methods

Use of lissamine rhodamine ceramide trihexoside as a functional assay for alpha-galactosidase A in intact cells

Christine R. Kaneski,[1,*] Raphael Schiffmann,[†] Roscoe O. Brady,[*] and Gary J. Murray[§]

National Institute of Neurological Disorders and Stroke,[*] National Institutes of Health, Bethesda, MD; Institute of Metabolic Disease,[†] Baylor Research Institute, Dallas, TX; and Division of Metabolism and Health Effects,[§] National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD

Abstract **Fabry disease is an X-linked disorder caused by mutations in the *GLA* gene encoding for α-galactosidase A (AGA, EC 3.2.1.22). Measurement of AGA enzyme activity using cell homogenates can easily identify men with Fabry disease, but in women, the degree of X-inactivation in the tested tissue may produce activities in homogenates that are indistinguishable from normal. Monti et al. developed a series of lissamine rhodamine-labeled glycosphingolipid substrates that can be used to measure clearance of these lipids in intact cells (1). We report here that one of these substrates, lissamine rhodamine ceramide trihexoside (LR-CTH), can be used as a probe for functional activity of AGA in intact fibroblasts, endothelial cells, and T-lymphocytes from patients with Fabry disease. By utilizing standard detection techniques, such as microscopic imaging, fluorescence microplate spectrophotometry, and flow cytometry, cells with impaired AGA activity can easily be distinguished from wild-type (WT) cells, and these two cell types can be isolated into separate populations using fluorescence-activated cell sorting (FACS). The assay we report here can be adapted to evaluate new therapies by high-throughput screening, can aid in the study of AGA activity in living cells, and can assist in the diagnosis of women with the Fabry trait.—** Kaneski, C. R., R. Schiffmann, R. O. Brady, and G. J. Murray. **Use of lissamine rhodamine ceramide trihexoside as a functional assay for alpha-galactosidase A in intact cells. *J. Lipid Res.* 2010.** 51: **2808–2817.**

Supplementary key words Fabry disease • lysosomal storage disorder • fluorescent substrate • globotriaosylceramide

Glycosphingolipids are important structural components of cellular membranes. Although cells are capable of synthesizing this class of lipids, recycling of components by degradation of preformed lipid molecules is a major contributor to intracellular pools of these lipids (2, 3). Glycosphingolipid molecules are degraded in lysosomes by a series of hydrolytic enzymes that sequentially remove their sugar moieties. Mutations in the genes encoding any of these enzymes result in accumulation of the undegraded substrate within the lysosomes, producing a number of systemic and neurological symptoms.

Fabry disease is an X-linked disorder caused by mutations in the *GLA* gene encoding for α-galactosidase A (AGA, EC 3.2.1.22). As the result of the reduced activity of this enzyme, α-galactosyl-terminal lipids, mainly ceramide trihexoside (CTH), also known as globotriaosylceramide, accumulate in the lysosomes of cells, impairing cardiac and renal function by mechanisms that are still not well understood. Several treatments for this disease, including enzyme replacement therapy, small molecule therapy for substrate reduction, pharmacological chaperones, and genetic therapy, are now available or are being developed (4, 5).

Since the gene is found on the X-chromosome, women with mutations in the *GLA* gene may also be symptomatic, depending on the degree of X-inactivation of the mutant allele in various tissues and on the nature of the mutation (6–9). Measurement of AGA enzyme activity using a fluorescent 4-methylumbelliferyl (4-MU) substrate in cell homogenates can easily identify men with Fabry disease, as residual activity in symptomatic males is usually reduced to less than 20% of normal activity. However, diagnosis in women is more problematic because the degree of X-inactivation in the tissue tested could result in activities that are indistinguishable from normal. Since there are currently over 520 identified mutations that cause Fabry dis-

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Abbreviations: 4-MU, 4-methylumbelliferone; 4-MUG, 4-methylumbelliferyl-α-D-galactopyranoside; AGA, alpha-galactosidase A; CTH, ceramide trihexoside; FACS, fluorescence-activated cell sorting; HI-FBS, heat-inactivated FBS; HTZ, heterozygote; IL-2, interleukin 2; ITS, insulin-transferrin-selenium supplement; LR-CTH, lissamine rhodamine ceramide trihexoside; RFU, relative fluorescence unit; UNK, unknown; WT, wild type.

[1]To whom correspondence should be addressed.
e-mail: Christine.Kaneski@baylorhealth.edu

ease (10), genetic screening of women suspected of having the disorder requires a complete sequencing of the *GLA* gene unless the familial mutation is known. In addition, Filoni et al. recently reported a patient and his sister with symptomatic disease but with no mutation in the *GLA* gene (11). Overproduction of a splice variant of the AGA resulted in interference of the function of the normal enzyme with resultant accumulation of CTH.

Monti et al. developed a series of fluorescent glycosphingolipid analogs labeled with lissamine rhodamine (LR) linked to the sphingosyl moiety of these lipids (1) that can be used as probes for trafficking of these lipids in intact cells. Cells with defects in the degradation of glycosphingolipids retain the fluorescently-labeled substrate, whereas normal cells process these analogs to fluorescent ceramide, which is released into the medium (12). This series of probes has been used to study the functional properties of the defective enzyme in Gaucher disease using thin-layer chromatography and microscopy (13–15), as a label for transgenically corrected cells in Niemann-Pick type B disease (16) and Fabry disease (17), and as a general detection assay for lysosomal storage disorders (12). Since lissamine rhodamine has peak excitation and emission wavelengths of 564 and 583, respectively (18), this compound is easily detectable by standard fluorescent techniques such as microscopy, spectrophotometry, and flow cytometry. We report here that labeling cultured cells with lissamine rhodamine CTH (LR-CTH) can be used as a functional assay of AGA activity in intact cells of various types, including fibroblasts, endothelial cells, and T-lymphocytes; and that mutant and normal cells can be easily distinguished in a mixed populations of cells, such as those found in Fabry heterozygotes.

MATERIALS AND METHODS

Preparation of substrate

LR-CTH (MW = 1502) was custom synthesized by Matreya, LLC (Pleasant Gap, PA) according to the method of Monti (1). It was provided in chloroform-methanol solution at a concentration of 1 mg/ml. The substrate was prepared in two lots designated 20638 and 20696. Because fluorescence intensity differed significantly between lots, lot 20696 was used for all experiments.

Cell cultures

Primary cell cultures from patients diagnosed with Fabry disease, female relatives of Fabry patients, and normal volunteers were established at the Developmental and Metabolic Neurology Branch, National Institute of Neurological Disorders and Stroke under IRB-approved protocols. All subjects gave written informed consent. Fibroblast cultures were obtained by explant culture of skin punch biopsies and were maintained on MEM-Alpha medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (HI-FBS, Hyclone, Logan, UT). Heat-inactivated serum was used to reduce uptake of active α-galactosidase present in untreated serum. For culture during loading experiments, medium was changed to DMEM without phenol red supplemented with 10% HI-FBS (experimental medium). Dermal microvascular endothelial cells were established from skin biopsies by the method of Normad and Karasik (19) and were maintained on Vasculife VEGF-Mv cell culture medium without phenol red (Life Technologies, Walkersville, MD) using the manufacturer-supplied supplements that included 5% FBS. Endothelial origin was confirmed by cobblestone morphology and positive staining for PECAM and von Willebrand factor. T-cell lymphocyte cultures were established from phytohemagglutinin-stimulated white blood cells isolated from heparinized blood by centrifuging over Lymphocyte Separation Medium (Lonza, Walkersville, MD) according to the manufacturer's instructions. Phytohemagglutinin-M was obtained from Gibco Invitrogen and used at the manufacturer-recommended concentration. Cultures were maintained in RPMI-1640 medium (Gibco Invitrogen) supplemented with 2 mM L-glutamine, 10 ng/ml interleukin-2 (IL-2, PreProTECH, Rocky Hill, NJ), and 10% HI-FBS.

Preparation of LR-CTH/BSA complexes

BSA complexes of LR-CTH were prepared by a modification of the method of Monte (1). For use in culture, 100 nmoles of LR-CTH in chloroform-methanol was dried under nitrogen in a glass vial and resuspended in 25 μl of DMSO or ethanol. This solution was heated to 60°C for 10 min, and then 300 μl of fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO, 20 mg/ml in PBS) was added to give a solution of 308 nmoles/ml LR-CTH in a 1:1 molar ratio with BSA. After incubation at 37°C for 20 min, the complexes were added as a stock solution to prewarmed (37°C) serum-free medium at the required concentration.

Loading of LR-CTH into adherent cells

To load LR-CTH into fibroblasts or endothelial cells, confluent cultures were washed once with PBS, and then refed with 0.150 ml/cm^2 LR-CTH/BSA at the indicated concentration (3.08 nmol/ml for most experiments). For fibroblasts, LR-CTH/BSA was diluted in a serum-free DMEM medium without phenol red and with a 1% insulin-transferrin-selenium supplement (ITS, Sigma Chemical Co., St. Louis, MO). For endothelial cells, LR-CTH/BSA was diluted in human endothelial-SFM (Invitrogen) supplemented with 10 ng/ml basic fibroblast growth factor (PreProTECH, Rocky Hill, NJ). The cultures were then incubated overnight (approximately 18 h), washed twice with PBS, and processed according to the type of fluorescence determination required. In all experiments, culture medium was changed daily to prevent reuptake of fluorescent metabolites. Cultures were allowed to equilibrate in a 37°C, 5% CO_2 incubator for at least 1 h after feeding before fluorescence determinations.

Unless otherwise noted, for experiments utilizing a fluorescence plate reader, cells from each well of a 24-well plate were trypsinized, neutralized with experimental medium, and pelleted (200 *g* for 5 min) in individual microfuge tubes. The cell pellet was washed once with PBS and resuspended in 500 μl of experimental medium. Then 150 μl of cell suspension was transferred to each of 3 wells of a 96-well, clear bottom, black microplate (Optiplate, Perkin-Elmer, Waltham, MA). To suspend cells for flow cytometry, cultures were trypsinized, neutralized with experimental medium, pelleted, and resuspended in serum-free DMEM without phenol red at a concentration of 250,000 to 500,000 cells/ml. For imaging experiments, loaded cells were trypsinized, pelleted, and seeded onto 2-well glass chamber slides (NUNC, Rochester, NY) in experimental medium.

Loading of LR-CTH into suspension cultures

For experiments with T-lymphocytes, suspension cultures in log-phase growth were pelleted and resuspended at 1×10^6 cells/ml in RPMI-1640 medium (Gibco Invitrogen) supplemented with ITS and IL-2. After 18 h incubation, the cells were collected by centrifugation, washed twice with PBS, and resus-

pended in fresh RPMI-1640 medium with 10% HI-FBS at a concentration of 1×10^6 cells/ml. For plate reader experiments, 500 μl of cell suspension were pelleted in a microfuge tube, and then resuspended in an equal volume of PBS plus 5% FBS. Then 200 μl of cell suspension were transferred to each of 2 wells of a clear bottom, black, 96-well Optiplate. The cells from the parent culture were counted, and results were expressed as relative fluorescence units (RFU) per one million cells. For flow cytometry, 0.5 ml of cell suspension was pelleted and resuspended in an equal volume of PBS with 5% FBS.

Fluorescence measurements

For tissue culture plates, total fluorescence of individual wells was measured using a Cytofluor II fluorescence microplate scanner (Applied Biosystems, Norwalk, CT) equipped with a low-pressure mercury vapor lamp. Fluorescence was measured with an excitation filter of 530 ± 25 nm combined with an emission filter of 590 ± 35 nm. In each experiment, background fluorescence of cells was determined using control wells containing parallel cultures in medium without substrate. Results are reported as RFUs minus the blank. In some experiments, results are reported as percentage of initial values to adjust for possible variability in cell numbers.

Fluorescence-activated cell sorting (FACS) analysis of cell suspensions was performed using either a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) or a Guava PCA (Millipore, Billerica, MA) as indicated. The FACSCalibur was equipped with a green laser with an excitation of 488 nm. Fluorescence was monitored in the FL-2 channel at 585 ± 42 nm. The Guava PCA was equipped with a 532 diode laser for excitation. Fluorescence was monitored in the PMT-1 channel using a PMT detector at 580–583 nm. Cells not treated with fluorescent substrate were used to establish baseline fluorescence. At least 10,000 cells were collected for the FACSCalibur and 2,000 cells for the Guava PCA. Analysis of data for both systems was performed using WinMDI software. Results are reported as either arbitrary fluorescence units or as percentage of the cell population above normal. The fluorescence gate for normal was set to exclude 95% of the fluorescent cells in the most fluorescent normal control in the experiment.

Microscopic images of fluorescent cells were taken using a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) with rhodamine filter set consisting of a 546 nm broadband excitation filter, 580 nm interference filter, and 590 nm emission filter. Images were acquired using a SpotRT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Measurement of AGA activity in cell homogenates

The standard fluorometric assay for AGA in cell extracts was performed as previously described (20). Briefly, cell pellets were homogenized with citrate-phosphate buffer (28 mM citric acid/44 mM disodium phosphate/5 mg/ml sodium taurocholate, pH 4.4). After centrifuging at 14,000 *g* for 10 min, aliquots of the supernatant were incubated with 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (4-MUG, Research Products International, Mount Prospect, IL) at pH 4.5 in the presence of 0.1 M N-acetylgalactosamine (Research Products International), a specific inhibitor of α-galactosidase B (21). Fluorescence in samples was compared with 4-MU standards in 0.1 M glycine stop buffer using a Cytofluor II plate reader with excitation filter of 355 nm and emission filter of 490 nm. Protein levels in extracts were determined using the MicroBCA kit (Pierce, Rockford, IL) according to the manufacturer's instructions. AGA activity is expressed as nmoles 4-MU formed/hour/mg protein.

Statistics

Z-scores of measurements were calculated as the difference between the value of the sample and the value of the normal divided by the standard deviation of normal. Z-scores above 3.0 were considered to be significant.

RESULTS

Loading conditions

Previously published techniques for the use of LR substrate for the evaluation of enzyme activity in Gaucher disease and other lysosomal storage disorders used incubation times from 6 h to 96 h (1, 15). To determine the optimal incubation time needed to load cells with fluorescent substrate, normal and affected fibroblasts were incubated with 3.1 nmol/ml LR-CTH in DMEM-SF for 30 min, 4.5 h, and 18 h. Appearance of fluorescence in the cells was monitored by fluorescence microscopy. Although intracellular fluorescence was detectable after 30 min of loading, longer incubations provided the largest visible difference in fluorescence between normal and Fabry fibroblasts (data not shown); therefore 18 h was selected as a standard loading time for subsequent experiments.

Dose response curve

A typical dose response curve in normal and Fabry fibroblasts measured by microplate reader at 6 h postloading is shown in **Fig. 1**. At all concentrations tested, Fabry cells showed increased cellular fluorescence compared with normal. To optimize loading conditions, addition of substrate complexed with BSA was compared with direct addition of an identical concentration of LR-CTH in ethanolic solution to serum-free medium. When measured in a fluorometric plate reader, the fluorescence signal from cells in two normal and two affected cells lines averaged 1.7 times higher (range 1.15–1.99) in the cells loaded with BSA complexes compared with the signal obtained from cells exposed to the same concentrations of substrate dissolved in ethanol alone (data not shown). As differences in fluorescence between normal and Fabry fibroblasts could be readily detected as early as 6 h postloading at concentra-

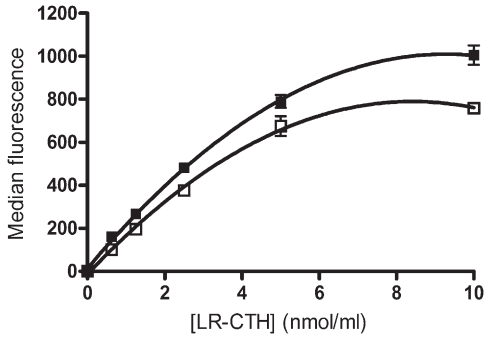

Fig. 1. Dose response curves of normal (□) and Fabry (■) fibroblasts exposed to LR-CTH in culture medium. After 18 h incubation, cultures were fed with fresh DMEM-10% HI-FBS. After 6 h, duplicate cultures were trypsinized and suspended in PBS plus 5% Cosmic Calf Serum. Median fluorescence of 2,000 cells was determined using the PM-1 channel of a Guava PCA. Data represent the median fluorescence of duplicate wells. HI-FBS, heat-inactivated FBS; LR-CTH, lissamine rhodamine ceramide trihexoside.

tions of 3–4 nmol/ml, this range was selected as the standard loading dose.

The necessity for transfer of cells to fresh plates before analyzing them with the microplate reader was determined by reading the plates before and after trypsinization. In 48-well plates, an average of 32% of the total fluorescence (range, 22-41%) remained in the wells after the cells had been removed by trypsinization and the wells had been washed twice with PBS. Assaying for residual protein in the same plates by addition of 25 μl NaOH followed by 150 μl of MicroBCA reagent resulted in no detectable protein reaction, indicating that the cells had been completely removed. These experiments demonstrated that a significant amount of fluorescent substrate attached to the plastic and was not removed by a simple PBS wash. Therefore, in all experiments, the cells were replated into fresh wells after loading with LR-CTH.

Time course of LR-CTH degradation

Because LR-CTH is labeled on the lipid moiety, normal fibroblasts are able to sequentially remove the attached saccharides, leaving fluorescent ceramide (LR-ceramide), which is released into the medium (1, 12). In cells with deficient AGA activity, the glycolipid cannot be degraded and remains in the cell. To determine the rate of intracellular degradation of the substrate under our standard experimental conditions, a pulse-chase experiment was performed. Fibroblasts were seeded in 24-well plates, labeled with LR-CTH for 18 h, and transferred to fresh 96-well plates as described in "Materials and Methods." Fluorescence measurements of individual wells were taken 2.5 h after replating, daily for 4 days, and then at 2–4 day intervals for a total of 14 days. Viability of cells was monitored by microscopic observation. The results are presented in **Fig. 2**.

As intracellular loading is a balance of uptake and degradation, initial intensity of normal cell lines after the initial 18 h load was much lower than that of the Fabry lines after the pulse period. After the initial pulse, there was a rapid loss of intracellular fluorescence in all cell lines once the substrate was removed (Fig. 2). In normal controls (n = 3), 82% ± 1% of the initial intracellular fluorescence was lost in the first 24 h. By 48 h after the initial loading phase, fluorescence intensity in normal controls was indistinguishable from background. In Fabry fibroblasts (n = 4), 53% In order to 6% of the initial fluorescence was lost in the first 24 h; however, in three of the four Fabry lines tested, the decrease in fluorescence intensity reached a plateau by day 4. These cell lines maintained a fluorescence intensity of 44% ± 4% of the initial value over the next 10 days without further decline. In the fourth cell line, there was a slow decrease in fluorescence, from 56% of the initial value on day 2 to 14% on day 14, indicating that degradation of substrate in this patient was impaired but not blocked.

Correlation with residual enzyme activity

Results of the time course experiment suggested that the amount of intracellular fluorescence lost in the first 48 h could be correlated with the residual AGA activity mea-

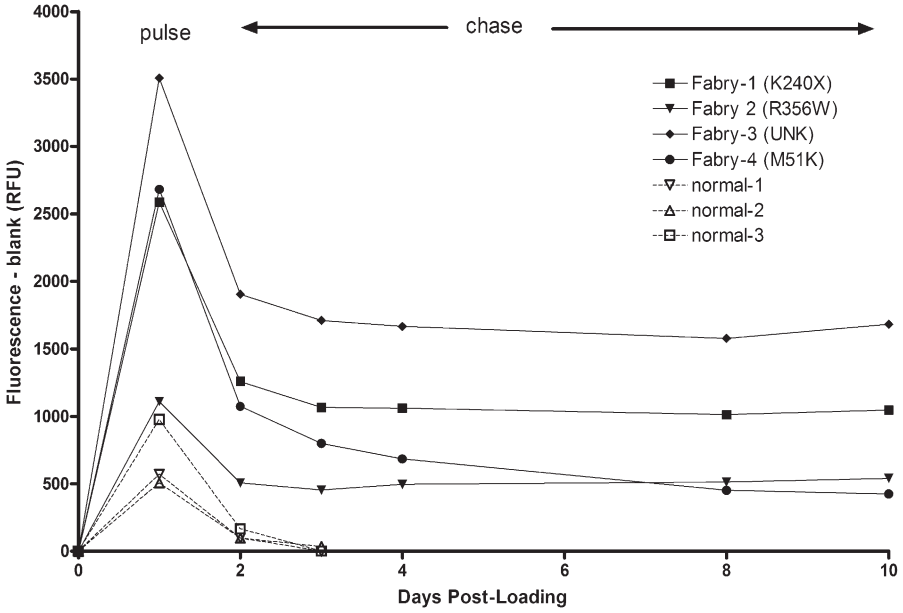

Fig. 2. Time course of LR-CTH degradation after loading. Normal (n = 3, dashed lines, open symbols) and Fabry (n = 4, solid lines, closed symbols) fibroblasts were loaded for 18 h in 3.1 nmol/ml LR-CTH and transferred to 96-well plates as described in "Materials and Methods." After 2.5 h, medium was replaced with fresh DMEM-10% HI-FBS without phenol red, and total fluorescence was determined using a Cytofluor II plate reader. Background fluorescence was determined using triplicate wells plated with the corresponding untreated cells. At each time point indicated, medium was replaced with fresh medium, and fluorescence was determined. Each point represents the average of triplicate wells. HI-FBS, heat-inactivated FBS; LR-CTH, lissamine rhodamine ceramide trihexoside.

sured by the 4-MUG assay. Therefore, loss of intracellular fluorescence intensity was compared with the standard 4-MUG activity assay in cellular homogenates from normal and Fabry fibroblasts. These results are presented in **Table 1**. Fluorescence intensity from LR-CTH at 48 h after the 18 h loading period corresponded well with residual activity measured by the standard 4-MUG assay in homogenates of Fabry and normal cultures grown in parallel. Fabry cells could easily be distinguished from normal with a mean Z-score of 37.4 ± 1.60. These results indicate that metabolism of LR-CTH in intact cells was correlated with enzymatic activity of AGA in normal and mutant cells.

One Fabry cell line (A97V mutation) with high residual AGA activity (12.9% of normal control) showed rapid degradation of the LR-CTH substrate compared with other Fabry lines (data not shown). Although residual fluorescence was still significantly higher than normal in this patient (Z-score = 5.1), these results indicate that the assay may need further optimization to detect patients with higher residual activities. Attempts to improve discrimination by decreasing incubation time or increasing substrate load by the addition of unlabeled CTH did not significantly increase retention of labeled substrate in this patient (data not shown).

Processing of LR-CTH in female heterozygotes

Because Fabry is an X-linked disorder, women with the Fabry trait have a mixed population of cells, both those expressing the mutant enzyme and those expressing the normal enzyme. Loading with LR-CTH was used to examine heterozygote status in several cell lines derived from female relatives of Fabry patients. Fibroblast cells from three mother and son pairs and two additional obligate heterozygotes were probed with LR-CTH. As shown in Table 1, fluorescence intensity from LR-CTH measured by a microplate reader at 48 h after loading was intermediate between normal and affected cell lines in four out of five heterozygous lines tested. In the fifth heterozygous patient, AGA activity against 4-MUG in fibroblast culture was 56% of normal; however, LR-CTH processing did not appear to be impaired and fell within the normal range. These results indicate that AGA activity in homogenates from heterozygotes may not necessarily reflect the intracellular activity found by LR-CTH probing. Because the cultures were grown in parallel, it is possible that subtle differences in culture conditions might have affected intracellular enzyme activity in the two cultures or that the proportions of normal and mutant cells may have differed. However, when used together, these two assays could detect heterozygosity in all females tested.

When male affected and female normal cells were mixed in known proportions and labeled with LR-CTH, total fluorescence intensity was proportionate to number of affected cells in the mixed population (**Fig. 3**). In this experiment, fluorescence intensity in the wells with even the lowest concentration of mutant cells (12.5% of the total population) was greater than the background by more than 2.5 standard deviations. Measurements of activity against 4-MUG in homogenates of parallel cultures did not achieve this level of significance until mutant cells composed 50% of the cell population. Cultures containing mixtures that were 12.5%, 25.0%, and 37.5% mutant cells had AGA activities against 4-MUG in homogenates that were 103.5% ± 1.1%, 94.2% ± 7.8%, and 82.3 ± 3.3% of the activity in normal fibroblasts, respectively.

Imaging experiments show that as long as five days after labeling with LR-CTH, fibroblast cultures from the female relative of a Fabry patient contain a mixed population of labeled and unlabeled cells (**Fig. 4A**). A more precise analysis of proportions of normal and mutant cells was possible using FACS analysis. Fig. 4B shows a dot plot of fluorescence distribution in normal, mutant, and heterozygous fibroblast populations. When the fluorescence distribution was gated to exclude 95.0% of the normal cells (negative), 3.3% of affected (R409C) fibroblasts were in the

TABLE 1. Comparison of AGA activity in fibroblast homogenates with residual LR-CTH fluorescence in intact cells after 48 h postloading

	Mutation	AGA Activity (nmol/h/mg)	LR-CTH (% initial)	Z-Score
Fabry Male				
Fabry-aff-5	S345X	3.6	34.0%	39.1
Fabry-aff-6	G144V	4.0	35.1%	37.3
Fabry-aff-7	UNK	4.9	36.6%	35.9
Fabry Female				
Fabry-htz-5-mo	S345X/WT	10.4	24.5%	24.5
Fabry-htz-6-mo	G144V/WT	38.9	4.3%	0.3
Fabry-htz-7-mo	UNK/WT	no data	15.7%	13.9
Fabry-htz-8	W162X/WT	33.8	19.5%	18.5
Fabry-htz-9	UNK/WT	35.3	15.3%	13.5
Normal Control				
NML-1	WT	99.8	3.4%	−0.8
NML-2	WT	46.7	5.0%	1.11
NML-3	WT	61.9	3.9%	−0.3
Mean Normal		69.5 ± 27.3	4.1% ± 0.8%	

aff, affected; AGA, alpha-galactosidase A; htz, heterozygous; LR-CTH, lissamine rhodamine ceramide trihexoside; mo, mother; NML, normal control; UNK, unknown; WT, wild type.

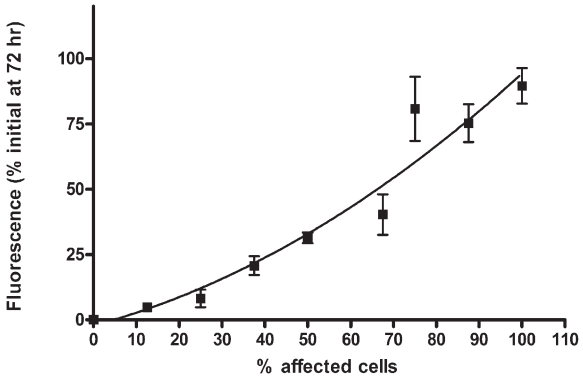

Fig. 3. Detection of mixed populations of normal and Fabry fibroblasts. Cultures of female normal and male Fabry fibroblasts were trypsinized, counted, mixed in known proportions, and seeded in 24-well plates. Cells were loaded with LR-CTH for 18 h in serum-free medium, then transferred to 3 wells of a 96-well plate. Fluorescence was determined at 4.5 h (initial) and again at 72 h. The results are expressed as the mean and standard deviation of the percent of the initial fluorescence remaining. LR-CTH, lissamine rhodamine ceramide trihexoside.

negative range, and 96.7% were in the positive (affected) range. In the heterozygous population, 68.6% of the cells were found to be in the normal range, with the remaining 31.4% in the affected range (Fig. 4B). The initial activity against 4-MUG in homogenates from fibroblasts from this patient was 82% of normal, which was nondiagnostic for carrier status. This patient was subsequently found to have a *GLA* mutation (Q283P). When LR-CTH loading was used in combination with the 4-MUG assay, more accurate diagnosis of heterozygous females could be obtained.

Separation of normal and mutant populations

As two populations could be distinguished by FACS analysis, heterozygous fibroblasts were labeled with LR-CTH, and the cells were separated and grown as two populations, each enhanced for cells expressing either the normal or mutant trait. Fibroblasts from a heterozygous female (W162×/WT) were loaded with LR-CTH for 18 h and then incubated without substrate for 72 h. Approximately 25,000 cells from the upper 1/3 (bright) and lower 1/3 (dark) of the fluorescent populations were collected by FACS using a FACSCalibur flow cytometer as described in "Materials and Methods" (**Fig. 5A**). Fig. 5B shows the labeling of cells in the unsorted and the two sorted populations at two days postsorting (five days postloading). Fig. 5C shows the results of the 4-MUG enzyme assay performed in the fibroblasts from this heterozygous patient at the second passage postsorting. Activities were compared with normal fibroblasts from two controls and fibroblasts from a related affected male (W162×). In fibroblasts from the heterozygous patient, AGA activity in cells that retained the label (bright cells) was severely reduced to 11.7 nmol/h/mg compared with the average normal activity (162.7 nmol/h/mg). AGA activity in cells that lost the fluorescent label (dark cells) was 154.5 nmol/h/mg, 94.9% of the normal average. These results indicate that retention of the fluorescent label was correlated with impaired intracellular AGA activity.

Evaluation of intracellular AGA activity in T-lymphocytes and endothelial cells

As a functional assay, loading with LR-CTH could also be used to determine functional AGA activity in cell types

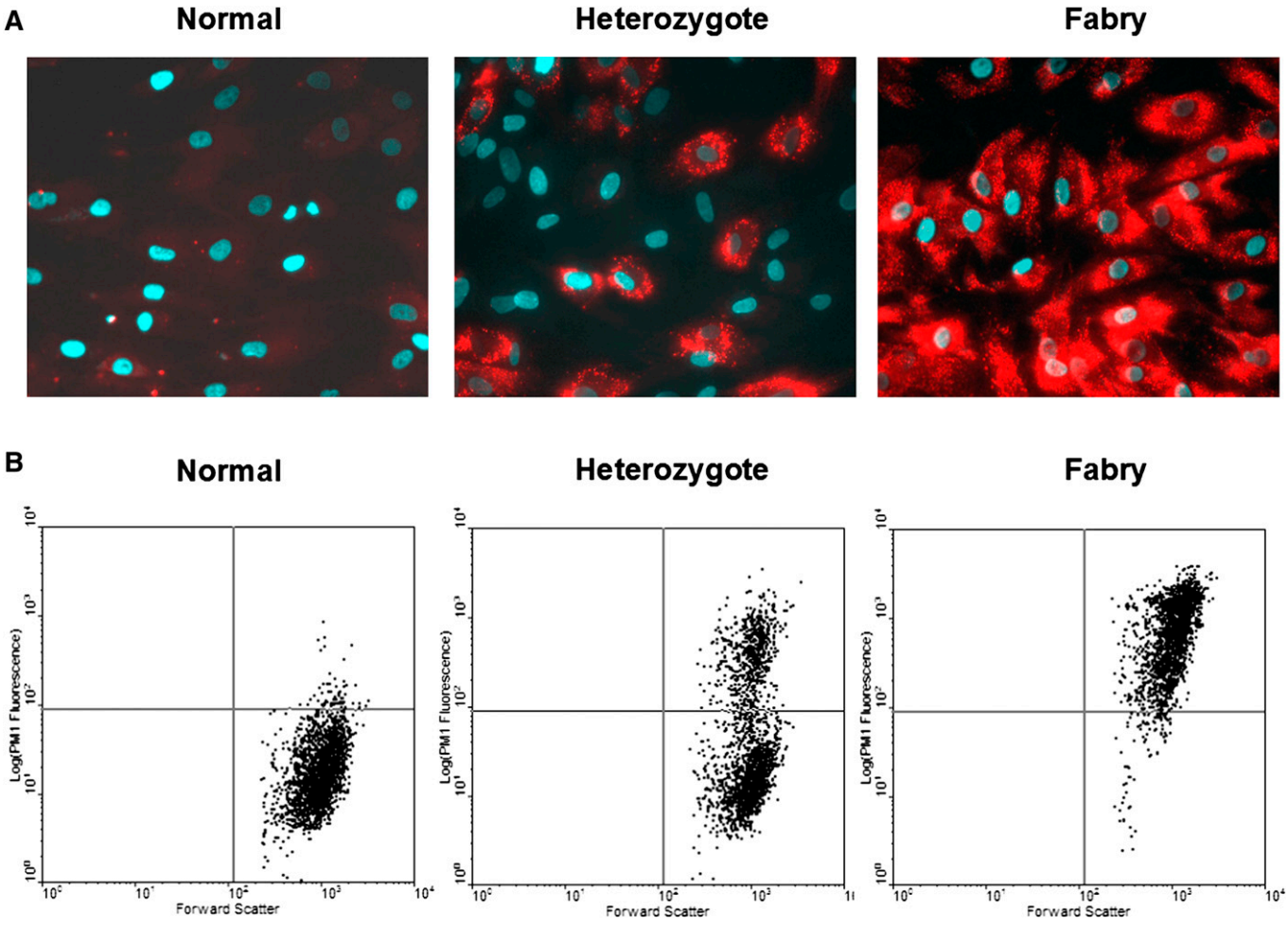

Fig. 4. A: Fibroblasts from normal, heterozygous, and Fabry fibroblasts were seeded on glass slides, probed with LR-CTH for 18 h, then fed with fresh media and imaged with fluorescence microscopy as described in "Materials and Methods." At 5 days postloading, images were taken at 40×. B: Parallel cultures of the same cell lines were probed with LR-CTH, and 5,000 cells were analyzed for fluorescence intensity using a Guava PCA. Quadrants were set to exclude 95% of normal cells from the top left (positive) quadrant. Results are expressed as relative fluorescence units. LR-CTH, lissamine rhodamine ceramide trihexoside.

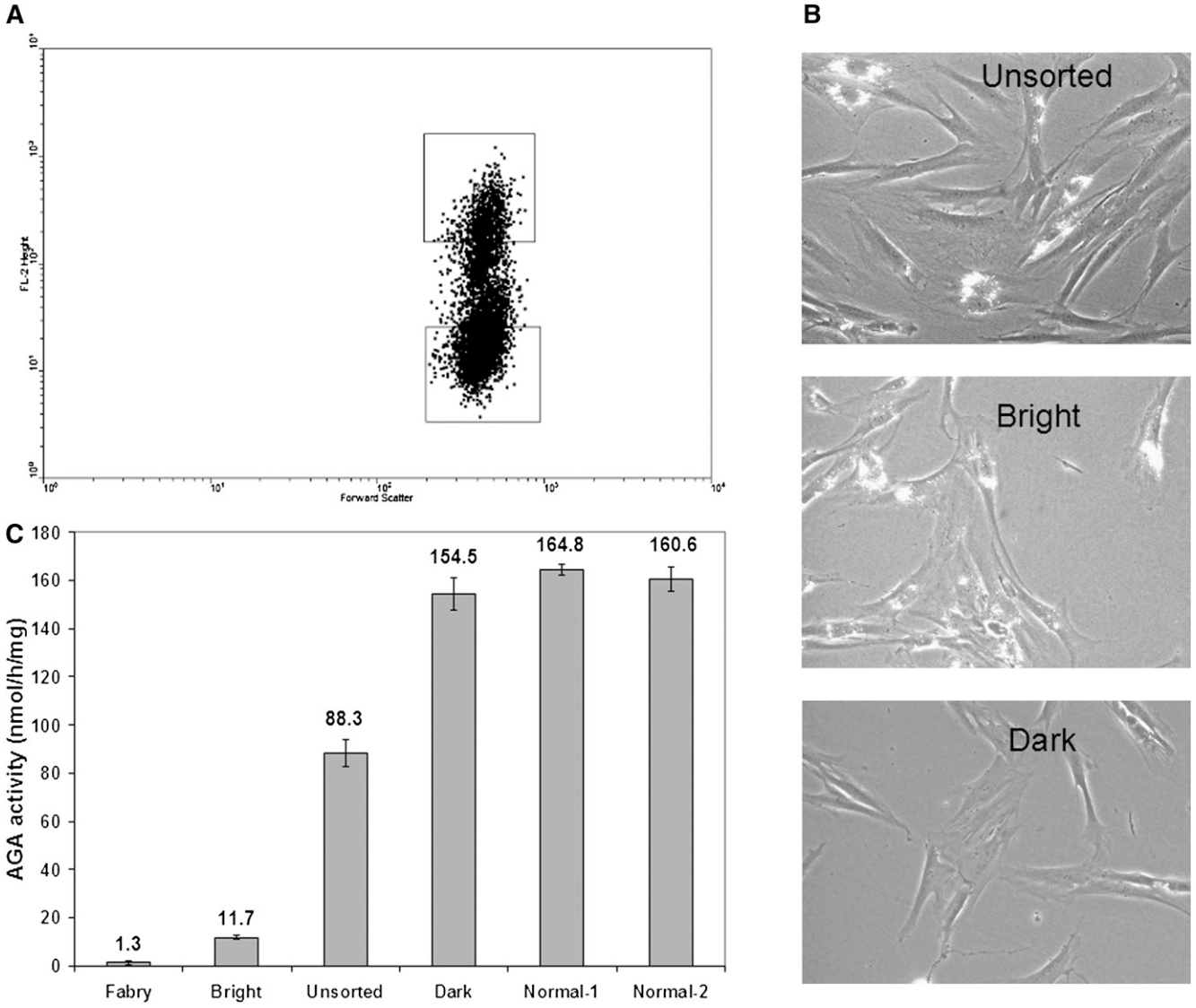

Fig. 5. FACS sorting of normal and mutant fibroblasts from a Fabry heterozygote. Cultured fibroblasts were loaded with LR-CTH for 18 h and incubated in fresh medium for three days as described in "Materials and Methods." A: Distribution of fluorescent cells. Fluorescence was determined in the FL-2 channel of a FACSCalibur flow cytometer. Boxes indicate the sort regions collected. The top region was designated as "bright" and the bottom as "dark." B: Fluorescence imaging of the parent (unsorted) bright and dark populations at two days postsorting (five days postloading). C: AGA activity against 4-MUG in homogenates of sorted cells after two passages. Bars indicate mean activity ± standard deviation of triplicate measurements. 4-MUG, 4-methylumbelliferyl-α-D-galactopyranoside; AGA, alpha-galactosidase A; FACS, fluorescence-activated cell sorting; LR-CTH, lissamine rhodamine ceramide trihexoside.

other than fibroblasts. **Fig. 6** shows measurement of intracellular fluorescence in cultured T-cell populations from normal, mutant, and heterozygous individuals. Distribution of fluorescence in the labeled cell population is shown in Fig. 6A. In contrast to fibroblasts, separation of mutant and wild-type populations was not as definitive in the T-cell population, possibly due to dilution of label by cell division. In a second experiment, cultured T-cells from three Fabry, three normal, and one obligate heterozygote were labeled and analyzed by FACS. When fluorescence was gated to exclude 95% of normal cells from the positive range, cells from Fabry patients and the obligate heterozygote contained significantly higher proportions of positive cells than normal (Fig. 6B). Residual fluorescence in this same culture of labeled cells was also measured in a Cytofluor II plate reader. Cells were counted 48 h after loading, and then 200 μl of cell suspension were transferred to each of two wells of a 96-well plate and fluorescence intensity was determined. Unlabeled cells were used as a blank. As shown in Fig. 6C, T-cells from Fabry patients retained significantly more fluorescence than T-cells from normal patients. T-cells derived from the obligate heterozygote were more fluorescent than normal, but the difference was not statistically significant.

Microvascular endothelial cells also took up and processed LR-CTH. **Fig. 7** shows labeling of dermal microvascular endothelial cells derived from a Fabry heterozygote. Immediately after loading, all cells were brightly labeled. Over the next four days, some cells became progressively nonfluorescent until, by day 4, there was an obvious mix-

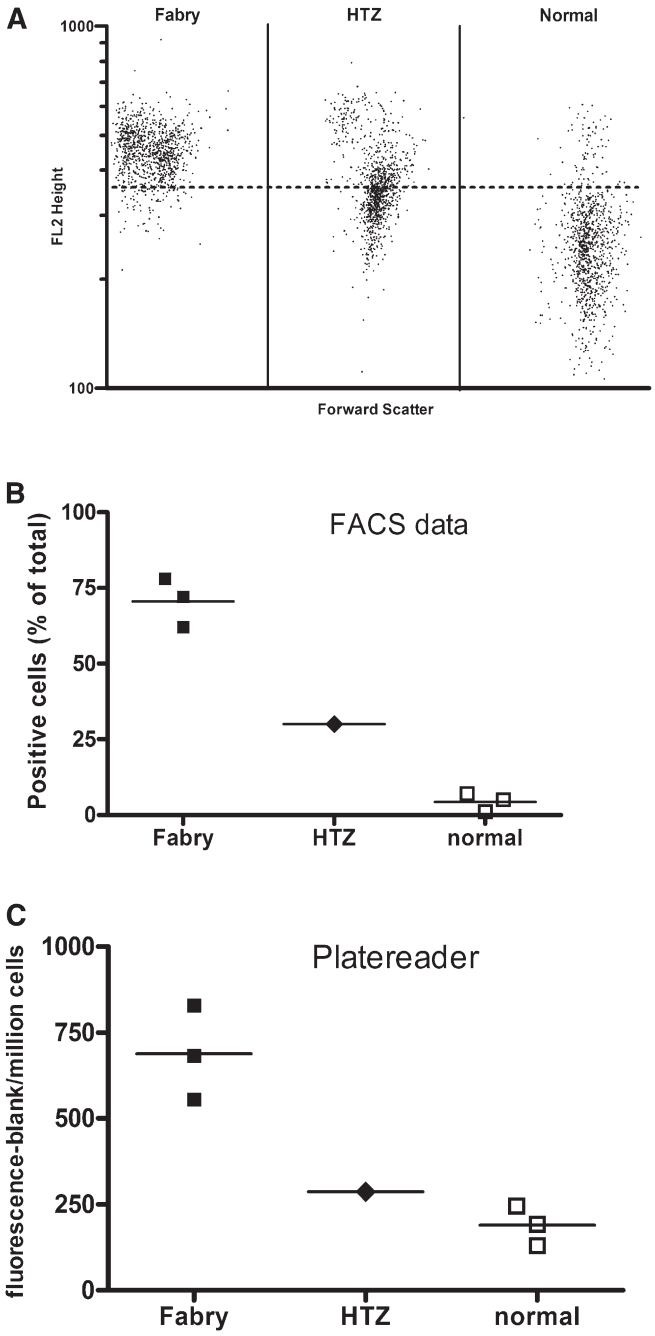

Fig. 6. Fluorescence of LR-CTH in T-lymphocytes. Cells were loaded with LR-CTH for 18 h and then refed with fresh medium as described in "Materials and Methods." A: Distribution of cellular fluorescence in Fabry, heterozygote, and normal populations. Fluorescence data in the FL-2 channel was collected using a FACSCalibur flow cytometer. The quadrant lines were drawn to exclude 95% of normal cells from the upper (positive) quadrant. B: Percentage of positive cells by FACS analysis. T-lymphocytes cultures from Fabry (n = 3), heterozygous (n = 1), and normal (n = 3) were labeled with LR-CTH for 18 h and resuspended in fresh medium as described in "Materials and Methods." FACS analysis using the FL-2 channel of a FACSCalibur flow cytometer was performed to determine the percentage of positive cells. C: The same population of cells from Fig 6B was resuspended in PBS with 5% FBS and total fluorescence was determined with a Cytofluor II microplate reader. Results are expressed as RFU-blank per million cells. FACS, fluorescence-activated cell sorting; LR-CTH, lissamine rhodamine ceramide trihexoside; RFU, relative fluorescence unit.

ture of cells, some of which had processed the label and some that had retained it.

DISCUSSION

We report here use of LR-CTH, a fluorescent probe that can be used for functional analysis of intracellular AGA activity. Several authors have reported use of these probes for measurement of intracellular enzyme activity in Gaucher disease and other lysosomal disorders (12, 13, 15). However, we are the first to report that, with direct measurement of intracellular fluorescence with a fluorescence plate reader or flow cytometer, LR-CTH can be used as a functional probe for AGA activity in intact cells without employing TLC or other labor-intensive methods.

Because the intracellular levels of the probe can be detected with standard methods of fluorescence detection, this assay has many applications. One-step detection using a standard fluorescence plate reader or a cell imager makes it useful for high-throughput screening for developing new therapies. The cells of interest can be loaded with LR-CTH and seeded in multi-well plates. After addition of the test compounds, change in fluorescence can be monitored with a fluorescence plate reader. Both short- and long-term experiments can be performed.

The fact that loss of LR-CTH label reaches a plateau in confluent cultures from some patients suggests that the accumulated lipid may be sequestered by the cell in storage vesicles that may not be accessible to normal cellular degradative pathways, such as the nonacidic, hydrolase-poor, postlysosomal vesicles demonstrated by Hirota et al. in NRK cells (22). However, extensive intracellular localization was beyond the scope of this study.

The physiological relevance of the long-term storage of labeled CTH found in the cells of some patients is unclear. The present study differs from the physiological state by the introduction of substrate entirely from an exogenous source, whereas in vivo the accumulated substrate results from cell turnover or from a combination of endogenous biosynthesis and degradation of exogenous lipids entering the cell through lipoproteins. Further study of the interplay between biosynthesis, transport, and degradation of this glycolipid and the parent globosides and glycoconjugates that may compete as substrates for the same limited enzyme activity may provide insights into the observation that some patients with even small amounts of residual activity have a milder clinical course (23).

Probing with LR-CTH can be used as an additional assay in cases where the standard 4-MUG assay provides ambiguous results. For example, as shown in Fig. 4, the heterozygous status of a female patient could be determined even though the activity against 4-MUG in homogenates of her fibroblasts was found to be in the low normal range. In addition, studies have shown that patients can be symptomatic despite the presence of a normal AGA gene (11, 24). In contrast, at least one known AGA variant (D313Y) can produce a deficiency of AGA activity in plasma in otherwise asymptomatic patients (25). Loading cells with LR-CTH can be used as an additional screening tool in such

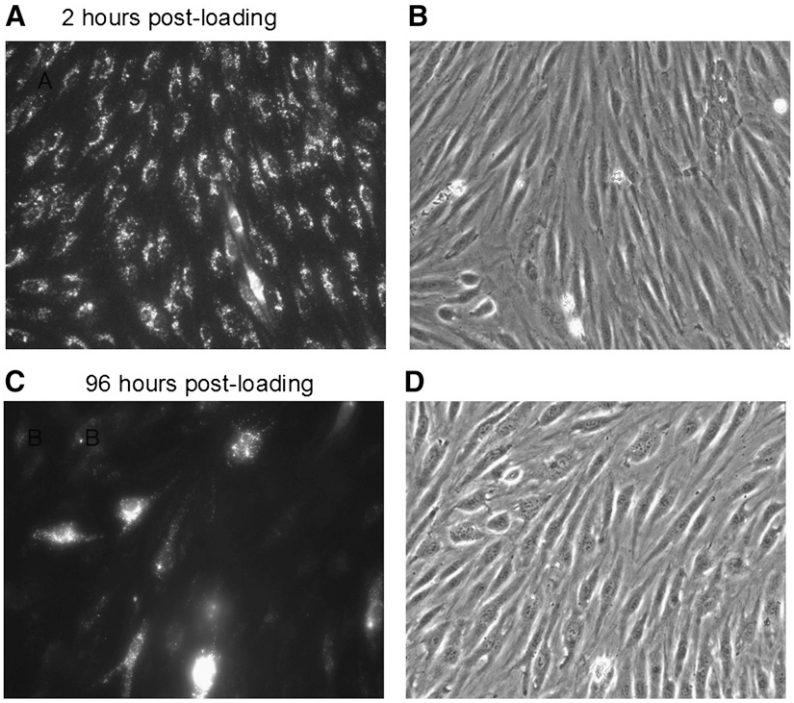

Fig. 7. Endothelial cells from a heterozygous Fabry female were grown on glass chamber slides and loaded with LR-CTH as described in "Materials and Methods," followed by incubation with fresh VEGF-Mv medium. Living cells were imaged at 20× with a Zeiss Axioplan microscope at 2 h postloading and 96 h post loading as described. Rhodamine fluorescence (A) and phase image (B) of cells at two h postloading. Rhodamine fluorescence (C) and phase image (D) at 96 h postloading. LR-CTH, lissamine rhodamine ceramide trihexoside.

patients because a reduction in intracellular function of AGA in the intact cell would result in retention of the fluorescent label.

Of particular note is the use of LR-CTH in the determination of heterozygote status in women with the Fabry trait. As the proportion of mutant cells is determined by random inactivation of one of the X-chromosomes in the cells of females (lyonization), traditional enzyme assays will not detect AGA deficiency in approximately 33% of heterozygous women (26). There are an estimated 527 known mutations in the *GLA* gene resulting in Fabry disease (10). Since many mutations are private, genetic screening in the absence of a known family history requires sequencing the entire gene. The addition of a functional assay provides a method for determining the presence and number of mutant cells in culture from a suspect patient. In addition, this method can be used to collect mutant cells for analysis.

As suggested by Yeyati et al. (16), FACS sorting can be used to retrieve corrected cells for assessing the success of gene therapy. In addition, probing with LR-CTH can be used to develop in vitro disease models by gene silencing techniques using a variety of available cell types. Cells in which *GLA* is successfully inhibited could be easily sorted by FACS analysis based on the retention of the LR-CTH label. Yeyati et al. found that genetically corrected fibroblasts over-expressing sphingomyelinase in Niemann-Pick type B secrete the enzyme and show significant cross-correction of untransduced cells. When transduced and untransduced cells were loaded with lissamine rhodamine sphingomyelin and cocultured, the result was a single homogeneous population with low fluorescence. In addition, bone marrow cells genetically transduced with the *GLA* gene to overexpress the enzyme have been shown to secrete AGA enzyme and to correct lipid storage in multiple organs in mice (27). In contrast with these experiments with overexpressing cells, in our study of cultures from Fabry heterozygotes, cells with normal levels of AGA do not appear to correct the mutant cells in the same population (Fig. 4). This observation provides an important insight into the pathology in females with the Fabry trait, suggesting there is not a significant bystander effect in these cells.

FACS separation of normal and mutant populations from female heterozygotes can provide an excellent opportunity for genomic, proteomic, and biochemical studies. Currently, experiments are in progress to develop matched sets of normal and mutant endothelial cells and fibroblasts derived from affected female patients. These cells will have completely identical genetic backgrounds, with the exception of the expression of the genes from the normal and mutant X-chromosome. Such matched pairs will aid in the further understanding of the metabolic consequences of mutant AGA activity.

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