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Atypical (Mild) Forms of Dihydropteridine Reductase Deficiency: Neurochemical Evaluation and Mutation Detection

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ABSTRACT. We investigated two patients with an atypical (mild) form of dihydropteridine reductase (DHPR) deficiency. Both responded to the loading test with tetrahydrobiopterin; their plasma phenylalanine levels were lowered from 278 μmol/L to 85 and 48 μmol/L and from 460 μmol/L to 97 and 36 μmol/L after 4 and 8 h, respectively. In one of the patients, a combined loading test with phenylalanine followed by tetrahydrobiopterin was also carried out and showed a profile typical for DHPR deficiency. The phenylalanine hydroxylation rate was calculated to be 43 and 87%, 4 and 8 h after cofactor administration, respectively. Diagnosis was confirmed by the absence of DHPR activity in the patient's erythrocytes. In cultured fibroblasts, residual activity of 4 and 10%, respectively, was found. Excretion of urinary pterins was essentially normal, and the biopterin to neopterin ratio in cerebrospinal fluid was increased. Although in both patients cerebrospinal fluid homovanillic acid was found to be normal, and 5-hydroxyindoleacetic acid was substantially reduced, there was no sign of neurologic alterations until the age of 2 y. However, one of the patients recently developed deceleration of head growth, whereas psychomotor development continued to be normal for age. Using the chemical cleavage method on the amplified cDNA, mismatches of T to G at nucleotide 659 and of G to A at nucleotide 475, respectively, were identified. These results also demonstrate that screening for tetrahydrobiopterin deficiency by urinary pterin analysis alone can miss some newborns with mild DHPR deficiency and that all children with tetrahydrobiopterin defects need full neurochemical evaluation together with analysis of the enzyme activity. (*Pediatr Res* 32: 726–730, 1992)

Abbreviations

DHPR, dihydropteridine reductase
BH_4, tetrahydrobiopterin
PKU, phenylketonuria
PCR, polymerase chain reaction
CSF, cerebrospinal fluid
5HIAA, 5-hydroxyindoleacetic acid
HVA, homovanillic acid

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Hyperphenylalaninemia (1) comprises a group of autosomal-recessively inherited disorders caused either by a defect of the phenylalanine-4-hydroxylase (classic PKU) or by deficiency of the cofactor BH_4. Two defects are known to occur in the biosynthesis of BH_4, *i.e.* GTP cyclohydrolase I deficiency and 6-pyruvoyl tetrahydropterin synthase deficiency. The latter is known to be the most common form of BH_4-dependent hyperphenylalaninemia (2). In DHPR deficiency, the second most common form of BH_4 deficiency, quinonoid-dihydrobiopterin formed during the hydroxylation of phenylalanine to tyrosine accumulates, and the amount of BH_4 synthesized by the *de novo* pathway is insufficient for the normal function of hepatic phenylalanine-4-hydroxylase (3). Because BH_4 is also the essential cofactor of tyrosine-3-hydroxylase and tryptophan-5-hydroxylase, these patients are generally characterized by depletion of the monoamine neurotransmitters dopamine, norepinephrine, and serotonin (4, 5).

In contrast to patients with classic PKU, which can be successfully treated with the low-phenylalanine diet, patients with DHPR deficiency need different treatment. They do not respond to the phenylalanine-restricted diet and, if not diagnosed early and treated properly, are subject to severe myoclonic epilepsy and mental retardation, which often lead to early death (6). Furthermore, several reports demonstrate partial and mild cases of DHPR deficiency (7, 8); however, no neurochemical evaluations were performed in these patients and there are no data on the type of mutation at the molecular level.

In this article, we describe the clinical and biochemical data of two patients with atypical (mild) forms of DHPR deficiency, one treated with BH_4 supplementation and the other not treated. Data presented clearly demonstrate that: *1*) measurement of DHPR activity in erythrocytes is essential for detection of DHPR deficient patients; *2*) despite favorable clinical presentation, these patients show a discrepancy between serotonin and dopamine turnover; and *3*) mRNA analysis performed on the patients' cultured fibroblasts revealed two different mutations despite identical clinical and biochemical presentation.

MATERIALS AND METHODS

Measurement of pterins in urine and CSF was performed after oxidation with manganese dioxide using reverse-phase HPLC with fluorescence detection (9). For the determination of tetrahydrobiopterin, samples were pretreated by differential oxidation (10). Neurotransmitter metabolites were measured by ion-pair HPLC (11). Amino acid analysis was performed by ion-exchange chromatography using Biotronik LC 5001.

The detection of enzyme mutations was performed by a chem-

ical cleavage reaction of mismatches in heteroduplexes formed between normal and patients' RNA (12). After mRNA isolation from the patients' fibroblasts and cDNA synthesis, the PCR was used to amplify the coding region of the cDNA. Oligonucleotides used for the amplification were complementary to the cDNA from nucleotides 34 to 45 and 784 to 803. The former also contained a 10-nucleotide tail with a *Bam*HI restriction site. Hence, all except the first seven of the 244 amino acids were screened. The mutant PCR product was then screened for mismatches with the wild type cDNA (13).

DHPR activity in erythrocytes was determined by a spectrophotometric measurement of the quinonoid-6-methyldihydropterin-dependent oxidation of ferricytochrome C (14). Cultured fibroblasts were assayed for DHPR activity (15). Additionally, both assay procedures were adapted using BH_4 instead of 6-methyltetrahydropterin.

(6R,S)-BH_4 and 6-methyltetrahydropterin were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland).

CASE REPORTS

Patient 1. M.LJ. is the third child of healthy, unrelated parents of Yugoslav origin. Family history, pregnancy, and birth (at term) were uneventful; birth weight was 2930 g; length, 48 cm; and head circumference, 34 cm. The Apgar scores were 9, 10, and 10 at 1, 5, and 10 min, respectively. In the postnatal period, hyperbilirubinemia (306 μmol/L), due to A0 incompatibility, was noted and treated with phototherapy. Hyperphenylalaninemia was detected by the Guthrie test in the course of the national screening program. It took 6 wk to get a serum for quantitative amino acid analysis due to noncompliance of the parents. At the age of 2 mo, plasma phenylalanine concentration was 284 μmol/L. Until the age of 21 mo, plasma phenylalanine ranged from 73 to 399 μmol/L while the patient was on a free diet. So far, the child shows a normal psychomotor development on a normal diet without any phenylalanine restriction and without substitution with BH_4 or neurotransmitter precursors, growing along the 25th percentile for all body measurements. At the age of 18 mo magnetic resonance imaging of CNS revealed no abnormalities.

Patient 2. G.A. is the first child of Turkish parents, who are first cousins. Family history, pregnancy, and birth (weight, 3200 g; length, 50 cm; head circumference, 34 cm, and Apgar scores, 9, 10, and 10 at 1, 5, and 10 min, respectively) were uneventful. Hyperphenylalaninemia was detected by the neonatal screening program. At the age of 2 wk, the plasma phenylalanine concentration was 500 μmol/L. The CSF concentration of the serotonin metabolite was substantially reduced, and cofactor replacement therapy was started with 10 mg BH_4/kg body weight, given in a single dose. Tetrahydrofolate concentrations in serum measured at the age of 1, 2, 3, and 9 mo were in the normal range (between 10.3 and 22.4 ng/mL). There had been no restriction of phenylalanine in the diet. No abnormalities were found in cranial magnetic resonance imaging scans performed at the age of 1 and 2 y. Examination of auditory-, visual-, and sensory-evoked potentials performed every 6 mo was always normal. The psychomotor development up to the age of 30 mo was completely normal, but during the last year a deceleration of the head growth was observed. At the age of 30 mo, the patient was microcephalic with a head circumference 0.5 cm below the 3rd percentile. Body weight was between the 3rd and 10th percentile; length was between the 10th and 25th percentiles. Because of the reduction of serotonin biosynthesis in CSF and because of the developing microcephaly, additional therapy with 5-hydroxytryptophan (2.5 mg/kg body weight/d) and folinic acid (15 mg/d) was started.

RESULTS

BH_4 loading test. In patient M.LJ., both oral administration of BH_4 (7.5 mg/kg body weight) as well as combined oral loading with phenylalanine (100 mg/kg body weight) and, 3 h later, with BH_4 (20 mg/kg body weight) showed a decrease of plasma phenylalanine after 4 and 8 h (Table 1). The hydroxylation rate of the administered phenylalanine after loading with BH_4, calculated as the amount of phenylalanine converted to tyrosine, was found to be 43% (4 h) and 87% (8 h). In patient G.A., an oral loading test with 20 mg BH_4/kg body weight showed similar normalization of plasma phenylalanine.

Pterins in urine and CSF. Urinary total neopterin and total biopterin were slightly increased in both patients. The pterin pattern was similar to that found in patients with classic PKU (Table 2). Percentage of biopterin (of the sum of neopterin and biopterin) was between 34 and 62% in the urine of patient M.LJ. and between 31 and 82% in the urine of patient G.A. However, patient G.A. had been on BH_4 monotherapy (10 mg/kg body weight/d) since the age of 3 mo. In the second patient, neopterin excretion decreased with age, whereas biopterin excretion increased. A percentage of biopterin in urine greater than 80 usually is characteristic of DHPR deficiency. The percentage of BH_4 (of the sum of BH_4, dihydrobiopterin, and biopterin) in the urine of patient M.LJ. was 7%, and in patient G.A., 17%, both markedly below the normal range of ~60%.

In the CSF, total neopterin was normal and total biopterin increased (Table 2), a pattern typical for DHPR deficiency. High neopterin in the CSF of patient G.A. at the age of 1 mo was probably due to a viral infection.

Neurotransmitter metabolites in CSF. The most interesting finding is that 5HIAA was markedly decreased, whereas HVA was in the normal range in both patients (Table 2). With increased age, values remained in the same range, and there was no correlation with either the changes in neopterin and biopterin content in the CSF or the plasma phenylalanine and tyrosine concentrations. These findings indicate diminished serotonin and normal catecholamine biosynthesis in the CNS, an unusual finding for patients with BH_4 deficiency. Trial of cofactor replacement therapy (10 mg/kg body weight/d) over 7 d in the patient G.A. resulted not only in a favorable control of hyperphenylalaninemia, but also in a slightly increased production of serotonin in the CSF, as was indicated by the CSF levels of 5HIAA (Table 2). Surprisingly, interruption of BH_4 treatment for 4 d resulted in neither a substantial increase of plasma and CSF phenylalanine nor a drop of CSF neurotransmitter metabolites (data not shown). After 8 wk without any medication, 5HIAA in CSF was further reduced and HVA lowered to borderline. Despite excellent psychomotor development of the patient, treatment with BH_4 was reintroduced at the age of 6 mo, resulting in a good control of the plasma phenylalanine. HVA completely normalized, whereas 5HIAA remained below the control value.

DHPR activity in erythrocytes and skin fibroblasts. Diagnosis of DHPR deficiency was confirmed by the measurement of

Table 1. *Plasma phenylalanine and tyrosine after BH_4 loads performed with and without oral administration of phenylalanine (Phe) in two children with atypical form of DHPR deficiency*

		Plasma (μmol/L)	
	Time (h)	Phe	Tyrosine
Patient M.LJ.			
Phe load (100 mg/kg)	−3	288	73
BH_4 load (20 mg/kg)	0	635	60
	4	363	93
	8	85	82
BH_4 load (7.5 mg/kg)	0	278	105
	4	97	99
	8	36	105
Patient G.A.			
BH_4 load (20 mg/kg)	0	460	88
	4	97	148
	8	36	93

Table 2. *Urinary and CSF pterins, CSF neurotransmitter metabolites, and plasma amino acids in two patients with atypical form of DHPR deficiency*

	Urine (mmol/mol creatinine)		CSF (nmol/L)				Plasma (μmol/L)		Therapy BH_4 (mg/kg/d)
Age (mo)	Neopterin	Biopterin	Neopterin	Biopterin	5HIAA	HVA	Phenylalanine	Tyrosine	
Patient M.LJ.									
2	10.2	5.2	13	57	66	464	288	73	
7	6.1	3.6	20	36	21	278	115	61	
10	5.1	4.7	25	51	21	307	103	55	
14	3.1	4.8	19	52	22	381	211	95	
Patient G.A.									
1	15.7	7.8	44	37	33	390	793	6	
3	5.5	6	17	59	59	571	194	99	10
4	4.5	7.1	14	44	34	377	133	66	
13	1.7	7.7	12	24	38	440	36	26	10
18	1.5	6.1	10	42	33	507	36	138	10
24	1.1	3.1	8	26	30	477	54	50	10
Controls	1.1–4.1	0.5–3.1	9–20	10–30	70–400	300–1000	<70	<120	

enzyme activity in the patients' erythrocytes and cultured skin fibroblasts (Table 3). In the erythrocytes of both patients, no DHPR activity was detectable if either the synthetic (6-methyltetrahydropterin) or the natural (BH_4) cofactor was used in the assay. Both parents were diagnosed to be heterozygous for DHPR deficiency, showing a reduced enzyme activity of ~50% of controls. In the cultured skin fibroblasts of the patients M.LJ. and G.A., a severely reduced DHPR activity of 4 and 10%, respectively, was found.

Screening for nucleotide substitution. cDNA screening of the patients with DHPR deficiency was performed on PCR products that encompassed the coding region of the cDNA. In the patient G.A., a mismatch, reacting with osmium tetroxide at base 659, corresponded to the conversion of T to G (Fig. 1), changing amino acid no. 212, phenylalanine (T*T*C), to cystine (T*G*C). In the patient M.LJ., a mismatch reacting with hydroxylamine at base 475, corresponded to the conversion of G to A (Fig. 2), changing amino acid no. 151, glycine (G*G*C), to serine (G*A*C). Both of the mutations are homozygous.

DISCUSSION

Two patients with DHPR deficiency described here demonstrate that every newborn with even slightly elevated plasma phenylalanine should be screened for BH_4 deficiency. The first patients with BH_4 deficiency were already identified in 1969 (16). Siblings with mild hyperphenylalaninemia, at that time described as "a genetic variant of phenylketonuria," were later characterized as DHPR deficient (17). Another child with severe neurologic symptoms, despite treatment with a low-phenylalanine diet,

Table 3. *DHPR activity in erythrocytes and skin fibroblasts of two patients with atypical form of DHPR deficiency and their parents*

	Erythrocytes (mU/mg Hb)*	Fibroblasts (mU/mg protein)†
Patient G.A.	<0.5	5
Father	1.4	ND‡
Mother	1.5	ND
Patient M.LJ.	<0.5	2
Father	1.2	ND
Mother	1.1	ND
Controls	2–5 (n = 29)	22–49 (n = 9)

* nmol cytochrome *c* reduced/min/mg hemoglobin.
† nmol NADH oxidized/min/mg protein.
‡ ND, not determined.

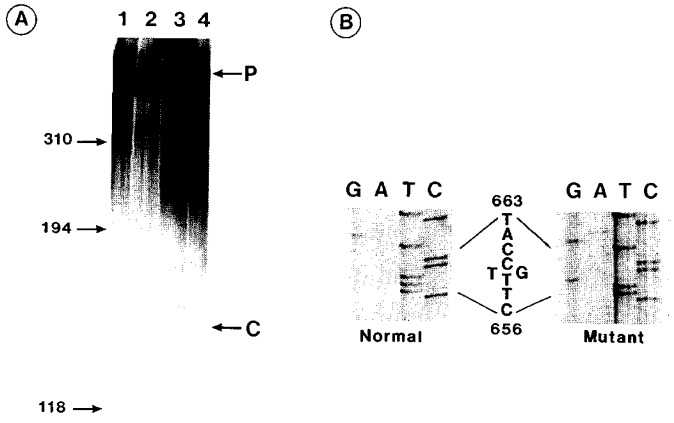

Fig. 1. Identification of a mutation in patient G.A. *Panel A* shows an autoradiogram of the chemical cleavage method gel. *Lanes 1* and *2* are normal controls; *lanes 3* and *4* are from the patient. *Lanes 1* and *3* are the result of hydroxylamine reaction; *lanes 2* and *4* are the result of reaction with osmium tetroxide. The probe band is marked as *P*, and cleavage product can be seen in *lane 4* (*C*). The numbers on the left-hand side of the gel refer to molecular weight markers, in bp. *Panel B* shows the nucleotide sequence around the estimated point of the mismatch. In the patient, the cDNA has a T to G substitution at nucleotide 659.

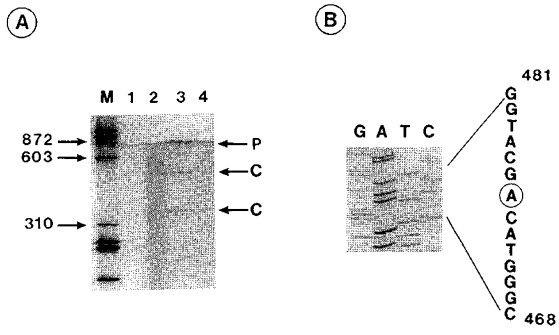

Fig. 2. Identification of a mutation in patient M.LJ. *Panel A* shows an autoradiogram of a chemical cleavage method gel (abbreviations and labels are the same as in Figure 1*A*). A cleavage is evident in *lane 3*. In this case, both cleavage products are visible. *Panel B* shows the nucleotide sequence around the estimated point of the mismatch. Only the sequence from the patient is shown. There is a G to A substitution at nucleotide 457 (*circled*).

was found to have normal phenylalanine-4-hydroxylase but no DHPR activity in the liver (3). Other patients with DHPR deficiency have been described later (18–20), and, in one family, two subjects were found to have no DHPR activity in blood but were clinically normal (21). Unfortunately, this family refused further investigations aimed at defining the reason for this finding.

The BH_4 loading test in patients with DHPR deficiency is not as effective as in patients with 6-pyruvoyl tetrahydropterin synthase deficiency. Because some of the DHPR-deficient patients did not respond to the dose of 7.5 mg BH_4/kg body weight (22), loading with 20 mg BH_4/kg body weight was introduced (23). However, one patient, described as a nonresponder even to this high dose of BH_4, died despite an early diagnosis and treatment and may represent a "malignant" form of DHPR deficiency (24). There is a remote possibility that this patient may have had both phenylalanine hydroxylase and DHPR deficiency, which could also explain the lack of a response to the BH_4 loading test. The differences in the response to oral loading with BH_4 may be due to the gene dosage effect or more likely to the heterogeneity of the molecular defect as demonstrated previously (22, 25). In our patients, in both loading tests (7.5 mg/kg body weight in patient M.LJ. and 20 mg/kg body weight in patient G.A.) plasma phenylalanine normalized 8 h after oral administration. The combined phenylalanine-BH_4 loading test performed in patient M.LJ. at initially normal plasma levels showed hydroxylation rates typical for DHPR deficiency.

Distribution of CSF 5HIAA (a metabolite of serotonin) and HVA (a metabolite of dopamine) in patients with DHPR deficiency is similar to that observed in 6-pyruvoyl tetrahydropterin synthase deficiency. However, the decrease of 5HIAA seems to be more dramatic than that of HVA (6). Absence of DHPR activity in the erythrocytes of our patients confirmed the diagnosis, and we expected to find the abnormal profile of the neurotransmitter metabolites in the CSF. This expectation was only partially confirmed by very low levels of 5HIAA but normal HVA in the CSF of both children. Despite a low serotonin biosynthesis, a favorable clinical course, good psychomotoric development, and high phenylalanine tolerance were found in our patients. Furthermore, mild hyperphenylalaninemia and normal CSF phenylalanine levels (data not shown) in both patients exclude the competitive inhibition of tryptophan-5-hydroxylase by phenylalanine. Because there were no signs of neurologic abnormalities, both children were kept on a normal diet. A trial with BH_4 (10 mg/kg body weight/d) in patient G.A. resulted, as expected, in complete normalization of plasma phenylalanine levels. Surprisingly, production of serotonin increased toward normal, and HVA increased substantially. Because the penetration of BH_4 into the brain is known to be poor, cofactor replacement therapy in DHPR deficiency is usually inefficient. Furthermore, in one of our patients reintroduction of BH_4 therapy normalized only catecholamine production, whereas serotonin biosynthesis remained low. A partial DHPR deficiency associated with mental retardation was documented by abnormally low levels of 5HIAA in the CSF from patients with neurologic and mental disorders (7). The residual activity of DHPR in erythrocytes purified by affinity chromatography was found in a patient with only mild mental retardation without neurotransmitter therapy (8), suggesting that in mild forms of DHPR deficiency neurologic symptoms may occur at a later stage. Therefore, therapy has been started in one patient and will be started in the second despite normal development so far.

Recently, patient G.A. showed deceleration of head growth and, despite the good psychomotoric development and normal blood folate concentrations, we intend to introduce therapy with the neurotransmitter precursor 5-hydroxytryptophan and folinic acid. Folinic acid supplementation was shown to improve neurologic symptoms in some patients with DHPR deficiency (26), with prevention of intracranial calcification (19, 20, 27). Because there is strong support for the belief that DHPR plays a role in maintaining tetrahydrofolate levels in the brain (28), folinic acid was recommended to be a part of the treatment in DHPR-deficient patients.

Clinical and neurochemical evaluation in our patients strongly supported the hypothesis that such atypical (mild) forms of DHPR deficiency may be due to the same type of mutation. While screening the cDNA of the DHPR-deficient patients, different types of mutations such as neutral mutation (polymorphism) of the leucine codon to another leucine codon and insertion of an extra codon for threonine were detected (13). In patient G.A., we found a mutation of a phenylalanine codon (T*T*C) to cystine codon (T*G*C), and in patient M.LJ., a mutation of a glycine codon (G*G*C) to serine codon (G*A*C). These two mutations occur 61 amino acids apart in a protein region not responsible for the NADH binding. However, there is no direct evidence that this may be the BH_4 binding domain. Therefore, it seems difficult to predict the severity of the disease on the basis of the type of mutation, at least in patients with DHPR deficiency.

The study of our patients with atypical DHPR deficiency demonstrates the importance of exact neurochemical evaluation of all children with BH_4 deficiency. This includes the measurement of not only pterins in urine but also neurotransmitter metabolites in CSF. Furthermore, these patients should be controlled periodically during the first years of life regardless of their clinical status.

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10. Extent and Nature of Circulation *(See instructions on reverse side)*	Average No. Copies Each Issue During Preceding 12 Months	Actual No. Copies of Single Issue Published Nearest to Filing Date
A. Total No. Copies *(Net Press Run)*	4395	4137
B. Paid and/or Requested Circulation **SEE BELOW 1. Sales through dealers and carriers, street vendors and counter sales	251	259
2. Mail Subscription *(Paid and/or requested)*	2957	3163
C. Total Paid and/or Requested Circulation *(Sum of 10B1 and 10B2)*	3208	3422
D. Free Distribution by Mail, Carrier or Other Means Samples, Complimentary, and Other Free Copies	53	24
E. Total Distribution *(Sum of C and D)*	3261	3446
F. Copies Not Distributed 1. Office use, left over, unaccounted, spoiled after printing	1134	691
2. Return from News Agents	NONE	NONE
G. TOTAL *(Sum of E, F1 and 2—should equal net press run shown in A)*	4395	4137

11. I certify that the statements made by me above are correct and complete
Signature and Title of Editor, Publisher, Business Manager, or Owner
Publisher

PS Form 3526, January 1991 *(See instructions on reverse)* **Paid Subs, mailed by other means