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"Hyperfolic-acidemia with Formiminoglutamic-aciduria Following Histidine Loading"

Suggested for a Case of Congenital Deficiency in Formiminotransferase

By

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As regards congenital disturbance in folic acid metabolism, an infantile case with relapsing megaloblastic anemia due to a specific defect in gastrointestinal absorption of folic acid, reported by Luhby *et al.*[1] in 1962, has been the only one of the kind.

In the present paper an infant was presented who was characterized by clinical findings such as round face, obesity, retardation of mental and physical development, and by hematologically a tendency to hypersegmentation of neutrophil nuclei on one hand, and biochemically by peculiar findings such as an abnormally high level of serum folic acid activity and an excessive excretion of formiminoglutamic acid (FIGLU) following histidine loading test on the other hand.

The results of our present experimentation revealed that a defect (or deficiency) in formiminotransferase in the liver of our own patient was a primary lesion, of possibly hereditary origin, responsible for the development of clinical and biochemical abnormalities above quoted.

REPORT OF CASE

C.K., a 8-month-old girl was admitted into our Clinic on June 15, 1962, with a chief complaint of occasional fits of convulsion. She was born at full term after an uneventful pregnancy and delivery. She was fed on mother's milk for the first 3 months of life, but thereafter had supplementary feeding with rice gruel because of hypogalacty. With about the 5th month of life, generalized edema and fits of convulsion developed. She was then admitted into the City Hospital at Kuroishi and treated under a diagnosis of 'starch injury' for about 3 months.

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The convulsion and edema had improved during hospitalization, but a tendency to hypersegmentation of neutrophils was noticed by Dr. Kudo of the City Hospital at Kuroishi, who then sent the patient to our University Clinic for further examination.

On admission physical examination revealed a fatty girl with round face of the age of 8 months and 22 days. Bodyweight was 10,200 g, height 67.5 cm. The chest and heart were clear to auscultation. The abdomen was distended. The liver was palpable 1.5 fingerbreadths below the costal margin and its edge was not firm on palpation. The spleen was not palpable. There was no lymphadenopathy. Knee jerks were not increased. Neither ankle clonus nor Babinski's sign was observed. The patient was unable to sit alone, support her head steadily, acknowledge her own mother, or say any word.

Laboratory findings

Urinalysis: tests for sugar and for protein were negative and there were no red cells and white cells in sediments.

X-ray examination revealed no calcification of soft tissues, no exostosis and no abnormalities of bones and chest.

Liver function tests were negative for serum transaminase, thymol turbidity and zinc sulfate tests, Takata's reaction, and serum levels of bilirubin and cholesterol.

Blood picture and bone marrow picture (cf. Tables I & II)

There was no reduction in both red cell counts and hemoglobin concentration, but there was a slight tendency to macrocytosis in an early stage of hospital day.

Respecting nuclear segmentation of neutrophils, a slight tendency to hypersegmentation of neutrophil nuclei was noticed. In normal children[2] the distribution of neutrophils according to number of nuclear lobes is that the proportion (I+II+III): (IV+V+VI) is 84%: 16% on average. In our own patient it was found that the proportion was 70%: 30%, so that she had surely a tendency of hypersegmentation of neutrophil nuclei.

In bone marrow pictures there was no megaloblasts and no increase in macroblasts (cf. Table II).

Chromozomal analysis revealed no abnormality in number and configuration.

Fasting blood sugar and urinary output of 17KS and 17OHCS were found to be within normal limits.

I^{131} uptake test gave normal result (42%).

Paper chromatography of urinary amino acids revealed no abnormality.

Serum amino acid pattern, analyzed by using Automatic Amino acid Analyzer revealed no abnormality other than a slight increase of alanine (cf. Table III).

TABLE I. Blood Pictures

Date of examination	Age (months)	Red cell count (10^4/mm^3)	Hemoglobin (gl/dl)	Hematocrit (%)	MCV (μ^3)	Total count of white cells (/mm^3)
1962						
18/VI	8	473	16.3	56	118	12,200
3/VII	9	429	12.3			13,750
15/VIII	10	432	10.0	46	106	8,600
10/XII	14	401	15.0	42	104	7,700
1963						
29/III	17	423	15.0	45	106	8,650
6/V	19	549	11.2	41	75	8,850
14/VI	20	445	11.4	43.5	98	15,250
27/VII	21	556	12.4	44	79	13,500

TABLE II. Bone Marrow Picture of Our Own Patient (August 9, 1963)

Nucleated cell count			27.3×10^4 (per mm^3)	
Megakaryocytes			83 (per mm^3)	
M : E			2.3 : 1	
Myeloblasts		1.4	Proerythroblasts	1.6
Promyelocytes		2.0	Macroblasts	0.4
Neutrophils	Myelocytes	9.8	Normoblasts	
	Metamyelocytes	12.4	Basophilic	7.4
	Stab	21.8	Polychromatic	8.4
	Segmented	9.2	Orthochromatic	8.8
Eosinophils	Myelocytes	1.6	Reticulum cells	0.4
	Metamyelocytes	0.2	Lymphocytes	11.6
	Stab	0.2	Mitotic figures	0.2
	Segmented	1.0		
Basophils		0		
Monocytes		1.6		

Serum electrolytes were found to be within normal limits except for a transient hypocalcemia accompanied with an elevation of phosphorus in an early hospital day (cf. Table IV).

Electroencephalography revealed localized slow waves at the parietal area, which disappeared later when serum calcium increased up to the normal level.

Serum folic acid activity and vitamin B_{12} in serum:

Serum folic acid activity was determined by Herbert *et al.*'s method[3] with use of L. casei. Vitamin B_{12} in serum was measured by the method[4] adopted by Vitamin B Committee of Japanese Society of Vitaminology with use of L. leichimannii ATCC 4797.

of Our Own Patient

Differential count of white cells (%)					Distribution of neutrophils according to number of nuclear lobes (%)							Remarks
B	E	L	M	N	I	II	III	IV	V	VI	IV+V+VI	
0	1.5	30	7.5	60	8	32	40	15	5	0	20	
0	2.5	29.5	6.5	61	7	29	43	13	7	0	20	
0	1.5	40.5	11.5	46.5	4	18	46	26	6	0	32	
0	0	32	3	65	10	21	41	18	9	1	28	
0	1	38	4.5	56.5	6	26	39	19	6	0	25	
0	2	59	7	32	3	26	33	32	6	0	38	
0	2	60	1	34								
0	2.5	54.5	8	35	6	29	36	23	6	0	29	Drumstick 9%

TABLE III. Serum Amino Acid Pattern of Our Own Patient

Amino acids	mg/100 cc of serum
Lysine	3.40
Histidine	0.61
Threonine	1.05
Serine	2.35
Glutamic acid	1.47
Proline	1.08
Glycine	1.75
Alanine	4.92
Valine	3.00
Isoleucine	0.88
Leucine	2.12
Tyrosine	1.18
Phenylalanine	1.14

TABLE IV. Serum Electrolyte Pattern of Our Own Patient

Date of examination	Age (months)	Calcium (mg/dl)	Phosphorus (mg/dl)	Chloride (mEq/L)	Sodium (mEq/L)	Potassium (mEq/L)
1962						
18/VI	8	7.6	6.8	105		
7/VII	9	4.9	6.5	107	142	5.6
25/VII		8.0	5.3	108		
28/VII		10.0	5.8	107		
11/IX	11	9.3	5.7	111		
12/XI	13	10.2	6.4	111		
24/XI		10.7	6.2	105		
1963						
7/II	16	10.0	4.7	113		
28/III	17	7.8	7.0	109		
28/V	19	10.0	5.9			

TABLE V. Serum Folic Acid Activity of Our Own Patient

Date of examination	Age (months)	Serum folic acid activity (mγ/cc)	Remark
1962			
29/VI	8	300 ~	
25/VII	9	300 ~	
28/VII		280	
19/VIII	10	300 ~	
11/IX	11	11	
22/IX		10	
28/XI	13	28	
1963			
15/II	15	80	
25/III	16	300 ~	
30/IV	17	120	
28/V	18	112	
7/VI	19	96	
15/VI		300 ~	
26/VI		250	Serum B_{12} 0.47 mγ/cc
28/VI		200	
5/VII	20	260	
10/VII		214	

As will be seen from Table V, abnormally high levels of folic acid activity in serum (more than 100 mγ/cc) were found during hospital day of about one year except for a short period of from September to November, 1962.

Vitamin B_{12} value was found to be normal.

Urinary formiminoglutamic acid (FIGLU) following histidine loading:

Histidine loading test[5,6] was carried out in March, 1963. Histidine, 0.25 g per kg of bodyweight, was given orally and urine specimens were collected for 8 hours following the dose, and FIGLU excreted in urine was measured enzymatically according to Tabor and Wyngarden's[7] procedure.

As will be seen from Table VI, abnormally large amounts of FIGLU were excreted into urine after the dose in our own patient.

Formiminotransferase activity of the liver of our own patient:

Liver specimens from our own patient and from an adult without hepatic disorder were removed surgically and kept at –20°C until assay. All the following operations were carried out at 0–4°C.

Then slices were made from about 100 mg of the frozen liver specimens and washed with a few cc of chilled physiologic saline. To the washed liver slices 3.0 cc of 0.1 M phosphate buffer of pH 7.2 were added and the content was homogenized by Potter's homogenizer for 15 seconds. The homogenate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was diluted up to protein level of 4.8 mg/cc with an addition of 0.1 M phosphate buffer of pH 7.2—

TABLE VI. Urinary Excretion of Formiminoglutamic Acid (FIGLU) Following Histidine Loading Test (0.25 g per kg of bodyweight orally)

Subjects examined	Date of examination	FIGLU excreted in urine for 8 hours following the dose (μmoles)
Our own patient (C.K.) (16 months)	1963 6/III	66.6
〃	19/III	13.3
Control	1963	
K.G. (epilepsy, 24 months)	13/III	6.2
T.S. (epilepsy, 31 months)	19/III	1.7

TABLE VII. Reaction Systems for Assay of Formiminotransferase of the Liver of Our Own Patient

Composition	For test	For blank
Tetrahydrofolic acid in mercaptoethanol*	0.1 cc	0.1 cc
Formiminoglutamic acid	0.2 μmoles	0.2 μmoles
Crude enzyme extract	0.25 cc	—
0.1 M phosphate buffer of pH 7.2	—	0.25 cc
1.0 M phosphate buffer of pH 7.2	0.1 cc	0.1 cc
Aq. distill.	0.55 cc	0.55 cc
Final volume	1.0 cc	1.0 cc

* 88 mg of tetrahydrofolic acid were dissolved in a mixture of 18.0 cc of 1 M mercaptoethanol and of 2.0 cc of 0.2N-KOH.

"crude enzyme extract".

For assay of formiminotransferase activity of the liver extract, a reaction system was prepared in the same way as described in Tabor and Wyngarden's report[7] (cf. Table VII). The enzymatic reaction was carried out at room temperature (28°C), and the reaction was stopped at $^1/_2$, 1, 2, and 3 minutes by an addition of 0.3 cc of 10% perchloric acid. Immediately after that, the contents were kept in a boiling water bath for 55 seconds, then cooled in an iced-water. After cooling, the contents were centrifuged at 2,500 rpm for 10 minutes and the clear supernatant was used for determination of optical density of 5, 10 methenyltetrahydrofolic acid thus formed. Differences of the optical density between 355 mμ and 420 mμ, measured by using a recording spectrophotometer, were taken as amounts of 5, 10 methenyltetrahydrofolic acid, in other words, activity of formiminotransferase which is indispensable[7] for formation of 5, 10 methenyltetrahydrofolic acid in these reaction systems. The results were given in Table VIII and Fig. 1, which showed a marked decrease in the formiminotransferase activity in the liver from our own patient.

Folic acid reductase systems:

It is now clear that folic acid must be reduced first to tetrahydrofolic acid before it can function in single-carbon transfer reactions. Reduction of folic acid

TABLE VIII. Formiminotransferase Activity of the Liver* of Our Own Patient

Duration of the enzymatic reaction	Difference of optical density between 355 mμ and 420 mμ	
	Our own patient	Control
For 30 seconds	0	2.8
For 1 minute	0.8	8.9
For 2 minutes	2.0	14.5
For 3 minutes	3.0	21.3

* Enzyme extracts of liver specimens were prepared so as to be of protein levels of 4.8 mg/cc in both our patient and control.

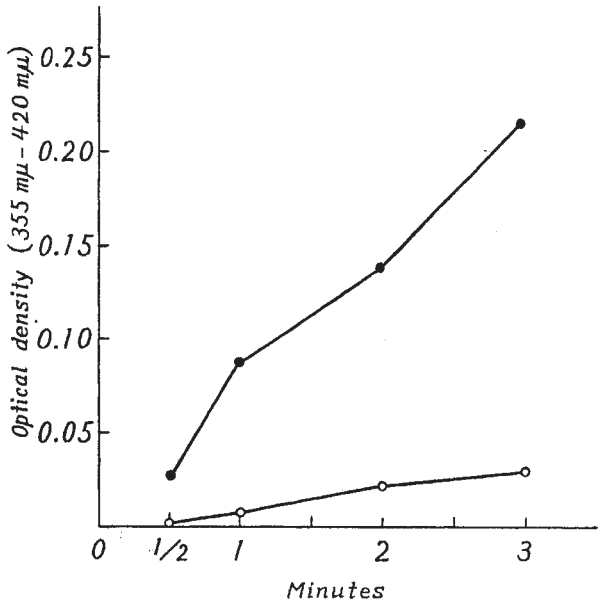

Fig. 1. Activity of formiminotransferase in the liver.
Our own patient o——o
Control •——•

into tetrahydrofolic acid by chicken liver was demonstrated by Futterman and Silverman in 1957. The activity in the folic acid reductase systems was investigated upon the liver biopsied from our own patient in the same way as Futterman and Silverman's[8] experimentation upon chicken liver. The liver specimens were removed surgically from our own patient and from an adult without hepatic disorder, and kept at –20°C until assay. The following operations were carried out at 0–4°C.

To 0.38 g of the frozen liver specimens were added 3 volumes of 0.1 M potassium phosphate buffer of pH 6.0, and the contents were homogenized by Potter's homogenizer for 2 minutes. The homogenate was centrifuged at 22,500× g for 15 minutes. The supernatant was lyophilized, thus about 45 mg of

Errata

Table VIII in page 376, Tohoku J. Exp. Med., **80**, 1963,
Should be corrected as follows:

TABLE VIII. Formiminotransferase Activity of the Liver* of Our Own Patient

Duration of the enzymatic reaction	Difference of optical density between 355 mμ and 420 mμ	
	Our own patient	Control
For 30 seconds	0	0.028
For 1 minute	0.008	0.089
For 2 minutes	0.020	0.145
For 3 minutes	0.030	0.213

* Enzyme extracts of liver specimens were prepared so as to be of protein levels of 4.8 mg/cc in both our patient and control.

TABLE IX. Reaction System for Assay of Folic Acid Reductase Activity

Composition	Amounts per tube
Enzyme preparation to be tested	15 mg of lyophilized powder
1 M potassium phosphate buffer of pH 6.0	0.2 cc
1% DPN aqueous solution	0.1 cc
ATP aqueous solution (5 mg/cc)	0.2 cc
1% $MgSO_4$	0.1 cc
3.8% sodium citrate	0.1 cc
Folic acid solution (1 mg/cc)	0.2 cc
Aq. distill.	1.1 cc

lyophilized powder were obtained. 15 mg of this lyophilized powder were used for assay of folic acid reductase activity. The reaction systems for this assay were constructed in the same way as those of Futterman and Silverman,[8] except that lyophilized powder of human liver was used in place of chicken liver extract (cf. Table IX).

The enzymatic reaction was carried out at 37°C for 90 minutes, then the reaction was stopped by an addition of 2.0 cc of 10% trichloroacetic acid. After an addition of 1.0 cc of water, the content was mixed well and centrifuged. The supernatant was used for colorimetric estimation of p-aminobenzoyl-glutamic acid thus formed by means of Bratton and Marshall's[9] method.

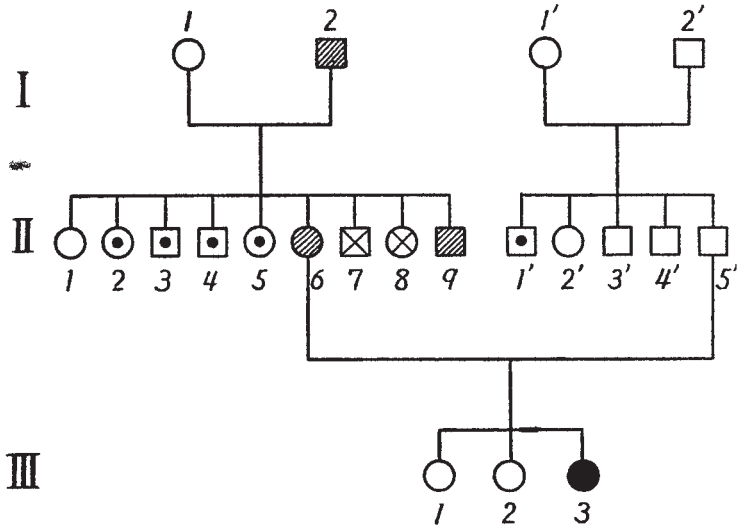

Fig. 2. The pedigree of the "C" family.

● ; *the patient*
◍, ▨ ; *subjects with a tendency of hypersegmentation of neutrophils*
⊗, ⊠ ; *subjects with an abnormally high level of serum folic acid activity*
⊙, ▣ ; *subjects not examined*

TABLE X. Blood Pictures, Serum Vitamin B_{12}

	Relation to the patient		No. in Fig. 2	Red cell count $10^4/mm^3$	Hemoglobin (g/dl)	White cell count (mm^3)	Differential count of neutrophils (%)				
							B	E	L	M	N
Paternal side	Grandfather	♂	I_2'	392	10.3	7,000	0	0	30	3.5	66.5
	Uncle	♂	II_3'	443	12.2	4,250	0.5	1	15	3.5	80
	Aunt	♀	II_2'	529	11.4	4,500	0.5	0.5	42	4	53
	Uncle	♂	II_4'	421	11.7	4,050	0	0	40	2	58
	Father	♂	II_5'	569	12.3	4,500	0.5	0.5	34	3	62
Maternal side	Grandfather	♂	I_2	498		5,040	0	0	33	5	62
	Grandmother	♀	I_1	370	8.3	3,900	1	0	44	3	52
	Aunt	♀	II_1	381	10.2	4,900	0	2	35	5	58
	Uncle	♂	II_7	460	12.5	5,050	0	1	37	8	54
	Aunt	♀	II_8	467	9.6	5,000	0	0.5	31	4	64
	Uncle	♂	II_9	451	13.1	5,900	0	1	24	3	72
	Mother	♀	II_6	364	9.6	6,150	0	1	36	4	59
	Sister	♀	III_1	362	7.9	7,150	0	2	41	3	54
	Sister	♀	III_2	479	8.9	7,150	0	0.5	44	3	53
	The patient		III_3	432		8,600	0	1	40	11	48

The results were that amounts of tetrahydrofolic acid, which were converted from folic acid in these reaction systems, were found to be 13 mμ moles in case of our own patient and 8 mμ moles in that of control. There was thus no significant difference in the activity of the folic acid reductase of the liver between our own patient and control.

Familial study for serum folic acid activity, vitamin B_{12} levels, and peripheral blood pictures:

The family tree of our own patient, C.K. — the "C" family — was showed in Fig. 2. Serum folic acid activity and vitamin B_{12} values of serum, and peripheral blood pictures were examined upon several members of the "C" family (cf. Table X).

Case with a tendency to hypersegmentation[2] of neutrophil nuclei (neutrophils with nuclear lobes of (IV+V+VI)>30%) or with abnormally high levels of serum folic acid activity (>100 mγ/cc) or a low concentration of hemoglobin (<10 g/dl) were found in members of only the maternal side, but not of the paternal (cf. Table X). Vitamin B_{12} levels of serum were found to be within normal limits (cf. Table X).

DISCUSSION

Clinical features:

Clinical characteristics of our own patient were retardation of mental and physical development, round face and obesity. The first impression of the patient

and Folic Acid Activity of the "C"-Family

Distribution of neutrophils according to number of nucler lobes (%)							Serum folic acid activity (mγ/cc)	Serum vitamin B_{12} (mγ/cc)
I	II	III	IV	V	VI	IV+V+VI		
5	46	37	10	2	0	12	18	0.22
9	46	38	7	0	0	7	24	0.10
6	40	30	18	6	0	24	84	0.43
3	51	37	9	0	0	9	80	0.20
10	41	41	6	2	0	8	61	
4	18	48	22	5	3	30	68	
6	31	40	18	5	0	23	68	
2	30	44	20	4	0	24	68	
13	43	32	11	1	0	12	300～	
10	42	39	7	2	0	9	124	
3	22	38	31	5	1	37	68	
4	24	40	27	5	0	32	20	0.30
9	37	32	18	4	0	22	40	0.13
9	35	40	16	0	0	16	20	0.25
4	18	46	26	6	0	32	300～	0.47

reminded us somewhat of pseudohypoparathyroidism or pseudo-pseudohypoparathyroidism. According to Papaioannou and Matsas[10] pseudohypoparathyroidism cannot be differentiated clinically from pseudo-pseudohypoparathyroidism, because both of them are, as they state, characterized by the same clinical features such as retardation of mental and physical development, obesity, round face, deformity of bones, calcification of soft tissues and abnormality in electroencephalogram. But low calcium and high phosphorus in serum are found consistently in pseudohypoparathyroidism, but these are absent, or transiently present, in pseudo-pseudohypoparathyroidism.[10] This is what they further state.

In our own case, obesity, round face, retardation of mental and physical development, abnormality in electroencephalogram, and a transient decrease in calcium accompanied with an increase in phosphorus in serum were found, and these findings seemed to support the diagnosis for pseudo-pseudohypoparathyroidism. But deformity of metacarpal or metatarsal bones, ectopic calcinosis, exostoses and short stature, which are found with considerable frequency in the cases with pseudo-pseudohypoparathyroidism, were not found in our own case. Still, this case is very much like of pseudo-pseudohypoparathyroidism despite the lack of these findings.

Hypersegmentation of neutrophil nuclei:

According to Herbert[11] occurrence of hypersegmented neutrophils in the peripheral blood suggests deficiency in folic acid or in vitamin B_{12}.

In our own case, neutrophils with nuclear lobes of IV or more were found in

30%, while they are found in less than 20% in normal children.[2] This finding suggests that there is a slight tendency to hypersegmentation of neutrophils in our own case. Such a tendency to hypersegmentation of neutrophils was also found among several members of the maternal side. In this respect, it should be mentioned of Undritz's[12] hereditary, constitutional hypersegmentation of neutrophils, which is characterized by familial occurrence of an extreme hypersegmentation of neutrophils—neutrophils with nuclear lobes of IV or more are found in more than 70%. In our own case the degree of hypersegmentation of neutrophils was far less striking than in the cases of Undritz's anomaly, so our own case is different from Undritz's hematological disorder.

Biochemical findings: Abnormally high levels of serum folic acid activity were found in our own case. According to Herbert *et al.*[3] "serum folic acid activity" for L. casei is due to mainly triglutamyl derivatives of reduced forms of folic acid, including 10-formyl-FH_4, 5, 10-methenyl FH_4, FH_4 carrying one-carbon units other than formyl, and FH_4 carrying no one-carbon unit. There was probably an abnormal accumulation of FH_4 or FH_4 derivatives in serum of our own patient.

On the other hand it was found that, after the histidine loading test, excessive amounts of FIGLU were excreted into urine of our patient. To such a case of our own we may suggest "*Hyperfolic-acidemia* with Formiminoglutamic-aciduria following Histidine Loading".

It is, therefore, reasonable to suppose an occurrence of deficiency of formiminotransferase which is indispensable[7] for interaction between FH_4 and FIGLU (cf. Fig. 3).

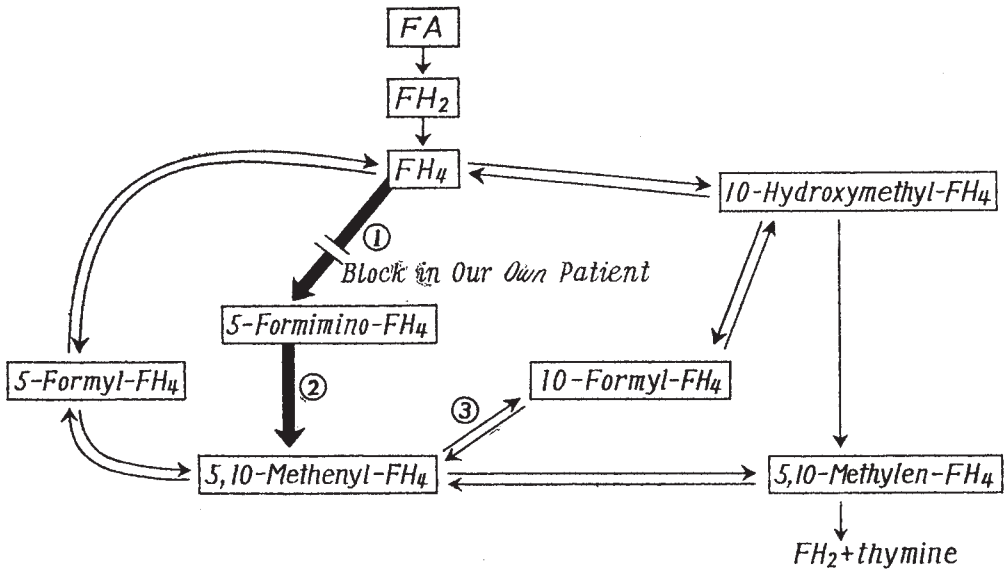

Fig. 3. Site of a block in our own patient, in biosynthesis of functional forms of folic acid (Greenberg, D.M.)[13]. FH_2, dihydrofolic acid; FH_4, tetrahydrofolic acid; FIGLU, formiminoglutamic acid.

①, formiminotransferase (Enzyme I): ②, cyclo-deaminase (Enzyme II): ③, cyclohydrolase (Enzyme III) (Tabor, H. & Wyngarden, L.)[7]

In fact, by our actual experimentation, marked decrease in formiminotransferase activity was demonstrated in the liver biopsied from our own patient.

Basing upon our results above mentioned, it is highly probable abnormally high levels of serum folic acid activity and excessive excretion of FIGLU following histidine loading are due to a deficiency in the activity of formiminotransferase in the liver of our own patient.

Now it remains unsolved what relationship should exist between the hypersegmentation of neutrophils and the block of formiminotransferase, even though a disturbance of formation of "hematologically active forms of folic acid"[14] might possibly, to some extent, result from a block in the pathway of folic acid → 5-formiminotetrahydrofolic acid → 5, 10 methenyltetrahydrofolic acid, due to the defect of formiminotransferase (cf. Fig. 3).

An abnormally high level of serum folic acid activity and a tendency to hypersegmentation of neutrophils were abnormalities identified in members of only the maternal side, and this will suggest that the disorder in question might be of hereditary origin.

SUMMARY AND CONCLUSIONS

A peculiar disorder of a female, aged 8 months old, was presented.

Clinical characteristics were round face, obesity, retardation of mental and physical development, and a slight tendency to hypersegmentation of neutrophils in the peripheral blood.

Biochemically, an abnormally high level of serum folic acid activity and an excessive excretion of formiminoglutamic-acid into urine following histidine loading were confirmed. These abnormalities were considered to be due to a marked decrease in formiminotransferase which was demonstrated in the liver biopsied from our own patient.

This defect in formiminotransferase in the liver may be of hereditary origin.

A term "hyperfolic-acidemia with formiminoglutamic-aciduria following histidine loading" may be suggested for this new entity of inborn error of folic acid metabolism.

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