Epimerase-Deficiency Galactosemia Is Not a Binary Condition

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Epimerase-deficiency galactosemia results from the impairment of UDP-galactose 4′-epimerase (GALE), the third enzyme in the Leloir pathway of galactose metabolism. Originally identified as a clinically benign "peripheral" condition with enzyme impairment restricted to circulating blood cells, GALE deficiency was later demonstrated also to exist in a rare but clinically severe "generalized" form, with enzyme impairment affecting a range of tissues. Isolated cases of clinically and/or biochemically intermediate cases of epimerase deficiency have also been reported. We report here studies of 10 patients who, in the neonatal period, received the diagnosis of hemolysate epimerase deficiency. We have characterized these patients with regard to three parameters: (1) GALE activity in transformed lymphoblasts, representing a "nonperipheral" tissue, (2) metabolic sensitivity of those lymphoblasts to galactose challenge in culture, and (3) evidence of normal versus abnormal galactose metabolism in the patients themselves. Our results demonstrate two important points. First, whereas some of the patients studied exhibited near-normal levels of GALE activity in lymphoblasts, consistent with a diagnosis of peripheral epimerase deficiency, many did not. We detected a spectrum of GALE activity levels ranging from 15%–64% of control levels, demonstrating that epimerase deficiency is not a binary condition; it is a continuum disorder. Second, lymphoblasts demonstrating the most severe reduction in GALE activity also demonstrated abnormal metabolite levels in the presence of external galactose and, in some cases, also in the absence of galactose. These abnormalities included elevated galactose-1P, elevated UDP-galactose, and deficient UDP-glucose. Moreover, some of the patients themselves also demonstrated metabolic abnormalities, both on and off galactose-restricted diet. Long-term follow-up studies of these and other patients will be required to elucidate the clinical significance of these biochemical abnormalities and the potential impact of dietary intervention on outcome.

In both prokaryotes and eukaryotes, galactose is metabolized predominantly via the Leloir pathway, a series of reactions catalyzed by the enzymes galactokinase (GALK [EC 2.7.1.6]), galactose-1-phosphate uridylyltransferase (GALT [EC 2.7.7.12]), and UDP-galactose 4′-epimerase (GALE [EC 5.1.3.2]) (fig. 1) (Holton et al. 2000). Impairment of any of these three enzymes in humans results in galactosemia, although the symptoms and severity depend on the identity of the enzyme impaired and the extent of the deficiency, among other factors.

The first clinically recognized form of galactosemia was profound transferase deficiency (MIM 230400), reported by Goppert (1917). This so-called classic galactosemia occurs with a frequency of 1/30,000 to 1/60,000 live births in the United States and in other multiethnic populations, and it is panethnic. If untreated, classic galactosemia can lead to rapid neonatal demise. Symptoms progress from vomiting and diarrhea to cataracts and failure to thrive to hepatomegaly, liver dysfunction with bleeding diathesis, and *Escherichia coli* sepsis, culminating in neonatal death (Holton et al. 2000). Classic galactosemia is often detected presymptomatically in most industrialized nations by newborn screening and is treated by lifelong dietary restriction of galactose. Although this intervention resolves the acute and potentially lethal symptoms of classic galactosemia, unfortunately, a spectrum of complications persist, including ataxia, learning disabilities, and verbal dyspraxia in >30% of patients and primary ovarian failure in >80% of females (Waggoner et al. 1990). The pathophysiology of classic galactosemia remains unclear, hindering the development of more-effective therapies.

The second form of galactosemia to be recognized

Received August 26, 2005; accepted for publication October 11, 2005; electronically published November 14, 2005.

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Am. J. Hum. Genet. 2006;78:89–102. 0002-9297/2006/7801-0010$15.00

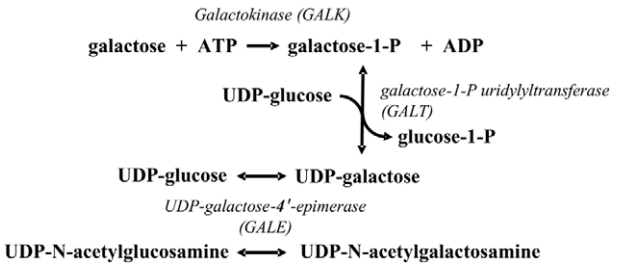

Figure 1 Reactions catalyzed by human enzymes of the Leloir pathway of galactose metabolism

clinically was GALK deficiency (MIM 230200), first reported by Gitzelmann (1967). Patients with GALK deficiency demonstrate none of the potentially lethal developmental, cognitive, or ovarian complications seen in classic galactosemia but do present with neonatal cataracts, which generally self-resolve upon dietary restriction of galactose. On the basis of biochemical ascertainment of carriers in a select white population, GALK deficiency was estimated to affect 1/40,000 to 1/50,000 newborn infants (Mayes and Guthrie 1968). One large population study, however, indicated that the frequency of detectable GALK deficiency at birth is significantly <1/100,000 (Levy 1980).

The third and most poorly understood form of galactosemia is epimerase deficiency (MIM 230350). First hypothesized by Kalckar (1965) and first reported by Gitzelmann (1972), epimerase-deficiency galactosemia was originally described as a benign condition in which human GALE (hGALE) impairment was restricted to the circulating red blood cells (RBC) and white blood cells (Gitzelmann 1972; Gitzelmann and Steimann 1973). Fibroblasts, liver, phytohemagglutinin (PHA)-stimulated leukocytes, and Epstein Barr virus (EBV)–transformed lymphoblasts from these patients all demonstrated normal or near-normal levels of hGALE activity (Gitzelmann 1972; Gitzelmann and Steimann 1973; Mitchell et al. 1975; Gitzelmann et al. 1977), which lead to the designation of this condition as "peripheral" epimerase deficiency (Holton et al. 2000).

Soon thereafter, however, a second form of epimerase-deficiency galactosemia became apparent when Holton and colleagues (1981) reported a patient who, despite normal GALT activity, presented with symptoms reminiscent of classic galactosemia and demonstrated severely impaired GALE activity in both RBCs and fibroblasts. This patient, whose acute clinical symptoms responded to dietary restriction of galactose, was said to have "generalized" epimerase deficiency. Subsequent studies revealed four additional patients with generalized epimerase-deficiency galactosemia, ostensibly derived from two different families (Sardharwalla et al. 1988; Walter et al. 1999), although all five patients later proved to be homozygotes for the same mutation, V94M (Walter et al. 1999; Wohlers et al. 1999), raising the suspicion that they might share common ancestry. Together, these reports supported the conclusion that epimerase-deficiency galactosemia was a binary condition, with a benign peripheral form occurring at a population frequency of 1/6,700 to <1/60,000, depending on the racial group (Alano et al. 1998), and a severe generalized form occurring at an extremely low frequency (Holton et al. 2000).

The conclusion that hGALE deficiency is a binary condition held until the 1990s, when groups in both Europe and the United States reported individual patients with partial or tissue-specific GALE impairment and who exhibited isolated clinical abnormalities. For example, Shin and colleagues (Endres and Shin 1990; Schulpis et al. 1993; Shin et al. 2000) described patients with GALE impairment in the lens who presented with cataracts, and both Quimby et al. [1997] and Alano et al. [1998] described a patient with partial GALE deficiency who was clinically well as an infant but who gradually manifested notable developmental/cognitive delay, starting after his first year of life. This patient exhibited only ~15% of normal levels of GALE activity in his transformed lymphoblasts (Quimby et al. 1997). It is important to point out that, although lymphoblasts are clearly derived from peripheral cells, with regard to GALE activity they more closely resemble nonperipheral cells (Mitchell et al. 1975) and, as such, offer a convenient tool for distinguishing peripheral from nonperipheral forms of epimerase-deficiency galactosemia. While these cases clearly raised concern that epimerase deficiency might not be a binary condition, the broader clinical significance of these isolated findings remained unclear.

We report here the biochemical and genetic studies of a cohort of 10 additional and unrelated epimerase-deficient patients accrued over a 4-year period and characterized, relative to controls, with regard to epimerase activity in transformed lymphoblasts as well as metabolic sensitivity of those lymphoblasts to galactose exposure in culture. Using these samples, we asked two fundamental questions: (1) Do all infants/children who receive the diagnosis of hemolysate epimerase deficiency exhibit normal GALE activity in their nonperipheral tissues, as represented by transformed lymphoblasts, and (2) is there any abnormal metabolic consequence to partial impairment of hGALE activity in patients or patient lymphoblasts exposed to environmental galactose?

The answer to the first question was "no." Our data clearly demonstrated that, at least in biochemical terms, epimerase deficiency is *not* binary; it is a continuum disorder. We observed GALE activity levels in lymphoblasts from patients that ranged from a low of ~15% of control levels to a high of 64% of control levels.

The answer to the second question was "yes." Our results demonstrated that lymphoblasts with as much as 35% residual GALE activity nonetheless exhibited metabolic abnormalities upon exposure to environmental galactose. Furthermore, some of the patients from whom those lymphoblasts were derived also demonstrated abnormal galactose metabolites. The relationship between these metabolic abnormalities and the possibility of clinical abnormalities later in life remains unclear. Long-term clinical follow-up studies of significantly larger numbers of patients with partial GALE deficiency will be required to address this question.

Material and Methods

Study Subjects

All patients were ascertained by referral, and we obtained their consent in accordance with Emory University Institutional Review Board Protocol 618–99 (primary investigator: Fridovich-Keil). Controls were ascertained as anonymous blood samples from nongalactosemic individuals. All hemolysate biochemical data were generated in clinical labs, as noted, and all clinical follow-up data were reported by referring health care professionals.

Lymphoblast Culture

EBV-transformed lymphoblasts were prepared and cultured from patient or control blood samples as described elsewhere (Neitzel 1986), with a substitution of interleukin-2 in place of cyclosporine. Transformed lymphoblasts were maintained in RPMI 1640 medium containing 11.1 mM glucose (2 g/liter) and 0.3 g/liter L-glutamine and were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 25 mM HEPES (Cellgro), and 10% (v/v) fetal bovine serum (Gibco). All cells were maintained at 37°C in a humidified 5% CO_2 incubator (NuAire).

GALT and GALE Enzyme Analyses

Soluble protein lysates were prepared from 0.15 g transformed lymphoblast cell pellets as follows. Cells were harvested by centrifugation, were washed with 1 × PBS to remove residual medium, and were resuspended in 300 μl of 100-mM glycine buffer (pH 8.7) containing protease inhibitor cocktail (Roche). Cells were disrupted on ice by three 15-s exposures to a Misonix Sonicator 3000 with microtip at output level 1.0. Insoluble debris was removed from each sample by centrifugation in an Eppendorf 5415D at 13.2 rpm at 4°C, and the supernatant was passed over a P-30 Bio-Spin Column (Bio-Rad) to remove small molecules before further analysis. Protein concentrations were determined using the Bio-Rad protein assay reagent with BSA as the standard, as recommended by the manufacturer.

Both GALT and GALE activities were measured in samples of each cell lysate by use of enzymatic assay procedures, essentially as described elsewhere (Ross et al. 2004; Schulz et al. 2005), with separation and quantification of reactants and products achieved by high-performance liquid chromatography (HPLC). GALE assays were performed at 37°C with the use of both UDP-galactose (UDP-Gal) and UDP-*N*-acetylgalactosamine (UDP-GalNAc) substrates, with a 30-min incubation time and 7.5–30 μg total protein in a final volume of 12.5 μl. UDP-Gal GALE activity assays contained 40 mM glycine, 0.4 mM UDP-Gal, and 8 mM NAD^+. UDP-GalNAc GALE activity assays contained 40 mM glycine, 0.4 mM UDP-GalNAc, and 8 mM NAD^+. GALT assays were performed at 37°C for 60 min with the use of 50–100 μg total protein in 125 mM glycylglycine (pH 7.5) and 4.1 mM UDP-glucose (UDP-Glc) in a final volume of 50 μl. GALT activity assays for each sample were performed in both the presence and absence of 1 mM galactose 1-phosphate (Gal-1P) to reveal background production of UDP-Gal (from UDP-Glc) by epimerase. To determine GALT activity, formation of UDP-Gal in the absence of Gal-1P was subtracted from the amount of UDP-Gal produced in the presence of Gal-1P. All reactions were initiated by the addition of protein and were monitored to ensure linearity. GALE and GALT reactions were stopped by the addition of 237.5 μl or 450 μl, respectively, of chilled sterile water and were then filtered (0.2-μm nylon filters [Alltech]) and subjected to HPLC separation and quantification of substrates and products. Enzyme activity was defined by picomole of product formed per microgram of protein per minute, under the conditions described. HPLC separation and quantification of enzymatic substrates and products was achieved as described elsewhere (Ross et al. 2004; Schulz et al. 2005).

Analysis of Metabolites from Lymphoblasts Cultured in the Presence versus the Absence of Environmental Galactose

Cells were cultured in the absence of galactose, as described above, until time zero, at which point ($t = 0$) the samples were harvested for both external and internal metabolites, and galactose was added to the remainder of the culture for a final concentration of 0.5 mM. Cultures were harvested 24 h after galactose addition. At each time point, 50 μl of cell suspension was removed for protein determination by use of the BioRad DC system, as recommended by the manufacturer. To monitor external galactose, 250 μl of culture medium was added directly into 500 μl 60% MeOH at −20°C, was mixed, and was centrifuged briefly at 13,200 rpm at 4°C in an Eppendorf 5415C microcentrifuge to remove cellular debris. Then, 375 μl of supernatant was dried under vacuum without heat, was rehydrated in 390 μl sterile Milli-Q water, and was filtered before HPLC fractionation. Internal metabolites were extracted from 10-ml samples of cell suspension harvested by centrifugation at 3,500 rpm in Sorvall RT6000B at room temperature and were washed once with PBS before further manipulation. Samples were prepared using a modified form of the procedure described elsewhere by our laboratory (Ross et al. 2004; Schulz et al. 2005) and originally described by Smits et. al (1998).

In brief, each washed cell pellet was resuspended in 1.1 ml cold PBS, of which 100 μl were used for protein determination via the Bio-Rad DC protein assay, as recommended by the manufacturer. Cells from the remaining 1 ml of suspension were collected by high-speed centrifugation at 4°C in an Eppendorf 5415C microcentrifuge. Intracellular metabolites were extracted by vigorous agitation of the cells for 45 min at 4°C

Table 1

PCR Primers Used

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

in a final volume of an 875-μl 4:2:1 mixture of $CHCl_3$/MeOH/water. The aqueous layer was collected after repeated high-speed centrifugation for 10 min at 4°C. The remaining organic phase was back-extracted a second time with 125 μl MeOH and 125 μl water. Aqueous layers were combined and dried under vacuum without heat (for ~6 h). Each dried metabolite pellet was rehydrated with a volume of sterile Milli-Q water calculated from the protein level measured for that cell suspension, with the use of 1 μl of water for every 5 μg of protein detected. Rehydrated samples were filtered through 0.2-μm nylon filters (Alltech) before being loaded into the HPLC autosampler.

HPLC analysis was performed using a DX600 or ISC2500 HPLC system (Dionex), each consisting of a Dionex AS50 autosampler, a Dionex GP50 gradient pump, and a Dionex ED50 electrochemical detector. As described elsewhere, carbohydrates were separated on a CarboPac PA10 column (250 × 4 mm) with an amino-trap (50 × 14 mm) placed before the analysis column and a borate-trap (50 × 4 mm) placed before the injector port to remove trace amounts of borate from the mobile phase buffers (Ross et al. 2004; Schulz et al. 2005). For all samples, 20 μl were injected into a 25-μl injection loop. Samples were maintained at 4°C in the autosampler tray, and the chromatography was performed at room temperature.

Mobile phase buffers for the separation of carbohydrates were used as described above. Flow rate was maintained at 0.8–1.0 ml/min. To prevent carbonate contamination of the analysis column, a 50% sodium hydroxide solution containing <0.04% sodium carbonate was used to prepare mobile phase buffers (Fisher). Buffers were degassed and maintained under a helium atmosphere.

All carbohydrate metabolites were separated as described elsewhere (Ross et al. 2004; Schulz et al. 2005). In brief, hexoses and hexose-phosphates were separated using buffers A (15 mM NaOH) and B (50 mM NaOH/1 M NaAC) mixed using a low-salt gradient procedure with flow rate of 1 ml/min. The sample injection occurred at $t = 0$. *Gradient 1*: 98% A and 2% B (−10 to 8 min), a linear increase of B to 30% (8–15 min), a linear increase of B to 50% (15–25 min), hold 50% A and 50% B (25–30 min), and a linear decrease of B to 2% (30–35 min). External galactose was determined using a low-salt gradient procedure with a flow rate of 1 ml/min. *Gradient 2*: 98% B and 2% B (−5 to 5 min), a linear increase of B to 50% (5–10 min), hold 50% A and 50% B (10–15 min), and a linear decrease of B to 2% (15–17 min). UDP-hexoses were separated using a high-salt gradient procedure with a flow rate of 0.8 ml/min. *Gradient 3*: 50% A and 50% B for (−5 to 1 min), a linear increase of B to 70% (1–22 min), hold 30% A and 70% B (22–27 min), and a linear decrease of B to 50%. Carbohydrates were detected and were quantified as described elsewhere (Ross et al. 2004; Schulz et al. 2005).

Mutational Analysis of the hGALE Locus

Genomic DNA was isolated from lymphoblast cells, as described elsewhere, by use of phenol/chloroform extraction (Davis et al. 1994). The GALE locus, including all introns and segments stretching from 430 bp upstream of the start codon to 491 bp downstream of the stop codon, were PCR amplified in seven overlapping fragments by use of an MJ Research PTC-150 minicycler with Eppendorf TripleMaster enzyme (Brinkmann), in accordance with the manufacturer's recommendations. PCR primer sequences are presented in table 1. All PCR programs included a 5-min initial denaturation (at 94°C) followed by 32 cycles of denaturation, annealing, and extension. The annealing temperatures and extension times used to amplify each fragment varied according to primer melting temperature and anticipated product size (see table 2). After amplification, PCR products were verified on 1% agarose gels and were purified using the PureLink PCR Purification Kit (Invitrogen), in accordance with the manufacturer's instructions. Sequencing reactions were performed by Macrogen.

Statistical Methods

We conducted tests of normality for enzyme activity levels separately in cases and controls, using a Shapiro-Wilk (Shapiro and Wilk 1965) test, as implemented in SAS PROC UNIVARIATE. We also estimated and tested the correlation between the activity levels of different enzymes separately in cases and controls, using a Spearman correlation coefficient (Hotelling and Pabst 1936), as implemented in SAS PROC CORR. Finally, for a particular enzyme, we examined whether the activity levels differed across different groups, using either a Kolmogorov-Smirnov test (Kolmogorov 1941) (assuming two groups) or a Kruskal-Wallis test (Kruskal 1952) (assuming more than two groups), as implemented in SAS PROC NPAR1WAY. For the Kolmogorov-Smirnov and Kruskal-Wallis tests, we determined exact *P* values to ensure valid inference in the presence of a small sample size.

Results

Defining the Normal Range of hGALE Activity in Human Lymphoblasts

Before initiating studies of hGALE activity in lymphoblasts derived from patients with RBC hGALE impairment, we first sought to define the range of hGALE activity in lymphoblasts derived from control subjects. Toward this end, we established 10 control lines and characterized each with regard to both hGALE and hGALT activities. Because normal hGALE catalyzes both the interconversion of UDP-Gal/UDP-Glc and the

Table 2

PCR Programs Used to Amplify Segments of the hGALE Gene

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

interconversion of UDP-GalNAc/UDP-GlcNAc (Piller et al. 1983; Schulz et al. 2004), we measured both reactions. Our results, presented in figure 2, demonstrate three important points.

First, while hGALE activity values with regard to any one substrate (UDP-Gal or UDP-GalNAc) and any one cell line were highly reproducible, even across separate cell harvests, these values varied from line to line. For example, GALE activity values with regard to UDP-Gal differed by up to almost a factor of two in pairwise comparisons between samples. The height of each bar plotted in figure 2 represents the hGALE specific activity for that sample; the number listed over each bar represents the same value normalized to the mean of the entire set. Results from Shapiro-Wilk tests ($P = .0083$) suggest that these values are not normally distributed, although these tests are likely sensitive to the small sample size considered in the study. With the assumption that these control lymphoblast lines biochemically reflect the individuals from whom they were derived, as appears to be the case for patient lymphoblasts characterized elsewhere with regard to hGALE (Quimby et al. 1997), this observation suggests that healthy subjects exhibit levels of hGALE activity that vary by up to a factor of two. In comparison, activities measured for a single cell line harvested and assayed at different times varied by no more than 15% (data not shown). The genetic, environmental, or other factors that underlie this individual-to-individual variation remain unclear.

The second important point illustrated in figure 2 is that, whereas hGALE activity levels in different cell lines varied for both substrates, they varied much less with regard to UDP-GalNAc than with regard to UDP-Gal. Furthermore, there was modest, albeit not statistically significant, evidence to suggest that the hGALE activities toward the two substrates were correlated (Spearman correlation 0.507; $P = .130$).

The third point illustrated in figure 2 is that, despite variations in hGALE activity, the hGALT activities detected in these same cell lines varied by <1.5-fold. Furthermore, as expected, there was no evidence for positive correlation between either the UDP-Gal or the UDP-

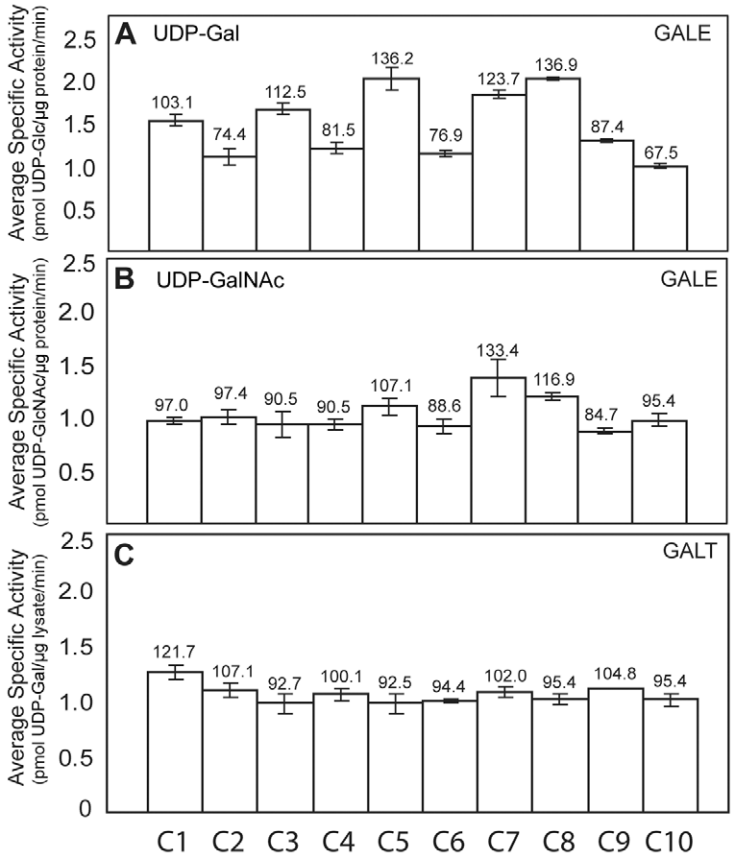

Figure 2 Enzyme activities in control lymphoblasts. Values plotted represent average specific activity ± SEM ($N \geq 3$). Numbers listed above each bar are normalized to the mean of the set. C1–C10 designate the individual control cell lines studied. *A,* hGALE activity with regard to UDP-Gal. *B,* hGALE activity with regard to UDP-GalNAc. *C,* hGALT activity.

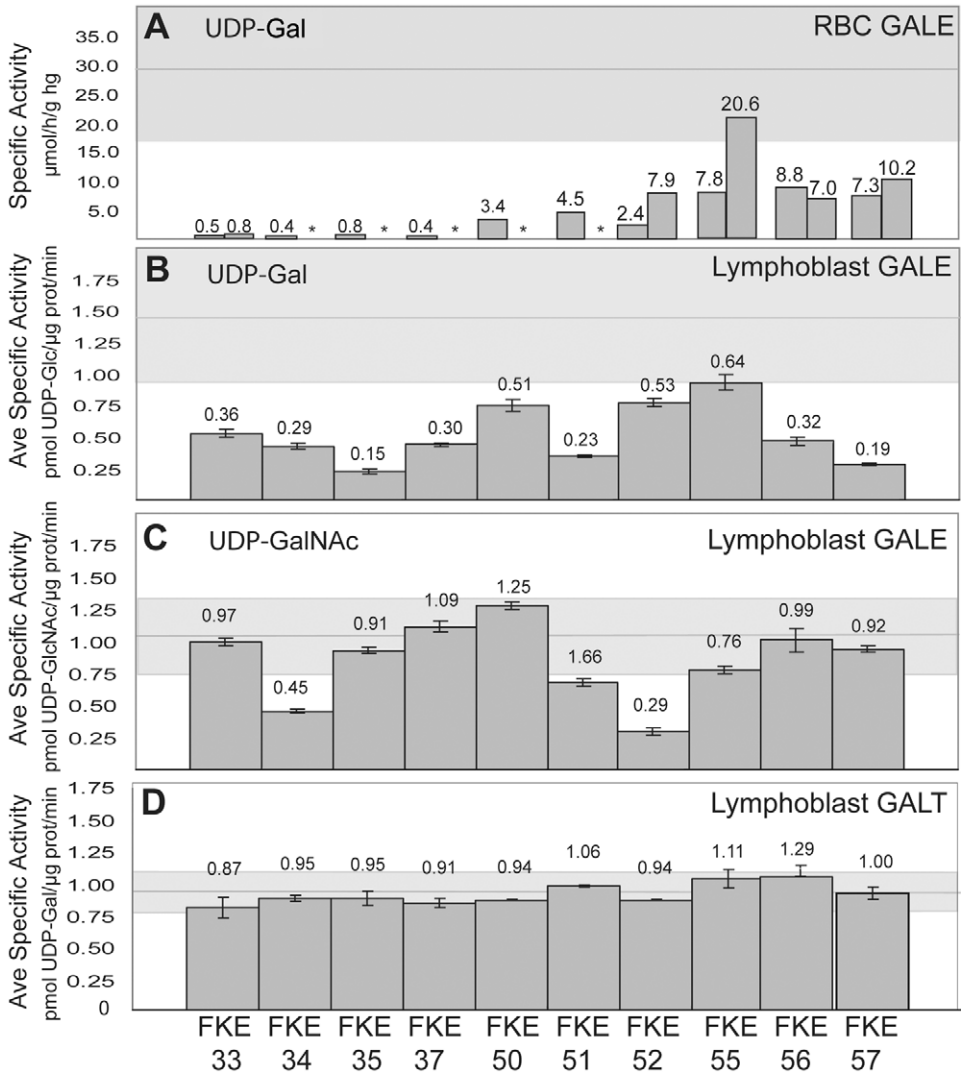

Figure 3 Enzyme activities in patient hemolysates and lymphoblasts. FKE numbers designate the individual patients enrolled in this study. *A,* RBC hGALE. Values plotted represent individual measurements of hemolysate hGALE specific activity with regard to UDP-Gal. Each asterisk (*) represents a missing value. Shaded area represents the control range for the assay, as reported by the clinical laboratory performing the assay. *B,* Lymphoblast hGALE. Values plotted represent average specific activity ± SEM ($N \geq 3$) with regard to UDP-Gal. Numbers listed above each bar are normalized to the mean of the corresponding control set. Shaded area represents the range of corresponding control activities. *C,* Lymphoblast hGALE. Values plotted represent average specific activity ± SEM ($N \geq 3$) with regard to UDP-GalNAc. Numbers listed above each bar are normalized to the mean of the corresponding control set. Shaded area represents the range of corresponding control activities. *D,* Lymphoblast hGALT. Values plotted represent average specific activity ± SEM ($N \geq 3$). Numbers listed above each bar are normalized to the mean of the corresponding control set. Shaded area represents the range of corresponding control activities. Ave = average; prot = protein.

GalNAc activities of hGALE and hGALT activity (Spearman correlation −0.232 and −0.031, respectively; $P = .520$ and $P = .920$, respectively).

A Spectrum of GALE Impairment in Patient Lymphoblasts

To define the extent of hGALE impairment in nonperipheral cells from infants with RBC hGALE impairment, we repeated the study described above with the use of lymphoblasts from 10 unrelated patients previously identified as RBC hGALE deficient in a clinical lab. Available RBC GALE data on these patients are presented in figure 3*A*.

The definition of peripheral epimerase deficiency dictates that, whereas hGALE activity is impaired in hemolysates, it is normal or near-normal in nonperipheral cells, including fibroblasts and transformed lymphoblasts (Gitzelmann 1972; Mitchell et al. 1975). Nonetheless, our results clearly demonstrated a spectrum of lymphoblast hGALE activities in the cohort of patients

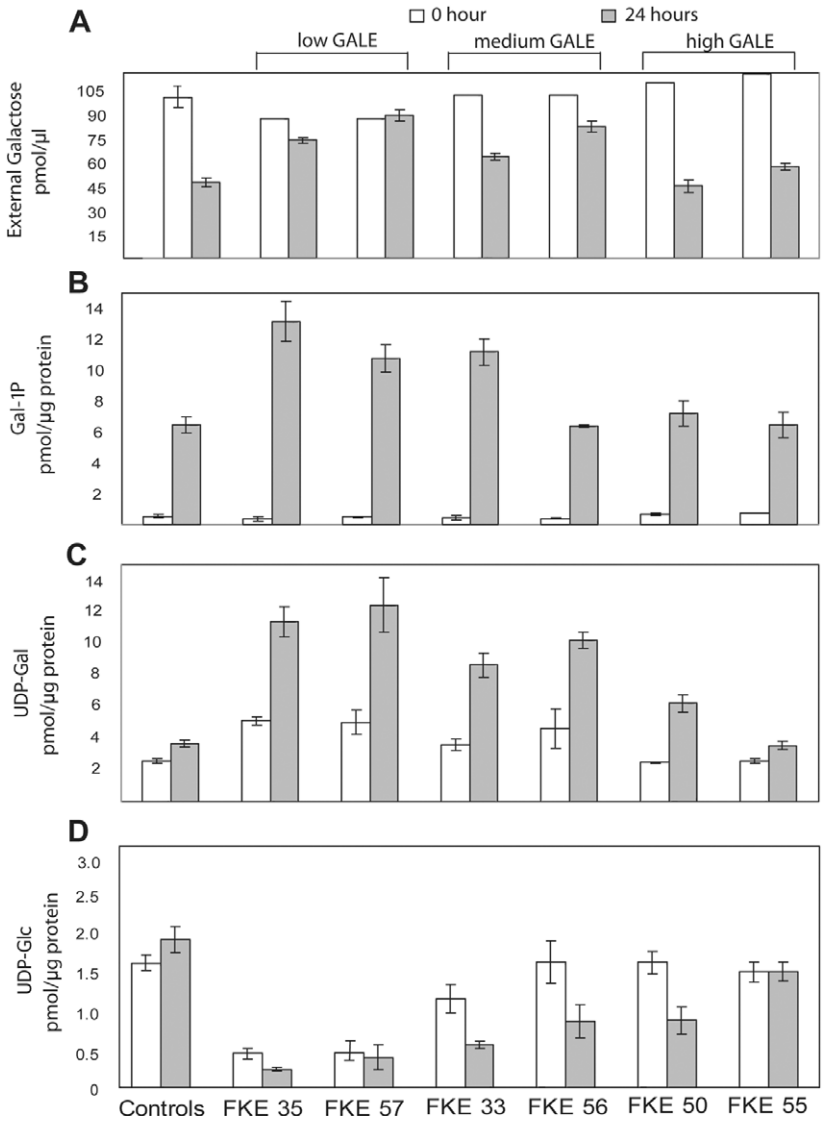

Figure 4 Metabolites in control and patient lymphoblasts exposed to galactose. *A,* External galactose in the medium of control and patient lymphoblasts at $t = 0$ (*open bar*) and at $t = 24$ h after addition of 0.5 mM galactose to the medium (*shaded bar*). Values plotted are average ± SEM ($N \geq 3$). Control values were averaged from each of six different control cell lines (C1, C2, C3, C5, C7, and C10), each assayed three times. Patient cell lines designated "low," "medium," and "high" hGALE had <20%, 30%–40%, and >50%, respectively, of control (mean of the control set) hGALE activity with regard to UDP-Gal. *B,* Internal Gal-1P. Values plotted are average ± SEM ($N = 3$) for each cell line. Open bars represent $t = 0$; shaded bars represent $t = 24$ h after addition of 0.5 mM galactose to the medium. *C,* Internal UDP-Gal. Values plotted are average ± SEM ($N = 3$) for each cell line. Open bars represent $t = 0$; shaded bars represent $t = 24$ h after addition of 0.5 mM galactose to the medium. *D,* Internal UDP-Glc. Values plotted are average ± SEM ($N = 3$) for each cell line. Open bars represent $t = 0$; shaded bars represent $t = 24$ h after addition of 0.5 mM galactose to the medium.

studied, ranging from a low of ~15% of the control mean to a high of ~64% of the control mean (fig. 3*B*). The integrity of each lysate was confirmed by analysis of hGALT, which demonstrated consistently normal activity (fig. 3*D*). Whereas small sample size and nonrandom ascertainment prevent extension from these data to estimate the population distribution of lymphoblast hGALE in RBC hGALE–deficient individuals as a group, the conclusion that many of these patients have hGALE impairment that extends to their lymphoblasts is unavoidable. Indeed, a Kolmogorov-Smirnov test of difference between controls and cases for UDP-Gal activity produced an exact P value of 1.09×10^{-5}. Unexpectedly, comparison of the hGALE deficiency in these patients with regard to both substrates demonstrated that enzyme activities with regard to UDP-Gal and UDP-

GalNAc were not significantly correlated (correlation 0.030; $P = .9338$. Indeed, the patient exhibiting the most severe impairment with regard to UDP-Gal (FKE35, ~15% of control) demonstrated essentially normal activity with regard to UDP-GalNAc (~90% of control). Conversely, the patient exhibiting the most severe impairment with regard to UDP-GalNAc (FKE52, ~29% of control) exhibited one of the higher activities with regard to UDP-Gal (~53% of control).

Finally, data presented in figure 3*A* and 3*B* illustrate that, whereas the RBC hGALE values reported for these 10 patients varied by >20-fold, they showed no significant evidence of correlation with the lymphoblast hGALE activities detected (correlation 0.280; $P = .434$). There are many possible explanations for this lack of correlation among the RBC hGALE activity values determined in the clinical labs, ranging from differences in the expression or function of different hGALE alleles in different cell types to the potential impact of diet or other biological factors or even to varying shipping and handling conditions or differing assay conditions. Indeed, the fact that repeat clinical assays on two of the patients in this study varied internally by close to a factor of 3 (FKE52 and FKE55) demonstrates the inherent variability in these values.

Metabolic Consequence of Partial GALE Impairment in Human Lymphoblasts

Whereas studies of GALE enzyme activity in vitro are useful, the more important parameter to measure is the metabolism of galactose in living cells. Toward that end, we monitored the metabolic response of selected control and patient lymphoblasts cultured in the presence and absence of 0.5 mM galactose. In brief, cells cultured in standard RPMI 1640 medium (containing 11.1 mM glucose) were divided into duplicate flasks at time zero, and 0.5 mM galactose was added to one flask from each set. External galactose and internal Gal-1P, UDP-Gal, and UDP-Glc were then monitored in samples harvested at 0 h and 24 h from these cultures (fig. 4). Twelve lines were studied, including six control and six patient lines. The control lines were selected to represent the spectrum of hGALE values evident in the control population studied (C1, C2, C3, C5, C7, and C10). The patient lymphoblasts were selected to include the two "lowest hGALE UDP-Gal activity" lines (FKE35 and FKE57), two "intermediate hGALE activity" lines (FKE33 and FKE56), and the two "highest hGALE activity" lines (FKE50 and FKE55).

With regard to external metabolites, by 24 h, all the control lines had consumed ~50% of their galactose. In contrast, the two patient lines with the lowest hGALE activity had each consumed less than ~15% of their galactose. The patient lines with the intermediate and highest level hGALE activity had each consumed between ~19% and ~50% of their galactose (fig. 4*A*). A Kruskal-Wallis test of difference between the four groups monitored (control and patient low, medium, and high) demonstrated that, whereas, as expected, there was no significant difference in the starting ($t = 0$) external galactose values for the four cultures (exact $P = .3817$), by 24 h, the levels of galactose remaining for the different groups of cells were significantly different from one another (exact $P = .0088$).

With regard to internal metabolites, many of the patient lines again displayed abnormalities. For example, whereas all of the control cell lines accumulated 6–7 pmol Gal-1P/μg protein after exposure to galactose, the most severely hGALE-impaired patient lines accumulated nearly twice that amount (fig. 4*B*). Similarly, whereas galactose exposure had little effect on UDP-Gal accumulation in control lymphoblasts, UDP-Gal levels in the patient cells essentially doubled in the presence of galactose for all but those with the highest hGALE activity. It is interesting to note that even basal UDP-Gal levels (in the absence of galactose exposure) were higher in the more severely affected patient lines, perhaps reflecting the altered metabolism of sugars scavenged from serum glycoproteins and glycolipids. Finally, whereas every control line demonstrated either unchanged or elevated UDP-Glc in response to galactose exposure, almost every patient line demonstrated depressed UDP-Glc after exposure to galactose. A Kruskal-Wallis test of difference between the four groups at 24 h was highly significant (exact $P = .0046$). Although the quantitative distinctions between control and patient lines for these internal metabolites were not huge, given the limited nature of the hGALE impairment, they were nonetheless remarkable.

RBC Metabolic Consequences of Partial GALE Impairment in Patients on Normal versus Galactose-Restricted Diets

Our lymphoblast studies clearly suggested that partial impairment of hGALE can result in metabolic abnormalities for cells exposed to galactose in culture. Nonetheless, how well do these results mimic the metabolic reality for patients? To address this question, we collected as much dietary and metabolic information as possible with regard to the patients enrolled in this study. Although this information was unavailable for some patients, for others we were able to learn if and when dietary restriction of galactose was initiated and/or discontinued and how RBC Gal-1P values responded to these changes in diet. The results were striking. In brief, whereas all the patients presumably exhibited elevated RBC galactose plus Gal-1P levels as neonates on normal diet—which is, in part, how they were ascertained by

the newborn screening programs—these Gal-1P values all essentially normalized in response to dietary restriction of galactose. Many of these children were later challenged with dietary galactose or were removed from dietary restriction altogether, and, whereas some demonstrated normal RBC Gal-1P values on relaxed diets later in childhood, others did not (table 3). Of note, one patient in this study (FKE37) demonstrated five consecutive abnormal RBC Gal-1P values following discontinuation of dietary galactose restriction. Another patient with marked lymphoblast hGALE impairment (FKE35) demonstrated inconsistent Gal-1P levels on dietary restriction but later demonstrated normal Gal-1P values despite a diet that contained galactose. This same patient also demonstrated abnormally *low* levels of RBC galactitol and galactonate while on dietary restriction (e.g., 0.16 μmol/liter [normal ± SD = 1.25 ± 0.35 μmol/liter] and 0.18 μmol/liter [normal ± SD = 3.72 ± 1.16 μmol/liter], respectively) (Ficicioglu et al. 2005); these values rose slowly back toward the normal range (0.62 μmol/liter and 2.26 μmol/liter, respectively) when galactose was restored to her diet.

A Study of GALE Heterozygotes

In addition to studying hGALE-impaired patients, we also explored the possibility of enzyme or metabolic impairment in the lymphoblasts of obligate hGALE heterozygotes—namely, the parents of one of the most severely affected patients in our group (FKE35). As predicted, the hGALE enzyme activities detected in these lines (65% of control and 70% of control) were intermediate between the mean value for controls and the level seen in their child, which placed them at the low end of the normal range. The RBC GALE activity level in one parent was 13.1 μmol UDP-Glc/h/g hemoglobin (hgb), which is within the reported carrier range (12.0–20.0 μmol UDP-Glc/h/g hgb) (Shin 1991), and for the other parent it was 17.5 μmol UDP-Glc/h/g hgb, which is also within the reported carrier range, although clearly there is overlap between the high end of this range and the low end reported as normal (17.1–40.1μmol UDP-Glc/h/g hgb) (Shin 1991). Consistent with data from "low hGALE activity" control lines or "high hGALE activity" patient lines, we observed no statistically significant metabolic abnormalities in these cells, after their exposure to environmental galactose (data not shown).

Mutations Detected in Patients with Partial GALE Impairment

Finally, as a step toward addressing the molecular basis of differential hGALE impairment in different patients, we sequenced the hGALE alleles of eight patients (16 alleles) in our study. As described in the "Material and Methods" section, the regions sequenced included both coding and noncoding domains stretching from 430 bp upstream of the hGALE translation initiation site to 491 bp downstream of the hGALE stop codon. In parallel, we also sequenced the hGALE alleles from three normal controls (six alleles). Our results, summarized in table 4 and table 5, demonstrated two main points.

First, by comparing our sequences with those in the reference sequence (GenBank accession number AF022382), we identified 49 positions (table 5) at which all our sequences, both control and patient, differed from the reference sequence. It is important to point out that, while distinct from the reference sequence, these base changes were nonetheless identical in all 22 hGALE alleles sequenced for this study, including 6 control alleles and 16 patient alleles representing at least four

Table 3

Gal-1P Levels Detected in the Hemolysates of Patients

Patient	Birth Year	Sex[a]	Race[b]	Gal-1P Values[c]	Normal Range
FKE33	1999	M	L	.15*, .04*, ***1.13***, .05*, .09* μmg/g hgb	0–.17 μmg/g hgb
FKE34	2001	F	H	4.5, 3.6*, .25*, .59*, .47 mg/100 ml RBCs	<1 mg/100 ml RBCs
FKE35	2002	F	MR	.0*, .94*, ***3.2****, .07*, .07*, .0, .0 mg/100 ml RBCs	<1 mg/100 ml RBCs
FKE37	2001	F	H	.02*, .06*, .06*, .01*, ***0.28****, .05*, .00*, ***.24, .24, .52, .24, .34*** μmg/g hgb	0–.17 μmg/g hgb
FKE50	2003	F	AA	***31.8***, .00*, .06 mg/100 ml RBCs	<1 mg/100 ml RBCs
FKE51	2003	M	AA	Unknown	…
FKE52	2003	F	AA	***2.1***, .7*, .26* mg/100 ml RBCs	<1 mg/100 ml RBCs
FKE55	2004	UK	AS	***11.2***, <1 mg/100 ml RBCs (diet unknown)	<1 mg/100 ml RBCs
FKE56	2004	M	AA	.0 (diet unknown), <1* mg/100 ml RBCs	<1 mg/100 ml RBCs
FKE57	2004	M	AA	<.1* mg/100 ml RBCs	<1 mg/100 ml RBCs

[a] UK = sex unknown.

[b] L = Latin American, H = Hispanic, MR = mixed race, AA = African American, and AS = Asian.

[c] Gal-1P values are listed chronologically in terms of the order in which the test was performed. Values listed in bold italics are abnormal. An asterisk indicates that the patient was on a galactose-restricted diet at the time of testing. Values without an asterisk indicate that the patient was on a normal diet at the time of testing.

Table 4

Base Changes Found in hGALE-Deficient Patients

Patient and Base Change	Amino Acid Substitution	Intron	Reference(s)
FKE34[a]:			
C3192T (het, unk)	P293L	...	This article; S. M. Huguenin and T. Cowan, personal communication
FKE35:			
A2712G (het, pat)	K257R	...	Alano et al. 1997; Maceratesi et al. 1998; Wasilenko et al. 2005
C1623T (het, mat)	Y136Y	...	Present article
C1639T (het, mat)	L142 L	...	Present article
FKE37[b]:			
C1664T (hom)	T150M	...	Present article
FKE50:			
G1698T (het, unk)	K161N	...	Present article
G253A (het, unk)	...	A	Present article
C839A (het, unk)	...	C	Present article
FKE51:			
G3270A (het, unk)	G319E	...	Alano et al. 1997; Maceratesi et al. 1998; Wasilenko et al. 2005
G3226A (het, unk)	N304N	...	Present article
A142T (het, unk)	...	A	Present article
C232T (het, unk)	...	A	Present article
T250C (het, unk)	...	A	Present article
Ins TA after 250 (het, unk)	...	A	Present article
A252G (het, unk)	...	A	Present article
G349T (het, unk)	...	A	Present article
FKE52:			
G1267A (het, unk)	Q99Q	...	Present article
G1590A (het, unk)	K125K	...	Present article
G1738A (het, unk)	D175N	...	Present article
FKE56[a]:			
A2712G (het, unk)	K257R	...	Alano et al. 1997; Maceratesi et al. 1998; Wasilenko et al. 2005
FKE57:			
C1213G (het, unk)	S81R	...	Present article
G3270A (het, unk)	G319E	...	Alano et al. 1997; Maceratesi et al. 1998; Wasilenko et al. 2005
G1017C (het, unk)	...	C	Present article
C1018A (het, unk)	...	C	Present article
A1020G (het, unk)	...	C	Present article

NOTE.—As indicated, some coding region base changes resulted in missense mutations, whereas others resulted in silent mutations. het = heterozygous mutation; hom = homozygous mutation; pat = paternal origin; mat = maternal origin; unk = parental origin unknown.

[a] Second mutation not identified despite completed sequence.

[b] Consanguineous.

different racial groups (Hispanic, African American, mixed race including white, and Asian). Although 48 of these changes impacted noncoding sequence, one was a missense substitution (T2298C, encoding V180A) that we had also reported elsewhere (Quimby et al. 1997). At least 13 other hGALE cDNA or partial cDNA sequences available in GenBank agreed with our sequence at this position (accession numbers NM_000403, NM_001008216, BC001273, CR616589, CR611350, CR602422, CR596991, CR592671, BC050685, L41668, CR592211, CR616462, and CR601378). A second human genomic GALE sequence (GenBank accession number AL031295) also varied from the AF022382 reference sequence at all 49 positions noted here, although, in three positions, the discrepancies were distinct from those we observed. There were also nine locations, all noncoding, at which our sequence agreed with AF022382 but not with AL031295. Considering these data together, we conclude that most, if not all, of the 49 base changes we identified in both patients and controls represent either errors in the deposited sequence or, alternatively, polymorphisms for which the sequence reported here is by far the predominant allele. We have deposited our normal hGALE genomic sequence and also a corrected version of the coding sequence in GenBank for future use as reference alleles (accession numbers DQ233667 [gene] and DQ233668 [coding sequence]).

The second conclusion resulting from our sequencing data is that most nonconsanguineous patients are compound heterozygotes who carry a mix of coding and noncoding base substitutions. Whereas some of these substitutions have been reported elsewhere (e.g., K257R or G319E [Alano et al. 1997; Maceratesi et al. 1998;

Table 5

Base Changes Relative to the hGALE Reference Sequence Found in All Patient and Control Alleles

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Wasilenko et al. 2005] and P293L [S. M. Huguenin and T. Cowan, personal communication]), the majority identified in this study (table 4) were novel. Future studies will explore the functional significance of these substitution mutations.

Discussion

The data reported here demonstrate two fundamental points with regard to epimerase-deficiency galactosemia. First, biochemical hGALE deficiency is not a binary condition; it is a continuum disorder. Not all the infants ascertained for this study with RBC epimerase deficiency exhibited normal hGALE activity in nonperipheral cells. We propose to apply the term "intermediate GALE deficiency" to patients who exhibit partial impairment of hGALE activity in nonperipheral cells, such as lymphoblasts. It is also important to note that many patient lines demonstrated impairment of hGALE activity, not only with regard to UDP-Gal but also with regard to UDP-GalNAc. Considering the importance of both reactions to the maintenance of normal UDP-sugar pools in mammalian cells, it remains unknown which loss of activity plays the greater role in defining patient outcome in epimerase-deficiency galactosemia. More in-depth and long-term studies of these and other patients may help to address this question. Finally, it is important to stress that, whereas all the patients ascertained exhibited RBC hGALE impairment, there was no statistically significant correlation between the degree of RBC hGALE impairment and the degree of lymphoblast hGALE deficiency observed. This is an important point, because it underscores the reality that RBC hGALE activity level cannot be used to distinguish peripheral from intermediate or other forms of epimerase-deficiency galactosemia. This point further raises the possibility of tissue-specific factors that influence either expression or function of hGALE. This concept is fully consistent with previous reports of tissue-specific GALE impairment in patients with peripheral epimerase deficiency (Gitzelmann 1972; Gitzelmann and Steimann 1973; Mitchell et al. 1975; Gitzelmann et al. 1977). One likely factor that may contribute to loss of GALE activity in RBCs relative to fibroblasts or lymphoblasts is compromised stability. Indeed, we have previously demonstrated evidence of instability in the proteins encoded by a small number of hGALE alleles derived from patients with ostensibly peripheral GALE deficiency (Wohlers et al. 1999). The possibility of other factors that contribute as tissue-specific modifiers of hGALE expression or function remains an open question.

Second, many of the intermediate hGALE deficiency lymphoblast lines studied here and at least some of the patients from whom these lines were derived demonstrated metabolic evidence of impaired galactose metabolism both in vitro and in vivo, in the presence of environmental galactose, and, in some cases, also in the absence of galactose. The most notable metabolic abnormalities observed in the presence of environmental galactose included markedly elevated Gal-1P and UDP-Gal and depressed UDP-Glc. The most notable metabolic abnormalities observed in patient cells cultured in the *absence* of environmental galactose included mildly elevated UDP-Gal and depressed UDP-Glc. It is important to point out that, whereas no free galactose was intentionally added to the medium bathing these cells, their medium did contain 10% fetal bovine serum, which is rich in glycoproteins and glycolipids that could serve as substrates for a salvage pathway (Krieger et al. 1989), thereby providing a "backdoor" supply of low levels of exogenous galactose to the cells. The ratio of UDP-Glc/UDP-Gal detected in control lymphoblasts cultured in both the presence and absence of galactose was between 0.5 and 0.67; this value is low compared with the 1.4 ratio reported elsewhere for human lymphoblasts (Wehrli et al. 2001). The basis for this disparity remains unknown but may reflect differences in the culture conditions used or in the procedures used for cell harvest, sample preparation, or analysis. What is striking is that the UDP-Glc-to-UDP-Gal ratio in the more severely hGALE-impaired patient cells was close to 0.1 in the absence of galactose and close to 0.02 in the presence of galactose. This is an extremely abnormal ratio and could have significant implications for the ability of these cells to synthesize normal glycoproteins and glycolipids.

As mentioned above, one patient with low lymphoblast hGALE activity was also monitored with regard to RBC galactitol and galactonate, both while on dietary restriction of galactose and then again after the introduction of dietary galactose. This patient demonstrated abnormally low levels of both metabolites in the absence of dietary galactose, and both values essentially normalized in response to treatment with dietary galactose. This result is striking not only because it serves as further evidence of abnormal galactose metabolism in vivo in a patient with intermediate GALE deficiency but also because this result is the opposite of what is seen for patients with transferase-deficiency galactosemia. Patients with even partial (Duarte/classic compound heterozygous galactosemia) GALT deficiency often demonstrate *elevated,* not depressed, levels of RBC galactitol and galactonate relative to age-matched controls (Yager et al. 2003; Ficicioglu et al. 2005).

At present, the clinical significance of all these metabolic abnormalities remains unclear but, given that some of the patients on normal diets (e.g., FKE37 and FKE52) demonstrated Gal-1P levels indistinguishable from those seen in treated patients with classic galactosemia, the concern remains that at least some patients with intermediate hGALE deficiency who are not treated may be at risk for long-term complications. Given that hGALE-deficiency in most patients is never diagnosed or, if it is diagnosed, those patients are lost to follow-up at an early age, the long-term outcome for most patients is never ascertained. Future large-scale, longitudinal studies will be required to address this issue. Unfortunately, states that drop total galactose (Gal + Gal-1P) screening from their newborn profiles will further exacerbate the current problem, since all patients with epimerase-deficiency galactosemia will demonstrate normal GALT activity and will, therefore, slip through an abbreviated screen undetected.

Impact of Impaired GALE in Mammalian Cells

Few studies have explored the biochemical or phenotypic impact of GALE impairment in mammalian cells cultured in the presence or absence of exogenous galactose (Robinson et al. 1963, 1966; Kingsley et al. 1986*a*, 1986*b*; Krieger et al. 1989; Schulz et al. 2005). Perhaps the best studied of these cells are the Chinese hamster ovary (CHO) cell–derived ldlD line, originally selected on the basis of impaired LDL receptor function by Krieger and colleagues (1981). Subsequent studies (Kingsley et al. 1986*a*; Krieger et al. 1989) defined this phenotype as secondary to a complete loss of GALE activity, resulting in the abnormal processing of both *N*- and *O*-linked glycoproteins, including the LDL receptor. These studies further demonstrated that, whereas supplementation of the culture medium with low levels of galactose and GalNAc restored both glycoprotein processing and LDL receptor function, supplementation of the medium with >125 μM galactose resulted in impaired ldlD cell growth. Normal CHO cells exposed to galactose in parallel demonstrated no growth abnormalities (Krieger et al. 1989).

Recent studies from our laboratory both confirmed and extended these earlier reports (Schulz et al. 2005). In particular, we demonstrated that not only ldlD cells alone, which exhibit no detectable GALE activity, but also ldlD cells transfected to express low (5%–10% of wild-type) levels of hGALE are growth impaired in the presence of 250 μM galactose. We further demonstrated that both ldlD cells and ldlD cells expressing low levels of hGALE accumulate abnormally high levels of Gal-1P and UDP-Gal and abnormally low levels of UDP-Glc upon exposure to 250 μM galactose. Both wild-type CHO and ldlD cells transfected to express wild-type levels of hGALE did not display these abnormalities (Schulz et al. 2005).

The quantitative relationship between the level of GALE expression and both the requirement for and the sensitivity to exogenous galactose in mammalian cells remains unclear. The most severely hGALE-impaired patients reported to date (Walter et al. 1999) are all homozygous for the V94M mutation associated with at least 5% residual activity (Wohlers et al. 1999; Wohlers and Fridovich-Keil 2000). These cells fail to demonstrate abnormal LDL receptor structure or function in culture (Kingsley et al. 1986*b*), although these patients clearly display a spectrum of metabolic and other abnormalities, some likely related to hGALE dysfunction, others likely not related (Walter et al. 1999). Finally, results reported here demonstrate that patients and/or their cells with intermediate levels of hGALE impairment can display abnormally high levels of Gal-1P in the presence of galactose and abnormally high levels of UDP-Gal and low levels of UDP-Glc even in the absence of galactose. These data clearly support the conclusion originally put forward by Kalckar (1965) that, unlike patients with classic galactosemia, GALE-deficient patients may require at least a limited amount of dietary galactose for normal homeostasis.

Our studies of ldlD cells demonstrated that uridine supplementation resolved some but not all of the growth and metabolic abnormalities in these GALE-deficient cells upon exposure to exogenous galactose (Schulz et al. 2005). Studies are currently under way to test the impact of uridine supplementation on patient cells.

Mutations in hGALE

From hGALE sequence analysis of eight of the patients in this study, we identified 23 distinct base changes, of which 20 were novel (table 4). All of the changes identified were found in the heterozygous or compound heterozygous state, except for a T150M missense substitution, which was found in the homozygous state in one patient of consanguineous origin. Of the six novel missense substitution mutations reported here, only one, K161N, impacts a residue that is fully conserved among the human, rat, yeast, and *E. coli* GALE sequences. Nonetheless, most of the amino acid substitutions are themselves nonconservative.

Surprisingly, most patients did not demonstrate precisely two mutations. Five patients (FKE35, FKE50, FKE51, FKE52, and FKE57) each demonstrated three or more base changes, the majority of which were either noncoding or silent substitutions. Future studies will be required to dissect the functional significance of each of these base changes and to define each as either a causal mutation or a neutral polymorphism. Two additional patients (FKE34 and FKE56) each demonstrated only a

single heterozygous mutation, despite complete sequencing of the hGALE locus. The absence of a second detectable mutation in each of these patients remains unexplained, although either of two possibilities may apply. First, there may be a second mutation that was not detected by our sequencing strategy. Extreme upstream or downstream regulatory mutations outside the targeted area, for example, would not have been detected. Alternatively, a partial gene deletion may have precluded PCR amplification of portions of one GALE allele, although the fact that one mutation was detected in the heterozygous state in each of these patients rules out the possibility of a full hGALE gene deletion. Future studies will address this question. The second possible explanation is that the two heterozygous "patients" are actually heterozygote carriers, and there is no second mutation. Parental studies will be required to address this possibility.

Acknowledgments

Our first and most important acknowledgment is to the patients and families who participated in this study. Without their selfless generosity, none of this work would have been possible. We also thank Stanton Segal, Stephen Shear, Deborah Boylan, Virginia Proud, Heather Creswick, Rebecca Sanders, and Uriel Castaneda for their contributions to this work. This project was supported by grant awards from the National Institutes of Health (to J.L.F.-K.), as well as by funds from the Achievement Rewards for College Scientists Foundation (to J.M.S.). This work is respectfully dedicated to the memory of Dr. Michael Palmieri.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

ExPASy, http://us.expasy.org/ (for GALK [EC 2.7.1.6], GALT [EC 2.7.7.12], and GALE [EC 5.1.3.2])

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for hGALE reference sequences [accession numbers AF022382, NM_000403, NM_001008216, BC001273, CR616589, CR611350, CR602422, CR596991, CR592671, BC050685, L41668, CR592211, CR616462, CR601378, AL031295, DQ233667, and DQ233668])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/entrez/Omim/

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