltransferase II (galactosylxylosylprotein 3-β-galactosyltransferase, EC 2.4.1.134), was also observed. Fibroblasts of patient's parents exhibited galactosyltransferase I activities that were half of the normal values suggesting that the parents were heterozygous. In spite of the decreased activities of the two galactosyltransferases the patient's fibroblasts retained the capacity to synthesize mature decorin molecules. Galactosyltransferase I gene was probably mutated as the enzyme was more thermolabile and exhibited a lower temperature optimum than that from normal fibroblasts. It should be pointed out, however, that in types I-III of Ehlers-Danlos syndrome the molecular defects are either unknown or involve the structure of collagen.

In patients with type VI of the syndrome a deficiency of lysine hydroxylase (EC 1.14.11.4) and a reduced content of hydroxylysine in collagen was described [50]. Hydroxylysine residues are essential for the formation of stable intermolecular cross-links between collagen chains and also for the attachment of carbohydrates. In osteogenesis imperfecta an increased content of hydroxylysine and glycosylated hydroxylysine was observed [51]. In some patients there was an overproduction of hyaluronate [52, 53] while in others type I procollagen was found to be less soluble and its C terminal propeptide excessively mannosylated [54]. Thus, glycosylation may be aberrant in several heritable disorders of the connective tissue though its contribution to the pathogenesis of the diseases is difficult to assess at present.

Paroxysmal nocturnal hemoglobinuria. In contrast to the former diseases PNH is an acquired condition [55–57]. Patients suffer from hemoglobinuria that in some of them occurs irregularly while in others only during sleep. Many patients have a prior history of aplastic anemia. Generally PNH is believed to be a clonal disorder resulting from a mutation or successive mutations in the hemopoietic stem cell [58]. Subsequently, the mutated clone expands presumably because of a selective advantage over normal stem cells. The affected erythrocytes exhibit increased sensitivity to complement-mediated lysis. Therefore they may be divided into subpopulations according to this sensitivity. These subpopulations circulate in blood along with normal erythrocytes. PNH erythrocytes do not have or have reduced amounts of at least three complement regulatory proteins, i.e. the decay accelerating factor (DAF), membrane inhibitor of reactive lysis (MIRL) and C8 binding protein [54–61]. Under normal conditions these proteins protect cells against the action of accidentally activated complement. They are attached to cell membranes through GPI-anchors. Structure of the mammalian GPI-anchor is depicted in Fig. 3. Biosynthesis of GPI-anchors (for refs. see [62, 63]) involves multiple genes that were identified by complementation analysis of mouse and human cell lines which do not express GPI-linked proteins [64, 65]. Three different classes of genes A, C, and H are required for linking GlcNAc in α-glycosidic linkage to carbon atom 6 of the inositol moiety of phosphatidylinositol. This step (catalysed by UDP-GlcNAc:PIGlcNAc transferase, EC 2.4.1.198) is followed by N-deacetylation of the GlcNAc residue and fatty acid acylation of the inositol ring. Subsquently, three mannose residues and phosphoethanolamine are added. The latter biosynthetic steps are controlled by genes belonging to classes B, E, and F. The completed GPI is then transferred *en bloc* to the COOH termini of proteins. Blood cells of PNH patients are unable to incorporate glucosamine to phosphatidylinositol [66, 67] and this precludes further biosynthetic steps including the addition of protein. Lacking the GPI anchor, the protein is exported rather than targeted to the plasma membrane. In all patients analysed so far, PNH resulted from different mutations in a recently cloned class A gene, termed PIG-A for phosphatidylinositol glycan class A [68, 69]. Function of the gene has not been elucidated

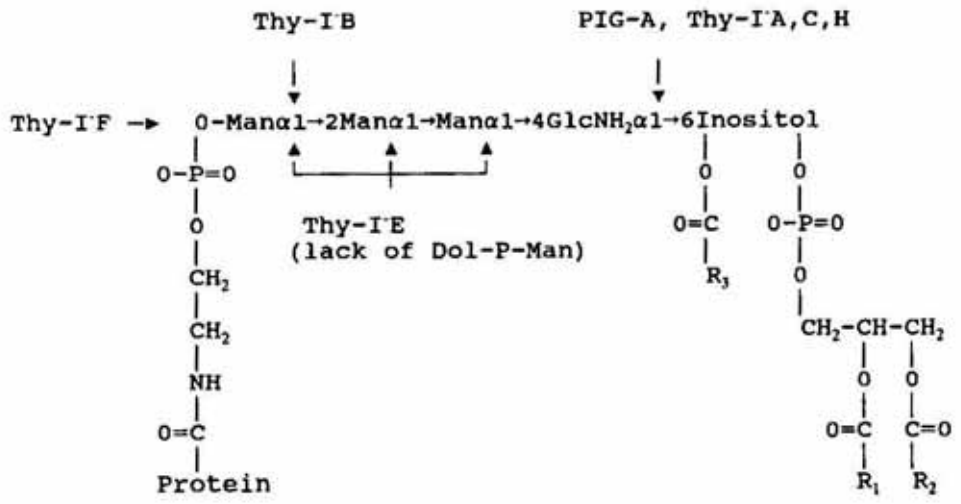

Fig. 3. Structure and biosynthesis of glycosylphosphatidylinositol-anchor. Large arrows indicate biosynthetic blocks due to the absence of particular genes. R_{1-3} represent aliphatic residues of fatty acids.

but it restored the biosynthesis of GPI-anchored proteins when transfected to GPI-deficient cell lines [69]. PIG-A gene has been mapped to chromosome Xp22 [69] while genes F and H have been mapped to autosomes [70]. Equal incidence of PNH in males and females should be attributed to the inactivation (lyonisation) of one X chromosome in females [71]. The inactivation of a particular X chromosome occurs early during embryonal development and persists for life. It is not known why the defective GPI-anchor biosynthesis in humans is always caused by mutations in PIG-A (in all 26 patients so far tested) [72] and never in genes B, C, E, H, and F.

Tn syndrome. Like PNH the Tn syndrome is an acquired disorder of erythropoiesis (for refs. see [73, 74]). The syndrome is described by the presence in circulation of a subpopulation of defective erythrocytes that can be agglutinated by ABO-compatible sera. The affected subjects often exhibit hemolytic anemia, thrombocytopenia, and leukopenia. The underlying cause is most likely a somatic mutation in the stem cell that precludes the expression of a gene encoding Gal3transferase. The enzyme links galactose with *N*-acetylgalactosamine that is attached directly to serine residues of proteins according to the equation: UDP-galactose + GalNAcα1→3serine-protein = UDP + Galβ1→3GalNAcα1→3serine-protein. Sialylated chains with this structure are present mostly in erythrocyte glycophorins but also in glycoproteins of other blood cells. In Tn cells these chains are unsubstituted with galactose and consist of single, terminal non-reducing GalNAc or sialylated GalNAc residues. Cells of all lineages including lymphocytes may be affected. It is assumed that hemolytic anemia and other pathological symptoms in Tn syndrome are mediated by natural anti-α-GalNAc antibodies that are present in the serum. Recently it has been demonstrated that 5-azacytidine, an inhibitor of DNA methylation, reactivated the expression of Gal3transferase in cloned, enzyme deficient, Tn lymphocytes [75]. Sodium butyrate displayed a similar effect. 5-Azacytidine had no effect on cloned PNH lymphocytes [76]. Previously it was established that 5-azacytidine restored the expression of several genes in the inactivated X-chromosome [71]. Sex of the cell donor in experiments on reactivation of Gal3transferase in Tn cells was not given while the donor of PNH lymphocytes was male [76]. Thus, he did not have the inactive X chromosome.

CONCLUSION

Recognition that aberrant glycosylation may cause disease has been made possible due to recent progress in methods of carbohydrate analysis, techniques of molecular biology, as well as better understanding of the biosynthesis and functions of glycans. More diseases of these type will likely be discovered in near future. It should be pointed out that the diseases of aberrant glycosylation provide important *in vivo* evidence for functions of glycans in glycoconjugates. CDA II illustrates a general physicochemical function of glycans, such as conferring water solubility upon protein moiety. The I-cell disease and leukocyte adhesion deficiency illustrate the role of glycans in targeting, homing and recognition. Antigenic and autoantigenic functions of glycans are supported by the analysis of Tn syndrome. In PNH the function of glycan is structural in that it provides a link between functionally important proteins and the plasma membrane. CDG syndrome will probably widen the scope of our knowledge on functions of glycans in glycoconjugates including interaction of bioactive molecules with their receptors in the plasma membrane. It is probably significant that, in general, defective or missing glycoproteins or proteoglycans, but not glycosphingolipids, are singled out as causes of the diseases of aberrant glycosylation. It has been proposed that most glycosphingolipids merely provide a carbohydrate cover for the membrane space not occupied by functional glycoproteins [77]. Thus, the basic function of glycosphingolipids would not be specific and, hence, absence of a particular glycosphingolipid would produce the disease only rarely.

Recent experiments have indeed suggested that glycosphingolipids are not indispensable substances for development of medaka embryo [78] or survival and proliferation of cultured mutant melanoma cells *in vitro* [79].

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