Am J Hum Genet 30:575–582, 1978

Iduronate Sulfatase Analysis of Hair Roots for Identification of Hunter Syndrome Heterozygotes

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INTRODUCTION

The biochemical defect in Hunter syndrome (mucopolysaccharidosis II) is a deficiency of iduronate sulfatase, an enzyme required in the stepwise degradation of dermatan sulfate and a heparan sulfate [1–3]. The assay of this sulfatase has been greatly facilitated by the introduction of radioactively labeled sulfoiduronosyl sulfoanhydromannitol, a disaccharide derived from heparin, as a substrate [4]. Because the enzyme is normally present in lymphocytes, serum, cultured fibroblasts [5, 6], cultured amniotic fluid cells, and amniotic fluid [7], routine testing for this X-linked recessive disorder is now possible. A reliable method for detecting heterozygotes, however, is still not available, despite the importance of being able to identify this genotype in families at risk. Although isolated cases of X-linked disorders, such as Hunter syndrome, may be new mutations, these cannot be distinguished by family history alone [8]. Serum enzyme levels of mothers of Hunter syndrome patients could not be distinguished from those of controls [5, 6]. Although the lymphocyte sulfatase levels of a few obligate heterozygotes (mothers with two or more affected sons or with one affected son and a family history of Hunter syndrome) were quite low, most were in the normal range [5, 6]. Carrier identification is possible by cloning fibroblasts [9–12], but this technique is too complex for diagnostic purposes.

Previous studies of mosaicism in hair roots of heterozygotes of glucose-6-phosphate dehydrogenase deficiency [12], Lesch-Nyhan disease [13, 14], and Fabry disease [15] indicated that hair roots are a good source of cloned material. Gartler et al. [12] suggested that individual hair roots are the product of three or four progenitor cells. Thus, if there is random inactivation of X chromosomes and no selection for either type of cell, 6%–12% of the hair roots should have the hemizygous phenotype. There will also be a skewing of the distribution toward low enzyme levels because of hair roots with mixed cell types. Since the amount of tissue associated with each hair follicle is limited, a highly sensitive assay system is necessary. We found that sulfoid-

Received January 18, 1978; revised May 12, 1978.

This study was supported by grants HD-8855, HD-4331, and HD-4612 from the National Institutes of Health.

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uronosyl sulfoanhydromannitol provided the necessary sensitivity for estimating iduronate sulfatase activity in individual hair roots, and we report here a preliminary investigation of its potential for diagnosing carriers of Hunter syndrome.

MATERIALS AND METHODS

Hair Root Samples

Hairs were plucked from three patients with Hunter syndrome, their mothers, and six controls. Mothers of patients 1 and 2 are considered possible rather than obligate heterozygotes because they have no other affected offspring nor family histories of this disorder. The mother of patient 3 is considered an obligate heterozygote because the fetus of a subsequent pregnancy which was terminated was affected.

Hair with visible sheaths and bulbs were trimmed just above the sheath and placed in individual disposable 1.5 ml polyethylene microcentrifuge tubes. Samples were processed within several hours of plucking, although no loss of enzyme activity was noted after 48 hr at room temperature; however, samples stored overnight at −20°C demonstrated no activity. Extracts were prepared by adding 0.1 ml of Tris-chloride buffer (25 mM, pH 7.5) supplemented with bovine serum albumin (1 mg/ml) and by subjecting the tissue to five freeze-thaw cycles. Extracts could be frozen without loss of activity.

Leukocytes and Plasma

Leukocytes were prepared from 10 ml of blood with dextran-heparin by the method of Kampine et al. [16] and suspended in 0.4 ml of Tris-chloride buffer. Cells were lysed by six freeze-thaw cycles and homogenized by hand in a glass homogenizer. The homogenate and plasma were dialyzed against five changes, 4 liters each, of Tris-chloride buffer over a period of 20 hr at 4°C. The dialyzed material was centrifuged for 2 min at 14,000 *g*, and the supernatant solution was analyzed for protein [17].

Substrate

O-(α-L-idopyranosyluronic acid 2-sulphate)-(1→4)-2,5-anhydro-D-[^3H-1] mannitol 6-sulfate was prepared by deaminative cleavage of heparin with butyl nitrite and reduction of disulfated disaccharide with sodium [^3H]borohydride by the procedure of Lim et al. [4]. The sulfoiduronosyl sulfoanhydromannitol isolated by high voltage paper electrophoresis was analyzed by the borate modified carbazole method [18] and estimated to have a specific activity of 2.5 mCi/mmol.

Enzyme Assays

The assays for iduronate sulfatase were patterned after the procedures of the Neufeld group [4–7]. For assays of leukocyte extract and plasma, 5μl of dialyzed extract (10–20 μg protein) was included in 50μl of reaction mixture composed of 100 mM sodium acetate buffer, 5mM sodium azide, 12 μM substrate (~1,500 cpm), and 0.5 mg/ml bovine serum albumin and incubated 2 hr at 37°C. The buffer pH was 3.5 for leukocytes and 4.5 for sera. For hair roots, 70μl of freeze-thaw extract was included in 100 μl of reaction mixture composed of 100 mM sodium acetate buffer, pH 3.7, 5 mM sodium azide, 6 μM substrate (~1,500 cpm), and 0.5 mg/ml bovine serum albumin and incubated for 17 hr at 37°C. Enzyme parameters for hair roots were tested by using a pooled sample of hair root extracts from several normal individuals.

At the end of the reaction period, each incubation mixture was diluted with 0.6 ml of cold water, and the radioactive product, iduronosyl sulfoanhydromannitol,* was separated from the

* With enzyme preparations containing α-L-iduronidase, the product may be further degraded to yield [^3H]sulfoanhydromannitol. The elution scheme was formulated so both products would be recovered in the same fractions.

unreacted substrate by fractionation on the anion exchanger ECTEOLA-cellulose (Cellex E, Bio-Rad Richmond, Calif.) in formate form. Diluted reaction mixture (0.2 ml) was passed over a column of resin (0.6 ml) packed in a tuberculin syringe, and the column washed with 3 ml of water. The desulfation product was eluted with two 5 ml portions of 0.115 M ammonium formate, pH 3.0. The unreacted substrate was then eluted with two 5 ml portions of 0.23 M ammonium formate. Eluate increments were collected directly into scintillation vials, 10 ml of Aquasol (New England Nuclear, Boston, Mass.) added, and the samples were counted.

β-*N*-Acetylhexosaminidase (HEX) activity was determined on 5 μl of hair root extract added to 0.1 ml of 1 mM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Pierce, Rockland, Ill.) in the McIlvaine citrate-phosphate buffer, pH 4.4 [19], and incubated for 30 min at 37°C. The reaction was stopped with 1 ml of 0.4 M glycine-NaOH, pH 10.5, and fluorescence was determined at an excitation wavelength of 365 and emission wave length of 450 nm.

RESULTS

Iduronate sulfatase activity in hair roots from 12 individuals is given in table 1. Activity in single hair root samples from controls was easily measured by a combination of the radioisotopic method for measuring desulfation and an extended (17 hr) incubation period. Hair roots from three Hunter syndrome hemizygotes showed no iduronate sulfatase activity. The mean sulfatase activities for mothers of Hunter patients 1, 2, and 3 were 42%, 77%, and 23%, respectively, of the average normal mean.

Activity in hair roots from each control subject demonstrated a wide range. When the sulfatase activity was referred to that of another lysosomal hydrolase, HEX, the

TABLE 1

HAIR ROOT IDURONATE SULFATASE ACTIVITIES

Subject	No.	Iduronate Sulfatase*	Sulfatase β-Hexosaminidase
Controls:			
1	11	0.32 ± 0.05	42 ± 21
2	11	0.30 ± 0.13	43 ± 31
3	9	0.23 ± 0.07	41 ± 23
4	9	0.37 ± 0.12	34 ± 21
5	8	0.35 ± 0.11	39 ± 8
6	8	0.27 ± 0.09	49 ± 8
Average of mean	···	0.31	41
Hunter hemizygotes:			
1	8	0.005 ± 0.009	0.26 ± 0.41
2	9	0.016 ± 0.018	3.08 ± 3.70
3	6	0.005 ± 0.011	1.35 ± 3.30
Average of mean	···	0.008	1.56
Mothers of Hunter hemizygotes:			
1	18	0.13 ± 0.10	16 ± 10
2	18	0.24 ± 0.11	27 ± 10
3	20	0.07 ± 0.06	17 ± 13

* nmol substrate hydrolyzed/17 hr/hair root

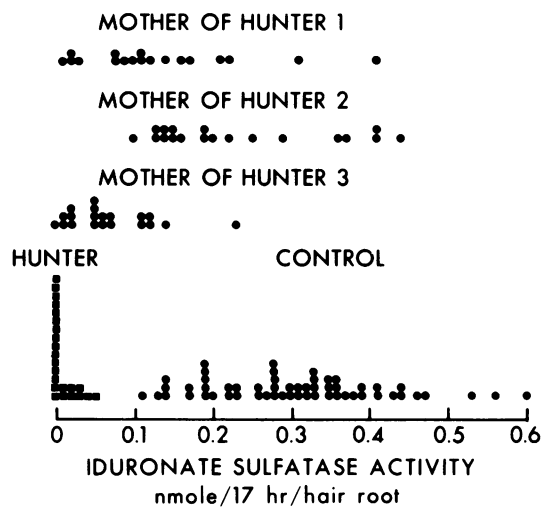

FIG. 1.—Iduronate sulfatase activity of individual hair roots. Individual results of three mothers of Hunter syndrome patients are presented, while results of the three Hunter syndrome patients and six control subjects are shown as composites. Each point represents one hair root.

spread of the data did not decrease as anticipated; in fact, it increased in several cases (cf. table 1). Therefore, the reference enzmye was used only to eliminate samples devoid of HEX activity, and iduronate sulfatase activity was expressed on a per hair basis.

The iduronate sulfatase activity for individual hair roots from controls, Hunter patients, and their mothers are presented in figure 1. Hair roots from mothers 1 and 3 fall into three groups: (1) some are within the Hunter hemizygote range and have essentially no iduronate sulfatase activity; (2) an intermediate group has detectable activity below the normal range; and (3) some are distributed within the range of the normal controls. The approximate proportion in the deficient, intermediate, and control ranges are 22%, 17%, and 61% for mother 1, and 30%, 40%, and 30% for mother 3. In the case of mother 2, all values are within the control range.

Plasma and leukocyte iduronate sulfatase levels are tabulated for each subject (table 2). Hunter syndrome was effectively diagnosed from either plasma or leukocyte analyses as reported by Liebaers and Neufeld [6]. For heterozygote identification, plasma levels were uninformative, ranging from 63% to 137% of controls. The leukocyte enzyme level was 45% of control for mother 1, 70% for mother 2, and 30% for mother 3. These values are consistent with the mean sulfatase activity from hair roots, indicating heterozygote levels for mothers 1 and 3, and a low normal level for mother 2.

DISCUSSION

This investigation established that iduronate sulfatase activity can be measured in single hair root samples, using tritiated sulfoiduronosyl sulfoanhydromannitol as substrate and an extended reaction period. The enzyme activity was quite stable and

TABLE 2

PLASMA AND LEUKOCYTE IDURONATE SULFATASE ACTIVITY

SUBJECT	IDURONATE SULFATASE*	
	Plasma	Leukocytes
Hunter hemizygotes:		
1	0.01	0.08
2	0	0.08
3	0	0
Mothers of Hunter hemizygotes:		
1	0.12	4.1
2	0.26	6.4
3	0.18	2.7
Controls	0.19 ± 0.03 (No. = 8)	9.1 ± 3.3 (No = 14)

* nmol substrate hydrolyzed/hr/mg protein

free from endogenous inhibitors. The reaction was proportional to the amount of extract added when less than half of the substrate was hydrolyzed. Individual hair roots with high activity tended to be underestimated; this procedure, however, enhanced the sensitivity for samples in the low activity range, which was the primary concern in this study.

Hair roots from Hunter syndrome patients showed little or no iduronate sulfatase activity, and there was no overlap between control and deficiency syndrome samples. Thus, this assay of hair root iduronate sulfatase can be used to diagnose Hunter syndrome, although it offers no advantage over assays of plasma or leukocyte iduronate sulfatase.

Hair roots from three mothers of Hunter syndrome patients showed two patterns of enzyme distribution. Mothers 1 and 3 had significant numbers of samples in the deficient and intermediate range, indicating that they are probably carriers of the Hunter mutation in agreement with their leukocyte enzyme levels. The carrier status of mother 3 had already been established by family history, but this was an original judgment for mother 1. All hair root samples from mother 2 were in the normal range as was her leukocyte enzyme level. We concluded that she is probably not a carrier of the Hunter gene; additional information, however, will be necessary before the continued risk of Hunter syndrome can be completely discounted in this family.

When only a small percentage of hair roots has no iduronate sulfatase activity, the heterozygote status of the individual is questionable. We, therefore, evaluated the distribution of values for each individual, as an alternative to comparison to an external control mean. Model distributions of hair root enzyme levels for heterozygotes were calculated assuming that each hair root was derived from three or four progenitor cells, as suggested by Gartler et al. [12]. Our models also assumed that an equal chance existed for either X chromosome being active in the precursor cells and that a normal spread of values would be obtained for each progenitor combination with a biological variation equal to that found in our control series. Thus, a few hair roots would possess

normal enzyme levels, a few would have no enzyme activity, and most would have intermediate levels of activity due to mixed cell types. Theoretical distributions predicted that 10%–20% of hair root samples from a heterozygote would fall below one-third of the mean of that individual. In contrast, the proportion below this one-third level in noncarriers would be only 2%–3%. Such a criterion permits mosaic hair roots with low enzyme levels as well as those with the mutant phenotype to be considered in heterozygote identification. When the present data were evaluated in this manner, no control subject had hair roots with an iduronate sulfatase activity below one-third of that individual's mean. Although there were too few samples from each control to constitute an effective test of the proposed distribution, when all samples were pooled, no sample fell into this category (table 3). Hunter mothers 1 and 3 had 22% and 25% of their hair roots below one-third of their mean, while mother 2 had none in this category. Conclusions parallel those derived from data analyzed by reference to the control mean and reinforce the view that the hair root iduronate sulfatase system can potentially identify Hunter syndrome heterozygotes.

The evaluation of heterozygosity by reference to each individual's mean enzyme level provides an internal control, which helps circumvent ambiguities caused by individual variations in hair morphology, nature and amount of tissue adhering to hair roots, developmental state of hair follicles, and cosmetic history. Recently, a subject with extremely fine hair illustrated the advantage of this approach (unpublished results). The mean hair root sulfatase level was less than 10% of the control mean. All samples were in the intermediate range, with none devoid of enzyme activity. The sulfatase to HEX ratios also suggested that all hair roots were an intermediate type. However, as there were no samples with an activity less than one-third the sample mean, heterozygosity was not indicated. Since there was no family history of Hunter syndrome, it is more likely that fine hair with correspondingly small hair roots was responsible for the low enzyme levels. Had this been an individual at risk, carrier status could have been diagnosed only by reference to the distribution of samples around its own mean.

The present findings suggest that the analysis of hair root iduronate sulfatase provides useful information for the differentiation of Hunter syndrome heterozygotes. However, a consideration of the results raises the following concerns: (1), more samples per individual need to be examined; (2), hair type may influence enzyme activity level, and (3), an appropriate reference system must be developed to normal-

TABLE 3

PROPORTION OF HAIR ROOTS WITH IDURONATE SULFATASE LESS THAN ONE-THIRD OF MEAN

Subjects	No.	% < 1/3 Mean
Controls	56	0
Mothers of Hunter hemizygotes:		
1	19	22
2	17	0
3	20	25

ize primary enzyme values. Others have used the ratio of activities of primary to reference enzymes to minimize variation due to differences in hair roots [13–15]. While the use of the individual's mean provides an internal control, it does not alleviate the problem of hair root variation within an individual. Either an improved HEX protocol or a different reference enzyme is needed. It is also essential that success of the system be demonstrated by a longitudinal follow-up of pregnancies in the test population. A reliable procedure for prenatal diagnosis of Hunter syndrome is available, and all test subjects must be encouraged to utilize prenatal monitoring even if hair root results suggest that they are not carriers. Only after a record of successful prediction has been established, should the need to monitor pregnancies be decided by the results of hair root enzyme studies.*

SUMMARY

Iduronate sulfatase, the enzyme deficient in Hunter syndrome, can be readily measured in individual hair roots. Samples from Hunter syndrome hemizygotes had activities at or near the limits of detection. Samples from two mothers of Hunter syndrome patients, one an obligate heterozygote, had lower average iduronate sulfatase activity than the normal mean, and a significant number of hair roots had activity in the pathognomic range. A third mother showed a normal distribution of enzyme activity, and no hair roots were in the range of those from an affected individual. These results are similar to studies on the distribution of other X-linked enzymes in individual hair root samples from heterozygotes. This suggests that hair root iduronate sulfatase assessment is useful in the detection of Hunter syndrome carrier status, but further refinement of the test system is necessary.

ACKNOWLEDGMENTS

We wish to express appreciation to Dr. Elizabeth F. Neufeld, National Institutes of Health, Bethesda, Maryland, for providing us with information on the iduronate sulfatase assay prior to publication. We thank Dr. Atsuko Fujimoto, Los Angeles County–University of Southern California Medical Center, Los Angeles, California; Dr. Larry J. Shapiro, Harbor General Hospital, Torrance, California; and Dr. Allan Murray, University of California, Irvine, Medical School, for allowing us to study their patients.

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* After the completion of the present studies, an abstract by Nwokoro et al. [20] appeared showing the feasibility of Hunter syndrome heterozygote identification by hair root iduronate sulfatase analysis. Their study included more subjects, used the sulfatase:β-hexosaminidase ratios for data analysis, and showed that heterozygotes had a substantial number of samples with an activity ratio below 40% of the mean control. The overall conclusions were similar to those reported here.

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