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Clinical and experimental studies on folic acid deficiency due to anticonvulsants. 2. Investigations on patients receiving anticonvulsants and experimental study on the effect of diphenylhydantoin on the absorption of folic acid in rats

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Clinical and experimental studies on folic acid deficiency due to anticonvulsants. 2. Investigations on patients receiving anticonvulsants and experimental study on the effect of diphenylhydantoin on the absorption of folic acid in rats*

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Abstract

A high incidence of subnormal serum folic acid levels was observed in 48 patients receiving anticonvulsants (75 %). In peripheral blood, macrocytosis was detected in 46 % and an increase of hypersegmented neutrophils was also seen in 24 % of the patients. Correlation existed between these signs and low serum folate levels. The growth response of Lactobacillus casei and L. leichmannii was not suppressed by the addition of various anticonvulsants to the medium of the bioassay systems. Administration of 5 mg of folic acid for a month corrected macrocytosis and an increase of hypersegmented neutrophils significantly. Folic acid supply also decreased mean diameters of the nuclei of oral epithelial cells significantly. It is concluded that subclinical folic acid deficiency is common among the patients receiving anticonvulsants. Absorption of 3H.folic acid from the small intestine of rats was inhibited by large dose of diphenylhydantoin (20 mg) not by 5 mg. This fact suggests that in patients on diphenylhydantoin, the quantity balance of folic acid and diphenylhydantoin in the intestine regulates the absorption of folic acid.

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CLINICAL AND EXPERIMENTAL STUDIES ON FOLIC ACID DEFICIENCY DUE TO ANTICONVULSANTS

2. INVESTIGATIONS ON PATIENTS RECEIVING ANTICONVULSANTS AND EXPERIMENTAL STUDY ON THE EFFECT OF DIPHENYLHYDANTOIN ON THE ABSORPTION OF FOLIC ACID IN RATS

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The occasional development of megaloblastic anemia during anticonvulsant drug therapy has been well documented (1, 2, 3, 4, 5, 6). Response to folic acid has invariably been complete and the folic acid deficiency was revealed by special examinations. Though the development of megaloblastic anemia is rare, subclinical folic acid deficiency was observed in patients receiving anticonvulsants (5, 6, 7, 8, 9, 10). The cause of the folic acid deficiency in these patients is still unknown. A role of nutritional factor for the devolopment of the megaloblastic anemia in two patients was reported in the previous study of the author (4). The present study reports the results of a survey of forty-eight patients who were being administered anticonvulsants and an experiment using ^{3}H-folic acid to study the effect of diphenylhydantoin (DPH) on the absorption of folic acid from the small intestine of rats.

MTERIALS AND METHODS

1. *Clinical study*

a. Patients studied: Forty-eight patients (Thirty-four males and fourteen females) taking anticonvulsants in the three psychiatric hospitals in Okayama Prefecture were investigated. The majority were on combined drug regimen of DPH, Primidone (Mysoline) and phenobarbital. Folic acid was not supplied for them. Some of them were supplied with vitamin B_{12} (B_{12}) orally. Duration of the anticonvulsant therapy ranged from one year to eighteen years (Table 1).

b. Laboratory methods: (1) Peripheral blood counts: one ml of preprandial venous blood was taken. "Anticlot ET" (Composed of EDTA -2Na and crude heparin) was used to prevent coagulation. The hematological methods used were those set by DACIE and LEWIS (11). The mean corpuscular diameter (MCD) was calculated using Price-Jones curves by measuring five hundred red blood

TABLE 1 RESULTS OF THE EXAMINATIONS ON PATIENTS RECEIVING ANTICONVULSANTS (1)

No.	Age, sex	Anticonvulsants, duration (year)	Hb (g/dl)	RBC (×10⁴)	C. I.	Ht (%)	MCV (cu. μ)	MCD (μ)	Macyocytes(%)	WBC	Hyper-seg. (%)	Ret (‰)	Platelet (×10⁴)	Iron (μg/dl)	Folate (ng/ml)	Vitamin B_{12} (pg/ml)
1	25 ♂	D+P 1	14.2 14.7	487 496	0.91 0.95	44.0 45.0	90.3 91	7.8 7.8	8.4 8.4	8,000 7,300	16 8	4 6	14.6 17.9	121 149	4.0 54.0	500 300
2	30 ♂	D+M+P 5	12.9 13.4	395 414	1.02 1.04	41.0 41.5	103.8 100	8.0 8.0	13.6 11.0	4,700 4,200	10 3	2 4	13.6 21.5	108 184	4.0 52.0	280 200
3	44 ♂	M+P 5	15.6 15.0	461 490	1.06 0.98	49.0 46.0	104 94	8.3 7.8	24.0 3.2	6,300 6,400	13 5	6 15	19.8 18.5	174 121	1.6 50.0	300 280
4	35 ♂	M+P 15	12.2 13.0	358 403	1.07 1.02	37.5 40.0	105 98	8.1 7.8	12.0 4.4	4,200 5,000	26 2	5 7	15.6 15.9	86 161	2.0 56.0	330 330
5	54 ♂	M+P 7	13.4 13.5	400 412	1.07 1.05	41.0 39.0	102.5 95	7.7 7.2	3.6 2.8	6,300 7,300	5 4	4 10	9.3 21.8	98 115	8.6 33.4	450 260
6	37 ♂	M+P 3	14.6 14.0	437 426	1.05 1.05	43.5 42.0	99.5 98	7.8 7.8	6.4 4.8	10,600 8,900	13 3	4 7	17.5 17.0	92 85	1.8 47.0	700 520
7	20 ♀	M+P 2	11.4 11.8	326 355	1.09 1.06	35.0 35.5	107.4 100	8.2 8.0	14.8 8.2	5,000 5,100	13 0	14 7	10.0 24.9	150 124	2.6 33.0	480 280
8	28 ♂	M+P 8	14.4 13.3	424 428	1.06 1.00	43.5 42.5	102.6 99	7.9 8.0	17.0 13.6	5,400 5,400	15 5	2 5	14.4 23.5	90	2.4 45.0	320 230
9	44 ♂	D+P 3	11.0 9.8	266 295	1.29 1.06	34.0 31.5	127.8 107	8.5 8.2	17.4 15.6	3,300 4,800	25 6	3 7	13.3 27.1	86 59	1.0 43.0	300 140
10	24 ♂	P 7	16.1 14.1	449 428	1.26 1.05	46.0 43.0	102 100	7.7 7.9	3.6 8.0	4,800 4,800	27 6	4 9	10.7 21.0	141 110	2.0 38.0	600 280
11	20 ♀	D+P 2	13.2 13.1	419 421	0.99 1.00	39.5 37.0	94.3 88	7.7 7.7	3.6 4.0	6,700 6,300	28 1	5 8	12.5 29.0	98 83	2.5 40.0	600 240
12	40 ♂	P 12	11.1 10.7	352 353	0.99 0.97	34.0 31.5	96.6 89	7.8 7.9	8.8 3.2	6,700 9,200	24 6	1 7	13.4 17.7	102 81	2.4 62.0	550 210
13	18 ♂	M+P 4	14.0 12.9	401 420	1.09 0.98	38.5 40.0	96 95	7.8 7.8	6.8 3.6	3,500 4,300	16 3	11 5	11.2 20.2	145 127	1.8 53.0	450 200
14	44 ♀	D+P 4	11.0 12.6	302 374	1.14 1.03	34 38.5	112.6 103	8.3 8.1	26.4 18.4	4,000 5,100	9 6	13 9	15.0 15.7	108 160	1.1 36.0	450 380

TABLE 1 RESULTS OF THE EXAMINATIONS ON PATIENTS RECEIVING ANTICONVULSANTS (2)

No.	Age, sex	Anticonvulsants, Duration (year)	Hb (g/dl)	REC (×10⁴)	C. I.	Ht (%)	MCV (cu. μ)	MCD (μ)	Macro-cytes (%)	WBC	Hyper seg. (%)	Platelet (×10⁴)	Folate (ng/ml)	Vitamin B_{12} (pg/ml)
15	57 ♀	D+P 6	10.3	328	1.00	32.5	99	7.92	7.4	5,700	6	20.4	4.5	400
16	22 ♀	D+P 5	13.5	435	0.99	41.0	94	7.80	4.4	5,700	3	19.6	7.0	340
17	42 ♀	D+M+P 17	12.0	413	0.93	37.5	91	8.04	12.8	4,900	0	31.8	6.3	430
18	63 ♂	D 3	14.0	476	0.94	41.5	87	7.62	2.6	7,100	13	16.7	7.2	340
19	21 ♂	D+P 2	14.6	488	0.96	43.0	88	7.78	4.8	6,300	10	38.0	5.0	800
20	35 ♂	D+P 8	15.7	504	1.00	47.0	93	7.83	6.0	7,500	7	19.2	3.0	510
21	39 ♂	D 13	13.7	480	0.92	45.0	94	8.19	18.4	5,800	6	33.6	2.9	400
22	29 ♂	D+P 1	16.1	488	1.05	49.5	101	8.01	10.8	6,000	2	25.4	1.6	380
23	34 ♂	D+P 17	14.1	455	0.99	42.0	92	7.81	7.0	9,200	6	18.2	2.5	720
24	40 ♂	D+P 8	16.8	531	1.01	51.5	97	8.01	10.8	7,600	4	20.2	4.8	330
25	37 ♂	D+P 4	14.3	417	1.07	43.0	103	7.90	8.8	5,200	25	8.3	1.7	350

TABLE 1 RESULTS OF THE EXAMINATIONS ON PATIENTS RECEIVING ANTICONVULSANTS (3)

No.	Age, sex	Anticonvulsants, duration (year)	Hb (g/dl)	RBC (×10⁴)	C. I.	Ht (%)	MCV (cu. μ)	WBC	Platelet (×10⁴)	Iron (μg/dl)	Folate (ng/ml)	Vitamin B_{12} (pg/ml)
26	42 ♀	D+M+P 17	12.5	366	1.2	35	95	8300	14.6	76	3.4	425
27	35 ♀	D+M+P 2	12.5	382	1.15	35	91	5200	15.4	76	2.1	638
28	45 ♀	D+P 2	12.6	384	1.15	36	94	3900	11.2	163	2.3	275
29	37 ♀	D+P 5	14.0	440	1.12	40	90	5200	14.0	135	6.0	425
30	33 ♀	D+M+P 2	13.4	392	1.16	35	88	4900	12.4	131	2.3	300
31	52 ♀	D 7	12.2	348	1.2	34	97	3800	8.9	178	1.6	325
32	45 ♀	D+M+P 3	13.0	403	1.13	38	92	3500	8.2	57	2.3	550
33	43 ♀	D+P 8	15.0	442	1.17	41	92	3800	9.4	66	2.8	325
34	18 ♂	D+M+P 1	15.8	475	1.11	45	94	6600	8.6	78	4.2	588
35	37 ♂	D+P 18	14.0	405	1.17	37	91	5000		183	1.2	925
36	20 ♂	D+P 1	17.0	528	1.09	43	90	4200	5.8	67	5.6	300
37	59 ♂	D+P 13	17.0	530	1.10	47	88	5700	7.8	76	2.1	3650
38	27 ♂	D+M+P 9	14.0	406	1.10	33	94	6300	16.0	115	2.2	413
39	39 ♂	D+M+P 5	17.0	523	1.03	47	90	5800	4.2	84	1.5	233
40	64 ♂	D 6	13.9	444	1.00	33	86	6000	5.4	124	3.2	563
41	32 ♂	D+M+P 5	15.1	442	1.09	40	91	5600	8.6	136	2.2	550
42	30 ♂	D+M+P 4	11.0	294	1.20	30	104	11600		55	2.0	875
43	25 ♂	D+P 2	14.8	476	0.99	42	88	5800	18.6	96	1.5	1425
44	41 ♂	D+P 8	16.0	505	0.99	44	88	4200	8.2	196	1.2	1750
45	29 ♂	D+M+P 2	16.2	467	1.11	46	93.5	6200	15.0	139	2.0	2200
46	41 ♂	D+P 1	16.5	530	1.00	46	87	5800	10.8	127	3.0	1125
47	20 ♂	D+P 2	14.3	420	1.09	41	97.5	5500	11.8	115	1.5	300
48	25 ♂	D+P 4	16.0	452	1.10	44	97.5	5300	7.9	150	2.2	550

D : Diphenylhydantoin, M : Mysoline (primidone), P : Phenobarbital

cells with an ocular micrometer.

(2) *Serum iron levels* : These were determined in thirty-seven patients by the bathophenanthrolin method.

(3) *Mean diameter of buccal cell nuclei* : In fourteen patients, oral mucosa were

taken by scraping the inside of the cheek with a piece of absorbent cotton. The samples were smeared evenly on microscope slides, air dried and stained with May-Grünwald-Giemsa stain. The mean diameter of two hundred nuclei in each cases was determined by measuring both the long and short axes with an ocular micrometer.

(4) *Serum folate levels*: These were assayed in 48 patients. The standard method of WATERS and MOLLIN (12) using *Lactobacillus casei* (*L. casei*) as the test organism was employed. The Difco "Folic acid casei medium" was used for the assay. Normal range in this laboratory is 3.5—20 ng/ml.

(5) *Serum B_{12} levels*: Serum B_{12} levels were determined in 48patients by the method of the Japan Vitamin Society (13). Normal range is 150—900 pg/ml.

(6) *The effect of folic acid supplements*: The effect of folic acid on the peripheral blood, the serum folic acid and B_{12} levels, the mean diameter of the buccal cell nuclei was studied after administration of 5 mg of folic acid to fourteen patients for a month.

2. *In vitro growth inhibition studies by anticonvulsants.*

Solution of DPH was obtained by diluting the "Aleviatin Na" solution (Dainippon Seiyaku Co. Ltd.) with water. The concentrations used were 10 μg/ml and 100 μg/ml. The saturated solution of Primidone (Mysoline; Dainippon Seiyaku Co. Ltd.) in water (about 500 μg/ml) was diluted to 10 μg/ml and 100 μg/ml. The phenobarbital solution was made by diluting a solution of 10% "Phenobal" (Fujinaga Seiyaku Co. Ltd.) with water. Growth inhibition was tested using *L. casei* and *L. leichmannii* by adding the anticonvulsants in various concentrations to the culture medium. The test was performed in duplicate. In one series, sera of known levels of folic acid (15 ng/ml) and B_{12} (250 pg/ml) were assayed by adding the anticonvulsants. In the other series, the standard solutions of folic acid (0.1 ng/ml) and B_{12} (4 μg/ml) were mixed with the solutions of anticonvulsants.

3. *Experimental studies*

(1) *Animals*: Mixed bred male rats weighing 150—240 g were used.

(2) *Radioactive materials*: ^{3}H-folic acid with specific activity of 1.5 μCi/mM was obtained from the Radiochemical Center, Amersham, England. Solution of 50 μg/ml of folic acid labelled with 5 μCi/ml of ^{3}H-folic acid was made by adding appropriate amount of nonradioactive folic acid to the original ^{3}H-folic acid solution. It was stored at −20°C.

(3) *Experimental procedures*: From the day before absorption test, rats were kept fasted. They were anesthetized with ether gas and abdominal incision was made. A polyvinyl catheter was placed in the duodenum via the stomach through a small incision. Then, ligatures were made at the pylorus and at a point midway between the pylorus and cecum, which was about 30 cm distal to the pylorus. Seventeen rats were given ^{3}H-folic acid 40 μg/kg animal through the catheter and then divided into four groups. Group A consisted of five rats and served as control. Group B consisted of five rats and they were administered 5 mg of DPH dissolved in 0.2 ml of the solvent, which consisted of 40 v/w% of propylene glycol and 10.5 v/w% of ethanol. Group C consisted of four rats and they were administered 20 mg of DPH dissolved in 0.8 ml of the solvent. Group

D consisted of three rats and they were given 0.8 ml of the solvent and also served as control. After administering these drugs through the catheter, the tube was washed once with one ml of 0.9% NaCl solution. Then the tube was pulled out and the ligature at the pylorus was tightened. The incision of the stomach and that of the abdomen were closed. The rats were killed two hours later. The small intestine including its contents was removed from the upper point of the ligature at the pylorus to the distal site of the second ligature at the mid-point of the small intestine. The livers and kidneys were also removed.

(4) *Counting technique*: All of the small intestine removed and its content were homogenized in a glass homogenizer with 10—40 ml of 0.1 M phosphate buffer (pH 6.0) containing 150 mg% of ascorbic acid. The livers and kidneys were also homogenized respectively. After autoclaving the homogenates for ten minutes at 121°C, 0.5 ml of the aliquots was dissolved in one ml of Hyamine hydroxide 10-X (1M/500 ml of p-(diisobutylcresoxyethoxyethyl dimethyl benzoyl ammonium hydroxide)). Then, 10 ml of the scintillator solution containing 500 mg of 2-5-diphenyloxazole (PPO) and 25 mg of 1-4-bis 2-(5-phenyloxazolyl) benzene (POPOP) in 100 ml of equivalent toluene ethanol solution (14) was added. The specimens were counted in a Shimazu liquid scintillation counter, LSG-11. Quenching was corrected by the external standard method. Half ml of the standard 3H-folic acid solution (5 μCi/ml) was counted in the same way for the standard. Radioactivity of the administered ^{3}H-folic acid in each rats were caluculated by multiplying the standard radioactivity with administered dosis. The radioactivity recovered from the intestinal wall and its content was subtracted from the administered radioactivity in each rats. The percentage of the remainder to the administered folic acid was expressed as the absorbed percentage of folic acid.

RESULTS

(1) *Hematological examinations*: The results of the hematological studies and serum folate and B_{12} assays performed in forty-eight subjects who were receiving anticonvulsants are presented in Table 1 and Fig. 1.

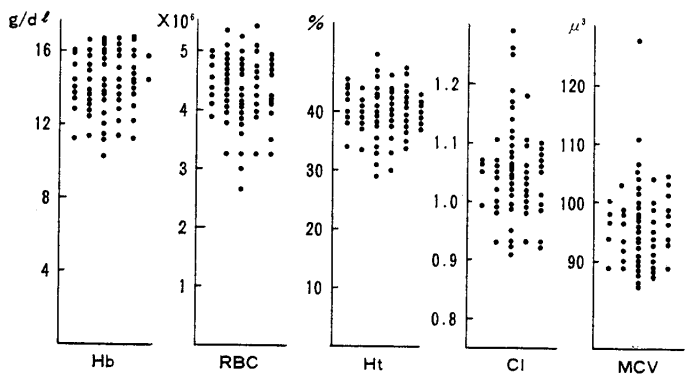

Fig. 1 Blood picture of patients receiving anticonvulsants

Slight degree of hyperchromic and macrocytic anemias were observed in eight of forty eight patients. Mean corpuscular volume (MCV, normal range 83-94 cu. μ) was over 100 cu. μ in twelve of forty-eight (25%) subjects on anticonvulants. In 22 patients (46%), MCV was over 95 cu. μ. MCD was over 8 μ in 9 of 25 patients (36%). Macrocytosis, that is over 5% of red cells are more than 9 μ in its diameter, was detected in 19 of 25 (76%) subjects. All had normal values in reticulocytes, platelets and white blood cells. The increased hypersegmented neutrophils, that is over 20% of neutrophils are more than four lobes, was observed in 6 of 25 patients (24%). The results of the hematological examinations after folic acid therapy were shown in Table 1 and Fig. 6. Decrease in MCV was significant ($t=5.13$, $p<0.01$). Also the percentages of macrocytes decreased ($t=2.60$, $p<0.05$) and hypersegmented neutrophils decreased in their percentages ($t=6.24$, $p<0.001$).

(2) *Serum iron levels*: 5 of 37 patients examined had high levels and low levels were observed in only 2 (Fig. 2).

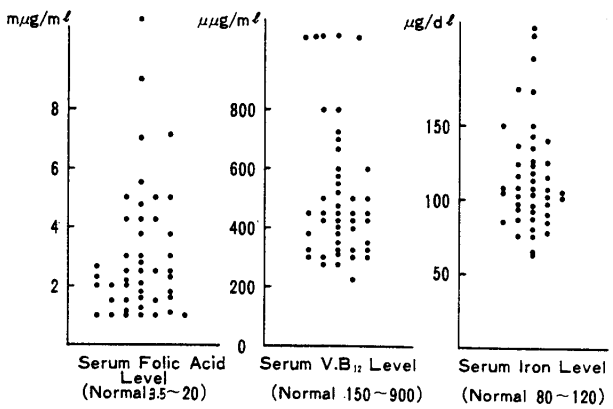

Fig. 2 Serum folic acid, vitamin B_{12} and iron levels of patients receiving anticonvulsants

(3) *Mean diameter of the buccal cell nuclei*: The results of the measurement of diameters of the nuclei of the buccal cells are presented in Fig. 3. The mean diameter of the long axes was 11.6 μ and that of the short axes was 9.2 μ in 14 patients examined. The effect of folic acid on the mean diameter of the oral cell neclei was also examined (Fig. 3). Decrease in the size of the long axes by the administration of folic acid was stastically significant ($t=3.43$, $p<0.01$). The short axes decreased in its size significantly ($t=2.688$, $p<0.05$).

(4) *Serum folic acid levels*: Low serum folic acid levels (under 3.4 ng/

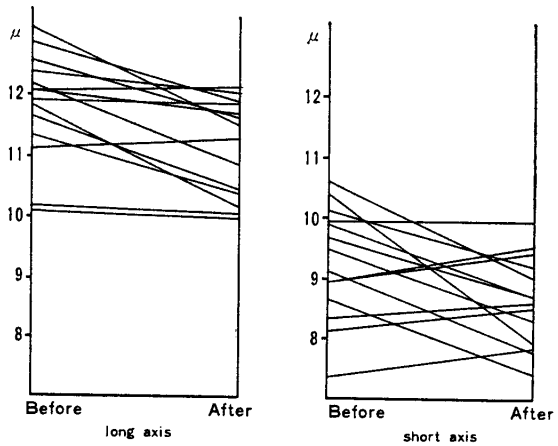

Fig. 3 Mean diameter of nucleus of oral epithelial cells in patients receiving anticonvulsants before and after administering 5 mg of folic acid for a month

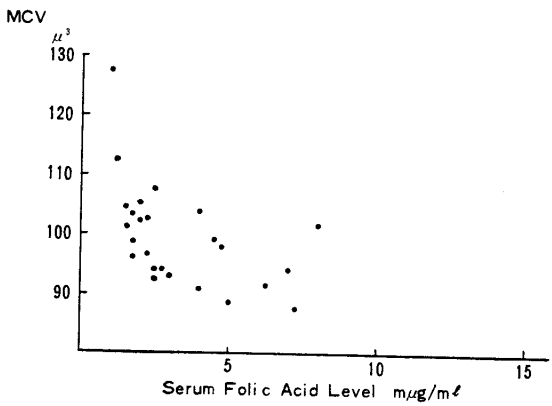

Fig. 4 Relation between serum folic acid and MCV

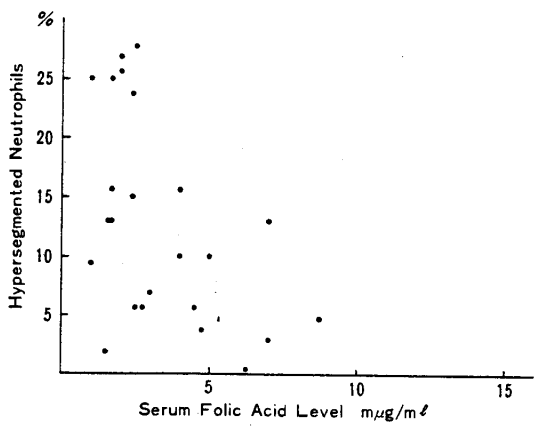

Fig. 5 Relation between serum folic acid and hypersegmented neutrophils

ml) were observed in 36 of 48 (75%) subjects receiving anticonvulsants (Fig. 2). In Fig. 4, correlation between the serum folic acid levels and MCV was presented. In Fig. 5, correlation between the serum folic acid levels and the percentages of hypersegmented neutrophils is shown.

(5) *Serum B_{12} levels*: No patient was found to have low serum B_{12}

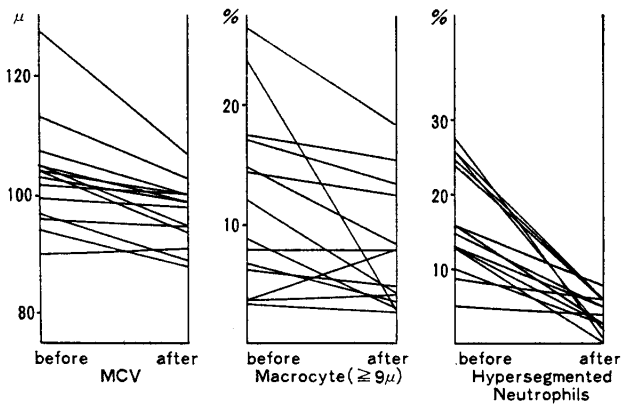

Fig. 6 Influence of folic acid administration on blood cells (Folic acid 5mg/day for a month)

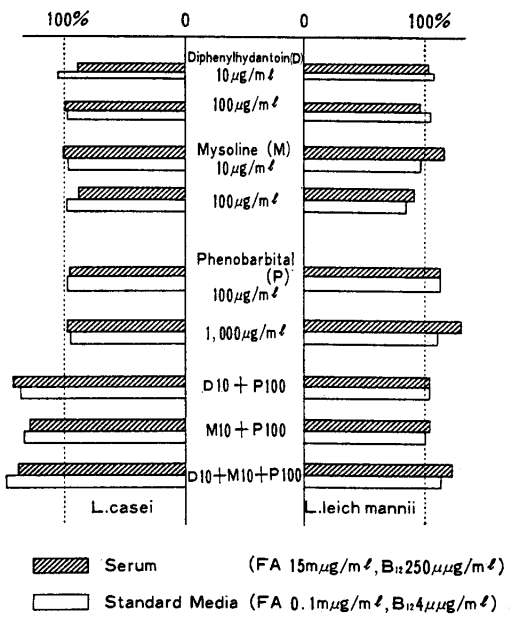

Fig. 7 Influence of anticonvulsants on microbioassay systems

(Fig. 2). Six patients whose levels were high were administered drugs containing B_{12}.

(6) *In vitro studies*: No suppressive effect was observed for the growth of *L. casei* and *L. leichmannii in vitro* by adding various anticonvulsants in the medium of the bioassay systems. (Fig. 7).

(7) *Results of experimental study using rats*: As was exhibited in Table 2, the mean percentage of the absorbed amount of folic acid in Group A was $56.1 \pm 6.8\%$. Only in the group C, significant decrease of the absorption was observed ($t=2.98$, $p<0.05$). No significant difference was demonstrated in group B and D in the present study. Hepatic and renal uptake of the absorbed folic acid were not different from each other among the four groups.

TABLE 2 FOLIC ACID ABSORPTION IN RATS

	Amounts absorbed (%)	Hepatic uptake (%)	Renal uptake (%)
Group A Folic acid 40 μg/kg alone	54.5	9.6	4.8
	43.6	15.3	5.2
	59.9	10.1	5.0
	62.6	21.7	6.1
	59.7	7.3	9.1
Mean±SD	56.1±6.8	12.8±5.2	6.0±1.6
Group B Folic acid 40 μg/kg and DPH 5 mg	64.0	7.3	7.3
	57.4	13.5	3.0
	68.8	8.2	4.3
	46.8	21.1	8.4
	65.7	21.8	11.9
Mean±SD	60.5±7.8	14.4±5.5	7.0±3.3
Group C Folic acid 40 μg/kg and DPH 20 mg	48.8	14.7	7.4
	40.9	12.1	7.9
	38.3	8.9	4.0
	46.6	9.2	7.1
Mean±SD	43.6±4.3	11.2±2.4	6.6±1.5
Group D Folic acid 40 μg/kg and the solvent	57.0	12.3	7.9
	52.0	11.7	8.2
	59.9	12.2	7.6
Mean±SD	56.6±3.3	12.1±0.3	7.9±0.2

Significant difference exists only in the amounts absorbed in Group A : Group C ($t=2.98$, $p<0.05$).

DISCUSSION

It is now well established that megaloblastic anemia during anticonvulsant drug therapy is due to folic acid deficiency (1, 2, 3, 4, 5). Notwithstanding the rare occurrence of megaloblastic anemia, subclinical folic acid deficiency has been frequently observed in nonanemic patients receiving anticonvulsants (6, 7, 8, 9, 10). High incidence of macrocytosis in nonanemic epileptic patients was first reported by HAWKINS and MEYNELL in 1958 (15). They confirmed that macrocytosis was a sign of folic acid deficiency by demonstrating a decrease of macrocytosis after folic acid administration. Though the incidence of macrocytosis was low (8–13 %) in the investigations reported by MULPAS *et al.* (7), REYNOLDS *et al.* (8) and CHILD *et al.* (10), correction of macrocytosis was made by folic acid administration (10). A correlation existed between serum folic acid levels and macrocytosis. Also a good correlation was demonstrated between subnormal serum folic acid levels and increase of hypersegmented neutrophils. Macrocytosis was also present as judged by diameter of the buccal cells before and after the administration of folic acid. Changes in epithelial cells covering a variety of body surfaces were observed in megaloblastic anemias (16, 17) and in nonanemic B_{12} deficient patients (20). Effect of the administration of folic acid on the macrocytosis (red blood cells and buccal cell nuclei) and the increased percentage of hypersegmented neutrophils were prominent in reversing these signs to normal. In the present study, hematologic study and direct assay of serum folic acid levels were performed. Anemia observed in 8 of 48 patients were very mild in its degree. Macrocytosis was observed in 46 % and subnormal serum folic acid levels in 75 % subjects investigated in the present study. The high incidence of subnormal serum folic acid levels found in this investigation is more in agreement with that found by REYNOLDS *et al.* (8) than with KLIPSTEIN (6) (53%), MULPAS *et al.* (7) (37 %), CHILD *et al.* (10)(27 %) and DAHLKE *et al.* (9) (54 %). Therefore the present study revealed that subclinical folic acid deficiency is common among the patients receiving anticonvulsants.

The mechanism of anticonvulsant-induced folic acid deficiency remains unsettled at the present time. There are three main hypothese about the mechanism. Attentions have been called to the structural resemblance of the drugs to that of folic acid (19, 20). The inhibitory effect of anticonvulsants to the growth of folic acid requiring microorganisms (*Trypanosoma crithidia fasciculata* (21) and *E. coli* mutant (22)) was presented as the evidence of this hypothesis. But HAMFELT *et al.* (23) could not

observe any inhibitory effect of the drugs on dihydrofolic acid reductase, methyltetrahydrofolate dehydrogenase of formyltetrahydrofolate synthetase. No inhibitory effect of the anticonvulsants to the growth of *L. casei in vitro* was observed by many workers (6, 9, 19, 24). The present study also revealed that anticonvulsants did not inhibit the growth of *L. casei* and *L. leichmannii* (Fig. 7). Therefore the competitive inhibition theory is not supported (25).

The second hypothesis is presented by HOFFBRAND and NECHELES (26) and ROSENBERG *et al.* (27). Most dietary folate are polyglutamates (28, 29). Gamma-glutamyl-carboxypeptidase (conjugase, which splits glutamic acid chain from folate polyglutamates at the small intestinal mucosa.) is required for the absorption of polyglutamates (30, 31, 32). Inhibitory effect of DPH on this enzyme was suggested by HOFFBRAND *et al.* and ROSENBERG *et al.* Although this hypothesis is attractive, recent studies (32, 33, 34) could not confirm this.

The third hypothesis is that anticonvulsants interfere with the absorption of free folic acid from the small intestine. MEYNELL (35) observed impaired absorption of 5 mg of folic acid when the anticonvulsants were given immediately before folic acid but not when they were given two hours after folic acid. DAHLKE *et al.* (9) demonstrated the malabsorption of folic acid by performing folic acid tolerance test at 0, 4, 12, 16, and 20 hours after DPH administration in man and showing a progressive rise in serum folic acid levels as DPH was withheld for longer periods prior to the administration of 600 μg of folic acid.

HEPNER (36) studied absorption of folic acid from the small intestine of rats and suggested that DPH inhibited absorption of folic acid. GERSON *et al.* (37) also reported the inhibition of folic acid absorption in man. Although the investigators who demonstrated conjugase inhibition by DPH found normal absorption of free folic acid (26, 27), the hypothesis that DPH interfere with the absorption of folic acid is most substantiated.

Absorption of folic acid occurs in the jejunum by an active process in man (38). In rats, folic acid is also absorbed in the jejunum (36, 39, 40, 41). In the present study, absorption of folate in rats were examined only at the jejunum. Absorbed amount of folic acid was revealed indirectly in the present study by subtracting the recovered radioactivity of the small intestine and its content from the administered one in each rats. Percentages of the subtracted radioactivity to the administered one are presented as the percentages of absorbed folate. Though the percentages would not be completely accordant with that of absorbed folic acid, they may be comparable among the four groups in this study as the technique used was

the same. Significant difference in the percentages between the group C (administered with 20 mg of DPH) and the control group (group A) is due to difference in the absorption of folic acid, because DPH did not interfere with folic acid utility in the present *in vitro* study using folic acid requiring microorganism (*L. casei*). In the present experiment using rats, DPH interfered with the absorption of folic acid only after the administration of large dose (20 mg), not after small dose (5 mg). This observation does not conflict with that of HEPNER (36) who demonstrated slight inhibition of folic acid absorption by 5 mg of DPH with 1.5 μg of ^{3}H-labelled folic acid. The dose of folic acid administered in the present study was 40 μg/kg, that was about 6 μg to 9.6 μg as the weights of rats were 150–240 g. HEPNER and HERBERT (42) indicated that DPH could inhibit the uptake of folic acid by mucosal cells only when it was given in a w/w ratio of 10,000 DPH : 1 folic acid. In this study, it was demonstrated that 5 mg of DPH did not interfere with the absorption of folic acid (w/w ratio of 10,000 DPH : 12–19.2 folic acid) but that 20 mg of DPH impaired folic acid absorption (w/w ratio of 10,000 DPH : 3–4.8 folic acid). The results are in accordance with that of HEPNER and HERBERT. Recently BENN *et al.* (43) investigated the effect of DPH and sodium bicarbonate on jejunal pH and on the absorption of folic acid in man.

Suggestion was made that changes in the bulk phase of intraluminal pH might have a profound effect on the absorption of folic acid in epileptics and in normal subjects. Although the solvent of DPH used in the present study had an alkaline pH, it did not inhibit the absorption of folic acid. DPH was essential for the malabsorption of folic acid. Therefore it is strongly suggested that interference of DPH on the absorption of folic acid depends on quantity balance of the two substances in the jejunum. This suggestion can explain well the fact that folic acid deficiency is so rare among patients taking enough foods and rather frequent among mentally defective patients taking inadequate diets.

As was suggested in the previous study of the author (4), the most important factor for the manifestation of megaloblastic anemia due to anticonvulsants is the amount of folic acid taken from diets.

CONCLUSION

A high incidence of subnormal serum folic acid levels was observed in 48 patients receiving anticonvulsants (75%). In peripheral blood, macrocytosis was detected in 46% and an increase of hypersegmented neutrophils was also seen in 24% of the patients. Correlation existed

between these signs and low serum folate levels. The growth response of *Lactobacillus casei* and *L. leichmannii* was not suppressed by the addition of various anticonvulsants to the medium of the bioassay systems. Administration of 5 mg of folic acid for a month corrected macrocytosis and an increase of hypersegmented neutrophils significantly. Folic acid supply also decreased mean diameters of the nuclei of oral epithelial cells significantly. It is concluded that subclinical folic acid deficiency is common among the patients receiving anticonvulsants.

Absorption of ^{3}H-folic acid from the small intestine of rats was inhibited by large dose of diphenylhydantoin (20 mg) not by 5 mg. This fact suggests that in patients on diphenylhydantoin, the quantity balance of folic acid and diphenylhydantoin in the intestine regulates the absorption of folic acid.

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