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Normal values and age-dependent changes in GTP cyclohydrolase I activity in stimulated mononuclear blood cells measured by high-performance liquid chromatography

Makoto Hibiya[a], Hiroshi Ichinose[a], Norio Ozaki[b], Kiyoshi Fujita[b], Teru Nishimoto[b], Tetsushi Yoshikawa[c], Yoshizo Asano[c], Toshiharu Nagatsu[a,*]

[a]*Division of Molecular Genetics, Institute for Comprehensive Medical Science, Graduate School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan*

[b]*Department of Psychiatry, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan*

[c]*Department of Pediatrics, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan*

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Abstract

GTP cyclohydrolase I (GCH1) activity in phytohemaglutinin (PHA)-stimulated mononuclear blood cells (MBCs) is a useful clinical marker for diagnosis of tetrahydrobiopterin (BH_4)-related genetic disorders such as recessively inherited GCH1 deficiency and dominantly inherited dopa-responsive dystonia (Segawa's disease). Since the assay is complex, including isolation of MBCs from blood, stimulation of MBCs by PHA under culture, isolation of the protein fraction from the PHA-stimulated MBCs, and the subsequent activity measurement, the reproducibility is problematic in its application to clinical study. We established a sensitive and reproducible method by high-performance liquid chromatography with fluorescence detection for clinical assay of GCH1 in PHA-stimulated MBCs, and measured the normal values of 91 healthy males and females of various ages (1–74 years). The mean normal values were 19.1±0.9 pmol/mg protein per h (mean±S.E., n=91). There were no significant differences between males and females. The activity tends to be higher in the first decade and to be decreased from the second to third decade and becomes almost stable from the third decade.

Keywords: Enzymes; GTP cyclohydrolase I

1. Introduction

GTP cyclohydrolase I (GCH1: EC 3.5.4.16; GTP 7,8-8,9-dihydrolase) catalyzes the conversion of GTP to D-*erythro*-7,8-dihydroneopterin triphosphate (NH_2P_3), which is the first step for the biosynthesis of (6*R*)-(L-*erythro*-1′,2′-dihydroxypropyl)-2-amino-4-oxo-5,6,7,8-tetrahydropteridine (tetrahydrobiopterin, BH_4) [1]. BH_4 acts as an essential cofactor for pteridine-dependent aromatic amino acid monooxygenases, i.e., phenylalanine hydroxylase for phenylalanine degradation, tyrosine hydroxylase for catecholamine biosynthesis and tryptophan hydroxylase for serotonin biosynthesis [2–4]. BH_4 also acts as a cofactor for nitric oxide synthase for the generation of nitric oxide from arginine [5,6]. Thus, BH_4 is

*Corresponding author. Tel.: +81-562-939-391; fax: +81-562-938-831.

E-mail address: tnagatsu@fujita-hu.ac.jp (T. Nagatsu)

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essential for biosynthesis of neurotransmitters and hormones such as dopamine, norepinephrine, epinephrine, serotonin and nitric oxide [1–6].

Changes in GCH1 activity affect the synthesis rate of BH_4, and consequently the rate of synthesis of catecholamines, serotonin and nitric oxide. In diseases, mutations in GCH1 cause autosomal recessive GCH1 deficiency [7–9], and autosomal dominant hereditary progressive dystonia with marked diurnal fluctuation/dopa-responsive dystonia (HPD/DRD, Segawa's disease) [10,11]. In autosomal dominant HPD/DRD, one allele of GCH1 gene is mutated and the other allele is normal [11]. As the result GCH1 activity is partially decreased to less than 20% of the normal values, and the resultant partial dopamine deficiency in the nigrostriatal dopamine neurons due to decreased tyrosine hydroxylase activity causes dopa-responsive dystonia [11]. The GCH1 activity in carriers were about 40% of the normal value [11]. In autosomal recessive GCH1 deficiency, the two alleles of the GCH1 gene are mutated, and GCH1 activity and BH_4 level are nearly undetectable [9]. As the result, severe decreases in catecholamines and serotonin due to decreased tyrosine and tryptophan hydroxylase activities, and hyperphenylalaninemia due to decreased phenylalanine hydroxylase cause severe neurological symptoms such as muscular hypotonia, epilepsy, fever episode, motor disturbance and brain developmental disorder [7–9].

GCH1 activity was measured with samples from liver biopsy [7]. The clinical monitoring of GCH1 activity is possible with phytohemaglutinin (PHA)-stimulated mononuclear blood cells (MBCs) [11–13]. The enzyme activity in unstimulated MBCs is not detectable, but is increased to be measurable after PHA-stimulation. Since GCH1 activity may affect important neurotransmitters and hormones such as catecholamines, serotonin and nitric oxide, assay of GCH1 activity in PHA-stimulated MBCs is of clinical interest in neuropsychiatric, endocrine and immune diseases. This assay can detect patients and carriers of HPD/DRD or heterozymotes and homozygotes of GCH1 deficiency [11,12], and can be used for differential diagnosis of HPD/DRD from juvenile Parkinsonism [13].

The assay of GCH1 activity in stimulated-MBCs is complex, and requires isolation of MBCs, their culture with PHA for stimulation, isolation of the protein fraction from the PHA-stimulated MBCs, and the measurement of the enzyme activity. Thus, the assay has not been used for clinical screening.

GCH1 activity is measured by radioenzymatic assay of ^{14}C-formic acid formed from 8-[^{14}C]-GTP as the substrate [14], by high-performance liquid chromatography (HPLC)–fluorimetry of either neopterin triphosphate (NP_3) after oxidation of the product D-*erythro*-7,8-dihydroneopterin triphosphate (NH_2P_3) [15], or neopterin (N) after oxidation and alkaline phosphate treatment of NH_2P_3 [11,13], or by radioimmunoassay [16] or enzyme-immunoassay [17] of N. Since the activity of GCH1 in stimulated MBCs is less than 1 pmol/mg protein per min, a highly sensitive method is required.

To our knowledge, there have been no reports published on the normal value of GCH1 activity in MBCs with a large number of healthy subjects, and the effects of ages and sex on the activity. In the present study, we established an HPLC method suitable for the clinical study of GCH1 activity in PHA-stimulated MBCs, and the normal value with 91 healthy subjects of various ages.

2. Experiments

2.1. Chemicals

All reagents were of commercial quality. High-purity Milli-Q water (Millipore, Bedford, MA, USA) was used throughout. D-*erythro*-N was synthesized and kindly donated by Dr. T. Sugimoto (Nagoya University, Nagoya, Japan). The RPMI 1640 cell culture medium was prepared as follows. RPMI Medium 1640 powder (Gibco Labs., Grand Island, NY, USA), 10.4 g, was dissolved in 800 ml of water, and sodium bicarbonate (2 g) was added. The pH of the solution was adjusted to pH 6.8 with 1 *M* HCl. Water was added to 990 ml, and 10 ml of a solution containing penicillin (10 000 U/ml) and streptomycin (10 000 μg/ml) were added. The culture medium was filtered through a filter (0.22-μm, Sterivex-GV, Millipore) for sterilization. The RPMI 1640 culture medium supplemented with heat inactivated fetal calf serum (FCS) was prepared by mixing one volume of FCS heated at 56°C for 30 min to nine volumes of RPMI 1640 culture medium.

2.2. Cell preparation

Whole-blood specimens were obtained from 91 healthy subjects of various ages. Whole blood (10 ml) was collected into Leuco PREP Direct DRAW Tubes containing sodium heparin or EDTA and Ficoll Hypaque (Becton Dickinson, Franklin Lakes, NJ, USA). The specimens were left for 1 h at room temperature, and centrifuged at 1800 *g* for 30 min at room temperature. The MBCs at the interface region were transferred into a 15-ml centrifuge tube and 10 ml of the RPMI 1640 culture medium was added. The number of living MBCs was counted after staining with trypan blue in phosphate-buffered saline (PBS) in a chamber under a microscope.

2.3. PHA stimulation

In each of two 60-mm culture dishes were added 4-ml of the RPMI 1640 culture medium containing FCS and MBCs ($6 \cdot 10^5$cells/ml). In one dish was added 8 μg PHA (Murex Diagnostics, Dartford, UK). The other dish without PHA was used for blank incubation. The cells were activated by HPA in culture at 37°C under 5% CO_2. After 72 h of culture, the cells were transferred to a 15-ml centrifuge tube for washing, suspended and centrifuged at 300 *g* for 15 min in PBS. The washing procedure was repeated with 10 ml PBS, and the cell precipitate was frozen at −80°C before GCH1 activity assay. The precipitated cells were suspended in 350 μl of ice-cold 50 m*M* potassium phosphate buffer (pH 7.0)–0.2 m*M* phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA), and sonication was carried out in ice under 15 cycles of 0.5 s sonication with 0.5 s interval by a sonicator (Model 250 Sonifier, Branson Ultrasonics, Danbury, CT, USA) at 50 W. The suspension was transferred to a 1.5-ml Eppendorf tube, and centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was transferred to another 1.5-ml Eppendorf tube.

2.4. Isolation of protein fraction

Protein fraction was isolated by LC using a SMART system with a fast desalting column (PC 100×3.2 mm, 0.8 ml) (Pharmacia, Uppsala, Sweden) and with a UV detector (LKB-UV-MII) under monitoring 280 nm absorbance at 15°C and at a flow-rate of 100 μl/min. A 40-μl volume of the soluble fraction was injected into the column, and 200 μl of the protein fraction was isolated. The protein concentration was measured by the method of Bradford using bovine serum albumin as standard [18].

2.5. Assay of GCH1 activity by HPLC

Each 100 μl or 75 μl of the protein fraction isolated by chromatography with the SMART System was placed in two 1.5-ml Eppendorf tubes. When a smaller volume of the protein fraction was used, total volume was adjusted to 100 μl with 0.1 *M* Tris–HCl (pH 7.8)–2.5 m*M* EDTA·2K–0.3 *M* KCl–10% glycerol. As control (blank), one tube was heated at 90°C for 5 min to inactivate the enzyme. To each of the two tubes, 4 μl of 25 m*M* GTP was added as substrate and incubated at 37°C for 60 min in the dark. After incubation, 10 μl of 1% I_2–2% KI–1 *M* HCl was added to terminate the reaction and to oxidize the product NH_2P_3 to NP_3 under gentle shaking in the dark for 60 min. After adding 10 μl of 2% ascorbic acid and 13 μl of 1 *M* NaOH, 15 μl of alkaline phosphatase [2 units/μl, 3.2 *M* ammonium sulfate (pH 7.0)–1.0 m*M* $MgCl_2$–0.1 m*M* $ZnCl_2$] (Sigma) were added, and the mixture was incubated at 37°C in the dark for 60 min for dephosphorylation of NP_3 to N. The reaction was terminated by adding 13 μl of 2 *M* acetic acid. The optimized procedure of incubation, oxidation and alkaline phosphatase treatments is graphically presented in Fig. 1. The mixture was centrifuged at 10 000 *g* for 15 min at 4°C. N formed from the product, NH_2P_3, was measured by HPLC–fluorimetry. The HPLC system (Tosoh 880-PU, Tokyo, Japan) was equipped with a degasser SD-8018 (Tosoh), a reversed-phase column (Inartsil ODS-3, 250×4.6 mm I.D.; GL Science, Tokyo, Japan), and a recorder (Shimadzu C-R6A, Kyoto, Japan). Isocratic elution was carried out with 10 m*M* sodium phosphate buffer (pH 7.0)–1 m*M* EDTA at a flow-rate of 1.0 ml/min. Using an autosampler, each 50 μl of the solution was automatically injected into the column. Fluorescence was recorded at 375 nm excitation/465 nm emission. N content was calculated from the standard curve of authentic N.

	Blank	Experiment
Enzyme solution	75 μl	75 μl
	Heat at 90℃ for 5 min.	*On ice*
25 mM GTP	4 μl	4 μl
0.1 M Tris HCl(pH7.8) - 2.5 mM EDTA 2K - 0.3 M KCl - 10% glycerol	21 μl	21 μl
Incubate at 37℃ for 60 min in the dark.		
1% I_2 - 2% KI - 1 M HCl	10 μl	10 μl
Shake for 60 min at room temperature in the dark.		
2% ascorbic acid	10 μl	10 μl
1 M NaOH	13 μl	13 μl
Diluted alkaline phosphatase(2 units/μl)	15 μl	15 μl
Incubate at 37℃ for 60 min in the dark.		
2 M acetic acid	13 μl	13 μl
Centrifuge at 10,000 g for 15 min at 4℃.		

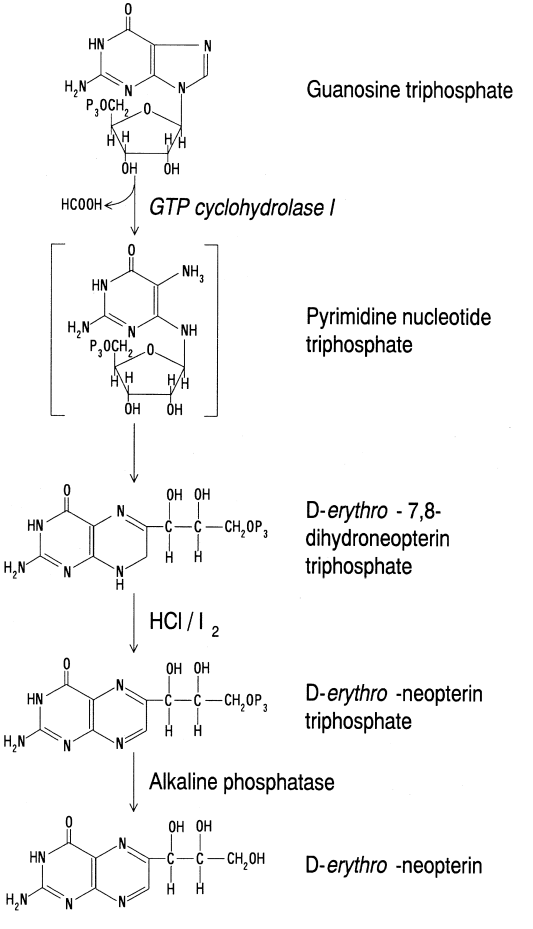

Fig. 1. Graphical presentation of the optimized procedure of incubation, oxidation and alkaline phosphatase treatments in the assay of GCH1 activity.

3. Results

The GCH1 reaction in terms of N formed was linear with protein concentration over the range of 0.1 to 1 mg and incubation times up to 60 min. GCH1 activity in stimulated MBCs was measured with the protein fraction. Desalting to remove endogenous N is necessary to get a high sensitivity by reducing the blank value. The limit of sensitivity of HPLC–fluorimetry for detection of N was approximately 30 fmol. The reproducibility of HPLC analysis for N was nearly 100% and thus calculation was made from the standard curve obtained from the values of authentic N.

A typical chromatogram of GCH1 activity in a PHA-stimulated MBC specimen from a healthy subject is shown in Fig. 2.

Effect of anticoagulant, heparin or EDTA, on GCH1 activity was examined with samples from six healthy subjects. GCH1 activity was not different between heparin (16.9±5.7 pmol/mg protein per h) and EDTA (16.1±2.9 pmol/mg protein per h). Heparin was used throughout.

The assays in this study were started 1 h after

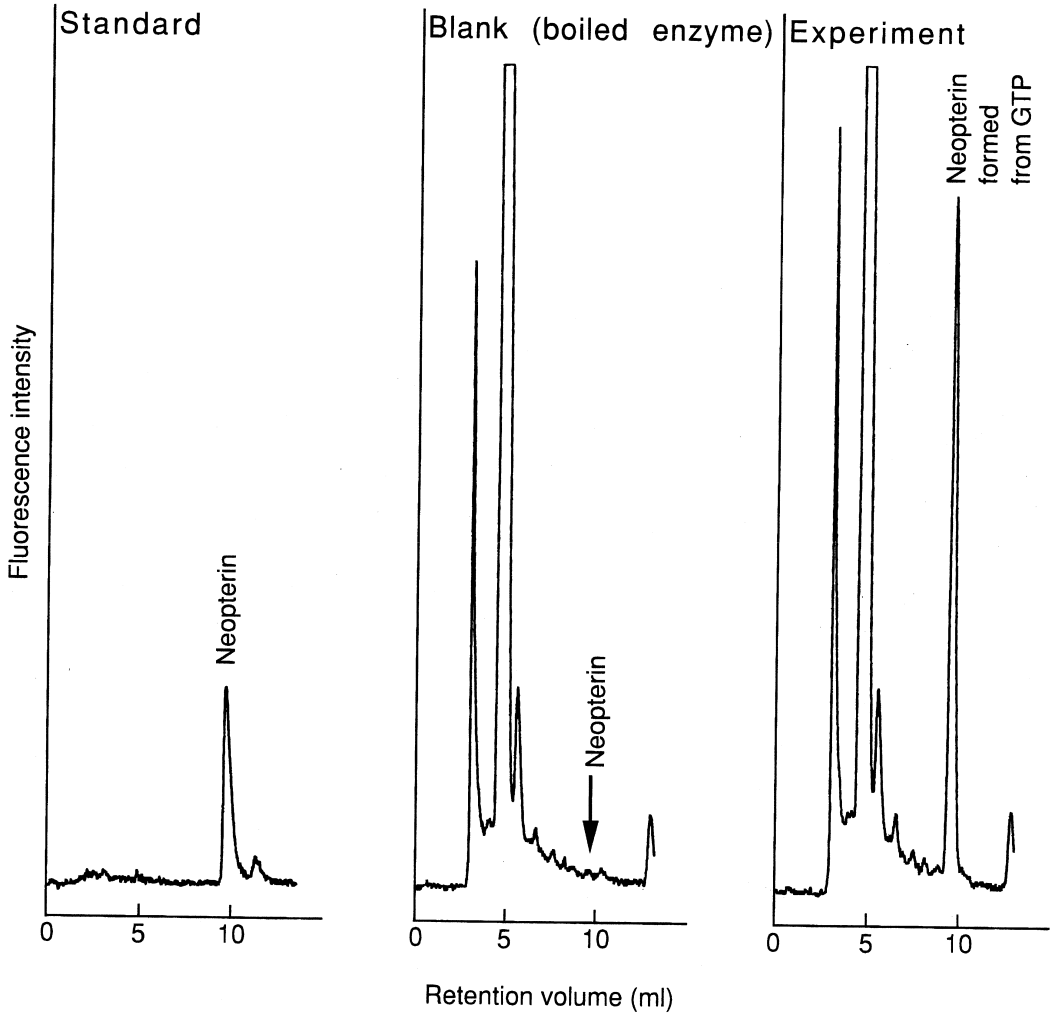

Fig. 2. A typical chromatogram in GCH1 activity in PHA-stimulated MBCs from a healthy subject. Standard N, 500 fmol; N in experiment, 1151 fmol; Calculated GCH1 activity, 23.7 pmol/mg protein per h.

Table 1
Reproducibility starting from cell culture all the way to the HPLC analysis of GCH1 activity in PHA-stimulated MBCs

Subject	GCH1 activity (pmol/mg protein per h)							
	Number of MBC assay samples					Mean	SD	RSD (%)
	I	II	III	IV	V			
M.H.	12.42	13.25	12.94	14.39	14.77	13.56	0.99	7.31
O.N.	10.59	10.91	12.50	11.45	12.49	11.59	0.88	7.63
F.K.	23.66	18.84	23.99	19.96	18.63	21.02	2.62	12.46

blood collection, but blood samples kept in ice gave identical activity within 20 h. Therefore, it is not necessary to do the assay immediately after blood collection.

The method starting from cell culture all the way to the HPLC procedure was reproducible with the same MBC specimens isolated from three normal subjects as shown in Table 1. The GCH1 activity in PHA-stimulated MBCs did not change with two subjects, when measured after 1 month, suggesting that it is constant with an individual subject during the periods of months (subject 1, 19.05 and 20.17; Subject 2, 12.03 and 10.08, pmol/mg protein per h, respectively).

GCH1 activity in PHA-stimulated MBCs (mean±S.E.) in 91 healthy subjects are shown in Table 2. Mean activity was 19.1±0.9 (9.0–48.3) pmol/mg protein per h. No significant difference

Table 2
GCH1 activity in PHA-stimulated MBCs from healthy subjects

Sex	No.	Age (years)		GCH1 activity (pmol/mg protein per h)	
		Range	Mean±SD	Mean±S.E.	Range
Male	8	<21	7.7±4.9	21.5±3.7	9.3–36.8
	12	21~30	24.9±2.9	16.6±1.8	9.8–29.1
	17	31~40	35.0±3.0	18.2±1.9	9.0–39.1
	16	41~50	45.3±2.7	19.2±2.3	11.9–48.3
	7	>50	62.6±7.7	17.7±3.5	10.5–37.3
Mean	60		35.2±15.8	18.5±1.1	9.0–48.3
Female	5	<21	6.4±7.0	27.7±3.4[a]	18.3–35.4
	5	21~30	23.6±3.0	15.8±2.4[b]	9.3–23.4
	15	31~40	34.7±3.7	19.9±2.2	11.7–42.4
	4	41~50	45.0±4.2	17.8±4.0	13.1–29.9
	2	>50	52.5±2.1	19.2±3.3	15.9–22.5
Mean	31		30.8±13.8	20.2±1.4	9.3–42.4
Total	13	<21	7.2±5.4	23.9±2.7[a]	9.3–36.8
	9	1–10	4.5±1.4	25.1±3.4[a]	9.3–36.8
	4	11–20	13.3±0.5	21.0±4.6	10.6–32.8
	17	21~30	24.5±2.9	16.4±1.4[b]	9.3–29.1
	32	31~40	34.8±3.3	19.0±1.4	9.0–42.4
	20	41~50	45.3±2.9	18.9±2.0	11.9–48.3
	9	>50	59.6±7.8	18.0±2.7	10.5–37.3
Mean	91		33.7±15.2	19.1±0.9	9.0–48.3

[a] Significantly different from the value.
[b] $P<0.05$.

was observed between males (18.5±1.1 pmol/mg protein per h, $n=60$) and females (20.2±1.4 pmol/mg protein per h, $n=31$).

The activity in terms of age was slightly higher during the first decade and decreased from the second to third decade and became almost stable from the third decade (Table 2).

GCH1 activity in PHA-stimulated MBCs had no significant correlation with N or B concentration in plasma (data not shown).

4. Discussion

We established a reproducible assay method of GCH1 activity in PHA-stimulated MBCs. The use of a vacuum tube for separating lymphocytes could simplify the isolation of MBCs. Use of the SMART System for isolation of the protein fraction from PHA-stimulated MBCs is rapid and reproducible, and as many as 16 samples can be treated in 5 h. If the SMART system is not available, protein fractions can be isolated by centrifugation (1000 *g* for 4 min) of the soluble fraction (75 μl) through a Micro Bio-Spin chromatography column (Bio-Rad, Hercules, CA, USA). The advantage of the SMART system is its automation and accurate chromatographic separation of the protein fraction. HPLC conditions are adjusted to optimal for rapid isolation of N and for high sensitivity. The HPLC system is automated. The used pre-column oxidation procedure of NH_2 with the iodine–potassium iodide system is simple and reproducible, as compared with post-column oxidation with $NaNO_2$ [19] or electrochemical oxidation [20]. The entire HPLC procedure except 72 h culture with PHA can be carried out within 1 day. The entire assay of 16 samples can be finished within 4 days.

N can also be measured by radioimmunoassay [16] or enzyme-immuno assay [17]. However, the advantage of HPLC is its automation, and markedly improved molecular specificity after chromatographic separation of the enzymatically formed N.

The present method is essentially similar to the previously published method in principle. However, the advantage of the present method is its automation in the SMART and HPLC systems. The previously reported method require several steps of manual procedures.

The normal value by the present method was 19.1±0.9 (9.0–48.3) pmol/mg protein per h ($n=91$). The value is similar to that reported by Ichinose et al. [18.7±2.3 (9.0–46.1) pmol/mg protein per h] with 18 healthy subjects [13]. Blau et al. [12] reported a higher normal value (63 pmol/mg protein per h, $n=15$). They stimulated MBCs for 120 h and measured NP_3 after oxidation of NH_2P_3. Our lower value may be due to differences in PHA-stimulation (72 h). We observed reproducible activity after 72 h PHA-stimulation.

Since the protocol of cell preparation and culture may affect the activity, the protocol should be strictly followed. It is recommended to carry out the assay of samples from patients simultaneously with samples from normal subjects.

Ichinose et al. [11] previously reported that the activity in females is slightly lower than that of in males without statistical significance, but the present results indicate that normal activity is similar between males and females.

Young children of less than 10 years of age showed higher GCH1 activity in PHA-stimulated MBCs. Aging did not affect the activity. However, since the numbers of aged subjects (>50 years) were small (12), more study will be required to confirm the effect of aging on the activity.

Bezin et al. [21] demonstrated that measurement of endogenous N levels in unstimulated lymphoblasts identified GCH1 dysfunction and can be a diagnostic test for autosomal dominant DRD. They suggest that PHA induction alone may result in incorrect identification of GCH1 dysfunction in DRD. This problem remains to be further examined.

The present method can be applied to the diagnosis of HPD/DRD or heterozygotes or homozygotes of GCH1 deficiency [11,12]. The patients of HPD/DRD had less than 20% of the normal activity, and the carriers about 40% [11]. A homozygote patient with GCH deficiency had less than 3% of normal activity, and the heterozygote parents had 30% and 40% of the activity, respectively [12]. The present method can be applied to screening of the enzyme activity in various neuropsychiatric, endocrinological and immunological diseases in which change in GCH1 activity are expected.

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