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Evidence for Alternate Galactose Oxidation in a Patient with Deletion of the Galactose-1-Phosphate Uridyltransferase Gene

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The persistent, dietary-independent elevation of galactose metabolites in patients with galactose-1-phosphate uridyltransferase (GALT) deficiency is probably secondary to *de novo* synthesis of galactose. Relatively constant steady-state levels of galactose metabolites in patients also suggest that non-GALT metabolic pathways must function to dispose of the galactose synthesized each day. The discovery of a patient with a rare deletion of the GALT gene provided a unique opportunity to examine the availability of any alternate galactose oxidative capacity both *in vivo* and *in vitro*. Utilizing genomic DNA from the patient, Southern blot data demonstrated that 10 of the 11 GALT exons were homozygously deleted. By measurement of $^{13}CO_2$ in expired air for up to 24 h after an oral bolus of [1-^{13}C]galactose, it was demonstrated that 17% of the galactose was metabolized, a value comparable to the 3-h elimination rate in a control subject. Furthermore, lymphoblasts prepared from the patient could also convert [1-^{14}C]galactose to $^{14}CO_2$. This unique study provides the first unambiguous evidence that another pathway exists in man that can be responsible for galactose disposal. Further knowledge of this alternate galactose oxidative route and its regulation may aid in formulating new strategies for the treatment of galactosemia.

Key Words: **galactosemia; galactose-1-phosphate uridyltransferase (GALT); [^{13}C]galactose breath test; alternate galactose pathway; galactose; galactose-1-phosphate; galactitol; galactonate.**

Hereditary galactosemia due to galactose-1-phosphate uridyltransferase (GALT) deficiency results in brain and ovarian complications that appear unrelated to dietary galactose restriction (1). We have recently shown that the persistent elevation of galactose metabolites in blood and urine of well-treated patients, a phenomenon of distinct importance to the pathogenesis of complications, is probably secondary to endogenous *de novo* synthesis of galactose (2). As this rate of endogenous galactose production may be in the order of 1 g per day in adults with galactosemia (2), there must be mechanisms whereby a comparable amount of galactose can be handled to maintain steady-state metabolite levels. Urinary galactitol and galactonate excretion are such examples but our data suggest that together they might only account for 30% of the daily synthetic load (2–5). A fraction or all of the remainder may be accounted for by oxidation as both carbon 1- and carbon 2-labeled galactose have been shown to be metabolized to CO_2 albeit at a fraction of the rate in controls, in patients with classic galactosemia undergoing 9–10 h of breath tests (6).

Whether patients with classic or severe galactosemia due to missense gene mutations such as the Q188R GALT mutation that results in absent or barely detectable GALT activity in erythrocytes possess residual hepatic GALT activity is controversial. If so, it might account for the low level of galactose oxidation to CO_2 and thus be the primary mechanism for maintenance of steady-state galactose metabolite levels in the treated state. Alternatively, this metabolism may be accomplished by other pathways such as the galactonate pathway that may

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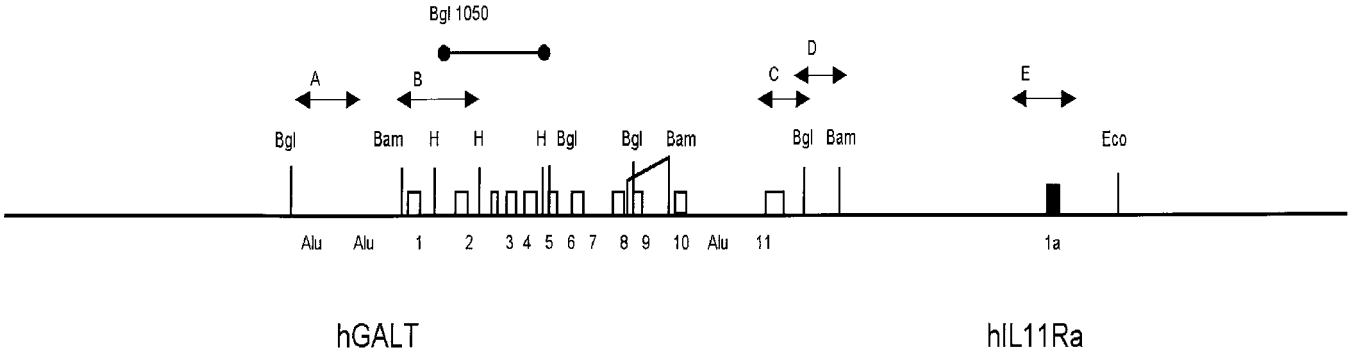

FIG. 1. Restriction map of the GALT gene and flanking regions. This composite map is drawn from analysis of the hGALT gene sequence (GenBank Accession No. M96264), the human hIL-11Rα sequence (GenBank Accession No. U32323), and RP-11BAC 195 F19 (GenBank Accession No. AL162231). Alu sequences were deduced using Repeat Masker (http://gtp.genome.washington.edu/cgi-bin/Repeat Masker). Sequences amplified by primer pairs A, B, C, D, and E are shown with ◀▶. The genomic probe, *Bgl* 1050, is shown by ●–●.

interface with the pentose phosphate shunt (7). In this report, we provide evidence that a pathway(s) exists enabling a patient to metabolize a substantial amount of galactose. In an Ashkenazi Jewish patient with a GALT gene deletion mutation (8,9) 17% of a bolus of [1-^{13}C]galactose was metabolized to $^{13}CO_2$ in 24 h. This amount was comparable to the 3-h elimination rate in a control subject. Although the alternate pathway(s) are slower than normal, they may account for the metabolism of the bulk of galactose synthesized *de novo* (2).

MATERIALS AND METHODS

Characterization of the GALT Deletion Mutation: Southern Analysis

Genomic DNA was isolated from transformed lymphoblasts by standard methods. For each restriction enzyme, 40 μg of DNA was digested with 100 units of *Bam*HI, *Bgl*II, *Eco*RI, or *Hin*dIII, separated on a 0.8% agarose-TBE gel, blotted onto Hybond N+ (Amersham-Pharmacia, Piscataway, NJ) membranes, and then hybridized with probes labeled with ^{32}P. The probes used are depicted on the restriction map of the GALT gene and flanking regions in Fig. 1. They include the PCR product B, encoding exons 1–2, amplified from control DNA, a 1050 *Sal-Bgl* fragment of cloned human genomic DNA encoding exons 2–5 (*Bgl* 1050), and PCR product D from the human interleukin-11 receptor α (hIL-11Rα) gene encoding exon 1A and amplified from control DNA.

PCR Analysis

PCR primers were designed using SeqWeb GCG software (Madison, WI) and purchased from Gibco-BRL (Grand Island, NY). Primer pairs B (Bf, GATCAAATGAATGATTGCAGCAAG; Br, GGGATATGACCCAGAAGGAG) and C (Cf, CCCTTATCCTCCTTAATTGCTC; Cr, AGCTATGCCTTTAGCTTTTAACC) were designed from hGALT genomic sequence (GenBank Accession No. M96264) and a primer pair E (Ef, TGGTGAGGAGTGGAAAGAG; Er, AGTGGTGAAGTGGATGGAG) was designed from the hIL-11Rα gene sequence spanning exon 1 (GenBank Accession No. U32323). Primer pairs A (Af, AGAAAACACTAGGCCCCAC; Ar, AGGATATGACCCTGGATCAAC) and D (Df, AGAGGAGTGTGAACCTTCAGAG; Dr, AGCCACTCACAAAGCCAGG) were designed from sequence corresponding to the 5′ and 3′ flanks of the hGALT gene, isolated, and sequenced from RP-11BAC 195F19 (GenBank Accession No. AL162231). For standard PCR, each pair was used to amplify 319- to 955-bp segments of genomic DNA from control or patient DNA using the following amplification cycle: 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, for 30 cycles.

[1-^{13}C]Galactose Breath Testing

Both the patient with galactosemia and the control subject were studied in the general clinical research center (GCRC) outpatient unit in the morning while fasting. An oral bolus of [1-^{13}C]galactose at 7 mg/kg/weight was given after 2 baseline breath samples were obtained for $^{13}CO_2$ enrichment analysis (11). Subsequently, a breath sample was ob-

tained at various intervals for up to 3 h in both subjects. In addition, the patient was tested at 12 and 24 h. She was on a regular lactose-restricted diet at the time of the study and a blood sample was obtained at the baseline for galactose 1-phosphate quantitation and a urine sample for galactitol quantitation. Both values were in the range typical for a patient with classic galactosemia on lactose-restricted diet.

[1-^{14}C]Galactose Oxidation in Lymphoblasts

A WBC pellet was prepared from whole blood obtained from a control subject, the patient with the GALT deletion mutation, and a galactosemic individual homozygous for the Q188R mutation. Lymphocytes were transformed by Epstein-Barr virus to produce a perpetual lymphoblastoid cell line. Oxidation of galactose to carbon dioxide by the lymphoblasts was studied after lymphoblasts were grown in RPMI 1640 with 20% fetal bovine calf serum containing 5 mmol/L D-glucose. Cells were incubated in RPMI 1640 without glucose with 4.5 μmol/L of [1-^{14}C]galactose (50 μCi/μmol) in a shaking 15-ml centrifuge tube that was sealed and contained a well filled with hyamine. The liberated $^{14}CO_2$ was trapped in the hyamine which was subjected to scintillation radioactivity counting. The incubations were carried out for 1 and 2.5 h for each cell line in triplicate. To account for any nonenzymatic release of $^{14}CO_2$, cells boiled for 2 min served as blanks and were incubated in parallel. The data are expressed as the percentage of galactose converted to CO_2 per 10^7 cells.

RESULTS

Description of Patient

The patient is a 2-year-old female. The birth weight was 2557 g and the length 45.7 cm. After blood was obtained for newborn screening, the baby was discharged on Day 2 of life at which time she was ingesting only small amounts of Similac associated with emesis. On Day 6, a positive newborn screen for galactosemia (absent GALT activity) was reported. At that time, the baby weighed 1903 g, but the history was only positive for poor intake and one episode of bradycardia. On physical examination, the infant was jaundiced, the liver was enlarged, and bilateral cataracts were detected. A sepsis workup was performed, and the infant was given antibiotics, intravenous fluids, and the formula Isomil. Phototherapy was initiated. The erythrocyte galactose 1-phosphate level was 53 mg% on Day 9 (N1: $<$1 mg%) and by quantitation the GALT activity was undetectable. The infant improved over the next week with a disappearance of jaundice, cultures were negative, and at 2 weeks of age the baby was discharged. At 1 month of age, the erythrocyte galactose 1-phosphate was 6.5 mg%. The hepatomegaly resolved and by 6 weeks of age the cataracts had disappeared. The appetite and dietary intake normalized and the infant's growth parameters achieved the 10–25th percentile (weight) and 25–50th percentile (length) by 6–12 months of age. She rolled over at 4 months, sat at 4–6 months, reached for objects at 4–5 months, transferred at 6 months, and said "da da" at 7 months. The erythrocyte galactose 1-phosphate level was 3.5 mg% and the urine galactitol excretion was 327 μmol/per mmole creatinine at 20 months of age (N1: $\leq$36 μmol/mmol creatinine). Based on a Bayley Scales of Infant Development (second edition) assessment, the infant was functioning at an age appropriate level of 20 months. However, some immaturity in fine motor and perceptual motor activities was noted. A brain MRI revealed ventricular prominence. At 21 months of age, a serum FSH was elevated at 87 mIU/ml (normal: 1.04–4.2), the serum LH was 4.6 mIU/ml (normal: 0.02–0.3), and serum estradiol was $<$0.5 mg/dl (normal: $<$1.5).

The GALT mutation analysis by multiplex polymerase chain reaction-mediated testing revealed an inability to amplify exons 6 and 9 using genomic DNA from the patient. Subsequently, Southern blotting and DNA sequencing were performed to characterize the large deletion. Both parents have GALT activity reduced to 50% of normal and are of Ashkenazi Jewish background. In addition, the mother is also a carrier for the Duarte N314D gene mutation.

An oral [1-^{13}C]galactose breath test was performed when the patient was 2 years old. Numerous control studies have been published establishing the range of oxidation of galactose to CO_2 (3,6,10,11). A 5-year-old female control subject was chosen to represent the nongalactosemic group because of the similarity in age. This study was approved by the IRB of the Children's Hospital of Philadelphia and the parents of both the patient and the control subjects provided written consent.

Characterization of the GALT Deletion Mutation

Southern analysis of DNA from the patient showed no hybridization with probes encoding the

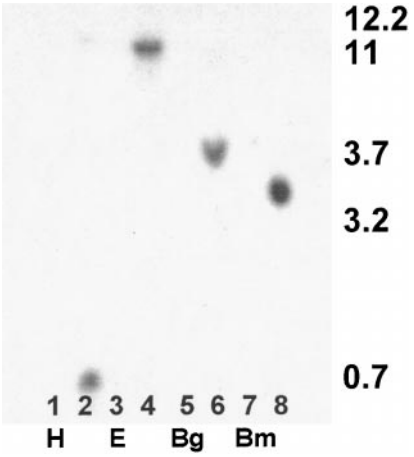

FIG. 2. Southern analysis of genomic DNA from patient and control DNA hybridized to a *Bgl* 1050-bp genomic probe encoding GALT exons 2–5. Lanes 1, 3, 5, and 7 are from the patient, while 2, 4, 6, and 8 are control DNA. Fragment sizes are in kb. Digests include *Hin*dIII (lanes 1, 2), *Eco*RI (lanes 3, 4), *Bgl*II (lanes 5, 6), and *Bam*HI (lanes 7, 8).

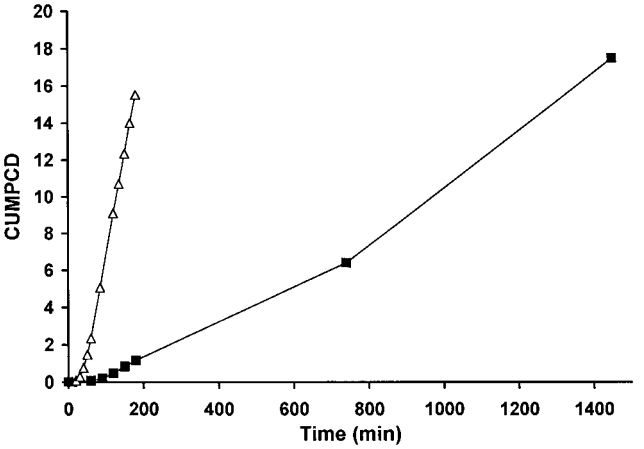

FIG. 3. The cumulative percentage of the dose (CUMPCD) of [1-^{13}C]galactose eliminated as $^{13}CO_2$ in expired air in a control subject (△) and the patient with a deletion of the GALT gene (■) following a 7 mg/kg oral bolus at Time 0.

hGALT promoter region as well as exons 1 and 2 (probe from primer pair B), nor from an overlapping genomic probe (*Bgl* 1050) corresponding to exons 2–5 (12). The Southern blot utilizing the *Bgl* 1050 probe, shown in Fig. 2, demonstrates the hybridizing fragments from control DNA, as compared to the lack of signal from patient DNA. The same blots, hybridized with genomic probes encoding exon 1 of the hIL-11Rα gene or with a PCR product derived from the far 5′ flank, showed clear hybridization signals from both DNA samples, with reduction in size of the normal 11 kb *Eco*RI fragment to 6 kb in the patient DNA.

PCR analysis of genomic DNA from the patient showed no amplification using primers B, C, or E, nor from intragenic primers spanning exons 2–6 or 8–10. However, primer pair A as well as several other pairs amplifying the 5′ flanking region, and primer pair D, just 3′ of exon 11, produced products of expected size, as did primer pair E, amplifying exon 1A of hIL-11Rα. From these data we deduce that the patient is homozygous for a 5-kb deletion spanning most if not all of the GALT gene, leaving the adjacent AL-11Rα intact.

[1-^{13}C]Galactose Breath Testing

The patient was able to oxidize the administered dose of galactose to a considerable extent. The data are shown in Fig. 3. The *y* axis shows the cumulative percentage of the dose (CUMPCD) fractionally eliminated as $^{13}CO_2$ in expired air with time displayed on the *x* axis. The control subject eliminated over 15% in 3 h, a value that corresponds to previously published data on breath testing in control subjects (3,11,12).

[1-^{14}C]Galactose Oxidation in Lymphoblasts

The percentage of galactose converted to CO_2 per 10^7 cells for the control cell line was 0.297 ± 0.008 (mean ± SE) and 0.573 ± 0.022 in 1 and 2.5 h, respectively. The patient with the GALT deletion mutation eliminated 0.014 ± 0.001 and 0.021 ± 0.002 in 1 and 2.5 h, respectively (Table 1). The cell line from the patient with the Q188R/Q188R genotype eliminated 0.013 ± 0.006 and 0.027 ± 0.007 of the galactose to CO_2 per 10^7 cells in 1 and 2.5 h, respectively.

TABLE 1
Oxidation of Galactose to CO_2 by Lymphoblasts

	Time	
Subjects	1.0 h	2.5 h
Normal	0.297 ± 0.008	0.573 ± 0.022
Q188R/Q188R	0.013 ± 0.006	0.027 ± 0.007
Del/Del	0.014 ± 0.001	0.021 ± 0.002

Note. Mean ± SEM percentage of [1-^{14}C]galactose converted to $^{14}CO_2$ per 10^7 cells in three determinations in one normal, one patient with the Q188R/Q188R genotype, and one patient homozygous for the GALT deletion mutation.

DISCUSSION

This is the first unambiguous demonstration both *in vivo* and *in vitro* of the existence of an alternate pathway for galactose oxidation. The availability of a patient with a large deletion of the GALT gene that not only abolishes GALT enzyme activity but also eliminates the potential for any protein expression allowed us to demonstrate the existence of one or more non-GALT oxidative pathways.

To date, the GALT gene deletion mutation has only been identified in Jewish individuals of Ashkenazi background (8,9). Our data demonstrate that the 5′ flank boundary of the deletion is in the 5′ flanking region of the gene and the 3′ boundary of the deletion leaves at most only a portion of exon 11 (12). The human interleukin 11 receptor α gene which is close enough to the GALT gene that intragenic splicing has been observed (13) appears to be intact. Therefore, the major pathological effect of this deletion is complete absence of the functional elements of GALT without evidence of contiguous gene deletion. Since the patient is homozygous for the same deletion and other patients of the same ethnic background have similar deletions (8,9), it appears that this is an inherited deletion. The most likely mechanism for the deletion event is recombination between the Alu elements 5′ to and within the GALT gene (8).

The degree of oxidation of [1-^{13}C]galactose to CO_2 in the control subject at 3 h was in line with our previously published data on the lower limit of the CUMPCD in children at 5–8 h being 20% of the dose (3,10,11). The data are striking in that it appears that if the patient with galactosemia is studied long enough the same degree of oxidation of the bolus occurs, but only after a much longer period of time. This is similar to the data that Segal and colleagues had published using [^{14}C]galactose loading in Caucasian patients with classical galactosemia who were studied for almost 10 h after an intravenous bolus of [1-^{14}C]galactose (6).

The *in vitro* data on galactose oxidation CO_2 on lymphoblasts support the presence of an oxidative pathway and are comparable to the results with *in vivo* breath testing. Our data on all three cell lines demonstrate that the appearance of $^{14}CO_2$ increased with time, an apparent doubling of CO_2 production between 1 and 2.5 h. The same relative increase occurred in the two classic galactosemic lines except at a rate that is less than 10% of the control line oxidation. The fact that the $^{14}CO_2$ production by Q188R/Q188R lymphoblasts was comparable to the production by the deletion/deletion lymphoblasts supports the hypothesis that most of the metabolism or oxidation of galactose produced by endogenous synthesis in patients with severe galactosemia employs alternate non-GALT pathways.

It is not surprising that there is evidence for galactose oxidation in the patient with classic galactosemia. Since there appears to be a substantial endogenous production of galactose (2), there must be a means to handle this daily load so that steady-state levels of metabolites such as galactose 1-phosphate and galactitol can be maintained in body tissues and fluids. Somewhat unexpected is the other finding that this oxidation, at least in the patient with deletion of the GALT gene, does not depend on the GALT pathway. The exact nature of the alternate pathway(s) remains to be demonstrated. But the existence of a galactonate pathway was shown many years ago by Segal and colleagues and, indeed, large amounts of galactonate are excreted in urine by patients even on a lactose restricted diet (5). This pathway at least allows for carbon dioxide to be generated following oxidation of galactonate at the level of carbon 1. The exact nature of this pathway in man remains to be delineated. Further knowledge of the alternate oxidative route and its regulation may aid in formulating new strategies for treatment of galactosemia.

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