Defects in degradation of blood group A and B glycosphingolipids in Schindler and Fabry diseases

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Abstract **Skin fibroblast cultures from patients with inherited lysosomal enzymopathies, α-*N*-acetylgalactosaminidase (α-NAGA) and α-galactosidase A deficiencies (Schindler and Fabry disease, respectively), and from normal controls were used to study in situ degradation of blood group A and B glycosphingolipids. Glycosphingolipids A-6-2 (Gal*N*Ac(α1→3)[Fucα1→2]Gal(β1→4)Glc*N*Ac(β1→3)Gal(β1→ 4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-*N*-acetylgalactosaminylneolactotetraosylceramide), B-6-2 (Gal(α1→3)[Fucα1→ 2] Gal(β1→4)Glc*N*Ac(β1→3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-galactosylneolactotetraosylceramide), and globoside (Gal*N*Ac(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1′) Cer, globotetraosylceramide) were tritium labeled in their ceramide moiety and used as natural substrates. The degradation rate of glycolipid A-6-2 was very low in fibroblasts of all the α-NAGA-deficient patients (less than 7% of controls), despite very heterogeneous clinical pictures, ruling out different residual enzyme activities as an explanation for the clinical heterogeneity. Strongly elevated urinary excretion of blood group A glycolipids was detected in one patient with blood group A, secretor status (five times higher than upper limit of controls), in support of the notion that blood group A-active glycolipids may contribute as storage compounds in blood group A patients. When glycolipid B-6-2 was fed to α-galactosidase A-deficient cells, the degradation rate was surprisingly high (50% of controls), while that of globotriaosylceramide was reduced to less than 15% of control average, presumably reflecting differences in the lysosomal enzymology of polar glycolipids versus less-polar ones. Relatively high-degree degradation of substrates with α-D-Galactosyl moieties hints at a possible contribution of other enzymes.**—Asfaw, B., J. Ledvinová, R. Dobrovolný, H. D. Bakker, R. Desnick, O. P. van Diggelen, J. de Jong, T. Kanzaki, A. Chabas, I. Maire, E. Conzelmann, and D. Schindler. **Defects in degradation of blood group A and B glycosphingolipids in Schindler and Fabry diseases.** ***J. Lipid Res.*** **2002.** 43: **1096–1104.**

Supplementary key words α-*N*-acetylgalactosaminidase deficiency • α-galactosidase A deficiency • skin fibroblasts • in situ metabolism • lysosome targeting • blood group glycolipids • secretor status

Glycolipids with blood group A or B specificity contain an α-*N*-acetylgalactosamine (α-Gal*N*Ac) or α-galactose (α-Gal) residue as the terminal sugar of their oligosaccharide chains, respectively. The blood group A determinant is characterized by the trisaccharide structure Gal*N*Acα1→3(Fucα1→2)Gal, blood group B by the structure Galα1→3(Fucα1→2)Gal, attached to the lacto-, neolacto-, or globo- series of glycoproteins or glycolipids (2).

Abbreviations: A-6-2, blood group glycolipid A-6-2 (Gal*N*Ac(α1→3)[Fucα1→2]Gal(β1→4)Glc*N*Ac(β1→3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-*N*-acetylgalactosaminylneolactotetraosylceramide); α-NAGA, α-*N*-acetylgalactosaminidase; B-6-2, blood group glycolipid B-6-2 (Gal(α1→3)[Fucα1→2]Gal(β1→4)Glc*N*Ac(β1→ 3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-galactosylneolactotetraosylceramide); GbOse$_3$Cer (Gal(α1→4)Gal(β1→4)Glc(β1→ 1′)Cer, globotriaosylceramide); globoside (Gal*N*Ac(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1′)Cer, globotetraosylceramide); mAb, mouse monoclonal antibody; the nomenclature used for neutral glycolipids follows the IUPAC-IUB recommendation (1).

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Manuscript received 11 December 2001 and in revised form 18 April 2002.
DOI 10.1194/jlr.M100423-JLR200

The oligosaccharide moieties of these compounds are ultimately degraded in the lysosomal compartment by exoglycosidases. These cleave off the sugars sequentially from the nonreducing end of the molecule. Inherited defects of α-*N*-acetylgalactosaminidase (α-NAGA, E.C.3.2.1.49; Schindler disease) and α-galactosidase A (E.C.3.2.1.22; Fabry disease) have been shown to block oligosaccharide degradation at the sites of normal hydrolysis by these enzymes. Thus, glycolipids with A determinants may contribute to the storage process in Schindler disease patients with blood group A, similarly as shown for glycolipids with blood group B specificity in Fabry disease patients (3, 4).

α-Galactosidase A has long been known to act predominantly on glycolipids. In addition to the major accumulated sphingolipids, globotriaosylceramide ($GbOse_3Cer$) and galadiosylceramide ($GaOse_2Cer$) in Fabry disease, the contribution of blood group B glycolipid antigens to the storage process has been shown in patients with blood group B (3, 4). Although the extent of accumulated glycolipid B-antigens detected in patients' tissues and urinary sediments was much lower than that of the classical "Fabry glycolipids," it may cause an additional metabolic burden.

α-NAGA deficiency is commonly regarded as a glycoproteinosis (5–7) because, with the exception of blood group glycolipids, almost all physiologically occurring α-Gal*N*Ac residues reside in the core structures of *O*-linked glycoproteins, forming the linkage between oligosaccharide chains and the polypeptide backbone; accordingly, the major accumulated compounds are glycopeptides. The glycopeptides excreted in urine carry α-Gal*N*Ac in an internal position and therefore probably are not identical to the primary lysosomal intermediates. The storage compounds have been characterized in cultured skin fibroblasts from patients by lectin histochemistry (8), but their chemical analysis is elusive to date. Glycosphingolipid storage in α-NAGA deficiency has not been shown. However, a block of the degradation of blood group A glycolipid antigens has been demonstrated recently in fibroblast cultures (9). Direct analysis of glycolipid storage in patients' tissues has not yet been possible. However, because blood group glycolipids are also expressed on epithelial cells of the urinary tract, a strongly elevated content of such compounds in urinary sediment would at least provide indirect evidence of their accumulation also in tissues.

α-NAGA deficiency is characterized by remarkable clinical heterogeneity (type I, severe infantile; type II, mild adult; type III, intermediate form) (10), and patients without overt clinical symptoms have been also reported (11). In many lysosomal storage diseases, such a variation could largely be explained by differences in the residual activities of the affected enzyme (12), although in some cases, such as α-L-Iduronidase deficiency [mucopolysaccharidosis (MPS) I, Hurler/Scheie syndromes], the differences are minute and are difficult to detect with standard enzyme assays (13). Loading studies with glycolipid substrates in cell culture were shown to provide a strongly magnified view of such residual activities (14). This approach was therefore used to assess any differences in residual α-NAGA activity in cell lines from almost all patients with α-NAGA deficiency diagnosed to date. For comparison, corresponding experiments were performed with blood group B glycolipids in fibroblasts from patients with Fabry disease.

MATERIALS AND METHODS

Materials

Outdated human blood concentrates (blood groups A and B) were obtained from the Institute of Hematology and Blood Transfusion, Prague. HPTLC plates (Silica gel 60) were from Merck AG, and Polygram Sil-G sheets were from Macherey and Nagel, Germany. The other chemicals, all of them reagent grade, were from Lachema, Czech Republic or Sigma, Germany. Organic solvents were distilled before use. DMEM, fetal calf serum, and trypsin were from Gibco, Germany. Viable Colostrum-Based Serum Replacement Media without lipoproteins (AC-2) was a product of Valio Bioproducts, Finland. Recombinant apolipoprotein (apo)E_3 was a kind gift from Dr. Tikva Vogel, Bio Technology General, Rehovot, Israel. Mouse monoclonal antibody (mAb) directed to blood group A and B determinants and peroxidase-conjugated anti mouse IgM secondary antibody were purchased from Exbio, Prague, Czech Republic. The latter was obtained also from Pierce, Rockford IL.

Cell lines. Patients' cell lines and their α-NAGA and α-galactosidase A activities are listed in **Table 1**. Cultured skin fibroblasts were obtained from almost all patients with α-NAGA deficiency diagnosed up to the present. For Fabry disease, only cell lines from patients with classical clinical phenotypes were selected. Several unrelated control fibroblasts were also included in the study.

Urine samples. Urine samples were obtained from eight of the ten α-NAGA-deficient patients. Two patients have blood group A (one with secretor status, one nonsecretor), six of them blood group O. Control samples from eight blood group A individuals (six secretors, two nonsecretors) were included, and all samples were kept frozen before use.

Methods

Preparation of glycolipids. Glycolipids A-6-2 and B-6-2 were isolated from human blood group A and B erythrocyte membranes, respectively (~4 mg of blood group active glycolipid antigens from 5 l erythrocyte concentrate) (9). Globoside was prepared from the same source. Their identities were confirmed by fast atom bombardment mass spectrometry (Dr. A. Suzuki and Dr. M. Suzuki, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and TLC-liquid secondary ion mass spectrometry (Dr. A. M. Lawson and Dr. W. Chai, The Glycoscience Laboratory, Harrow, UK).

Glycolipids (0.5 mg each) were labeled by catalytic hydrogenation of the sphingosine double bonds with tritium gas (15). Radiolabeled glycolipids were recovered from the reaction mixture and were purified by HPLC as described in details previously (9).

Loading experiments.

SUBSTRATE PREPARATIONS. Small unilamellar liposomes were prepared from phosphatidylcholine, α-tocopherol, phosphatidic acid, and the glycolipid substrate as described and were coated with apoE (9).

FEEDING ASSAYS. Unless stated otherwise, ~1.28 nmol of glycolipid (specific radioactivity 11 Terabecquerels/mol) were applied per 25 cm^2 flask, feeding time was 2–5 days, followed by 0–5 days chase, in standard experiments. To limit production of soluble radioactive products, 50–150 μM (final concentration) conduritol B-epoxide was added to the cell culture medium (DMEM with 5% serum replacement AC 2).

TABLE 1. Survey of patients included in the study

Patient	Ethnic Origin	In Vitro Enzyme Activity in Fibroblasts	Clinical Phenotype and Reference
		α-NAGA	
		nmol/mg/hr[a]	
Schindler disease			
D1.1	German	0.8	Infantile, severe neurological signs (6)
D1.2	German	0.5	Infantile, severe neurological signs (6)
D2.1	German	1.4	Infantile, severe neurological signs (8)
NL1.1	Dutch	3.2	Infantile, mild neurological signs (17)
E1.1	Spanish	0.2	Adult, lymphedema, angiokeratoma (18)
E1.2	Spanish	0.2	Adult, lymphedema, angiokeratoma (18)
J1.1	Japanese	0.3	Adult, angiokeratoma (19)
F1.1	French/Albanian	2.0	Infantile, mild psychomotor retardation (10)
NL2.1	Moroccan	1.0	No pathological signs (age 7 years) (11)
NL2.2	Moroccan	1.9	Infantile, mild psychomotor retardation, cataract (11)
Controls: n = 34		40–130	(8)
		α-Galactosidase A (21)	
		nmol/mg/hr[a]	
Fabry disease			
FD1	Czech	1.2	Classical[b] (20)
FD2	Czech	0.9	Classical (4)
FD3	German	deficient	Classical, not reported
FD4	German	deficient	Classical, not reported
FD5	Czech	1.0	Classical, not reported
FD6	Czech	0.9	Classical (20)
FD7	Czech	0.6	Classical (4)
FD8	Czech	0.7	Classical (4)
FD9	Czech	1.1	Classical (4)
Controls: n = 14		55–71	

[a] Enzymatic activities measured with synthetic substrates.
[b] Classical Fabry phenotype: renal and cardiac impairment, acroparesthesias, hypohidrosis, angiokeratoma, corneal and lenticular opacity.

ANALYSIS OF DEGRADATION PRODUCTS. Harvested cells were homogenized, and lipids were extracted with chloroform-methanol (2:1; v/v) and separated on HPTLC plates (Silica gel Merck, Germany). Chromatograms were evaluated by TLC-Linear Radioactivity Analyzer (Raytest, Germany) or by PhosphorImager (Molecular Dynamics SI, software Image Quant v.4.2).

Analysis of urinary lipids. Lipids were isolated from urine samples of patients and control individuals with corresponding blood groups by reverse-phase column chromatography as described previously (16). Aliquots of lipid extracts were applied to TLC plates (Polygram Sil G, Macherey-Nagel, Germany), and chromatograms were developed in chloroform-methanol-water systems (56:38:10 or 60:35:8; v/v/v). Orcinol detection and immunodetection with corresponding antibodies (mouse anti-A and anti-B mAb) were used for visualization (4).

RESULTS

Feeding conditions and evaluation of products

The glycolipids used in this study, A-6-2, B-6-2, and $GbOse_4Cer$, were targeted to lysosomes in apoE-coated liposomes via the apoB/E receptor. The substrate of α-galactosidase A ($GbOse_3Cer$) was formed directly in lysosomes from $GbOse_4Cer$.

In our previous work (9), it had become evident that differences in uptake rates might lead to a distorted representation of degradation rates, depending on how the latter were expressed. In our experience, dpm/mg protein/day reflects best the real situation, i.e., the capacity of normal and pathological cells to degrade the substrate. Therefore these units have been used for evaluation throughout the study.

Product formation in control cells is usually underestimated because complete degradation of the glycolipid generates radiolabeled fatty acids (note that radiolabeled dihydrosphingosine is degraded to palmitic aldehyde and hence to palmitic acid), which may either be used for resynthesis of other lipids, some of which may co-migrate with the substrate and thus escape detection, or may be β-oxidized to give tritiated water, which is lost to the culture medium. To minimize this problem, 50–150 μM conduritol B-epoxide was added to all cultures in order to arrest glycolipid hydrolysis at the penultimate step catalyzed by glucocerebrosidase.

Degradation of glycolipid A-6-2 by α-NAGA-deficient cell lines

Fibroblast cultures from almost all α-NAGA-deficient patients diagnosed up to the present (see Table 1) were used for feeding studies, with blood group A glycolipid A-6-2 as substrate. As expected, product formation was considerably slower than in normal or pathologic (Fabry disease) control cells (~7% of control rates; **Fig. 1**). The rates were equally low in the cells from all α-NAGA-deficient patients, with no significant difference between them, although the clinical pictures of the patients are very heterogeneous (from clinically normal phenotype to severely affected child).

Analysis of urinary glycolipids

Urine samples were available from two α-NAGA-deficient patients with blood group A (D1.2, blood group A

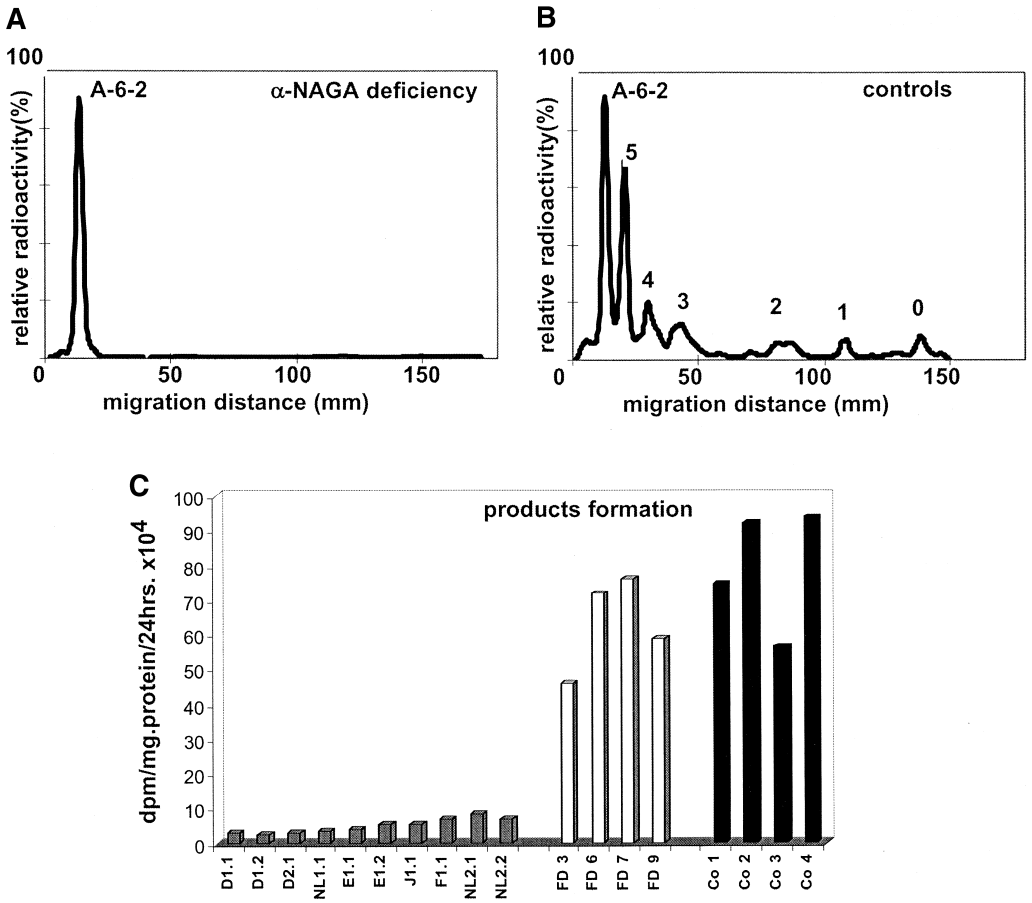

Fig. 1. Degradation of blood group glycolipid A-6-2 by skin fibroblasts from patients with α-*N*-acetylgalactosaminidase (α-NAGA) and α-galactosidase A deficiencies and from controls. Tritium-labeled glycolipid A-6-2 (5×10^6 dpm, 11.61 μCi, ~0.77 nmol), incorporated into liposomes coated with apolipoprotein (apo)E, was added into the culture medium of skin fibroblasts in 25 cm^2 flasks as described in the Materials and Methods. After 5 days, cells were harvested and the lipids extracted and separated by TLC. A: Lipid pattern of α-NAGA-deficient cells. B: Lipid pattern of control cells. The chromatographic positions of substrate and various products are indicated by number of sugar residues on the glycolipid, from 0 (ceramide) to 5 (pentahexosylceramide). C: Quantification of products (sum of all products on the TLC plate) formed by α-NAGA-deficient, α-galactosidase-deficient, and control cells. Values are average of at least three experiments. The reproducibility of method was evaluated from eight parallel experiments on a single control cell line. The sum of products (expressed as percentage of total radioactivity) was 80.7 ± 7.9% (mean ± SD).

secretor; J1.1, Le^{a+} nonsecretor). The rest of the patients had blood group O, and a urine sample from one of them was used as a pathologic control. Patient D1.2 was found to excrete an at least 5-fold elevated amount of group A immunoreactive glycolipids with six or more sugar moieties (**Fig. 2**, **Table 2**) when compared with the highest control value. This finding confirms that the metabolic defect in Schindler disease affects the catabolism not only of glycoproteins but also of glycolipids with α-*N*-acetylgalactosamine residue, including blood group A glycoconjugates. The other group A patient, nonsecretor J1.1, exhibited a value in the upper control range.

Degradation of glycolipid B-6-2 by Fabry cells

In a complementary study, the B blood group glycolipid B-6-2, which has a terminal α-galactose residue, was fed to α-galactosidase-deficient cells (Fabry disease). Surprisingly, the degradation rate was quite high, ~50% of that in normal and pathologic (α-NAGA-deficient) controls (**Fig. 3**). Also in this case, it was not possible to establish significant differences between the Fabry patients.

In contrast, the degradation of globotriaosylceramide $GbOse_3Cer$, formed in situ from the globoside fed to the cell cultures, was strongly reduced to ~15% of control average (**Fig. 4**).

DISCUSSION

In the study of lysosomal storage diseases, feeding radiolabeled glycolipids to cell cultures has been used for various purposes, e.g., for investigating general metabolic pathways (22), for diagnostic purposes such as discriminating between genuine metachromatic leukodystrophy and arylsulfatase A pseudodeficiency (23, 24), or for determination of

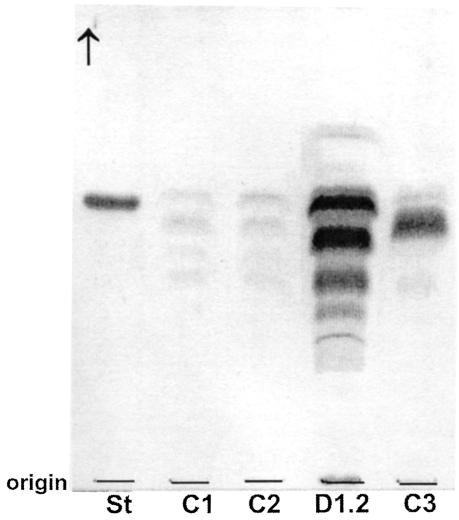

Fig. 2. Blood group A immunoactive glycosphingolipids in urine samples of α-NAGA-deficient patient D1.2 and of controls. Lipids were isolated by reverse-phase chromatography as described in Materials and Methods. Samples corresponding to 4 ml urine were applied on Polygram Sil G sheets and resolved in chloroform-methanol-water 56:38:10 (v/v/v). Immunodetection was performed as follows: 1 h blocking at 37°C [5% ovalbumin, 1% polyvinylpyrrolidon in PBS (0.135 M NaCl, 10 mM phosphate buffer, pH 7.4)], incubation with anti-blood group A mouse monoclonal antibody (mAb) over night (dilution 1:3 with 1% BSA in PBS) followed by 1h. incubation with peroxidase-conjugated secondary anti-mouse IgM Ab (dilution 1:500 with 1% BSA in PBS) at 37°C and colored reaction with 4-chloro-1-naphthol. Spots are evaluated densitometrically using a Camag TLC Scanner II (Cats3; Camag Scientific, Muttenz, Switzerland; reflection mode, 546 nm). St, standard (purified glycolipid A-6-2); C1, C2, and C3, controls, blood group A secretors; D1.2, α-NAGA-deficient patient blood group A secretor (see Tables 1 and 2).

residual activities in enzyme-deficient cells (14). Such assays were also particularly useful for identifying deficiencies of nonenzymatic protein cofactors of lysosomal glycolipid catabolism (sphingolipid activator proteins) (22, 25–28), including prosaposin deficiency (29, 30).

The advantage of feeding studies in situ is that they assess the entire degradation system, including, in addition to the relevant enzyme(s), any nonenzymic cofactors. Quantitative evaluation of such experiments, however, is for various reasons not as straightforward as would be desirable.The capacity of lysosomal enzymes in normal cells usually exceeds substrate influx by several orders of magnitude (12, 14). The measured turnover rates are therefore limited by substrate uptake and transport to the lysosome rather than by the activity of any degrading enzyme. It is next to impossible to saturate the enzyme and to determine its true capacity. Therefore, the degradative capacity in normal cells tends to be grossly underestimated.

In contrast, in enzyme-deficient cells, the affected enzyme's residual activity usually limits overall turnover, allowing the estimation of the remaining capacity. For this reason, residual activities determined by in situ feeding studies are always much higher than those determined by direct assay of the affected enzyme. They may, under certain conditions, even approach control values in spite of a substantial deficiency of the enzyme (14). On the other hand, cells from different patients with an enzyme defect can be compared directly because they are subject to the same limitations. The considerable magnification factor with which the residual activities are viewed in such assays permits the precise determination of even minute activities in cells with an almost complete deficiency of the enzyme.

Additional technical problems, such as loss of radioactive water to the medium or formation of products that migrate with the substrate peak in TLC and hence escape detection, may be reduced largely by appropriate procedures (e.g., arresting degradation at the level of glucocerebroside by adding conduritol B-epoxide to the culture medium) but cannot be eliminated completely. Their influence is, however, of minor importance.

Previously, we have shown that fibroblasts from patients with an inherited deficiency of lysosomal α-NAGA can practically not degrade glycolipids with an α-glycosidically bound *N*-acetylgalactosamine at the nonreducing termi-

TABLE 2. Excretion of blood group A glycosphingolipids (AGSL) in urine of Schindler patients

	Blood Group/Secretory Status	AGSL	
		arbitrary units[a]*/ml urine*	*arbitrary units/nmol SM*
Patients			
D1.2	A/secretor	4,907	5,492
J1.1	A/non-secretor	219	796
Pathologic Controls			
D1.1	O/secretor	0	0
Controls			
C1	A/secretor	100	588
C2	A/secretor	146	322
C3	A/secretor	412	935
C4	A/secretor	tr	—
C5	A/secretor	tr	—
C6	A/secretor	tr	—
C7	A/non-secretor	tr	—
C8	A/non-secretor	tr	—

Sphingomyelin (SM) was detected with primulin and quantified by densitometry using standards of known concentration (fluorescence at 365 nm). For experimental details see Fig. 2. tr, traces: concentration was under the limit of detection (less than 50 arbitrary units).

[a] Total area of A-immunopositive spots determined by densitometry.

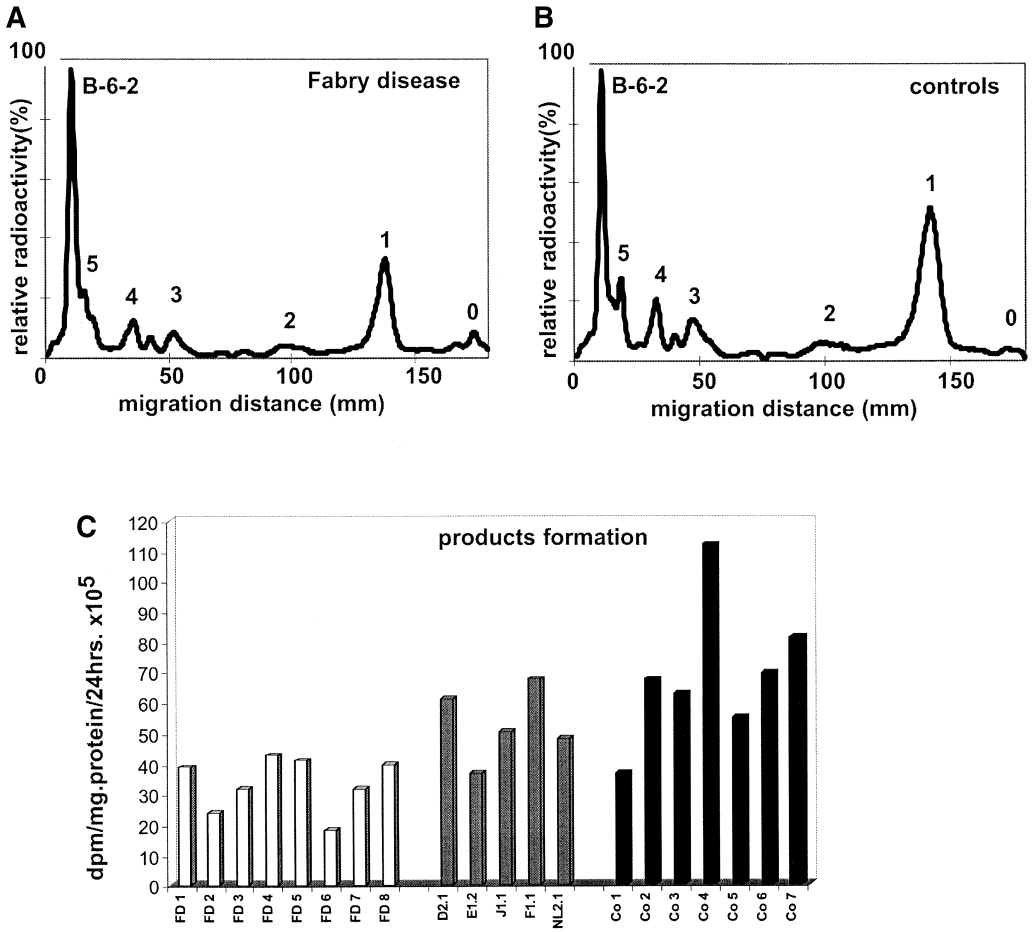

Fig. 3. Degradation of blood group glycolipid B-6-2 by skin fibroblasts from patients with α-galactosidase A and α-NAGA deficiencies and from controls. Tritium-labeled glycolipid B-6-2 (5×10^6 dpm) incorporated into liposomes coated with apoE, was added to the culture medium of skin fibroblasts in 25 cm^2 flasks as described in Materials and Methods. After 5 days, cells were harvested and the lipids extracted and separated by TLC. A: Lipid pattern of α-galactosidase A-deficient cells. The chromatographic positions of substrate and various products are indicated by number of sugar residues on the glycolipid, from 0 (ceramide) to 5 (pentahexosylceramide). B: Lipid pattern of control cells. C: Quantification of products (sum of all products on the TLC plate) formed by α-galactosidase-deficient, α-NAGA-deficient, and control cells, respectively. Values are average of at least three experiments. The reproducibility of method was evaluated from six parallel experiments on a single control cell line. The sum of products (expressed as percentage of total radioactivity) was 77.1 ± 5.3% (mean ± SD).

nus, such as blood group A active glycolipid A-6-2 (9). Meanwhile, 11 patients with NAGA deficiency have been diagnosed worldwide. Their clinical pictures are extremely heterogeneous, including two severe infantile cases with neuroaxonal dystrophy and a mild adult type of the disease (10), but also two individuals without overt clinical symptoms (11). In other lysosomal storage diseases, such as metachromatic leukodystrophy or the G_{M2} gangliosidoses, the mild, protracted, late-onset forms could be correlated with small but discernible residual activities of the affected enzyme, sufficient to maintain a still-considerable degradation rate of the substrate and thus to retard the storage process (14). It was of interest to see whether a similar explanation would hold for the different clinical forms of NAGA deficiency. Surprisingly, this was not the case. Cells from all 10 patients analyzed exhibited extremely low degradation rates, close to the detection limit and not significantly different from each other, irrespective of the patient's clinical picture. Obviously, all patients examined have a complete deficiency of α-NAGA. This raises the question as to what other factors determine the clinical course of the disease in the different patients. The major storage compounds in α-NAGA deficiency are not glycolipids but glycoprotein-derived glycopeptides. Possible candidates may therefore be the rate of glycoprotein turnover or the capacity of alternative ways of disposing of the accumulating glycopeptides.

Although glycolipids are not the major substrates of α-NAGA, their accumulation in α-NAGA deficiency, in addition to that of glycopeptides, may impose an additional burden on the affected organs or cell types. In this case, blood group and secretor status may be factors that influence the disease process. Blood group antigens are normally expressed on epithelial cells of the distal part of the nephron. Desquamated cells are components of urinary sediment, analysis of which is regarded as a "chemical biopsy." Analysis of the urinary sediments from three α-NAGA-deficient patients showed that in one case, secre-

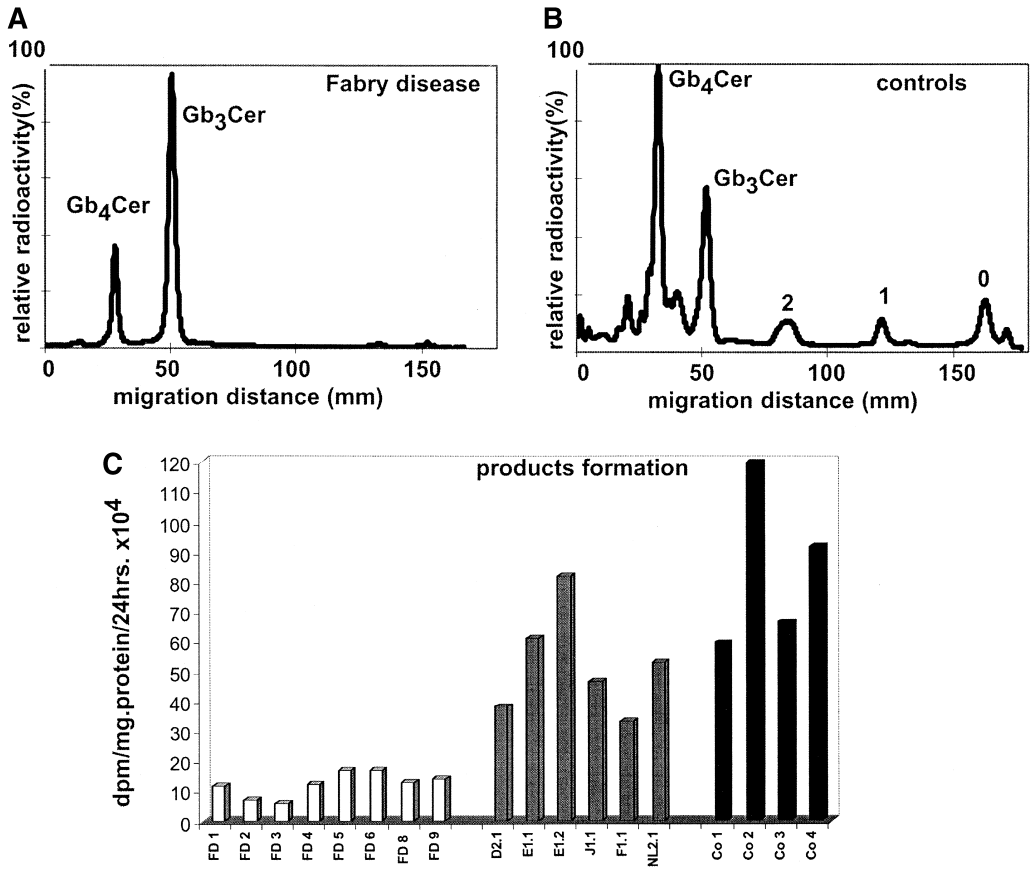

Fig. 4. Degradation of Gb_3Cer ($GbOse_3Cer$) by skin fibroblasts from patients with α-galactosidase A and α-NAGA deficiencies and from controls. Gb_3Cer was formed from Gb_4Cer (globoside) directly in the lysosomal compartment. Tritium-labeled glycolipid Gb_4Cer (5×10^6 dpm) incorporated into liposomes coated with apoE, was added to the culture medium of skin fibroblasts in 25 cm^2 flasks as described in Materials and Methods. After 5 days, cells were harvested and the lipids extracted and separated by TLC. A: Lipid pattern of α-galactosidase A-deficient cells. B: Lipid pattern of control cells. The chromatographic positions of substrate and various products are indicated by number of sugar residues on the glycolipid, from 0 (ceramide) to 2 (dihexosylceramide). C: Quantification of products (sum of all products on the TLC plate) formed by α-galactosidase-deficient, α-NAGA-deficient, and control cells. Values are average of at least three experiments. The reproducibility of method was evaluated from five parallel experiments on a single control cell line. The sum of products (expressed as percentage of total radioactivity) was 81.1 ± 6.1% (mean ± SD).

tor with blood group A, the concentration of a series of blood group A glycolipids was strongly elevated, indicating a storage process. This result proves that the inability to degrade this kind of lipid is not merely an in vitro finding but that these substrates also accumulate in those patients' tissues and cell types, where they are expressed under physiological conditions (31). Whether this additional metabolic load does indeed influence the development of the clinical phenotype is, of course, still hypothetical; further detailed investigation of different tissues from α-NAGA-deficient patients, when these become available, is needed.

In the converse experiment, i.e., feeding blood group B glycolipids to α-galactosidase A-deficient (Fabry disease) cells, substantial degradation was found in all cases. As discussed above, a nearly normal turnover rate does not necessarily mean a normal enzyme activity but may be reached at residual activities as low as a few percent of normal controls, depending on the rate of substrate uptake (14). In all Fabry disease cell lines employed, α-galactosidase was found to be completely absent when measured in vitro. The observed relatively high residual activity toward glycolipid B-6-2 was probably contributed by some other enzyme. The most likely candidate is α-NAGA, which is known to accept also α-galactosides as substrate, albeit with a high K_m value (32, 33). [The enzyme was indeed initially purified as α-galactosidase B (34)]. A high degree of structural and biochemical similarity between α-NAGA and α-galactosidase A, suggesting that these enzymes have evolved from a common ancestral gene, has been reported (35–37).

A much lower degradation rate was observed with $GbOse_3Cer$ as substrate. To some extent, this may be attributed to differences in the amounts of substrates taken up (influx rate), but it also reflects a particular mechanism of lysosomal glycolipid catabolism: most lysosomal glycosidases are water-soluble enzymes. Although they can directly interact with oligosaccharide and glycopeptide substrates, glycolipids are only directly accessible to them if the carbohydrate chain protrudes far enough from the

membrane into the aqueous phase, which appears to require at least a tetrasaccharide chain (38). Glycolipids with shorter carbohydrate chains, i.e., with only three or fewer sugars, are either degraded by membrane-associated enzymes (e.g., glucocerebrosidase) or must be solubilized by specific glycolipid binding proteins, so-called "activator proteins" (for review, compare 39, 40). These protein cofactors bind the glycolipid more or less specifically and extract it from the membrane to form a water-soluble complex (41, 42), which is the true substrate of the enzyme reaction. Interaction between activator protein and enzyme may be very specific.

Such a specificity was, at least in vitro, found for the interaction between the sulfatide activator (saposin B) and α-galactosidase A (43, 44), and for that of the α-subunit of hexosaminidase A with the G_{M2} activator protein (38, 45). Patients with a defect of saposin B do indeed excrete $GbOse_3Cer$, in addition to sulphatides, in their urine (46). It is thus quite plausible that the degradation of $GbOse_3Cer$ depends on the specific interaction between α-galactosidase A and saposin B, whereas blood group B glycolipids, with their longer carbohydrate chains, can be hydrolyzed without an activator.

α-NAGA-deficient fibroblasts seemed to have slightly reduced degradation of B-6-2 glycolipid in comparison to normal control cells. The statistical significance of this difference is, however, very speculative in view of the limited number of cell lines and, in particular, the wide biological heterogeneity of cells.

We thank Professor Milan Elleder for helpful discussions and critical reading of manuscript. We would also like to thank Drs. A. M. Lawson and W. Chai, The Glycoscience Laboratory, Harrow, UK, and Drs. A. Suzuki and M. Suzuki, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, for recording the mass spectra of purified lipids. We are grateful to Dr. Tikva Vogel, Bio Technology General, Rehovot, Israel, for recombinant $apoE_3$. This work was supported by grants from the Grant Agency of the Charles University (GAUK 37/2000/C) and from the Ministry of Education and Youth of the Czech Republic (Research Projects 111100003).

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