Uridine Diphosphate Hexoses in Leukocytes and Fibroblasts of Classic Galactosemics and Patients with Other Metabolic Diseases

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ABSTRACT

To examine uridine diphosphate hexose (UDPhexose) content of cells that have more complete metabolic patterns than erythrocytes, which have been commonly used in the study of galactosemia, the concentrations of uridine diphosphate galactose (UDPgalactose) and uridine diphosphate glucose (UDPglucose) were determined in white blood cells (WBC) and fibroblasts cultured from skin biopsies. Leukocyte UDPgalactose and UDPglucose values were determined in 60 normal individuals, 14 classic galactosemics, and 18 patients with other metabolic diseases on protein-restricted and low-lactose diets. There was no difference in the average concentration of these compounds between any of these groups. There was no relationship between age and WBC UDPhexose content or correlation of WBC and erythrocyte UDPhexose levels in the same blood specimens. WBC from galactosemic individuals differ from their red blood cells because the former do not show the low average UDPgalactose levels and abnormal UDPglucose to UDPgalactose ratio previously reported for erythrocytes from galactose-1-phosphate uridyltrans-

ferase-deficient individuals. Fibroblast cell lines from 10 normal and 10 galactosemic individuals, cultured and grown to confluence in glucose medium, also showed no difference in nucleotide sugar concentrations. Thus far, of the cell types easily available, red blood cells appear to be unique in showing an abnormality in nucleotide sugar metabolism. The fact that galactosemic fibroblasts demonstrate no abnormality in the concentration of these compounds suggests that the defective galactosylation that has been observed in galactosemic fibroblasts is not due to unavailability of UDPgalactose. (*Pediatr Res* 36: 613-618, 1994)

Abbreviations

TCA, trichloroacetic acid
UDPhexose, uridine diphosphate hexose
UDPgalactose, uridine diphosphate galactose
UDPglucose, uridine diphosphate glucose
WBC, white blood cell

Since Ng *et al.* (1) reported that UDPgalactose concentration was low in red blood cells, liver biopsies, and cultured fibroblasts of patients with classic galactosemia, much attention has been focused on cellular UDPhexose levels. It has been suggested that a deficiency of UDPgalactose, the obligate donor in galactosylation, could lead to a defect in this important cellular process. The postulate that impaired galactosylation may be the basis for the long-term complications seen in galactosemics,

who adhere to prescribed lactose-free diets, has made UDPgalactose a pivotal substance for investigation.

Several reports have recently compared erythrocyte nucleotide sugar levels in normal subjects and galactosemics (1-4). Using HPLC, a direct chemical assay for determination of UDPhexoses, we have found that the average concentration of erythrocyte UDPgalactose in patients with galactosemia is significantly different from that of normal subjects. This difference was significant despite the overlap of many individual values in the two populations (2, 4, 4a). A prominent feature of erythrocytes from most patients with galactosemia is the highly abnormal ratio of UDPglucose to UDPgalactose (2, 4, 4a).

Historically, erythrocytes have been used for analysis because of their ease of availability and their currency for diagnosis, diet monitoring, and clinical research of galactosemia. The first demonstration of the enzymatic defect

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in classic galactosemia was performed in erythrocytes (5). Subsequently, the diagnosis of this disorder has been routinely made by measuring galactose-1-phosphate uridylyltransferase in these cells, and erythrocyte accumulation of galactose-1-phosphate has been used for monitoring dietary therapy (6). Despite their usefulness in this regard, the repertoire of metabolic patterns and responses of the erythrocyte may be limited and may not be representative of the processes occurring in other cell types.

To broaden the scope of our studies of the metabolism of UDPhexoses, we examined nucleotide sugar concentrations in circulating leukocytes and in fibroblasts cultured from skin biopsies of normal individuals and patients with galactosemia. For comparison, leukocytes from patients with other metabolic diseases on protein-restricted and low-lactose diets were studied. Circulating leukocytes represent an available alternative tissue whose metabolic fluxes may be more representative of other body tissues. They contain the Leloir metabolic pathway involved in the metabolism of galactose. WBC from patients with galactosemia show similar galactose-1-phosphate uridylyltransferase defects (7) and accumulate galactose-1-phosphate to the same extent as erythrocytes (8). Palmieri *et al.* (9) have described a method for the accurate determination of nucleotide sugars in normal WBC. This procedure facilitates the examination of leukocytes and cultured fibroblasts from patients with galactosemia.

Fibroblasts are a traditional cell type for biochemical investigation *in vitro*, representing a renewable and easily manipulated biochemical resource. They are a model that has often been used to study both normal and abnormal galactose metabolism (7, 10–12). Galactosemic fibroblasts fail to replicate in galactose-containing medium, mimicking the untreated patient's failure to thrive (12). Fibroblasts are important for assessing nucleotide sugar concentrations because fibroblasts from galactosemic patients, when grown in glucose, show evidence of abnormal galactosylation (10, 11). This abnormality was demonstrated by an increased number of vacant galactose acceptor sites on complex glycoconjugates (11).

In this paper, we report that leukocytes from patients with galactosemia, in contrast to the published results in erythrocytes (2, 4), do not differ from WBC from normal individuals in their concentrations of UDPgalactose and UDPglucose or the ratio of UDPglucose to UDPgalactose. There is no correlation between the concentrations of UDPhexoses in erythrocytes and leukocytes. Patients with other metabolic diseases whose erythrocytes showed a low average UDPgalactose concentration (2, 4, 4a) also had normal leukocyte UDPgalactose levels. Fibroblasts from normal individuals and patients with galactosemia also exhibit no difference in UDPgalactose and UDPglucose values. In this regard, our data are in contrast to those of Ng *et al.* (1) and suggest that low concentrations of UDPgalactose are not responsible for the defective galactosylation in fibroblasts from patients with galactosemia.

METHODS

Subjects. The normal population consisted of 60 individuals aged birth to 64 y. There were 28 females and 32 males. The individuals with classic galactosemia consisted of 14 patients ranging in age from 21 d to 28 y. There were eight females and six males. Genotype information was known for 12 galactosemics, with six being homozygous for the Q188R mutation (13), five having a single copy of the Q188R mutation, and one not having a Q188R mutation. Eighteen subjects with other metabolic disorders, whose therapy requires a low-protein diet and consequently a low-lactose diet, were also studied. They ranged in age from 1.3 to 22 y and consisted of 13 males and five females. Informed consent for venipuncture was obtained from either the donors or their guardians in accord with study approval by the Children's Hospital of Philadelphia Institutional Review Board.

Blood specimen preparation. Leukocytes were isolated by the method of Skoog and Beck (14) from 6 to 8 mL of blood and prepared for HPLC by the method described by Palmieri *et al.* (9). Erythrocytes were prepared as previously described (15) from additional blood obtained at the same time from all galactosemics and patients with other metabolic disorders and from 56 of the normal subjects so that UDPhexose content could be compared between leukocytes and erythrocytes.

Growth and preparation of fibroblasts. Skin biopsies were performed by a punch technique after informed consent. Fibroblast lines were initiated for all 10 normal individuals and for three classic galactosemics. Seven other galactosemic skin fibroblast lines were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ. All lines from galactosemic individuals were shown to be deficient in galactose-1-phosphate uridylyltransferase. Genotypes were known or determined for nine lines; three were homozygous Q188R/Q188R, two had Q188R in a single copy, and four lines did not have Q188R mutations.

Cell lines were maintained in minimal Eagle's medium supplemented with 2 mM glutamine and 20% FCS in 25 cm² flasks at 37°C in a 5% CO₂/95% air atmosphere. Cells became confluent in 5–7 d and were passed in a 1:2 split by washing the cell layer with 4 mL of Puck's solution B-EDTA followed by trypsinization with 2 mL of Puck's solution B-EDTA containing 0.04% (wt/vol) trypsin. One mL of this cell suspension was transferred to each new flask, which contained 4 mL of the medium described above.

Cell harvest. Fibroblasts were harvested at cellular confluence as judged by inspection with phase microscopy. Two to four flasks were used for each analysis. The medium was removed by aspiration, and the cell layer of each flask was washed with 4 mL of Puck's solution B-EDTA. Cells were removed from the flask by trypsin treatment as described for cell passage. Each flask was rinsed with 2 mL of PBS without calcium or magnesium,

pH 7.4. The rinse solution was added to the trypsin fraction. The cell suspension was centrifuged at $800 \times g$ for 5 min at 4°C in a refrigerated centrifuge. The supernatant was removed by aspiration, the pellet was resuspended in 1 mL of PBS, pH 7.4, and recentrifuged to wash the cells. After three additional washes, the pellet was suspended in 1 mL of ice-cold deionized water and sonicated in a Branson Power Sonifier for 5 s at a power setting of 5. The cellular protein was precipitated by the addition of 70 μL of 100% TCA, then placed on ice for 15 min. The TCA removal and subsequent steps were performed as previously described for leukocytes (9).

The protein pellet was dissolved in 100 μL of 1.0 N NaOH, diluted 10-fold, and analyzed for protein according to the method of Lowry *et al.* (16).

Analytic procedures. UDPgalactose, UDPglucose, and TCA were obtained from Sigma Chemical Co. (St. Louis, MO). The purity and concentration of the UDPgalactose and UDPglucose were confirmed by spectrophotometric analysis. Other reagents for the HPLC were of the highest commercially available purity.

HPLC was performed on 20 μL of each sample with a Dionex CarboPac PA-1 anion exchange column (Dionex Corporation, Sunnyvale, CA) on the system described by Palmieri *et al.* (15) (first half of samples) or with the modifications outlined by Gibson *et al.* (17).

A series of external chromatographic standards was used to calculate the concentrations of UDPgalactose and UDPglucose. Standard curves for linearity of response and recovery were done with the commercially obtained compounds, taking into account the purity, water of hydration, counterions, and other solvents of each lot.

Hb determinations were performed on the packed erythrocytes using the commercially available kit (625-A) from Sigma Chemical Co.

All of the values are expressed as μmol of UDPhexose per 100 g of protein or per 100 g of Hb. Statistical testing was done with *t* test with a minimal level of significance of $p = 0.05$.

RESULTS

Leukocyte Sugar Nucleotides

Effect of age. Palmieri *et al.* (9) reported that in a group of 33 normal subjects there appeared to be no effect of the donor's age on leukocyte UDPgalactose or UDPglucose in contrast to age-related changes in erythrocytes (2). We have been able to verify that leukocyte UDPhexose con-

centration is not related to the donor's age by stratifying the results from 60 normal individuals by subjects' age in 5- to 10-y groupings up to age 25 and beyond. These results are shown in Table 1. There are few or no differences in the average UDPglucose or UDPgalactose values of the various groups. No age group's results differ from the average values of the whole studied population. In a group of young adults, 28 ± 5 y old, Borzi *et al.* (18), using a different HPLC technique, found total leukocyte UDPhexose levels similar to ours. These data led us to examine the values for 14 galactosemics and 18 patients with other disorders of various ages without regard to age.

Comparison of leukocyte UDPhexose concentrations in normal subjects, galactosemics, and patients with other disorders. Figure 1 presents the data for UDPgalactose and UDPglucose in leukocytes of normal subjects and galactosemics. It is readily apparent that there is complete overlap of values for these compounds as well as for their ratios. Table 2 shows that the leukocyte UDPgalactose concentration in galactosemics of 13.41 ± 6.45 (mean $\pm$ SD) $\mu\text{mol}/100$ g protein does not differ significantly from the 12.42 ± 4.84 value found in the normal population. The same conclusion is reached for UDPglucose and total UDPhexose. The ratio of UDPglucose to UDPgalactose in leukocytes is essentially the same in the two groups. Table 2 also shows that the mean leukocyte UDPgalactose concentration of patients with other metabolic disorders, 12.22 ± 4.97 $\mu\text{mol}/100$ g protein, is indistinguishable from the means of the other two groups. The individual leukocyte UDPgalactose values in this group of patients ranged from 4.8 to 24.6 $\mu\text{mol}/100$ g protein, which is comparable to those shown in Figure 1 for normal subjects or galactosemics. UDPglucose concentrations, total UDPhexose values, and the ratio of UDPglucose to UDPgalactose in leukocytes of the patients with other disorders did not differ from those of the normal population.

Leukocyte versus erythrocyte sugar nucleotide concentrations. The relationship of UDPgalactose content of leukocytes to that of erythrocytes isolated from the same blood specimen is shown in Figure 2a. The data show there is no significant correlation between leukocyte and erythrocyte UDPgalactose values for any of the studied populations. Additionally, no correlation existed between values for the two cell types for either UDPglucose concentration (Fig. 2b) or ratio (data not shown) in any studied population.

Table 1. UDPhexoses in leukocytes of normal subjects by age*

Age (y)	n	UDPgalactose ($\mu\text{mol}/100$ g protein)	UDPglucose ($\mu\text{mol}/100$ g protein)	Total UDPhexose ($\mu\text{mol}/100$ g protein)	Ratio UDPG/UDPGal
<5	12	12.28 ± 4.25	34.35 ± 10.29	46.63 ± 11.4	3.13 ± 1.35
5-10	6	15.00 ± 5.67	34.55 ± 8.17	49.51 ± 11.1	2.49 ± 0.60
10-15	10	11.82 ± 4.09	33.66 ± 6.12	45.48 ± 9.46	3.11 ± 1.00
15-25	12	12.88 ± 3.46	36.90 ± 10.82	49.78 ± 12.9	3.00 ± 0.95
>25	20	12.02 ± 5.61	28.75 ± 9.54	40.77 ± 14.1	2.65 ± 0.82

* Values are mean $\pm$ SD.

Figure 1. Uridine nucleotide sugar levels in galactosemic and normal leukocytes. UDPG, UDPglucose; UDPGal, UDPgalactose.

Nucleotide Sugar Concentrations in Cultured Fibroblasts

The levels of UDPgalactose and UDPglucose were determined in 10 normal and 10 galactosemic cell lines grown to confluence. Six of the 10 in each group were analyzed from four to nine times between the fourth and 10th passage, with the average of the determinations taken as the best estimate of the individual cell line values. Figure 3 shows the array of levels in the individual cell lines and indicates no difference in the distribution of the UDPhexose concentrations or the ratio of UDPglucose to UDPgalactose between normal and galactosemic fibroblasts. Table 2 shows the average UDPgalactose level of 42.0 ± 18.5 $\mu\text{mol}/100$ g protein (mean $\pm$ SD) in normal subjects was similar to the 39.7 ± 13.8 value determined in galactosemic cells ($p > 0.05$). Similarly, there was no statistical difference in the mean UDPglucose content, total UDPhexose value, or ratio of UDPglucose to UDPgalactose between these two groups of cell lines.

DISCUSSION

In contrast to the findings in erythrocytes of galactosemic patients that the average UDPgalactose level is lower than normal and the mean ratio of UDPglucose to

Figure 2. Relationship of UDPhexose levels between leukocytes and erythrocytes in normal subjects, galactosemics, and patients with other metabolic disorders. RBC, red blood cells; Hgb, hemoglobin. *a*, UDPgalactose. The solid line is the regression curve for normal subjects: $y = 10.37 + 0.98x$, $r = 0.21$. The dotted line is the regression curve for galactosemics: $y = 7.37 + 1.45x$, $r = 0.24$. The broken line represents the regression $y = 17.23 - 2.03x$, $r = -0.30$, for patients with other metabolic diseases. *b*, UDPglucose. The regression curves are $y = 32.9 + 0.26x$, $r = 0.05$; $y = 23.1 + 1.42x$, $r = 0.33$; and $y = 45.6 - 1.46x$, $r = 0.36$, respectively, for normal subjects, galactosemics, and other patients. + = normal subjects; ▼ = galactosemics; ■ = patients with other metabolic disorders.

UDPgalactose is higher than normal (2, 4, 4a), our data indicate that there is no abnormality in these parameters

Table 2. UDPhexoses in leukocytes and fibroblasts of normal subjects, galactosemics, and patients with other metabolic diseases*

	<i>n</i>	UDPgalactose ($\mu\text{mol}/100$ g protein)	UDPglucose ($\mu\text{mol}/100$ g protein)	Total UDPhexose ($\mu\text{mol}/100$ g protein)	Ratio UDPG/UDPGal
WBC					
Normal subjects	60	12.42 ± 4.84	32.44 ± 10.49	44.86 ± 13.5	2.84 ± 1.04
Galactosemics	14	13.41 ± 6.45	36.81 ± 14.3	50.23 ± 18.8	3.05 ± 0.95
Other metabolic diseases	18	12.22 ± 4.97	34.46 ± 11.29	46.67 ± 15.65	2.97 ± 0.84
Fibroblasts					
Normal subjects	10	42.0 ± 18.5	131.4 ± 54.1	181.5 ± 72.3	3.39 ± 1.06
Galactosemics	10	39.7 ± 13.8	130.1 ± 47.5	169.5 ± 59.2	3.43 ± 0.81

*Values are mean $\pm$ SD.

Figure 3. Uridine nucleotide sugar levels in galactosemic and normal fibroblasts. The mean values for each of the six galactosemic and normal cell lines that were assayed repeatedly are plotted along with the single determinations of the other lines. UDPG, UDPglucose; UDPGal, UDPgalactose.

in leukocytes or cultured fibroblasts. Additionally, no abnormality in UDPHexose level exists in the leukocytes of patients with other metabolic disorders, unlike the findings in their erythrocytes (2, 4a). The lack of correlation between erythrocyte and leukocyte sugar nucleotide levels from the same blood specimen in normal subjects, galactosemic patients, and patients with other metabolic disorders strengthens the conclusion that the metabolic regulation of UDPHexose levels differs in these blood elements. The difference also appears when age is examined as a factor. Red blood cells from both normal and galactosemic children under age 10 y have higher levels of UDPHexoses than older individuals (4, 4a), whereas WBC show no such age dependence.

Leukocytes, although containing the Leloir metabolic pathway of galactose metabolism (7) and having a more complete metabolic complement than erythrocytes, have not been used to the same extent as erythrocytes in galactosemia investigations. Erythrocytes are easily obtained, whereas leukocytes have to be isolated and manipulated in a more complicated fashion. However, the examination of leukocyte galactose metabolism in galactosemics may give a more accurate assessment of the defect than can be obtained in erythrocytes. Long-term lymphoid cell lines have been used in galactosemic studies (19). In this regard, our own findings that leukocyte sugar nucleotide content is not different in granulocytes or lymphocytes (9) suggest that immortalized lymphocytes may be a suitable model for study of UDPHexose metabolism.

Numerous studies have been made to assess the metabolism of galactose by cultured galactosemic fibroblasts (7, 8, 10–12). They grow poorly and die when cultured in galactose-containing medium but show normal growth on glucose or inosine, although there is accumulation of galactose-1-phosphate and galactitol (20).

Despite these abnormalities, our findings show that, when grown to confluence in glucose-containing medium, galactosemic fibroblasts have normal UDPgalactose content and a normal UDPglucose to UDPgalactose ratio. It is interesting to note that there is a 3-fold difference in UDPHexose content between normal as well as galactosemic leukocytes and fibroblasts, which may be related to the type-specific metabolic requirements of these cells.

Our results on the levels in fibroblasts are at variance with those of Ng *et al.* (1), who reported that in six galactosemic cell lines there was a marked lowering of UDPgalactose content compared with four normal cell lines. Although different growth conditions and the degree of confluence may contribute to the difference, it may be more related to Ng *et al.* using an inaccurate enzymatic method to assay for UDPgalactose and UDPglucose as we have demonstrated in erythrocytes (17).

The mechanism for maintaining the steady state level of cellular UDPgalactose and UDPglucose is complex and not totally understood. If galactose is being metabolized via the Leloir pathway, UDPgalactose is formed from galactose-1-phosphate via the action of galactose-1-phosphate uridylyltransferase. In the absence of this reaction in classic galactosemia, Ng *et al.* postulated cellular UDPgalactose would be low (1). UDPglucose is readily formed from uridine triphosphate and glucose-1-phosphate by the action of UDPglucose pyrophosphorylase. In contrast, the formation of UDPgalactose from galactose-1-phosphate via UDPgalactose pyrophosphorylase is minimal (21). On the other hand, UDPgalactose is readily formed from UDPglucose via UDPgalactose-4-epimerase. This enzyme normally maintains an equilibrium between the two UDPHexoses with the ratio of UDPglucose to UDPgalactose being approximately 3 to 1 (22, 23). This is the average ratio of the uridine sugar previously observed in normal erythrocytes (2, 4, 4a) and now reported in normal leukocytes and fibroblasts. The fact that erythrocytes from many galactosemic patients have abnormally high ratios of UDPglucose to UDPgalactose (2, 4, 4a) suggests that the bidirectional flux of UDPHexoses catalyzed by the epimerase is perturbed in the steady state milieu of the galactosemic erythrocyte. Leukocytes and cultured fibroblasts from galactosemics have a normal ratio of UDPglucose to UDPgalactose, which indicates such a dysequilibrium in the epimerization of UDPHexoses is not a common feature of all galactosemic tissues. Whether such a dysfunction exists in target organs to explain long-term complications of galactosemia involving the brain and ovary is not yet known (24), but it seems unlikely. Ng *et al.* (1) reported low UDPgalactose in several long-frozen liver biopsies from galactosemics, which raises this possibility. This observation needs further exploration and assessment by more appropriate HPLC analysis of target organ specimens.

Evidence exists that there is defective galactosylation of cell glycoconjugates in galactosemia. Haberland *et al.* (25) described an abnormal glycoprotein pattern in the

brain of a galactosemic patient. More recently, Dobbie *et al.* (10) reported an abnormal ratio of galactose to mannose in galactosemic fibroblasts, and Ornstein *et al.* (11) reported an increased number of vacant galactose-accepting sites in galactosemic fibroblasts. The fact that we demonstrate UDPgalactose levels to be normal in galactosemic fibroblasts suggests that defective galactosylation observed in these cultured cells is not due to the unavailability of UDPgalactose. Other etiologies, such as an intrinsic defect in UDPgalactose transfer to acceptor molecules, must be explored.

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