Degradation of blood group A glycolipid A-6-2 by normal and mutant human skin fibroblasts

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Abstract The degradation of blood group glycolipid A-6-2 (GalNAc(α1→3)[Fucα1→2]Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-N-acetylgalactosaminylneolactotetraosylceramide), tritium-labeled in its ceramide moiety, was studied in situ, in skin fibroblast cultures from normal controls, from patients with defects of lysosomal α-N-acetylgalactosaminidase, and from patients with other lysosomal storage diseases. Uptake of the glycolipid with apolipoprotein E-coated liposomes was linear with time and with the amount of glycolipid added. In normal cells, the expected array of less polar products and some lipids resulting from re-using the liberated sphingosine, mainly sphingomyelin and phosphatidylcholine, were formed. In α-N-acetylgalactosaminidase-deficient cells, the glycolipid was virtually not degraded; product formation was less than 2% of the normal control rate, suggesting that blood group A-active glycolipids contribute as storage compounds to the pathogenesis of this disease. The expected accumulation of degradation intermediates was seen in fucosidosis, and in Sandhoff, Gaucher, and Farber disease cells, whereas normal turnover rates were found in Tay-Sachs disease cells, G_{M2} activator-deficient (variant AB of G_{M2} gangliosidosis) and in sulfatide activator- (*sap*-B-) deficient cells. In G_{M1} gangliosidosis and in *sap* precursor-deficient cells, the lysosomal glycolipid catabolism was found to be strongly retarded; accumulation of individual products could not be seen. Skin fibroblasts from patients with α-N-acetylgalactosaminidase deficiency (Schindler disease) cannot degrade the major blood group A glycolipid.—Asfaw, B., D. Schindler, J. Ledvinová, B. Černý, F. Šmíd, and E. Conzelmann. **Degradation of blood group A glycolipid A-6-2 by normal and mutant human skin fibroblasts. *J. Lipid. Res.* 1998.** 39: **1768–1780.**

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Blood groups O, A, and B are determined by the oligosaccharide groups Fucα1→Gal, GalNAcα1→3[Fucα1→2]Gal, and Galα1→3[Fucα1→2]Gal, respectively, as the non-reducing termini of glycosphingolipids, mainly of the lacto, neolacto, and globo series, and of glycoproteins (1). Cells that carry blood group determinants include erythrocytes, vascular endothelial cells, cardiac and smooth muscle cells, pancreas and, in the case of secretors, epithelial cells of the gastrointestinal and urinary tracts. From the number of blood group determinants on an erythrocyte (estimated at 10^6 per cell (2), approx. 20% of them as glycolipids), total erythrocyte number (approx. 30×10^{12}), and the average half life of these cells of some 120 days, it can be estimated that approximately 0.5 mg of blood group glycolipids must be degraded every day.

Degradation of glycolipids takes place in the lysosome, catalyzed by an array of specific glycosidases. Practically all of these enzymes are exo-glycosidases, which can only remove monosaccharide moieties from the non-reducing end, therefore the degradation has to proceed sequentially. The deficiency of one of the enzymes involved

Abbreviations: A-6-2, blood group glycolipid A-6-2 (GalNAc(α1→3)[Fucα1→2]Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-N-acetylgalactosaminylneolactotetraosylceramide); H-5-2, blood group glycolipid H-5-2 (Fuc(α1→2)Gal(β1→4)GlcNAc (β1→3)Gal(β1→4)Glc (β1→1′)Cer, IV^2-α-fucosylneolactotetraosylceramide); Cer-5, pentaglycosylceramide; Cer-4, tetraglycosylceramide; nLc$_4$Cer, neolactotetraosylceramide; Lc$_3$Cer, lactotriaosylceramide; Lac Cer, lactosylceramide; GlcCer, glucosylceramide; Cer, ceramide (N-acylsphingosine); SM, sphingomyelin; PC, phosphatidylcholine; ApoE, apolipoprotein E$_3$; DMEM, Dulbecco's minimum essential medium; FAB–MS, fast atom bombardment mass spectrometry; FCS, fetal calf serum; mAb, monoclonal antibody; α-NAGA, α-N-acetyl-d-galactosaminidase; PBS, phosphate-buffered saline (0.135 m NaCl, 10 mm Na phosphate buffer, pH 7.4); sap, sphingolipid activator proteins; LSIMS, liquid secondary ion mass spectrometry.

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thus blocks the catabolism at the respective stage, leading to the accumulation of its substrates and, as a consequence, to a lysosomal storage disease. The manifestations and the severity of the disease depend largely on the site and the amount of substrate accumulation. As blood group glycolipids are not negligible in amount, their accumulation may conceivably contribute to the disease process. In those cases where removal of an internal sugar residue is blocked, all blood group glycolipids will be similarly affected and the patient's blood group will not matter. In contrast, defects of either α-galactosidase A (Fabry disease) or α-N-acetylgalactosaminidase (Schindler disease) may be expected to specifically impair the degradation of glycolipids with blood group B or A specificity, respectively, so that the patient's blood group may well make a difference. For Fabry disease, it has indeed been shown that patients with blood group B accumulate, in addition to the major storage compounds globotriaosylceramide and digalactosylceramide, also various blood group B glycolipids in pancreas, tonsils, and urinary sediment (3, 4), and that, statistically, these patients have a shorter life expectancy than those with blood groups O or A (3). It is not yet possible to do comparable studies on α-N-acetylgalactosaminidase deficiency because only a few patients with this disease have been detected so far and all of them are still alive. Even the basic pathogenesis of the disease is essentially unclear as the glycopeptides excreted by the patients have α-N-acetylgalactosamine at internal positions, not as the non-reducing termini (for review, see ref. 5). Glycosphingolipid storage could not be demonstrated in the patients' tissues or in urinary sediment (6). It is not even known whether blood group A glycolipids do accumulate at all in these patients or whether an alternative pathway exists for the degradation of these compounds. Studies on cell extracts or cultured fibroblasts with Forssman glycolipid had shown the expected block in degradation whereas some first studies with a blood group A determinant trisaccharide neoglycolipid had indicated the possibility of a substantial activity towards this substrate (7). It seemed therefore of interest to study the catabolism of blood group glycosphingolipids in normal probands and in patients with lysosomal enzyme deficiencies.

As a first step, we isolated the natural compound, glycolipid A-6-2 (IV^2-α-fucosyl-IV^3-α-N-acetylgalactosaminyl-neolactotetraosylceramide, shorthand nomenclature according to the suggestion of Holgersson, Breimer, and Samuelson (8)) in mg amounts, labeled it by catalytic reduction of the ceramide moiety with tritium gas and studied its turnover in situ, with skin fibroblast cultures from normal controls, from patients with α-N-acetylgalactosaminidase deficiency and from patients with other lysosomal storage diseases. A virtually complete block of blood group A glycolipid degradation in α-N-acetylgalactosaminidase-deficient cells could be demonstrated. In addition to this result, these studies also provided interesting information on the further degradation of this glycolipid and on the roles played by the different sphingolipid activator proteins in this pathway.

MATERIALS AND METHODS

Materials

Outdated human donor blood concentrates (blood group A) 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 from Macherey & Nagel, Germany. HPLC Separon SGX (silica) columns and Separon SGX-C18 cartridges were from Tessek, Czech Republic. The other chemicals, all of them reagent grade, were from Lachema, Czech Republic or Sigma, Germany. Organic solvents were distilled before use. Dulbecco's Minimum Essential Medium (DMEM), fetal calf serum (FCS), and trypsin were from Gibco, Germany. Viable Colostrum-Based Serum Replacement Media without lipoproteins (AC-2 and HC-3) were products of Valio Bioproducts, Finland. Bovine kidney α-l-fucosidase was purchased from Boehringer Mannheim, Germany. Recombinant human α-N-acetylgalactoaminidase was kindly supplied by Dr. Robert Desnick, New York. Recombinant apolipoprotein E_3 was a kind gift from Dr. Tikva Vogel, Bio Technology General, Rehovot, Israel. Mouse monoclonal antibody (mAbs) directed to blood group A determinants was purchased from Exbio, Czech Republic.

Isolation of blood group A-6-2 glycolipid

Isolation of erythrocyte stroma from outdated human blood concentrates was performed by the toluene flotation method according to Koscielak, Miller-Podraza, and Zdebska (9). Total lipids from erythrocyte membranes were extracted with 83% aqueous ethanol and chloroform–methanol–water 60:40:9 (v/v/v) (9, 10). Alkali-labile lipids were removed by mild alkaline methanolysis and extensive dialysis. Neutral and acidic sphingolipids were separated by ion-exchange chromatography on a DEAE-Sephadex column (11). Blood group A glycosphingolipids were separated from the crude neutral glycosphingolipid fraction by high performance liquid chromatography (HPLC) on silica gel column Sepharon SGX with a linear gradient from 83% chloroform/17% solvent B (methanol–water 19:1 by vol) to 100% solvent B. Further purification of crude glycolipid A-6-2 was performed as peracetylated derivatives by HPLC on the same type of column (smaller size) with a linear gradient of 0–6.3 % methanol in chloroform. The purified A-6-2 was deacetylated with sodium methoxide and desalted by reversed phase chromatography (Separon SGX-C18 cartridges) according to Schnaar (12). Isolation and purification steps were monitored by HPTLC with orcinol detection and by thin-layer chromatography (TLC)-immunostaining with monoclonal antibodies (mAbs) recognizing blood group A determinants. The identity of A-6-2 was confirmed by FAB-MS (Drs. A. Suzuki and M. Suzuki, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and TLC–liquid secondary ion mass spectrometry (LSIMS) (Drs. A. M. Lawson and W. Chai, The Glycoscience Laboratory, Harrow, U.K.).

Radioactive labeling

Radioactive labeling of A-6-2 with ^3H at the ceramide moiety was performed by catalytic hydrogenation of double bonds with tritium gas (13) using the apparatus for microhydrogenation (14). A mixture of 0.5 mg glycosphingolipid, 5.2 mg catalyst (10% $Pd/BaSO_4$) and 0.5 ml solvent (dioxane–methanol 2:1, v/v) were mixed together with tritium gas under 80 kPa of pressure. After 60 min the solvent was removed by lyophilization. Then the residue was redissolved in dioxane–methanol 2:1 (v/v), the catalyst was removed by centrifugation, and the supernate obtained was freeze-dried. Further purification (>99%) of the [^3H]A-6-2 glycosphingolipid was performed by HPLC using the same gradient system as above.

Preparation of [^{3}H]glycolipid H-5-2

N-acetylgalactosamine was removed from the [^{3}H]A-6-2 glycolipid by enzymatic degradation with human recombinant α-N-acetylgalactosaminidase and the remaining glycolipid was purified by preparative TLC.

Cell culture

Fibroblasts from patients and control subjects were cultured according to routine procedures in DMEM and 10% FCS in 25 cm^2 culture flasks (Nunc A/S, Denmark).

Protein determination

Protein content in cell homogenates was determined by the method of Bradford (15) using bovine serum albumin as standard.

Enzyme assays

Enzyme activities were assayed in cell homogenates prepared by sonication in water. The substrates were dissolved in citrate buffer of appropriate pH and concentration for each hydrolase (16). Reaction mixture of total volume 100 μl contained 5–10 μg protein of cell homogenates and was incubated according to the activity of each enzyme for 15–60 min. The reactions were terminated by addition of 600 μl of 0.2 mol/l glycine/carbonate buffer, pH 10.6. One enzyme unit is defined as the amount of enzyme that hydrolyzes 1 μmol of this substrate per min.

Cell feeding studies

Liposome preparation. Solutions of 28.8 μmol di-oleoyl-phosphatidylcholine (in chloroform), 0.6 μmol α-tocopherol in chloroform–methanol 2:1, and 0.6 μmol phosphatidic acid (Na salt) were mixed together and dried completely under a stream of N_2 and in a desiccator. After addition of 3 ml of PBS, the liposome suspension was prepared by sonication (Branson sonifier, 200 W, 20% output, microtip, for 15 min). The suspension was transferred to Eppendorf tubes and centrifuged in an Eppendorf centrifuge at maximal speed for 6 min. The supernatant was transferred to another vial and autoclaved (20 min, 121°C).

Preparation of glycolipids for feeding studies. For standard feeding studies, 40 nmol unlabeled and 12 μCi of ^{3}H-labeled A-6-2 glycolipid in chloroform–methanol 2:1 were mixed in 2-ml Eppendorf tubes and dried under vacuum in a desiccator (i.e., sp. act. 0.3 Ci/mmol). The desiccator was opened under sterile conditions, and 10 μl of liposome suspension and 990 μl of culture medium were added to the dried glycolipid. The mixture was sonicated in a cup-horn sonifier (80% output) for 10 min. Finally, 100 μl solution of apolipoprotein E_3 (apoE, 1 mg/ml) was added and mixed. After standing for 15 min at least, the preparation was used for feeding studies. For determination of residual activity in α-NAGA deficient cells, A-6-2 and H-5-2 with very high specific radioactivity (i.e., 15.1 Ci/mmol) were prepared by the same procedure as above and the same ratio of components was kept.

Feeding assays. Confluent monolayer fibroblasts were preincubated in the "application" medium, DMEM with 5% serum replacement AC-2. After 2 days, the glycolipid preparation was applied and cells were incubated for different durations of time as indicated in Results. In feeding experiments with chase, the medium containing the glycolipid preparation was replaced with fresh medium.

Cell harvesting. Incubation was terminated by removing the medium; the fibroblasts were quickly rinsed with 0.25% trypsin in PBS (2 ml for 25 cm^2 flasks) and then incubated with another portion of 0.25% trypsin in PBS (3 ml) for 15 min at 37°C. The cell suspension was transferred to centrifuge tubes, the culture flask was washed with PBS (2 ml), and the washing was added to the cell suspension. The cell pellet was isolated by centrifugation at room temperature at 600 *g*. This procedure proved to be sufficient to remove adsorbed glycolipids from the cell surface.

Extraction and TLC analysis of glycolipids. The cell pellet was transferred into an Eppendorf tube with 0.5 ml H_2O and sonicated for 3 min in a cup-horn sonifier (50% output). Aliquots of cell homogenate, medium, and trypsin-PBS were mixed with scintillation cocktail (Hewlett Packard, 5 ml) and radioactivity was measured in each fraction. The rest of the cell homogenate was extracted with chloroform–methanol 2:1 (2 ml). After vigorous mixing the mixture was centrifuged for 10 min at 1000 *g*. Both the upper and lower phases were aspirated and saved. The interphase was reextracted with another 2 ml of chloroform–methanol 2:1. The combined extracts were evaporated to dryness under N_2. The residues were redissolved in 50 μl chloroform–methanol 2:1 and applied on the HPTLC plate. Chromatograms were developed in chloroform–methanol–H_2O 70:30:5 (by vol) and evaluated by TLC-Linear Radioactivity Analyzer (Raytest, Straubenhardt, Germany).

Enzymatic analysis of pentaglycosylceramide

Bands of pentaglycosylceramide were scraped from the TLC plates and extracted with 3 × 3 ml of chloroform–methanol–water 60:40:9 (by vol). The radioactivity was determined on a small aliquot (90 μl). The solvent was evaporated under a stream of nitrogen and the residue was redissolved in 300 μl chloroform–methanol 2:1 (by vol). Aliquots of 100 μl were transferred to Eppendorf tubes (1.5 ml, "safe lock") and the solvent was evaporated. Ten μl of 10 mm aqueous sodium taurocholate and 10 μl of 100 mm sodium citrate buffer, pH 4.5, were added and the glycolipid was dissolved by mild sonication. After addition of 30 μl of suitably diluted enzyme solution (α-N-acetylgalactosaminidase or α-l-fucosidase, or water for the blanks), the mixtures were incubated overnight at 37°C. The same assays were run in parallel with a similar amount of glycolipid A-6-2 as substrate. After incubation, 200 μl of chloroform–methanol 2:1 (by vol) was added, the samples were mixed vigorously and then centrifuged in an Eppendorf benchtop centrifuge. Upper phases were washed with 150 μl of synthetic lower phase (chloroform–methanol–water 87:12:1.5, by vol), then the combined lower phases were reduced in volume by evaporation and applied to TLC plates (Kieselgel 60, 20 cm). Chromatograms were developed in chloroform–methanol–H_2O 70:30:5 (by vol) and evaluated by TLC-Linear Radioactivity Analyzer (Raytest, Straubenhardt, Germany).

Subcellular fractionation of fibroblasts

After harvesting the cells by trypsinization, homogenization, fractionation on a self-forming Percoll gradient, and assay of the marker enzymes β-hexosaminidase (lysosomes) and 5′-nucleotidase (plasma membranes) were performed essentially as described by Banerjee et al. (17).

RESULTS

Preparation of substrate

Blood group glycolipid A-6-2 (GalNAc(α1→3)[Fucα1→2]Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1′)Cer, IV^2-α-fucosyl-IV^3-α-N-acetylgalactosaminylneolactotetraosylceramide) was isolated from blood group A erythrocyte membranes; final yield was approx. 8 mg, from a pool of 10 L of erythrocyte concentrate. Molecular mass and sugar sequence of the compound were verified by mass spectrometry and the presence of the A blood group epitope by its cross-reactivity with a monoclonal antibody

against blood group A in TLC immunostaining. An aliquot of 0.5 mg was radiolabeled by catalytic reduction of the sphingosine double bond with tritium gas. After repurification of the product by HPLC, the final yield was approx. 0.3 mg of labeled material, with a specific activity of approx. 1.1 TBq/mmol (30 Ci/mmol). Radiochemical purity was >95% as judged from radioscans after TLC (not shown but cf. Fig. 2, panel a).

Establishment of feeding conditions

The glycolipid A-6-2 (1.28 nmol per experiment unless otherwise stated) was incorporated into preformed liposomes corresponding to 32 μmol total lipids, by mild sonication, then a solution of 32 μg apoE was added. This amount of apoE was found to be sufficient for optimal uptake of the liposomes by fibroblasts; higher amounts were also tried but did not further enhance uptake (results not shown). Any increase in the amount of liposomes used, while the amount of glycolipid was kept constant, led to a reduction of the uptake rate (not shown).

The amount of radioactive glycolipid stably associated with the cells increased in an essentially linear fashion with time (**Fig. 1a**) and with the amount of glycolipid added (Fig. 1b). The uptake rates varied to some extent between different series of experiments. In some cases, more than 20% of the radioactive lipid was taken up within 2 days, but in a series of experiments with 12 different cell lines under identical standard conditions (25 cm^2 flasks, 1.28 nmol glycolipid/flask, 2 days feeding time), an average of 4.0% ± 1.3% (2.5–6.2) was found. After 5 days feeding, in a parallel experiment, the average was 9.1% ± 4.2% (3.4–16.3). In both cases, uptake rates were only loosely correlated with the amount of cellular protein present (regression analysis gave r^2 values of 0.19 and 0.30 for the 2-day and 5-day feeding series, respectively).

Degradation of glycolipid A-6-2 by normal cells

Normal fibroblasts metabolized the glycolipid to the array of less polar products expected from lysosomal catabolism, and to some lipids resulting from re-using the liberated sphingosine, mainly sphingomyelin and phosphatidylcholine (**Fig. 2**).

The various peaks of radioactive products could be identified by comparison of their relative mobilities with those of authentic standards (sphingomyelin, phosphatidylcholine, lactosylceramide, glucosylceramide) and/or by their accumulation in cells with lysosomal enzyme deficiencies (see below). The most polar of the products (pentaglycosylceramide, Cer-5) was also isolated from several TLC plates and analyzed by enzymic degradation in vitro. Pure recombinant α-N-acetylgalactosaminidase (α-NAGA) converted the blood group glycolipid into Cer-5 but did not degrade the isolated Cer-5. Conversely, pure α-l-fucosidase did not appreciably attack the blood group glycolipid but converted the isolated Cer-5 efficiently into the next product (tetraglycosylceramide, Cer-4) (**Fig. 3**). Thus, the Cer-5 peak contains only the product of α-NAGA action on glycolipid A-6-2, i.e., glycolipid H-5-2.

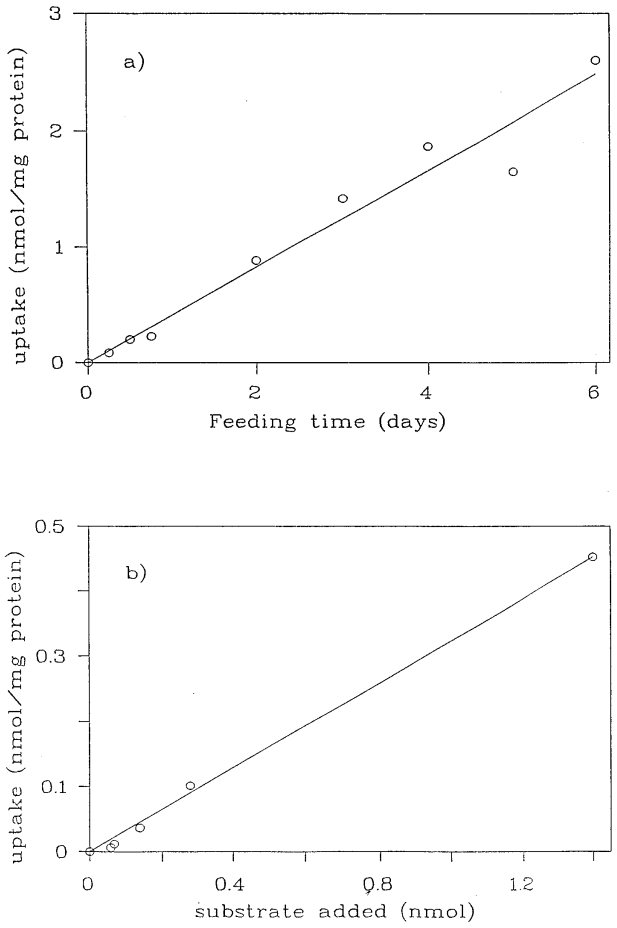

Fig. 1. Uptake of liposome-associated blood group glycolipid A-6-2 by cultured skin fibroblasts as a function of time (a) and of the amount of glycolipid (b). Tritium-labeled glycolipid A-6-2, incorporated into preformed phosphatidylcholine liposomes (1.28 nmol glycolipid/32 μmol phospholipid) with apolipoprotein E (1 μg/μmol total lipid), was added to the culture medium of confluent normal skin fibroblasts in 25-cm^2 flasks. After 48 h (panel b) or after the times indicated (panel a), the cells were harvested, washed, and homogenized in water and their protein content and radioactivity were determined. (For details, see Experimental Procedures.)

Formation of total products was essentially linear with time (cf. Fig. 5a).

The total amount of products formed within a given time was linearly proportional with the amount of substrate taken up (**Fig. 4**), indicating that the rate-determining enzyme (α-NAGA) was far from being saturated.

When turnover rates were expressed as % degradation (i.e., sum of all products as percentage of total radioactivity incorporated), they were fairly constant for each cell line but varied considerably among different cell lines. Incidentally, the one cell line that was used for the initial extensive series of studies (control #1) turned out to have by far the lowest turnover rate of all lines examined (7.8% ± 2.9% per day, n = 24). When the same series of experiments was repeated with another cell line (control #2), turnover rates were reproducibly twice as high (15.5% ± 4.9% per day, n = 10). This figure was still at the lower end of the variability seen in the standard feeding experi-

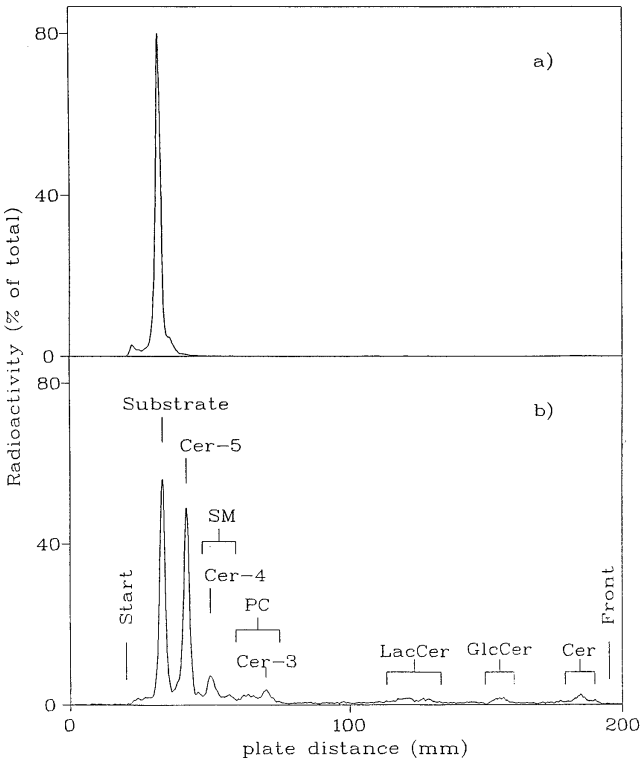

Fig. 2. Degradation of liposome-associated blood group glycolipid A-6-2 by normal skin fibroblasts. Tritium-labeled glycolipid A-6-2 (1.28 nmol), incorporated into preformed phosphatidylcholine liposomes with apolipoprotein E, was added to the culture medium of confluent normal skin fibroblasts in 25 cm^2 flasks. After 5 days, the cells were harvested, the lipids were extracted and separated by thin-layer chromatography. a) Substrate before feeding (blank). b) Lipids extracted from cells. Abbreviations: Cer-5, pentaglycosylceramide; Cer-4, tetraglycosylceramide (nLc_4Cer); Cer-3, triglycosylceramide (Lc_3Cer); SM, sphingomyelin; PC, phosphatidylcholine; LacCer, lactosylceramide; GlcCer, glucosylceramide; Cer, ceramide.

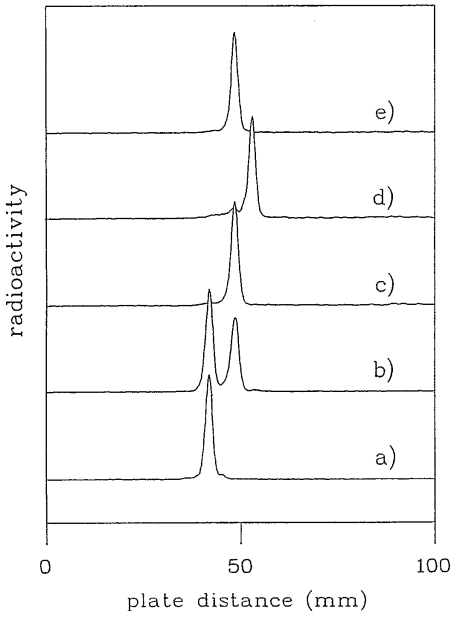

Fig. 3. Degradation of glycolipid A-6-2 and of the pentaglycosylceramide isolated from cells, by purified glycosidases in vitro. After feeding of radiolabeled glycolipid A-6-2 to normal cells for 48 h and TLC analysis of the lipid products (cf. Fig. 4), the Cer-5 bands were scraped from the plates and eluted with organic solvent. Aliquots of 2.8 nmol were incubated in a total volume of 50 μl 20 mm sodium citrate buffer, pH 4.5, containing 2 mm sodium taurodeoxycholate, with 1.5 μg α-l-fucosidase or 4 mU of recombinant human α-N-acetylgalactosaminidase at 37°C overnight. Parallel assays were performed with the same amount of glycolipid A-6-2 as substrate. Products were analyzed by TLC. (For details, see Experimental Procedures.) a) Glycolipid A-6-2 incubated with α-l-fucosidase. b) Glycolipid A-6-2 incubated with α-N-acetylgalactosaminidase. c) Pentaglycosylceramide before incubation. d) Pentaglycosylceramide incubated with α-l-fucosidase. e) Pentaglycosylceramide incubated with α-N-acetylgalactosaminidase.

ment with 12 independent cell lines (controls #3–14), mentioned above. For the 2-day feeding series, an average of 28.3% ± 6.6% per day (range: 18.9–38.4) was found.

Quantitation of individual products was possible only for those that could be well separated by TLC, i.e., H-5-2, lactosyl ceramide (LacCer), glucosyl ceramide (GlcCer), and ceramide (Cer). Tetra- and triglycosylceramide (nLc_4Cer and Lc_3Cer) overlapped with the products of resynthesis, sphingomyelin (SM) and phosphatidylcholine (PC), respectively. The amount of Lc_3Cer was also too small usually to be discerned. The most prominent product peak was nearly always that of H-5-2, indicating that even this unbranched product is a rather poor substrate for α-l-fucosidase (for a more detailed evaluation, see below, Discussion section). The concentrations of other degradation intermediates remained much lower even at prolonged feeding times. The time course of the amounts of individual products is shown for 7 days feeding time in **Fig. 5a**. Glycolipid H-5-2 reached a relatively high steady state concentration after approx. 2 days, the steady state concentrations of other products were considerably lower and were attained later. Resynthesis products (SM, PC) did not reach steady state but accumulated continuously. When the culture medium was replaced by unlabeled medium after 2 days feeding, substrate content in the cells diminished rapidly (Fig. 5b), with a concomitant decrease of the H-5-2 level. Other degradation intermediates still increased slightly, but accumulation of resynthesis products slowed down.

Degradation of glycolipid A-6-2 in enzyme-deficient cells

Degradation of glycolipid A-6-2 was also studied in cultured fibroblasts with deficiencies of lysosomal enzymes or protein cofactors required for glycolipid catabolism ("activator proteins").

In cells from two patients with α-N-acetylgalactosaminidase deficiency (Schindler disease), no significant formation of products could be seen in standard assays (**Fig. 6a**) or in feeding assays with a reduced amount of unlabeled glycolipid (0.77 nmol) and 2–5 days of chase (not shown). The intracellular distribution of the substrate was analyzed by subcellular fractionation of a parallel culture to ascertain that the lack of degradation was due to the enzyme deficiency and not to severely retarded intracellular transport. After 5 days feeding, more than 76% of the ra-

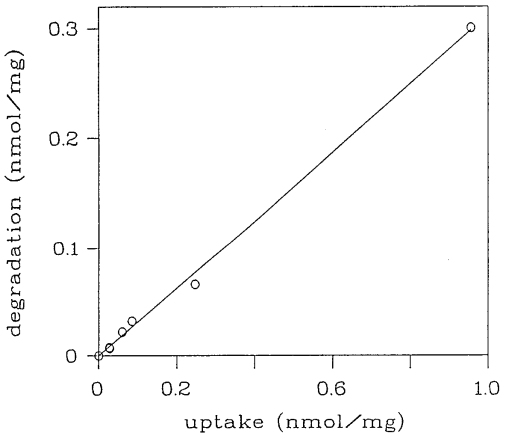

Fig. 4. Degradation of liposome-associated blood group glycolipid A-6-2 by cultured normal skin fibroblasts as a function of the amount taken up. Tritium-labeled glycolipid A-6-2 was incorporated into preformed phosphatidylcholine liposomes as detailed in Experimental Procedures (1.28 nmol glycolipid/32 μmol phospholipid) and apolipoprotein E was added (1 μg/μmol total lipid). Various amounts of glycolipid (see Fig. 1b) were mixed with culture medium and added to confluent normal skin fibroblasts in 25-cm^2 flasks. After 48 h, the cells were harvested, the lipids were extracted and separated by thin-layer chromatography. Bands of substrate and products were measured with a linear scanner.

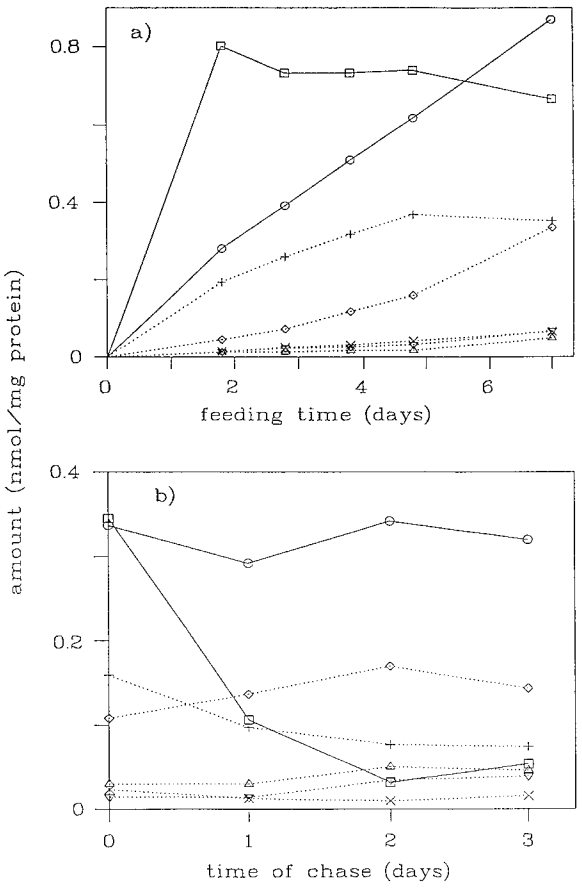

Fig. 5. Product formation from blood group glycolipid A-6-2 by cultured normal skin fibroblasts as a function of time. Tritium-labeled glycolipid A-6-2 (1.28 nmol), incorporated into preformed phosphatidylcholine liposomes with apolipoprotein E, was added to the culture medium of confluent normal skin fibroblasts in 25-cm^2 flasks. a) After the indicated times, the cells were harvested and the lipids were extracted and separated by thin-layer chromatography. b) After 2 days, the medium was replaced by fresh medium without lipids. Incubation was continued for the times indicated, then the cells were harvested and the lipids were extracted and separated by thin-layer chromatography. Bands of substrate and individual products were quantified with a linear scanner. (□—□), glycolipid A-6-2 (substrate); (○—○), sum of all products; (+·····+), glycolipid H-5-2 (pentaglycosylceramide); (◇·····◇), tetraglycosylceramide, sphingomyelin and phosphatidylcholine; (×·····×), lactosylceramide; (△·····△), glucosylceramide; (▽·····▽), ceramide.

dioactivity co-distributed with the lysosomal marker enzyme β-hexosaminidase (not shown but cf. Fig. 8). In a long term experiment with 0.77 nmol substrate per flask and 6 days feeding followed by 7 days chase, small amounts of the usual array of products were detected, corresponding to a degradation rate of some 2.2 to 3.0 pmol per day and per mg protein (0.26% and 0.50%, respectively, of incorporated substrate per day, which is about 1–2% of normal control average). When the same studies were done in the presence of 20 μm conduritol B epoxide, a very potent suicide inhibitor of glucosylceramidase (18), in the medium, a small but significant amount of radioactive glucosylceramide was found to accumulate, corresponding to some 3.4% of total after 5 days of feeding followed by 5 days chase (**Fig. 7**). In normal cells, under the same conditions, the percentage of glucosylceramide amounted to some 43% (plus 12% other products).

In fucosidosis cells, the expected accumulation of the first degradation product, H-5-2, was clearly evident (Fig. 6b). The same qualitative result was obtained with 3 different cell lines; the percentage of H-5-2 ranged between 49% and 68% of total radioactivity after 3 days feeding. Higher magnification of the chromatograms revealed the presence of traces of other, less polar, products (not shown), which together comprised some 10% of total radioactivity.

Cells from patients with G_{M1} gangliosidosis had uptake rates comparable to those of normal cells but degradation was strongly retarded in all three cell lines examined so that, within the usual incubation times (up to 5 days), a significant concentration of nLc_4Cer did not build up (see Fig. 6c for a typical example). On subcellular fractionation of two different cell lines, after 5 days feeding, some 95% of the radioactivity was found to co-distribute with the lysosomal marker enzyme, β-hexosaminidase (shown for one cell line in **Fig. 8**). In one experiment with 5 days feeding followed by 10 days chase, the nLc_4Cer peak accounted for some 11% of total radioactivity (substrate 52%, H-5-2 22%, other products 15%; data not shown).

In cells of the three variants of G_{M2} gangliosidosis, a clear accumulation of triglycosylceramide, Lc_3Cer, was only seen in Sandhoff disease cells (deficiency of both major β-hexosaminidase isoenzymes, A and B, Fig. 6d) whereas both Tay-Sachs disease (deficiency of β-hexosaminidase A only) and variant AB cells (deficiency of the G_{M2} activator protein) produced a normal pattern of products (Figs. 6e and 6f).

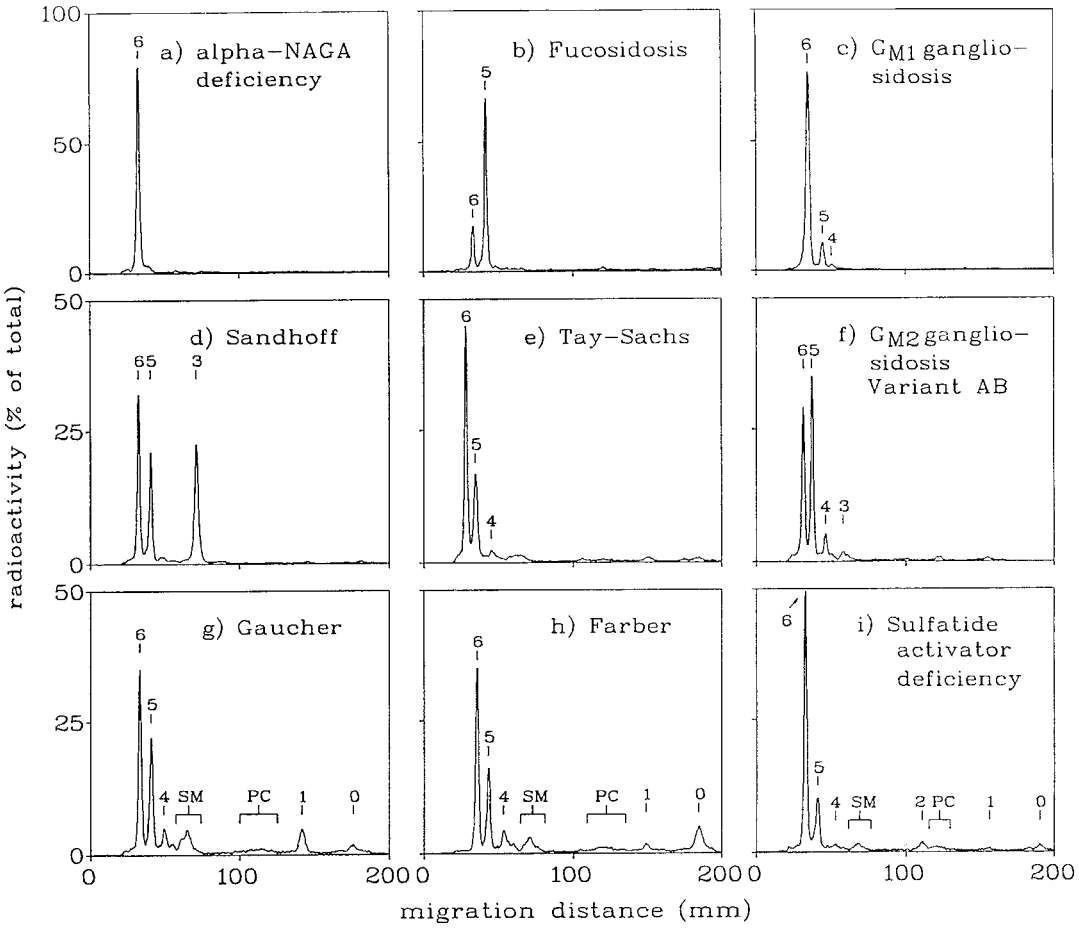

Fig. 6. Degradation of blood group glycolipid A-6-2 by cultured skin fibroblasts from patients with various lysosomal storage diseases. Tritium-labeled glycolipid A-6-2 (1.2 nmol) was incorporated into preformed phosphatidylcholine liposomes as detailed in Experimental Procedures. After addition of apolipoprotein E (1 μg/μmol total lipids), the liposome suspension was mixed with culture medium and added to the confluent skin fibroblasts in 25-cm^2 flasks. After 5 days (10 days in the case of Farber disease cells, panel h), the cells were harvested, the lipids were extracted and separated by thin-layer chromatography. The chromatographic positions of substrate and of the various products are indicated by the number of sugar residues on the glycosphingolipid, from 0 (=ceramide) to 6 (=substrate). PC, phosphatidyl choline; SM, sphingomyelin. a) α-N-acetylgalactosaminidase deficiency (Schindler disease); b) fucosidosis; c) G_{M1}-gangliosidosis; d) deficiency of β-hexosaminidases A and B (Sandhoff disease); e) deficiency of β-hexosaminidase A (Tay-Sachs disease); f) deficiency of G_{M2} activator protein (G_{M2} gangliosidosis variant AB); g) Gaucher disease; h) Farber disease; i) deficiency of sulfatide activator protein *sap*-B (variant metachromatic leukodystrophy).

Cells from patients with Gaucher disease, type 2 (severe infantile type), showed the expected accumulation of glucosylceramide, albeit at low levels (Fig. 6g).

In Farber disease cells (deficiency of ceramidase), after 5 days feeding a small amount of ceramide was found to accumulate. This peak increased significantly in size after another 5 days chase (Fig. 6h).

A normal degradation was found in Krabbe disease cells; in particular, no accumulation of lactosylceramide was seen (not shown).

Also included in this study were cells from patients with deficiencies of either the sulfatide activator protein (19) ("*sap*-B") or of *sap* precursor protein (20), from which four different but homologous *sap* proteins are derived by proteolytic cleavage (21, 22). In sulfatide activator-deficient cells, the degradation rate was on the low side of normal control range (44% of incorporated substrate within 5 days) but the product pattern was normal; no particular accumulation of any intermediate was seen (Fig. 6i).

Of the patients with *sap* precursor deficiency, primary fibroblast cultures were no longer available, owing to the very poor growth of these cells (K. Harzer, personal communication). However, permanent cell lines have been established by SV40 transformation (23) and were kindly supplied, together with three strains of transformed normal cells as appropriate controls, by Dr. T. Levade. The transformed control cells had a rapid uptake (21–30% of total within 2 days) and turnover of the glycolipid (25–50% of the incorporated material after 2 days feeding) with essentially the same product pattern as untransformed fibroblasts (**Fig. 9a**). The *sap* precursor-deficient cells had about the same uptake rate but degradation was considerably slower (2–5% of incorporated material) (Fig.

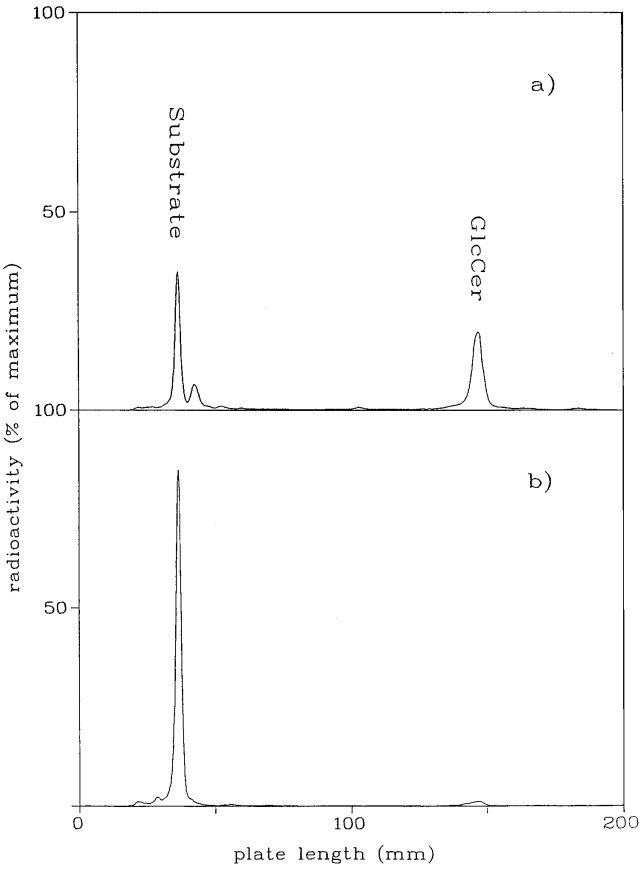

Fig. 7. Degradation of blood group glycolipid A-6-2 by skin fibroblasts in the presence of conduritol B epoxide. Tritium-labeled glycolipid A-6-2 (0.39 nmol) was incorporated into preformed phosphatidylcholine liposomes as detailed in Experimental Procedures. After addition of 30 μg apolipoprotein E, the liposome suspension was mixed with culture medium containing 20 μm conduritol B epoxide and added to confluent skin fibroblasts from normal controls (panel a) or from a patient with α-N-acetylgalactosaminidase deficiency (panel b) in 25-cm^2 flasks. After 5 days the cells were harvested, the lipids were extracted and separated by thin-layer chromatography.

9b). Therefore, the accumulation of less polar sphingolipids (LacCer, GlcCer, and ceramide) reported by Schmid et al. (24) was not seen.

Degradation of glycolipid H-5-2

A few feeding experiments were also carried out with radioactive H-5-2, which had been prepared in vitro from glycolipid A-6-2 by incubation with recombinant α-NAGA and purified by preparative TLC (see Methods section for details). In normal as well as α-NAGA-deficient cells, this substrate was metabolized to the expected array of products (**Fig. 10a** and **b**). In G_{M1} gangliosidosis cells, the turnover was again strongly reduced in comparison to normal cells (4% to 16% of incorporated substrate within 5 days [Fig. 10c] as compared to 30% to 52% for controls). Similarly, the turnover in *sap* precursor-deficient cells was approximately one third of the average in transformed control cells (not shown).

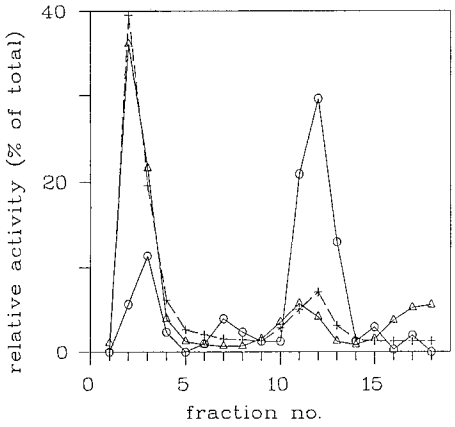

Fig. 8. Subcellular fractionation of G_{M1} gangliosidosis fibroblasts after feeding with liposome-associated blood group glycolipid A-6-2. Tritium-labeled glycolipid A-6-2 (2.56 nmol), incorporated into preformed phosphatidylcholine liposomes with apolipoprotein E, was added to the culture medium of confluent skin fibroblasts from a G_{M1} gangliosidosis patient, in 75-cm^2 flasks. After 5 days, the cells were harvested, homogenized, and subjected to subcellular fractionation in a self-forming isoosmotic Percoll gradient as detailed in Experimental Procedures. Fractions were collected from the bottom of the tube and assayed for β-hexosaminidase (△—△), 5′-nucleotidase (○—○) and for radioactivity (+---+).

DISCUSSION

Feeding radiolabeled glycolipids to cells in culture and analysis of their metabolism has frequently been used for a number of purposes such as general analysis of the metabolic pathways (25, 26), assessment of residual activities in enzyme-deficient cells (27, 28), or diagnosis of storage disorders. This technique was found particularly valuable for the distinction between metachromatic leukodystrophy and arylsulfatase A pseudodeficiency (29) and for the diagnosis of sphingolipid activator protein deficiencies such as variant AB of G_{M2} gangliosidosis (26), sulfatide activator deficiency (19), and *sap* precursor deficiency (20, 24). The initial intention of the present study was to examine whether cells from patients with deficiency of α-N-acetylgalactosaminidase (Schindler disease) would still be able to degrade blood group glycolipids with terminal α-N-acetylgalactosamine (blood group A) and, if so, to what extent. Previous studies with a blood group A active trisaccharide neoglycolipid had indicated the possibility of considerable residual activities in vitro and in situ (7), which might have been sufficient for normal catabolism in vivo. The natural human blood group lipids had not been used as substrates nor had their turnover been studied in intact cells.

Quantitative analysis of the degradation pathway is rather complex because it has to take into account a series of events: incorporation of the lipid into the plasma membrane, either from the micelle (25, 30) or from liposomes (by fusion or endocytosis) (31), intracellular transport to the digestive compartment (endocytosis), a sequence of enzymatic steps in the lysosome and possibly also efflux of products into the cytosol and their use for resynthesis

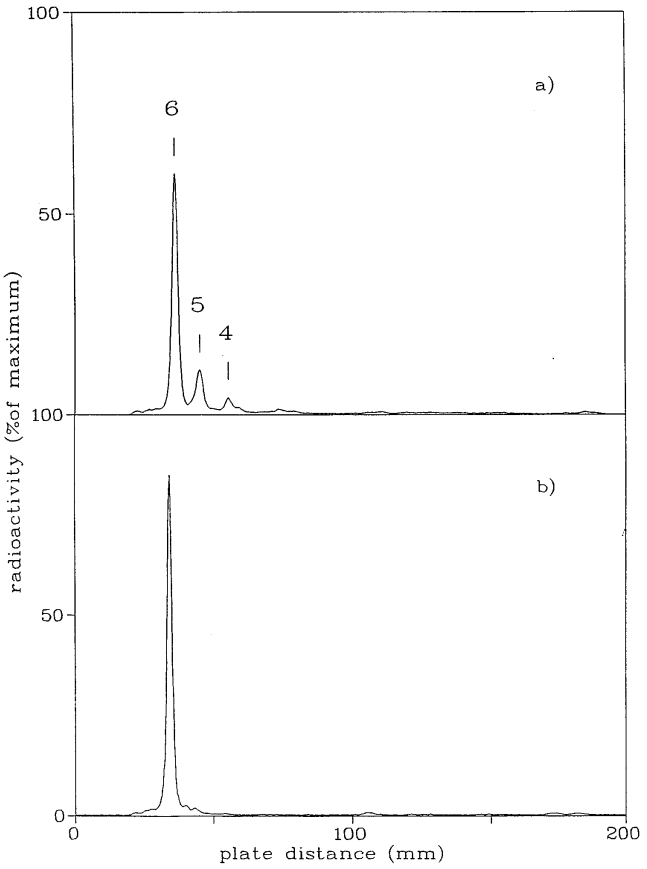

Fig. 9. Degradation of blood group glycolipid A-6-2 by SV40-transformed skin fibroblasts. Tritium-labeled glycolipid A-6-2 (1.2 nmol) was incorporated into preformed phosphatidylcholine liposomes as detailed in Experimental Procedures. After addition of apolipoprotein E (1 μg/μmol total lipids), the liposome suspension was mixed with culture medium and added to confluent cultures of SV40-transformed human skin fibroblasts from a normal control (panel a) or from a patient with *sap* precursor deficiency (panel b), in 25-cm^2 flasks. After 4 days, the cells were harvested, the lipids were extracted and analyzed by thin-layer chromatography.

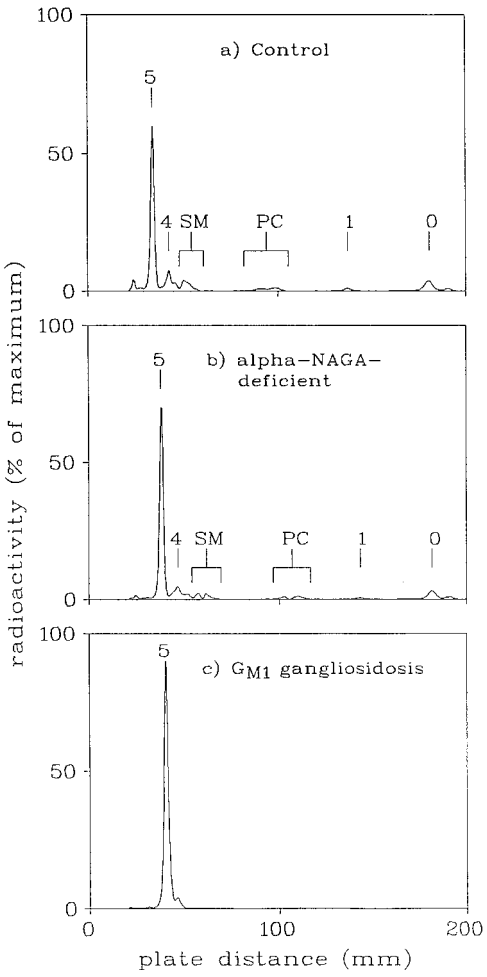

Fig. 10. Degradation of glycolipid H-5-2 by skin fibroblasts from patients with various lysosomal storage diseases. Glycolipid H-5-2 (0.58 nmol), prepared from A-6-2 by enzymatic degradation as described in Experimental Procedures, was incorporated into preformed phosphatidylcholine liposomes. After addition of 30 μg apolipoprotein E, the liposome suspension was mixed with culture medium and added to the confluent skin fibroblasts in 25-cm^2 flasks. After 5 days, the cells were harvested, the lipids were extracted and separated by thin-layer chromatography. Chromatographic positions of individual compounds are labeled as in Fig. 6. a) Normal control. b) α-N-acetylgalactosaminidase deficiency (Schindler disease). c) G_{M1}-gangliosidosis.

(26). Because understanding of at least some of these parameters is critical for the interpretation of the results of feeding studies, we tried to characterize uptake and intracellular transport rates. As vehicles for application of the glycolipids, small unilamellar liposomes with a defined amount of glycolipid and apolipoprotein E were used. If cells are kept in a lipoprotein-free medium for a few days, they express the appropriate receptors in high numbers, leading to efficient uptake of these liposomes (31, 32). In preliminary experiments with normal cells, it was found that uptake rates fluctuated considerably and depended on a number of variables. Particularly, uptake rates were lower when larger amounts of phospholipid vesicles were used and addition of apolipoprotein E beyond a certain amount did not further enhance uptake, suggesting that the number of apoE receptors on the cell surface is a rate-limiting parameter. Apparently, the cells can bind a certain amount of liposomes on their surface; any further binding then depends on endocytosis of receptor and ligand (and hence liposomes) and concomitant reappearance of free receptor. The substantial variation of uptake rates observed among different cell lines could be explained by different rates of endocytosis and intracellular transport and, possibly, different receptor densities on the surface.

For the rate of degradation (i.e., formation of all products observed), the rate-limiting step in normal cells is generally assumed to be not the activity of any lysosomal enzyme but the rate of intracellular transport to the lysosome (28, 33). In the present study, this was corroborated by two findings. *1*) In experiments with the same cell line, the amount of products formed after a given time increased linearly with the amount of substrate taken up (Fig. 4), without any indication of saturation. *2*) Turnover rates (expressed as % degradation per day) were fairly

constant for each of the cell lines but varied up to 5-fold among the different cell lines; variation of α-NAGA activity was less than 2-fold. Thus, the rate at which the substrate is delivered to the lysosomal compartment (and is degraded in normal cells) depends on the concentration of the glycolipid in plasma membrane and related structures and on the individual endocytosis rate. As long as internalized substrate is replenished by absorption from the medium, a rather constant degradation rate ensues. When the external supply is cut off ("chase period"), internalization and hence degradation follows essentially the expected first order kinetics (see Fig. 5).

These considerations also have profound consequences for the interpretation of the minute residual degradation rate seen in cells from Schindler disease patients. Reduction of the activity of any lysosomal enzyme will, within wide limits, have no influence on the turnover rate of its substrates but will merely result in a moderate increase of the substrates' steady-state concentration. Only when the activity falls below a critical threshold (below 5% of normal average in most cases) will it no longer be able to cope with substrate influx and become rate-limiting (28, 33). The turnover rate will, below that threshold, decrease linearly with the residual activity. The actual degradation rate of 1–2% of normal controls, observed in Schindler disease cells, thus means that the enzyme's residual activity is probably below 0.1% of control average. (The residual turnover does, of course, not even have to represent any residual α-N-acetylgalactosaminidase activity but may result from minor side activities of other enzymes such as α-galactosidase.)

A very interesting result was the rather poor action of lysosomal α-l-fucosidase on blood group glycolipids. The A blood group lipid was not attacked by α-l-fucosidase to any significant degree, either in cells with α-NAGA deficiency, or in vitro, with purified enzyme (from bovine kidney). In accord with these results, enzymatic analysis of the pentaglycosylceramide isolated from normal cells demonstrated only the H blood group lipid (H-5-2) but not the defucosylated A-6-2. (The latter might however have been subject to rapid further degradation and thus have escaped detection.) The inability of α-l-fucosidase to attack branched-chain substrates is reminiscent of a similar inability of lysosomal sialidase to degrade gangliosides with sialic acid residues in branching positions. The linear chain product, glycolipid H-5-2, was hydrolyzed by α-l-fucosidase but still appeared to be a rather poor substrate. Determination of the relevant kinetic constants is difficult, because in vitro conditions, particularly the detergents used to disperse the lipid substrates, may alter an enzyme's substrate specificity (34), and was not attempted. However, kinetic analysis of lysosomal degradation as a linear chain of enzyme reactions shows that, after some time, a dynamic equilibrium is reached where each intermediate attains a steady state concentration $[S]_{eq}$ which depends on the flux rate of substrate through the pathway (v_i) and on V_{max} and K_M of the degrading enzyme:

$$[S]_{eq} = K_M/(V_{max}/v_i - 1)$$

(33). In almost all experiments, the radioactivity of the H-5-2 band was much higher than that of any other intermediate (unless degradation had progressed so far that the substrate was depleted). Qualitatively, this is, for example, illustrated in Fig. 2. That this represents indeed a steady state and not an initial build-up of the concentration of the first product (while the others had not yet time to follow) is shown by the quantitative data on the time course for the various products (Figs. 5a and b). That a significant portion of the substrate had already passed through the degradative pathway is also seen in those cases where one of the intermediates was intercepted, particularly the experiments shown in Figs. 6d and 7a. Together these data suggest that α-l-fucosidase can attack the membrane-bound glycolipid substrates only with difficulty, which translates into a high K_M value for such substrates. These considerations cannot be applied to the substrate peak because an unknown share of the as yet undegraded substrate is still located outside the lysosomal compartment, as discussed above.

G_{M1} gangliosidosis cells reproducibly did not show the expected accumulation of the intermediate with terminal β-galactose, nLc_4Cer (except for one long-time incubation experiment), but had a generally very slow turnover. The results of subcellular fractionation showed that this was not due to retarded transport. It was also not restricted to glycolipid A-6-2 but was likewise observed with the fucosidase substrate H-5-2. It thus rather appears that the massive accumulation of other β-galactosidase substrates, mainly oligosaccharides and glycopeptides, strongly interferes with lysosomal catabolism in general. This storage may be much more pronounced in G_{M1} gangliosidosis cells than in the other enzyme-deficient cell lines used in this study because β-galactose is a component of almost all complex glycoconjugates and therefore β-galactosidase substrates are present in large amounts also in fibroblasts.

The accumulation of trihexosylceramide in Sandhoff disease cells was to be expected because these cells lack both major lysosomal β-hexosaminidase isoenzymes, A and B (35) (for review on G_{M2} gangliosidoses, see ref. 36). The normal turnover of the glycolipid in Tay-Sachs disease cells also is not too surprising because glycolipid G_{A2}, which is also a neutral triglycosylceramide, can to a small but significant extent also be hydrolyzed by β-hexosaminidase B (34). Less expected was the practically normal turnover in cells with G_{M2} activator deficiency (variant AB of G_{M2} gangliosidosis). In the few patients examined post mortem, storage of the neutral glycolipid G_{A2} ($GgOse_3Cer$) was comparable to that in Sandhoff patients (37) and feeding studies in cell culture also indicated that this substrate is not further degraded in variant AB cells (26). Possibly, glycolipids with terminal N-acetylglucosamine residues are better substrates than those with N-acetylgalactosamine because, owing to the better solubility of glucose derivatives over galactose derivatives, they are better hydrated and protrude slightly more into the aqueous space. Also, V_{max} of β-hexosaminidases is some 10-fold higher for glucosaminides than for galactosaminides (38). Both fac-

tors may lead to a small activity of β-hexosaminidase B towards the Lc_3Cer derived from glycolipid A-6-2, sufficient to deal with the low throughput rates in our studies.

Cells from patients with deficiencies of other protein cofactors of lysosomal glycolipid degradation were also included in this study because we hoped to get some additional information on the physiologic roles of these proteins. A deficiency of the sulfatide activator protein (also called sphingolipid activator protein B *sap*-B, for review, see ref. 39) leads to a variant form of metachromatic leukodystrophy (19). In vitro studies (40, 41) and the urinary glycolipid excretion pattern of the patients (42) suggest that this activator protein is required not only for the degradation of sulfatide by arylsulfatase A but also of some (less polar) glycolipids by β-galactosidase and by α-galactosidase A. In the present study, no particular storage of any intermediate was found. Apparently, β-galactosidase can degrade the tetraglycosylceramide sufficiently well without the activator. Lactosylceramide, on the other hand, can be hydrolyzed by galactocerebrosidase (43, 44).

Sap precursor protein is the precursor of several protein cofactors needed for lysosomal glycolipid catabolism. In the lysosome, it is cleaved proteolytically into four smaller polypeptides (sphingolipid activator proteins or *sap* A–D). *Sap* B is identical with the sulfatide activator discussed above. The other *sap* proteins were found to be required for activity of galactocerebrosidase (*sap* A), glucocerebrosidase (*sap* C), and ceramidase (*sap* D) (for review, see ref. 39). The deficiency of the precursor was found to block the catabolism of several lipids, including sulfatide, galactosylceramide, glucosylceramide, lactosylceramide, and ceramide (20, 45). In our studies, accumulation of any intermediate could not be seen because, similar to the case of G_{M1} gangliosidosis, overall turnover rates of both cell lines examined were vanishingly small in comparison to normal controls. This phenomenon probably does not reflect specific inhibition of α-N-acetylgalactosaminidase but rather a general impairment of lysosomal catabolism, at least of glycolipids, because it was also apparent with the pentaglycosylceramide, H-5-2. Conceivably, the block in the degradation of major membrane lipids (sphingomyelin, ceramide) also leads to a considerable accumulation of material in fibroblasts, similar to the case of G_{M1} gangliosidosis discussed above. In contrast to our observations, Schmid et al. (24) and Chatelut et al. (46) could show accumulation of catabolic intermediates of ganglioside G_{M1} in primary fibroblasts of these patients (24) as well as in transformed cells (46). The reason for this discordance is not yet clear. It may be related to the different types of substrates (negatively charged vs. neutral) or, more likely, to different processing of liposomes vs. micellar substrate. (From micelles, the glycolipids insert into the plasma membrane and thus become integral components of the endosomal membrane (25) whereas apolipoprotein E-coated liposomes are phagocytosed via receptors as intact particles, which may be more difficult to attack by lysosomal enzymes.)

In Gaucher and Farber cells, the accumulation of glucosylceramide and ceramide, respectively, was clearly visible but was smaller than expected. Possible reasons are that the turnover rates of those cells happen to be in the low normal range and not much substrate has reached these final stages, or that there is some residual activity (or side action of some other enzyme, e.g., unspecific amidase for ceramide). At least in the case of Gaucher cells, the appearance of a distinct ceramide peak and of a small but characteristic phosphatidylcholine peak (cf. Fig. 6g) argues for the latter possibility. In several long-term experiments (5 days feeding, 5 days chase) more than 90% of the incorporated substrate was degraded, indicating sufficiently high turnover rates, but the glucosylceramide peak still comprised less than 10% of total radioactivity. Apparently, even the severe type 2 Gaucher patients have some residual activity. Complete inactivation of β-glucosidase by conduritol B epoxide caused the expected considerable accumulation of glucosylceramide (Fig. 7a).

The results of this study clearly show that loading experiments in cell culture are a valuable tool to analyze general degradation pathways, assess the physiologic significance and the substrate specificity in vivo of the enzymes involved, and estimate their residual activities (or that of alternative degradation pathways) in cases of incomplete enzyme deficiencies. Quantitative interpretation of the results is possible, provided that a number of relevant parameters (uptake rate, intracellular transport, etc.) are taken into account and are carefully monitored. For the initial question of the present study, i.e., whether patients with α-NAGA deficiency can be shown to possess any significant alternative degradative mechanism for blood group A-active glycolipids, the loading experiments gave a clearly negative answer, at least for fibroblasts. As lysosomal enzymes are generally ubiquitous, it seems likely that those patients with blood groups A or AB do indeed accumulate the A-type glycolipids, e.g., in the tissues of the reticuloendothelial system. Whether this is indeed the case and, if so, whether it has any consequences for the pathogenesis remains to be shown.

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