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Correction in trans for Fabry disease: Expression, secretion, and uptake of α-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector

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ABSTRACT **Fabry disease is an X-linked metabolic disorder due to a deficiency of α-galactosidase A (α-gal A; EC 3.2.1.22). Patients accumulate glycosphingolipids with terminal α-galactosyl residues that come from intracellular synthesis, circulating metabolites, or from the biodegradation of senescent cells. Patients eventually succumb to renal, cardio-, or cerebrovascular disease. No specific therapy exists. One possible approach to ameliorating this disorder is to target corrective gene transfer therapy to circulating hematopoietic cells. Toward this end, an amphotropic virus-producer cell line has been developed that produces a high titer (>10^6 i.p. per ml) recombinant retrovirus constructed to transduce and correct target cells. Virus-producer cells also demonstrate expression of large amounts of both intracellular and secreted α-gal A. To examine the utility of this therapeutic vector, skin fibroblasts from Fabry patients were corrected for the metabolic defect by infection with this recombinant virus and secreted enzyme was observed. Furthermore, the secreted enzyme was found to be taken up by uncorrected cells in a mannose-6-phosphate receptor-dependent manner. In related experiments, immortalized B cell lines from Fabry patients, created as a hematologic delivery test system, were transduced. As with the fibroblasts, transduced patient B cell lines demonstrated both endogenous enzyme correction and a small amount of secretion together with uptake by uncorrected cells. These studies demonstrate that endogenous metabolic correction in transduced cells, combined with secretion, may provide a continuous source of corrective material in trans to unmodified patient bystander cells (metabolic cooperativity).**

Corrective gene transfer has been suggested as curative therapy for a number of human disorders that result from single enzyme defects. This strategy is especially attractive in lysosomal storage disorders that involve elements from the hematopoietic system (1–4). Indeed, Fabry disease is a compelling model to examine approaches to deliver corrective factors to compromised cells and tissues because no specific therapy currently exists despite the identification of the enzymatic defect in Fabry disease in 1967 (5).

Fabry disease is an X-linked recessive panethnic disorder caused by a deficiency of the enzyme α-galactosidase A (α-gal A). Patients with diminished enzyme activity accumulate high levels of incompletely metabolized glycosphingolipids that have terminal α-galactosyl residues (for review, see ref. 6). These lipids are deposited in many systemic organs as well as blood vessels and neurons (7). Patients have characteristic angiokeratoma, hypohidrosis, and episodic pain crises in the extremities, and they eventually succumb to renal disease, myocardial infarction, or stroke.

This study describes the construction and application of a high-titer recombinant retroviral vector to correct the enzymatic defect in hematopoietic and other cells derived from patients with Fabry disease. Moreover, cells engineered to overexpress α-gal A by retroviral transduction were found also to secrete the specific enzyme activity. Secreted α-gal A activity was found to be able to be taken up by uncorrected patient's cells in a mannose-6-phosphate (Man-6-P) receptor-dependent manner. These findings enhance prospects of therapy for Fabry disease by gene correction in concert with enzymatic correction of unmodified bystander cells.

MATERIALS AND METHODS

Vector Construction. The full-length α-galactosidase A cDNA was amplified by PCR from plasmid pGB78A using DNA oligonucleotide primers that added unique *Not*I and *Xho*I (New England Biolabs) restriction enzyme sites at the 5′ and 3′ noncoding ends of the sequence. The sequences of the primers GalA1 and GalA2 were 5′-GATCCTTTGCGGCCGC-3′ and 5′-CCTGGGCTCGAGTTAAAGTAAGTCTTTTAATG-3′, respectively. The resulting PCR product was digested and subcloned into retroviral vector PG1 (Genetic Therapy, Gaithersburg, MD) at the *Not*I and *Xho*I sites and sequenced to insure fidelity. Initial sequence results demonstrated no nucleotide alterations in the PCR product from the wild-type α-gal A cDNA sequence. However, repeated intensive sequencing showed that a single G nucleotide at position 531 was changed to an A. This alters the amino acid ^{178}Glu to a lysine residue. Contextual analyses predicted that this alteration would have little effect on the predicted hydrophilicity, surface probability, chain flexibility, helical structure, and antigenicity index for that region.

The PG1α-gal A DNA plasmid construct was then transfected into BOSC23 cells according to Pear *et al.* (8) and ecotropic retroviral supernatant was collected from multiple plates at 24 and 48 hr after transfection. The supernatant solution was used to infect amphotropic virus-producing GP + AM12 packaging cells (9) multiple times in the presence of polybrene (Sigma). The pool of infected AM12 cells was used as a producer virus source in some initial studies. Amphotropic virus-producing cell clones were also expanded from isolated single cells and assayed for α-gal A enzyme activity (see below). A single clone, AM12PG1α-gal A#8, was selected for subsequent studies.

Cell Culture, Virus Production, and Infection. GP + AM12, NIH 3T3 (American Type Culture Collection), and BOSC23 cells, were maintained under standard culture conditions. Cultured skin fibroblasts and leukocytes from peripheral blood from patients with Fabry disease were collected under approved National Institutes of Health protocols. Immortalized B cell lines were generated and maintained after Epstein–Barr virus (EBV) transformation of leukocytes derived from peripheral blood as described (10). The AM12PG1α-gal A#8

Abbreviations: α-gal, α-galactosidase; Man-6-P, mannose-6-phosphate.
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packaging cell construct was tested by sensitive marker rescue assays for the presence of helper virus (10). No helper virus was found at any dilution (data not shown). Viral infections of all target cells with viral supernatants were performed essentially as described (10). Briefly, cells as monolayers on plates or as suspension cultures, were infected with filtered supernatant from the AM12PG1α-gal A#8 producer line which had been incubated 16 hr previously with the media specific to the target cells of interest. Filter-sterilized protamine sulfate (Elkins–Sinn, Cherry Hill, NJ) at 4 μg/ml was added to each incubation. Virus (as supernatant) was added to target cells for four consecutive overnight applications.

Southern Blot Analysis. Genomic DNA, prepared from NIH 3T3 cells infected with supernatant from the AM12PG1α-gal A#8 producer line using an isolation kit (Qiagen, Chatsworth, CA) and the manufacturer's protocols, was digested with restriction enzyme *Nhe*I (New England Biolabs) and separated by electrophoresis on agarose gels. A single provirus band was detected after transfer to membranes and hybridization to a nonradioactive reporter system (Tropix, Bedford, MA). An effective viral titer of $>5 \times 10^6$ infectious units/ml was observed from two separate assays (data not shown).

Enzyme Assays. The fluorimetric assay of α-gal A activity (11) was modified as follows. Retroviral producer cells or confluent fibroblasts, harvested by cell scraping, or leukocytes and immortalized B cells, were pelleted by low-speed centrifugation. The cell pellets were washed 3 times with PBS, sonicated 2×5 sec on ice in homogenization buffer (28 mM citric acid/44 mM disodium phosphate/3 mg/ml sodium taurocholate, pH 4.4) and then centrifuged at $20{,}000 \times g$ for 30 min. Total α-gal activity was determined by incubating aliquots of the supernatant solutions for various times with 10 mM 4-methylumbelliferyl-α-D-galactopyranoside (Research Products International) in the homogenization buffer at 37°C without taurocholate but with bovine serum albumin (5 mg/ml). Media was collected, filtered, and assayed directly. Isoenzyme-specific α-gal A activity was determined by comparison of total α-gal activity with that observed in the presence of 0.1 M *N*-acetylgalactosamine, an inhibitor of α-gal B (12). As an additional control for specificity, β-hexosaminidase activity was measured in cell extracts and media (13). One unit of α-gal A activity is equivalent to the hydrolysis of 1 nmol of substrate in 1 hr at 37°C.

Uptake and Secretion Studies. Fresh media, appropriate to the type of recipient cell being examined, were added to source cells 12–16 hr before assay. This media was then collected and filtered through a 0.45 μm filter. The media was then assayed as above or added to recipient cells, which were then incubated for 3 hr at 37°C or at 4°C in the presence or absence of 1 mM Man-6-P. The recipient cells were then collected and assayed as above.

RESULTS

The Retroviral PG1α-Gal A Construct. A recombinant vector was designed for eventual therapy trials in patients with Fabry disease. Recombinant virus produced from the PG1 backbone, used in the present study, has been shown to have high titer (14) and lead to stable production of a corrective factor over time following provirus integration (15).

Intracellular α-gal A activity was measured in the total cell pool and in numerous AM12PG1α-gal A cell clones isolated after multiple infections with the transfected BOSC23 ecotropic viral supernatant. Specific activities for various clones were increased from 10- to 50-fold over background AM12 levels. The highest level of enzyme activity approximated 10^5 units/mg cell protein (data not shown). Based on this information, a single clone was selected for subsequent study. The effective retroviral titer of this clone was estimated by supernatant infection of NIH 3T3 cells followed by semiquantitative Southern blot analyses. In two independent assays, a titer of $>5 \times 10^6$ infectious particles per ml was observed (data not shown).

AM12PG1α-gal A#8 producer cells were also examined for the secretion of α-gal A activity. No secretion of α-gal A activity was observed from uninfected AM12 cells. In contrast, $\approx 2 \times 10^6$ AM12PG1α-gal A#8 cells secreted $\approx$100 nmol/hr/ml of α-gal A activity into the media (data not shown). This level represents $\approx$0.05 mg of α-gal A enzyme per liter of supernatant, based on a minimum specific activity of 2×10^6 units/mg for the purified enzyme (G.J.M., unpublished observations). No secreted β-hexosaminidase activity was measurable in the media of either parent AM12 cells or the producer cell clone (data not shown) indicating that this was not a broad lysosomal enzyme effect.

Enzymatic Correction of Cells Obtained from Patients with Fabry Disease. Cultured skin fibroblasts ($\approx 2 \times 10^6$ cells), derived from a moderately affected patient with Fabry disease, were infected four times with supernatant from the AM12PG1α-gal A cell pool. α-Gal A specific enzyme activity was then measured in the collected cells. Uncorrected cells exhibited an α-gal A enzyme specific activity of 210 units/mg cell protein ($\approx$29% of normal; Fig. 1). After transduction with supernatant from the uncloned AM12PG1α-gal A producer cell pool, α-gal A activity increased to more than 10-fold above the normal level (Fig. 1). No increase in α-gal A activity was observed in the mock-infected cells.

To generate a convenient hematologic cell model for Fabry disease, peripheral blood was gathered from Fabry patients other than the skin fibroblast donor and from a normal volunteer donor under an approved National Institutes of Health protocol. Leukocytes were assayed for total α-gal and specific α-gal A enzyme activity as were B cell lines generated from patients with Fabry disease and a normal control generated after EBV transformation (Fig. 2 *A* and *B*). No major changes were seen in the specific enzyme activities indicating that the immortalization procedure did not significantly alter α-gal A enzyme production or stability. Patients 1 and 2 had

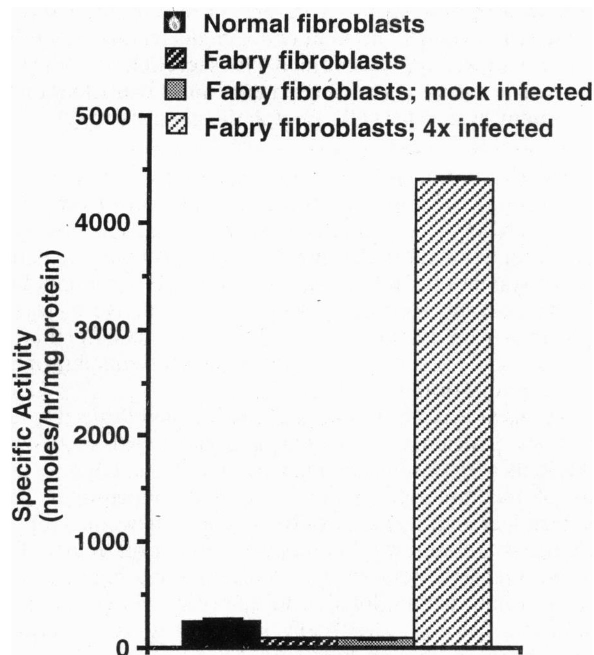

FIG. 1. Correction of the enzymatic defect in Fabry patient skin fibroblasts. Cells were infected 4× with supernatant from the AM12PG1α-gal A virus producer pool and assayed for enzyme activity. Bars represent α-gal A intracellular enzyme specific activity with standard error of the mean in all cases.

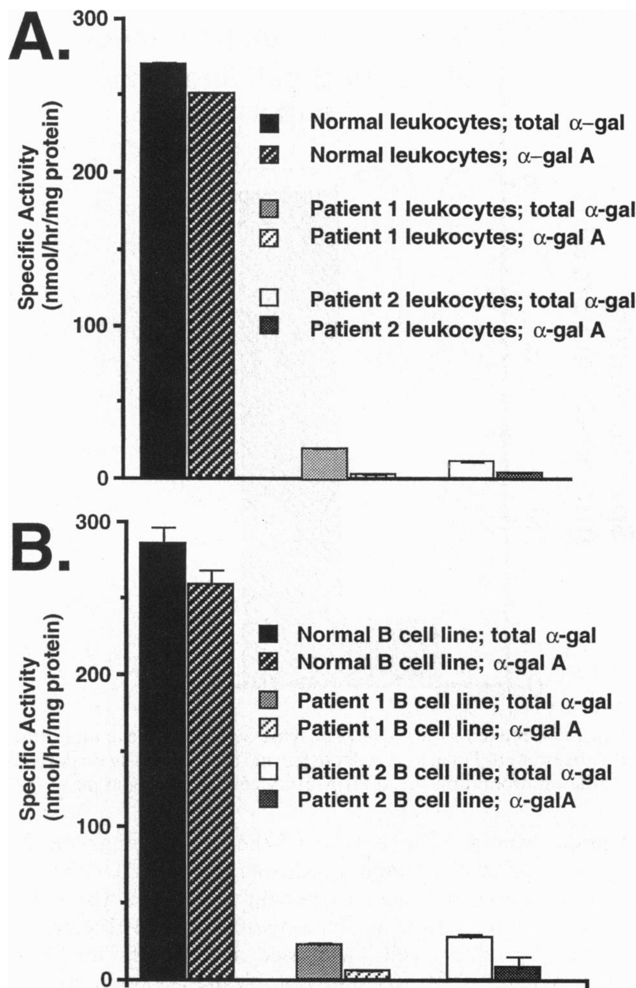

FIG. 2. Total α-gal and α-gal A intracellular enzyme activity levels in (*A*) patient peripheral blood leukocytes and in (*B*) patient immortalized B cell lines generated after EBV transformation.

total α-gal and α-gal A enzyme levels that were observed to be $\approx$1% of normal making these cells appropriate models for studies with recombinant transfer vectors or alternative DNA and enzyme delivery systems.

Significant increases in α-gal A activity in the patient B cell lines were observed after recombinant retroviral infection (Fig. 3). Correction to an enzyme level of 63% of normal was observed in one case, whereas correction to a level of greater than that seen in normal cells was observed in the other. Both of the corrected Fabry patient B cell lines showed greater than 100-fold increases in α-gal A activity levels. This finding is encouraging despite the fact that the infection of the patient B cell lines with the recombinant construct was likely quite inefficient (10), probably because of reduced amphotropic virus receptor present on these cells. In support of this concept, increasing the number of AM12PG1α-gal A infections of the patient B cell lines (up to 16$\times$) increased the amount of intracellular enzyme produced in a correlative fashion (data not shown). This increased enzyme activity also correlated with increased proviral copy number as measured by comparative Southern blot analysis (data not shown). As a control to gauge the effects on total lysosomal compartment enzyme levels, β-hexosaminidase activity was largely unaffected under these conditions and manipulations (data not shown).

Secretion Studies. To determine whether extracellular secretion of α-gal A activity occurred in the corrected cells, culture media of the infected fibroblasts and the corrected B cells from Fabry patients were assayed for enzyme activity.

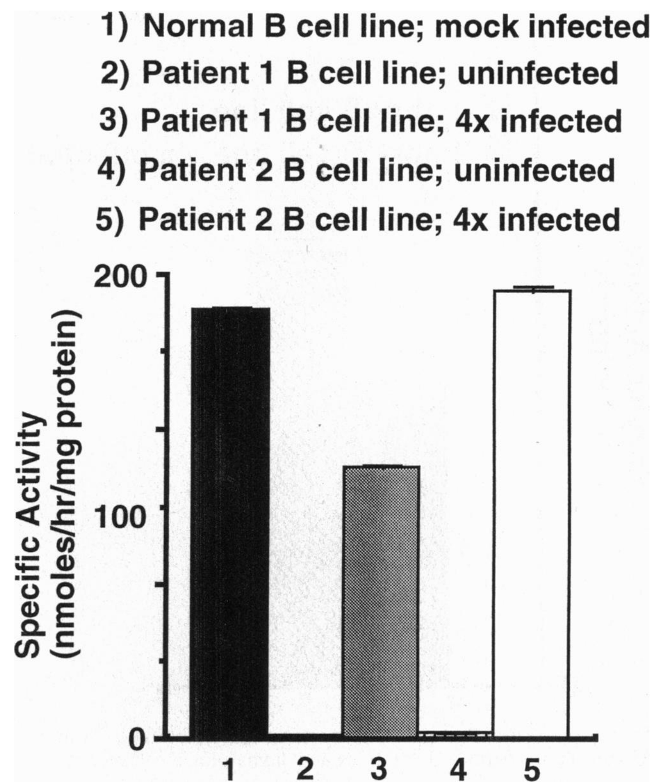

FIG. 3. Intracellular α-gal A enzyme specific activity levels obtained after infection of the the patient B cell lines with the recombinant retroviral supernatant from the AM12PG1α-gal A#8 virus producer line.

Media from normal, Fabry, and the Fabry mock-infected fibroblasts exhibited minimal enzyme activity (Fig. 4). In contrast, media from the pool of 4$\times$-infected fibroblasts (representing about 2×10^6 cells) contained 8.4 nmol/hr/ml of media. This (10-fold) increase over background correlates well with the intracellular levels observed in Fig. 1.

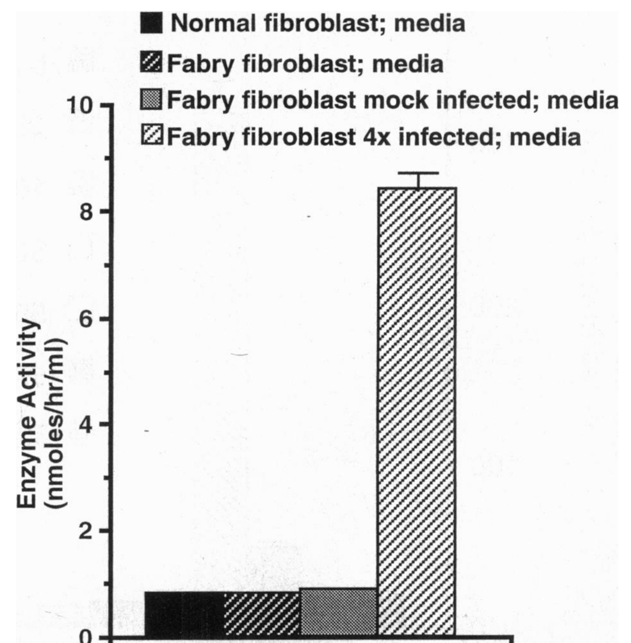

FIG. 4. Secreted α-gal A enzyme activity obtained from the infected patient fibroblasts.

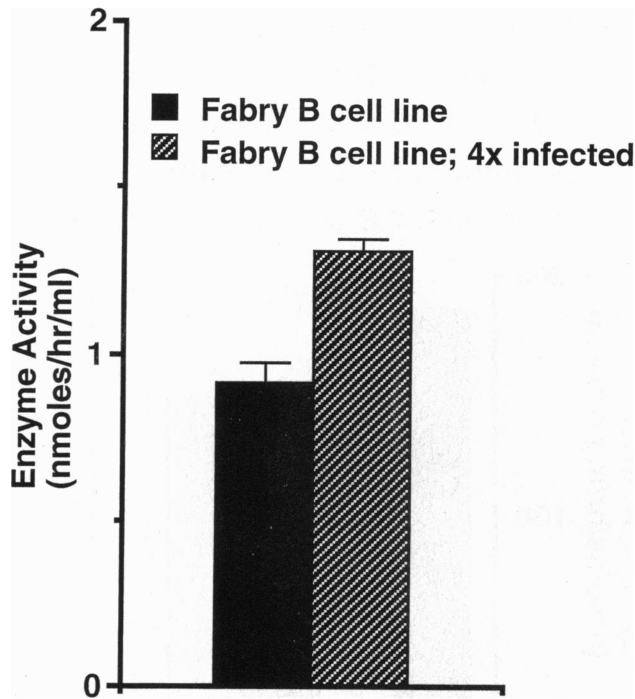

FIG. 5. Secreted α-gal A enzyme activity obtained from the infected Fabry patient B cell lines as a hematologic cell model.

A low, but significant ($P < 0.0001$ by t test; $n = 9$), amount of secreted α-gal A activity was observed in the media of the infected B cells grown at a density of $<10^6$ cells per ml (Fig. 5). The small amount of secreted enzyme from the infected B cells reflects the fact that the infection was likely less efficient than the fibroblasts. Differences in the lysosomal protein production and secretion pathways for each cell type may also play a role. As with the endogenous α-gal A activity, secreted activity increased linearly with increases in the transduction events of the patient B cell lines (data not shown).

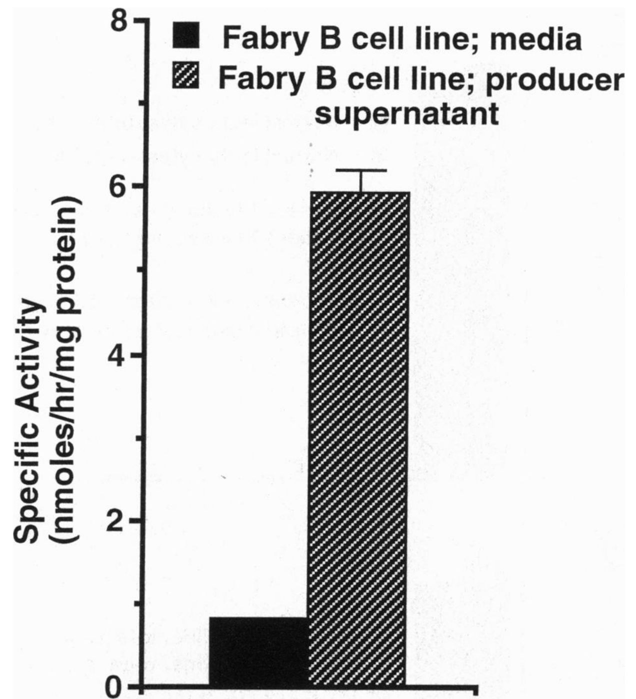

FIG. 7. Uptake of the α-gal A enzyme activity into the immortalized Fabry patient B cell line. Added enzyme activity was again derived from the multiply transduced virus-producer cell supernatant.

Uptake Studies. The uptake of the secreted enzyme by various uncorrected bystander cells was examined. Unconcentrated and unpurified media (specific to the cell type being assayed), obtained after incubation with the AM12PG1α-gal A#8 virus producer cells, was used as a source for these studies. When ≈2×10^6 skin fibroblasts, obtained from a patient with Fabry disease, were incubated with various amounts of the producer supernatant, increases >16-fold over background levels of enzyme were seen (Fig. 6). After mea-

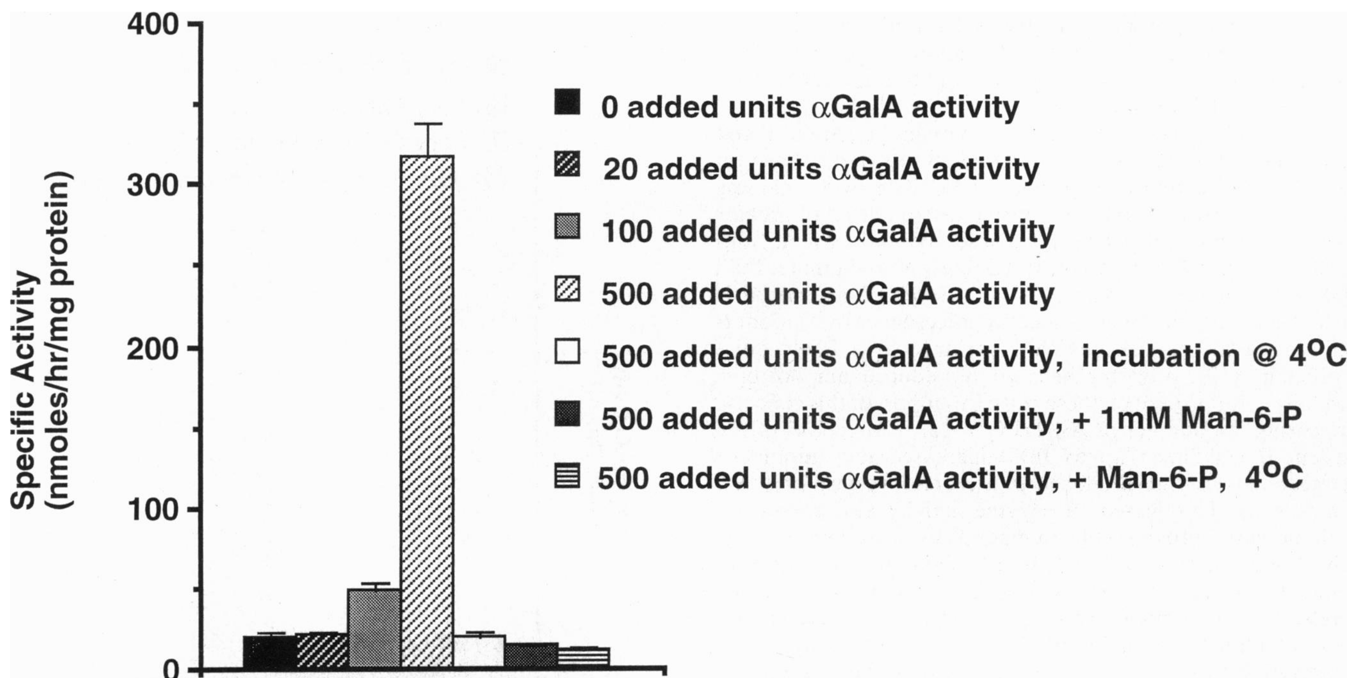

FIG. 6. Uptake of the α-gal A enzyme activity into uncorrected Fabry patient fibroblasts. Various units of secreted α-gal A activity from source supernatant were added to the cells and incubation, in the presence or absence of Man-6-P, was at 37°C unless noted.

surement of the total cell protein, it was established that about 6 units of enzyme were taken up in 3 hr out of the ≈500 units added. Additional studies revealed that this uptake was completely abrogated when cells and source supernatant were incubated at 4°C (to demonstrate specific uptake as opposed to extramembrane "sticking"). The uptake was nearly totally blocked when Man-6-P was added to the incubation indicating that the uptake was mediated through the Man-6-P receptor on these cells and was not due to a nonspecific import mechanism.

In the hematologic cell model, when $\approx 10^7$ uninfected patient B cells were incubated with 13 ml of the producer supernatant (representing about 1300 units of enzyme) and assayed for intracellular activity, more than a 7-fold increase of endogenous enzyme activity in the recipient cells was found (Fig. 7). Here it is estimated that 4 units of enzyme were taken up in 3 hr by these cells. Even at the lower per cell uptake of the soluble α-gal A for the Fabry B cell hematologic model ($\approx 10^3$ molecules per cell in 3 hr vs. $\approx 10^4$ molecules per cell for the Fabry fibroblasts), this finding further demonstrates that the metabolic cooperativity model is applicable to various types of bystander cells.

Uptake studies into uncorrected patient fibroblasts and the immortalized patient B cell models, were attempted with media collected from the 4×-transduced Fabry fibroblasts. No uptake, as measured by enzyme activity assays, above background was observed (data not shown). More sensitive detection procedures, i.e., radiolabeling of the added α-gal A protein, may be necessary to detect internalized enzyme in this case due to the relatively low amount of corrective enzyme secreted.

DISCUSSION

A recombinant retroviral vector has been constructed that engineers efficient transduction of cells and expression of human α-gal A activity. This vector is of high titer, and it corrects the enzymatic defect in multiple types of cells obtained from patients with Fabry disease. Further, the virus engineers expression of a form of α-gal A activity that is secreted and taken up, in a M-6-P receptor-specific manner, into unmodified bystander cells. Uptake of this secreted form of α-gal A was observed in fibroblasts and in hematologic cells derived from patients. Such transduced cells may provide a constant corrective function in trans. These findings represent the first observation of such a corrective effect for Fabry disease in these varied cell types.

Treatment for Fabry disease was initiated a number of years ago by infusion of normal human plasma (16) or partially purified preparations of α-gal A to patients (17, 18). The enzyme has a short half-life in circulation, however, and the corrective activity was rapidly cleared. A step toward the genetic correction of Fabry disease has recently been undertaken by the construction of a recombinant retroviral vector that delivers the α-gal A cDNA (19). Although efficient intracellular expression of the α-gal A was observed, the recombinant retrovirus produced was of low titer. This may have been due to the presence of the large insert in the vector added for the functional selection of corrected cells. In addition, no correction of patient cells was documented in that study.

Stable and marked overexpression of human α-gal A has been engineered in Chinese hamster ovary cells, and large amounts of secreted high-mannose forms of the enzyme were detected (20). This secretion was postulated to occur because of an aggregation of the overexpressed protein resulting in a reduced affinity for Man-6-P receptors in the trans Golgi network that directs protein to the lysosomes, causing some enzyme to be shunted to secretory pathways. It is possible that the overexpression observed in the present investigation is sufficient to cause a similar aggregation and secretion of α-gal A enzyme that has the requisite Man-6-P ligand for uptake by other cells. The lesser amount of secretion in the B cell model would then be consistent with the lower overall expression seen.

It is possible that corrected stem cells (and their progeny) from Fabry patients, after *ex vivo* transduction and reimplantation, may become a continual source of secreted α-gal A activity *in vivo*. This activity would then be circulating and able to be delivered and taken up by various target cell and tissue types. Corrected stem/progenitor cells may also have a growth advantage due to a reduction in their intracellular lipid load. If such an advantage is realized, it would increase the production of secreted enzyme and thereby increase the circulating amounts of protein available over a considerable period of time.

Metabolic cooperativity or "cross-correction" has been demonstrated in the context of other lysosomal storage disorders. Enzymatic cross-correction in mucopolysaccharidosis VII mice, derived from transduced stem/progenitor cells, reduced lysosomal storage in liver and spleen in this model (21). Effective correction of a mouse model for galactosialidosis was accomplished by transplantation of hematopoietic cells engineered to overexpress and secrete the corrective factor (22). Low but noticeable correction of the central nervous system from the overexpressing transplanted bone marrow was also observed in that study. These findings provide encouragement for the application of such correction to humans. Along these lines, lymphocytes from patients with Hunter syndrome were corrected endogenously for the enzymatic defect by retroviral-mediated gene transfer and cell-to-cell enzyme activity transmission occurred (23). Another recent study demonstrated sustained long-term expression and secretion of α-L-iduronidase, another lysosomal enzyme, in transduced human stem/progenitor cells (24). This further demonstrates the potential for metabolic cooperativity for many diseases of this type.

Studies where $CD34^+$ stem/progenitor cells from normal and Fabry patient donors are transduced are underway. These experiments are necessary to gauge the effectiveness of this viral construct in transducing these primitive repopulating cells and to establish whether long-term overexpression and secretion can be maintained in this system.

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1. Karlsson, S. (1991) *Blood* **78,** 2481–2492.
2. Wolfe, J. H. (1994) *Curr. Opin. Pediatr.* **6,** 213–218.
3. Barranger, J. A., Bahnson, A. B., Nimgaonkar, M. T., Ball, E. D., Robbins, P. D., Boggs, S. S. & Ohashi, T. (1995) *Int. Pediatr.* **10,** 5–9.
4. Medin, J. A., Migita, M. & Karlsson, S. (1995) in *Cell Therapy*, eds. Morstyn, G. & Sheridan, W. P. (Cambridge Univ. Press, Cambridge, U.K.), pp. 385–412,.
5. Brady, R. O., Gal, A. E., Bradley, R. M., Martensson, E., Warshaw, A. L. & Laster, L. (1967) *N. Engl. J. Med.* **276,** 1163–1167.
6. Desnick, R. J., Ioannou, Y. A. & Eng, C. M. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), pp. 2741–2784.
7. deVeber, G. A., Schwarting, G. A., Kolodny, E. & Kowall, N. W. (1992) *Ann. Neurol.* **31,** 409–415.
8. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8392–8396.
9. Markowitz, D., Goff, S. & Bank, A. (1988) *Virology* **167,** 400–406.
10. Medin, J. A., Migita, M., Pawliuk, R., Jacobson, S., Amiri, M., Kluepfel-Stahl, S., Brady, R. O., Humphries, R. K. & Karlsson, S. (1996) *Blood* **87,** 1754–1762.
11. Kusiak, J. W., Quirk, J. M. & Brady, R. O. (1978) *J. Biol. Chem.* **253,** 184–190.
12. Mayes, J. S., Scheerer, J. B., Sifers, R. N. & Donaldson, M. L. (1981) *Clin. Chim. Acta* **112,** 247–251.

13. Tallman, J. F., Brady, R. O., Quirk, J. M., Villalba, M. & Gal, A. E. (1974) *J. Biol. Chem.* **249,** 3489–3499.
14. Correll, P. H., Colilla, S. & Karlsson, S. (1994) *Blood* **84,** 1812–1822.
15. Correll, P. H., Colilla, S., Dave, H. P. G. & Karlsson, S. K. (1992) *Blood* **80,** 331–336.
16. Mapes, C. A., Anderson, R. L., Sweeley, C. C., Desnick, R. J. & Krivit, W. (1970) *Science* **169,** 987–989.
17. Brady, R. O., Tallman, J, F., Johnson, W. G., Gal, A. E., Leahy, W. R., Quirk, J. M. & Dekaban, A. S. (1973) *N. Engl. J. Med.* **289,** 9–14.
18. Desnick, R. J., Dean, K. J., Grabowski, G., Bishop, D. F. & Sweeley, C. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5326–5330.
19. Sugimoto, Y., Aksentijevich, I., Murray, G. J., Brady, R. O., Pastan, I. & Gottesman, M. M. (1995) *Hum. Gene Ther.* **6,** 905–915.
20. Ioannou, Y. A., Bishop, D. F. & Desnick, R. J. (1992) *J. Cell Biol.* **5,** 1137–1150.
21. Wolfe, J. H., Sands, M. S., Barker, J. E., Gwynn, B., Rowe, L. B., Vogler, C. A. & Birkenmeier, E. H. (1992) *Nature (London)* **360,** 749–753.
22. Zhou, X. Y., Morreau, H., Rottier, R., Davis, D., Bonten, E., Gillemans, N., Wenger, D., Grosveld, F. G., Doherty, P., Suzuki, K., Grosveld, G, C. & d'Azzo, A. (1995) *Genes Dev.* **9,** 2623–2634.
23. Braun, S. E .M., Pan, D., Aronovich, E. L., Jonsson, J. J., McIvor, R. S. & Whitley, C. B. (1996) *Hum. Gene Ther.* **7,** 283–290.
24. Fairbairn, L. J., Lashford, L. S., Spooncer, E., McDermott, R. H., Lebens, G., Arrand, J. E., Arrand, J. R., Bellantuono, I., Holt, R., Hatton, C. E., Cooper, A., Besley, G. T. N., Wraith, J. E., Anson, D. S., Hopwood, J. J. & Dexter, T. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2025–2030.