The homozygous M712T mutation of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase results in reduced enzyme activities but not in altered overall cellular sialylation in hereditary inclusion body myopathy

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Abstract Hereditary inclusion body myopathy (HIBM) is a neuromuscular disorder, caused by mutations in UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, the key enzyme of sialic acid biosynthesis. In Middle Eastern patients a single homozygous mutation occurs, converting methionine-712 to threonine. Recombinant expression of the mutated enzyme revealed slightly reduced *N*-acetylmannosamine kinase activity, in agreement with the localization of the mutation within the kinase domain. B lymphoblastoid cell lines derived from patients expressing the mutated enzyme also display reduced UDP-*N*-acetylglucosamine 2-epimerase activity. Nevertheless, no reduced cellular sialylation was found in those cells by colorimetric assays and lectin analysis, indicating that HIBM is not directly caused by an altered overall expression of sialic acids.

Keywords: GNE; Hereditary inclusion body myopathy; Lymphocyte; Sialic acid; Sialylation; UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase

1. Introduction

Hereditary inclusion body myopathy (HIBM) is a neuromuscular disorder characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology including cytoplasmic rimmed vacuoles and cytoplasmic or nuclear filamentous inclusions composed of tubular filaments. Various hereditary forms with similar presentations have been described in diverse ethnic clusters. This disease, occurring in various Middle Eastern population clusters, is mostly common in the Jewish Persian community with a prevalence of 1:1500 [1,2], and presents an unusual feature: the sparing of the quadriceps. Recently, a single homozygous missense mutation in all Persian and other Middle Eastern Jewish HIBM patients in the gene encoding the enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase/ManNAc kinase; GNE) was identified [3], confirming the founder effect hypothesis of this disorder in Middle Eastern Jews. Moreover, different missense mutations in this same gene have been identified worldwide in quadriceps sparing HIBM non-Jewish patients [3–5].

GNE is the key enzyme in the biosynthetic pathway of sialic acid [6]. Sialic acids are the most abundant terminal monosaccharides on glycoproteins and glycolipids in eukaryotic cells. They comprise a family of more than 50 naturally occurring carboxylated amino sugars with a scaffold of nine carbon atoms [7]. Sialic acids influence adhesion processes which play an important role in many cellular functions, such as cell migration, transformation of tissues, inflammation, wound healing and metastasis [8,9]. The first two steps of sialic acid biosynthesis are catalyzed by one of the two distinct functional domains of GNE. First, the UDP-GlcNAc 2-epimerase domain forms ManNAc from UDP-GlcNAc with simultaneous release of UDP. The ManNAc kinase domain then phosphorylates ManNAc to create ManNAc 6-phosphate [10,11]. In the following steps, sialic acid is formed by condensation of ManNAc-6-phosphate and phospho*enol*pyruvate and activated by CTP to form CMP–sialic acid. This nucleotide sugar is used as a substrate of sialyltransferases in glycoconjugate biosynthesis [12].

HIBM patients worldwide display mutations in both domains either in a homozygous form or, more commonly, in a

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Abbreviations: GNE, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; HIBM, hereditary inclusion body myopathy; ManNAc kinase, *N*-acetylmannosamine kinase; UDP-GlcNAc 2-epimerase, UDP-*N*-acetylglucosamine 2-epimerase

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compound heterozygous form. The homozygous M712T mutation, occurring in Middle Eastern Jewish patients, is located at the C-terminal end of the kinase domain. Although the role of GNE has been thoroughly recognized as a key enzyme in the biosynthetic pathway of sialic acid [6,13], the process by which HIBM mutations in the enzyme lead to this muscle disease is not yet understood. Here, we report the characterization of the recombinant M712T mutant GNE enzyme and of HIBM patient-derived lymphoblastoid cells expressing the mutated enzyme.

2. Materials and methods

2.1. Materials

All chemicals and enzymes, if not otherwise indicated, were from Sigma (Deisenhofen, Germany) or from Sigma, Israel.

2.2. Cloning of GNE cDNAs and generation of expression vectors

GNE cDNAs were generated from total RNA isolated from lymphoblastoid cell lines derived from a healthy individual and from an HIBM patient carrying the M712T mutation in *GNE* by reverse transcription (Superscript II reverse transcriptase; Gibco-BRL, Karlsruhe, Germany) with random hexamer primers. PCR was then performed to amplify GNE using primers BamX17F (CTATGGATCCTCGAGCCTCTCAAAACGAAACAAGC) and EcoK2251R (GATTGAATTCGGTACCAGGAGCTCTGGAGAG- AAG). These primers encompass *GNE* cDNA (Accession No. AJ238764) from nucleotide #17 to nucleotide #2251 and include at their 5′ ends sequential restriction sites for *Bam*HI–*Xho*I (New England Biolabs; Beverly, USA), and for *Eco*RI–*Kpn*I (New England Biolabs), respectively. PCR products were cloned in either pcDNA3.1 (Invitrogen; Karlsruhe, Germany) or pBluescript SK (Stratagene; La Jolla, USA) vectors.

2.3. Expression and purification of GNE from insect cells

Generation of baculovirus and protein expression in insect cells was done by the BAC-To-BAC system (Gibco-BRL). In brief, genes were cloned from the cloning vectors into the expression vector pFastBacHTa by *Xho*I and *Kpn*I. The vectors were then transformed into *Escherichia coli* DH10 BAC cells. Bacmid DNA was generated by homologous recombination in the DH10 cells, isolated and Sf9 cells were transfected with the DNA. After harvesting the first virus, it was amplified twice and the virus titer was determined after the last amplification step. For protein expression, 2×10^6 cells/ml were infected by the viruses with an MOI of 1 and incubated for 48 h. Cells were harvested, washed once with PBS and used for protein preparation.

For protein purification, cells were resuspended in 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and lysed by 20 strokes through a syringe with a 26 gauge needle. The lysate was centrifuged at $20\,000 \times g$ for 20 min and the supernatant applied to a Ni–NTA column (0.5 ml; Qiagen; Hilden, Germany). The column was washed with 5 ml of 10 mM NaH_2PO_4 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 20 mM imidazole (pH 8.0) and proteins were eluted in 2 ml of 10 mM NaH_2PO_4, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 100 mM imidazole (pH 8.0). Fractions containing protein were applied to a PD-10 gel filtration column (Amersham; Freiburg, Germany) and eluted with 10 mM NaH_2PO_4 (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM UDP. Fractions were checked for protein concentration by the Coomassie protein assay kit (BioRad; München, Germany) and for purity by SDS–PAGE using a Mini-Protean II system (BioRad).

2.4. Enzyme assays

The assay used to determine the epimerase activity of a recombinant GNE contained 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-GlcNAc and 0.2–1 μg of protein in a final volume of 200 μl. The reaction was performed at 37 °C for 30 min and stopped by heating at 100 °C for 1 min. ManNAc produced in this reaction was detected by the Morgan–Elson method [14]. In brief, 150 μl of sample was mixed with 30 μl of 0.8 M H_2BO_3, pH 9.1, and boiled for 3 min. Then, 800 μl of DMAB solution (1% (w/v) 4-dimethylamino benzaldehyde in acetic acid/1.25% 10 N HCl) was added and incubated at 37 °C for 30 min. The absorbance was read at 578 nm.

The assay used to determine the kinase assay of recombinant GNE contained 60 mM Tris/HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM ManNAc, 10 mM ATP, 0.2 mM NADH, 2 mM phospho*enol*pyruvate, 2 U pyruvate kinase, 2 U lactate dehydrogenase and 0.1–0.5 μg of protein in a final volume of 200 μl. The reaction was performed for 20 min at 37 °C and stopped by addition of 800 μl of 10 mM EDTA. The decrease of NADH was monitored at 340 nm.

UDP-GlcNAc 2-epimerase activity in lymphoblastoid cells was determined as previously described [10]. Cytosolic supernatant was prepared by using the procedure described above for insect cells. Assays were performed in a final volume of 200 μl, containing 35 mM sodium phosphate, pH 7.5, 4 mM $MgCl_2$, 0.5 mM UDP-GlcNAc, 50 nCi [^{14}C] UDP-GlcNAc and 300 μg of cytosolic protein. Incubations were carried out at 37 °C for 3 h and stopped by addition of 300 μl ethanol. Radiolabelled substrates were separated by descending paper chromatography and quantified by liquid scintillation analysis. All specific activities were given as U/mg of protein. One unit is defined as the formation of 1 μmol product per minute.

The overall reaction of recombinant GNE was determined by a similar assay. A final volume of 200 μl contained 35 mM sodium phosphate, pH 7.5, 4 mM $MgCl_2$, 0.5 mM UDP-GlcNAc, 12.5 nCi[^{14}C] UDP-GlcNAc, 10 mM ATP and 0.2 μg of purified protein. Incubations were carried out at 37 °C for 30 min and stopped by addition of 300 μl ethanol. Radiolabelled substrates were separated by descending paper chromatography and quantified by liquid scintillation analysis. The R_f values were 0.08 for UDP-GlcNAc, 0.55 for ManNAc and 0.17 for ManNAc-6-phosphate.

2.5. Cell cultures

Lymphoblastoid cells were grown in RPMI medium supplemented with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel). For bound sialic acid determination, sialic acid uptake was avoided by growing the cells in serum-free medium (DCCM, Biological Industries) for 4–5 days prior to performing the assay.

2.6. Sialic acid quantification

Membrane-bound sialic acid was quantified by the periodate/resorcinol method [15]. In brief, lysates from 20×10^6 cells obtained after lysis as described above were oxidized in 250 μl with 5 μl of 0.4 M periodic acid at 37 °C for 90 min, followed by 15 min boiling in 500 μl of 6% resorcinol/2.5 mM $CuSO_4$/44% HCl. After cooling for a few minutes, 500 μl *tert*-butyl alcohol was added, the samples were vortexed and centrifuged for 5 min to precipitate cell debris. Immediately after spinning, the supernatants were poured into OD cuvettes and read at 630 nm. Sialic acid concentrations were calculated by comparison with a standard curve (usually in a range of 0–250 μM sialic acid) and expressed in nanomoles per mg cytosolic protein.

2.7. FACS analysis

10^6 cells were incubated for 45 min directly with 1 μg of either FITC-*Maackia amurensis* agglutinin (MAA) (EY Laboratories, CA, USA), FITC-*Sambucus nigra* agglutinin (SNA) (EY Laboratories), or with biotin-TML (Calbiochem, CA, USA) followed by an additional 45 min incubation with 1 μg FITC–streptavidin (Sigma). Two washes with 1 ml PBS each were performed between all steps. Cell fluorescence was analyzed in a Becton–Dickinson FACS apparatus.

3. Results and discussion

3.1. Functional expression and characterization of human GNE and the M712T mutant in insect (Sf9) cells

The expression of GNE in most human tissues or cell lines is low making it very difficult to isolate and purify sufficient amounts of this enzyme for biochemical characterization from these sources. To avoid this limitation, we cloned the cDNA of wild-type and mutated GNE from patient-derived lymphoblastoid cell lines and expressed it in insect (Sf9) cells by the baculovirus expression system. This system is well suited for GNE expression, as previously shown for the rat enzyme [11].

The cDNAs were cloned into the pFASTBacHT vector, which fuses the resulting protein with an N-terminal 6×His-tag. Baculoviruses generated from these vectors were used to infect Sf9 cells, which then produced recombinant GNE. After cell lysis, proteins were purified from the cytosol by Ni–NTA chromatography. Imidazole used in this step affects GNE stability requiring that it be removed by chromatography on PD-10 gel filtration columns immediately after Ni–NTA agarose chromatography. GNE preparations obtained after this step were active for several days at 4 °C. SDS–PAGE analysis showed the presence of 80 kDa bands for the wild-type enzyme and the M712T mutant, and purity of the samples of more than 90% (data not shown).

The epimerase and kinase enzyme activities of the two forms of recombinant GNE were determined. First, both enzymes showed similar UDP-GlcNAc 2-epimerase activities (Fig. 1A). Furthermore, UDP-GlcNAc 2-epimerase activity of the wild-type as well as the M712T mutant enzymes was completely inhibited by 100 μM CMP-Neu5Ac, the feed-back inhibitor of this enzyme activity (data not shown). The ManNAc kinase activity of the M712T mutant was reduced by about 30% compared to the wild-type enzyme (Fig. 1A). These results are consistent with localization of the M712T mutation in the kinase domain of GNE. The oligomerization was also analyzed for both enzymes. Both wild-type and mutated GNE assemble as homohexamers of six subunits (data not shown). These results indicate, that the M712T mutation causes only subtle effects in vitro, specifically affecting the kinase domain of the recombinant enzyme.

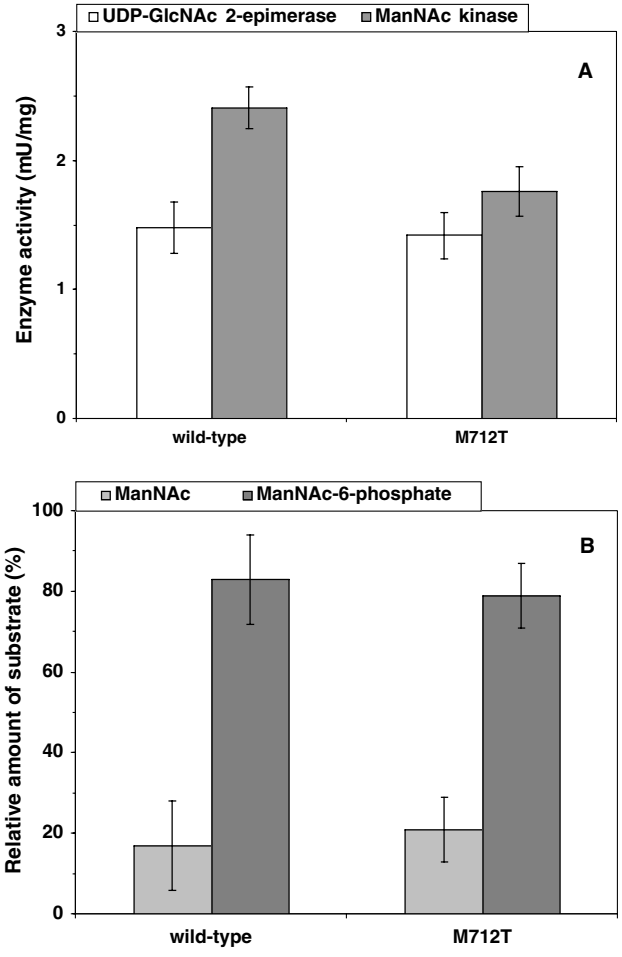

Fig. 1. Enzyme activities of recombinant wild-type and M712T GNE. (A) Specific enzyme activities. Enzymes were expressed in Sf9 cells, purified by Ni–NTA-chromatography and assayed for enzyme activities as described under Sections 2.3 and 2.4. Values are means ± S.D. of five independent enzyme preparations. (B) Overall reaction of GNE. Purified enzymes were incubated with radiolabelled UDP-GlcNAc in the presence of unlabelled ATP as described under Section 2.4. Relative amounts of ManNAc and ManNAc-6-phosphate were determined by paper chromatography. Values are means ± S.D. of three independent experiments.

In the wild-type enzyme, the specific activity of UDP-GlcNAc 2-epimerase is nearly 2-fold lower than the specific activity of ManNAc kinase (Fig. 1A). Therefore, the epimerase activity might be rate-limiting for the overall reaction of the bifunctional enzyme. We therefore developed an assay checking the two-step conversion of UDP-GlcNAc to ManNAc 6-phosphate. Wild-type and mutated GNE, incubated in the presence of radiolabelled UDP-GlcNAc and non-labelled ATP, were analyzed for production of ManNAc and ManNAc-6-phosphate. Under these assay conditions, wild-type GNE immediately converted more than 80% of the ManNAc generated by UDP-GlcNAc 2-epimerase to ManNAc-6-phosphate (Fig. 1B). This result confirmed that the ManNAc kinase domain of GNE has a higher metabolic rate than UDP-GlcNAc 2-epimerase. The same result was found for the M712T mutant GNE. So at least in this in vitro assay, the mutation does not result in a significant change of the production of ManNAc 6-phosphate by the enzyme.

The localization of the M712T mutation within the tertiary structure GNE is currently unknown because the crystal structure of this enzyme is not yet available. Sequence similarities with other sugar kinases reveal a conserved region between amino acids 410 and 684 [11], most likely representing the core of the kinase domain. We therefore speculate that M712 is located outside of the core region that determines kinase activity, thereby accounting for the minor effects of this mutation on enzyme activities.

3.2. Characterization of GNE activities in patient-derived lymphoblastoid cell lines

In order to characterize the M712T mutant in a cellular system, we used B lymphoblastoid cell lines derived from HIBM patients, healthy controls and heterozygous carriers of the mutation. For each group at least five individual cell lines were investigated for UDP-GlcNAc 2-epimerase activity (Fig. 2). The mean activities were 0.24 ± 0.03 mU/mg for control cells, 0.21 ± 0.04 mU/mg for cells of heterozygous carriers and 0.15 ± 0.02 mU/mg for cells from patients with clinical symptoms of HIBM. Whereas no significant reduction was found for cells from heterozygous carriers compared to normal cells, UDP-GlcNAc 2-epimerase activity was reduced by about 35% in patient cells. This result is in contrast to the findings for the recombinant GNE proteins produced in insect cells, which displayed no difference in UDP-GlcNAc 2-epimerase activity. In order to exclude the possibility that the decrease in epimerase activity results from alterations in the GNE expression level, cells were checked by Western blot analysis, and no expression differences were found between cell lines derived from HIBM patients and their healthy controls (data not shown). It was not possible to detect the kinase activity specifically resulting from ManNAc kinase in the cell lines we investigated from human patients because of the low

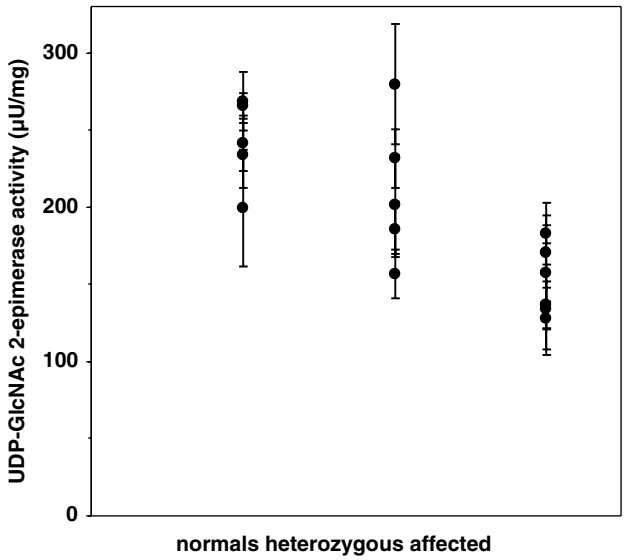

Fig. 2. UDP-GlcNAc 2-epimerase activity in B lymphoblastoid cell lines. Enzyme activities were determined in the cytosol of cell lines from healthy control individuals (normals), heterozygous carriers of the M712T mutation (heterozygous), and HIBM patients (affected) as described under Section 2.4. Values are means ± S.D. of three independent experiments.

expression of GNE and the high expression of several sugar kinases other than GNE in the lymphoblastoid cells. In particular, GlcNAc kinase has a high intrinsic ManNAc kinase activity [16], which would be capable of masking any kinase deficiencies of GNE in cells. Therefore, the question is still open whether cellular GNE also displays a 30% reduction of ManNAc kinase activity for the M712T mutation, as found for the mutated recombinant enzyme. Because specific inhibitors of GlcNAc kinase are not available to date, another potential method to measure ManNAc kinase activities may take advantage of the high molecular mass of hexameric GNE. The protein may be separated from other sugar kinases by gel filtration prior to enzyme assays. Nevertheless, for cells with very low expression of GNE, as the lymphoblastoid cells used in this study, this method is not promising.

Another factor that makes the analysis of the exact cellular effects of GNE mutations difficult is the ability of this enzyme to be regulated by various mechanisms. For example, it is known that phosphorylation of GNE by protein kinase C increases UDP-GlcNAc 2 epimerase activity [17]. Also the GNE protein can be modified by the addition of O-linked *N*-acetylglucosamine residues (R. Horstkorte, unpublished observation). It is possible that the M712T mutation impairs such cellular mechanisms and thus destabilizes the entire GNE molecule, thereby indirectly affecting both enzymatic activities. Ultimately, an understanding of both affected and unaffected cells will be required to fully understand the cellular basis of HIBM. Further studies, therefore, are underway to reveal if alterations of UDP-GlcNAc 2-epimerase activity are present in other cell types expressing the mutated enzyme. Of particular interest are myocytes or neuronal cells implicated in the muscle disorders found in HIBM.

3.3. Characterization of sialylation in patient-derived lymphoblastoid cell lines

Since we measured reduced UDP-GlcNAc 2-epimerase activity in all cell lines of HIBM patients, together with the reduced ManNAc kinase activity of the recombinant enzyme, we analyzed sialic acid expression of all cell lines. Membrane-bound sialic acids (including plasma membrane, membranes of the Golgi apparatus and lysosomal membranes) were quantified by the colorimetric periodate/resorcinol assay (Table 1). No significant difference was found between cells of healthy individuals and cells homozygously expressing the M712T mutant GNE. It is well known, that cells are able to take up sialic acids or its metabolic precursors from the culture medium and convert them to glycoconjugate-bound sialic acids [18]. Because these sugars are especially abundant in the supplemented serum, we therefore changed culture conditions and used serum-free medium with a very low content of sialic acids. The question was, whether these conditions may reveal an hyposialylated phenotype. No difference, however, was found in the overall membrane-bound sialic acids contents of control and patient-derived cells even when this sialic acid depleted media were used (data not shown).

To verify these results, cell surface expression of sialic acids was analyzed using several sialic acid specific lectins. *Tritrichomonas mobilensis* lectin (TML) binds to sialic acid in α2,3-, α2,6- and α2,8-linkages. Furthermore, its affinity is influenced by modifications in sialic acids, such as acetylation or the *N*-glycolyl modification [19]. No significant difference in TML binding was found between HIBM and healthy control cells when analyzed by FACS (Table 1; Fig. 3). These results confirm data from the periodate/resorcinol assay that showed unchanged overall sialylation and further indicated that there is no obvious altered expression of the differently linked types of sialic acid. Next we used two linkage-specific lectins, MAA for α2,3-linked sialic acids and SNA for α2,6-linked sialic acids, in order to investigate more subtle changes in sialic acid expression. Here again, the analysis of the cells by FACS with these lectins showed no significant differences between patient-derived and control cells (Table 1; Fig. 3). We then used MAA in lectin blot analysis of membrane glycoproteins. No reduction in MAA specific sialylation was found in the proteins of patient-derived cells in this experiment either (data not shown).

Our data do not reveal any influence of the M712T mutation on the expression of sialic acids on the lymphoblastoid cell lines. Obviously, the cells are able to compensate for the reduced enzymatic activities of GNE. Whereas a loss of

Table 1
Overall sialic acid detection in lymphoblastoid cell lines by the periodate/resorcinol assay and by FACS analysis

Cells	Membrane-bound sialic acids	TML	MAA	SNA
	nmol/mg protein	Mean fluorescence intensity	Mean fluorescence intensity	Mean fluorescence intensity
Normal	26 ± 6 (5)	18 ± 10 (5)	53 ± 21 (4)	179 ± 71 (5)
Affected	29 ± 9 (5)	10 ± 3 (5)	50 ± 14 (9)	155 ± 60 (6)

Values are means ± S.D. of the data of the indicated number of cell lines.

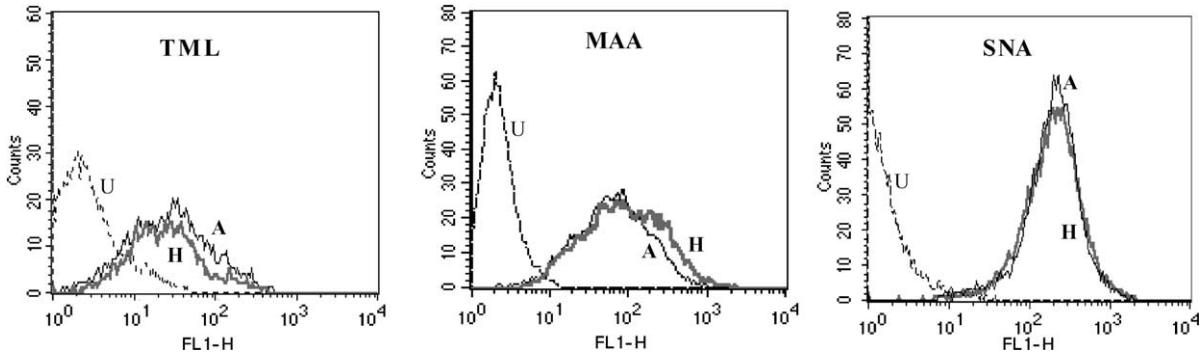

Fig. 3. Cell surface sialylation of B lymphoblastoid cell lines. Sialylation was FACS-analyzed by the sialic acid specific lectins TML, MAA and SNA as described under Section 2.7. U, Untreated lymphoblastoid cells; H, lymphoblastoid cells from a healthy individual; A, lymphoblastoid cells from an HIBM affected patient.

ManNAc kinase activity can be effectively compensated by GlcNAc kinase [16], there is no alternative known for UDP-GlcNAc 2-epimerase. Accordingly, the 35% reduction of epimerase activity found in M712T cells seems to be within the range of acceptable changes to enzyme activity needed to maintain intact sialic acid metabolism. This finding may also be due to the fact that lymphocytes required relatively low levels of sialic acids in contrast to, for example, hepatocytes, which synthesize large amounts of sialylated serum glycoproteins. On the other hand no liver failure has been reported in HIBM patients, indicating that reduced activities of GNE do not lead to reduced sialylation in most tissues. It is likely that the range of "acceptable changes" is variable in different tissues, with effects being more likely to be felt in cells with relatively low levels of GNE expression, such as muscle cells, but high requirements for sialic acid. In a very recent study, Noguchi et al. [20] showed that muscle cells from Japanese HIBM patients, carrying other mutations than M712T, may have slightly reduced sialic acid content and hyposialylation in primary cultured myofibers. It is also possible that a slight reduction of sialylation occurs in a specific protein which may not be relevant for lymphocyte metabolism, but that could be essential in muscle physiology, thus leading to the HIBM phenotype. Although in more than 120 patients with the M712T mutation no other consistent disorder was associated with the GNE defect, additionally it may be possible that GNE or one of the various enzymes acting downstream is involved in additional pathways, not related to sialic acid biosynthesis. The findings in this study indicate that the mechanism of HIBM cannot be explained easily through the well characterized sialic acid pathway in cellular metabolism and the role of GNE in this disease remains still enigmatic. Further studies on muscle cells will therefore be essential to give more insights into the pathophysiology of this disorder.

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