Elevation of Total Homocysteine in the Serum of Patients with Cobalamin or Folate Deficiency Detected by Capillary Gas Chromatography–Mass Spectrometry

Sally P. Stabler,* Paul D. Marcell,* Elaine R. Podell,* Robert H. Allen,* David G. Savage,‡ and John Lindenbaum‡
**Divisions of Hematology and Oncology, Department of Medicine, and the Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262; and ‡Departments of Medicine, Harlem Hospital Center, Columbia-Presbyterian Medical Center and College of Physicians & Surgeons, Columbia University, New York, New York 10032*

Abstract

To determine if levels of serum total homocysteine are elevated in patients with either cobalamin or folate deficiency, we utilized a new capillary gas chromatographic–mass spectrometric technique to measure total homocysteine in the serum of 78 patients with clinically confirmed cobalamin deficiency and 19 patients with clinically confirmed folate deficiency. Values ranged from 11 to 476 μmol/liter in the cobalamin-deficient patients and 77 of the 78 patients had values above the normal range of 7–22 μmol/liter as determined for 50 normal blood donors. In the cobalamin-deficient patients, serum total homocysteine was positively correlated with serum folate, mean corpuscular volume, serum lactate dehydrogenase, serum methylmalonic acid, and the degree of neurologic involvement, and inversely correlated with platelets and hematocrit. In the folate-deficient patients, values for serum total homocysteine ranged from 17 to 185 μmol/liter and 18 of the 19 patients had values above the normal range. Some patients with pernicious anemia who were intermittently treated with cyanocobalamin were found to have elevated serum levels of total homocysteine while they were free of hematologic and neurologic abnormalities. The measurement of serum total homocysteine will help define the incidence of cobalamin deficiency and folate deficiency in various patient populations.

Introduction

At present the diagnosis of cobalamin (Cbl,[1] vitamin B12) deficiency and folate deficiency are almost entirely dependent on the demonstration of low serum levels of the vitamins in patients with supportive clinical and laboratory findings. The limitations in using only the serum Cbl level to detect Cbl deficiency have been widely recognized (1–4). For example, the significance of the high incidence of low serum Cbl values in elderly subjects without hematologic abnormalities (5, 6), and in patients who have various neuropsychiatric abnormalities (7, 8), is not known. Other tests (9), such as the deoxyuridine suppression test, have not been widely used to assess patients for Cbl deficiency.

The diagnosis of folate deficiency can also be problematic for several reasons. For instance, after acute dietary deprivation, serum folate levels may be decreased, although tissue folate levels are adequate (10). In the setting of chronic alcoholism, the laboratory features of megaloblastic anemia due to folate deficiency may be confused by concurrent illness. Serum and red blood cell folate may be normal in patients with alcoholism and megaloblastic anemia (11). Finally, because many of the clinical and laboratory features of Cbl and folate deficiency are similar, it is often difficult to distinguish between them (12), and the administration of folic acid alone to a patient with Cbl deficiency is dangerous (13). Therefore, as in Cbl deficiency syndromes, additional diagnostic tests for folate deficiency would be useful.

In both Cbl and folate deficiency, it is likely that there is reduced activity of the Cbl-dependent enzyme, methionine synthetase (tetrahydropteroylglutamate methyltransferase), which simultaneously methylates homocysteine to methionine while demethylating N^5-methyltetrahydrofolate to tetrahydrofolate, as shown in Fig. 1. The reduced folates formed from this reaction are necessary for thymidine synthesis and ultimately DNA synthesis, and the methionine formed participates in the methylation of many compounds (14). If methionine synthetase activity is decreased by a deficiency of Cbl or folate, serum homocysteine levels might increase and methionine levels might decrease, although regulation of other enzymes in the pathway shown in Fig. 1 might maintain levels of one or both of these amino acids within normal limits. A few (15–20) but not all (21–24) children with severe Cbl deficiency have been reported to have increased levels of homocystine in their urine and plasma, along with decreased levels of plasma methionine. An early study (25) reported that plasma methionine levels were low in adults with Cbl deficiency. In addition, excretion of large amounts of homocystine in the urine has been reported in patients with various inherited defects affecting the activity of methionine synthetase such as the inability to form methylcobalamin (Cbl C and Cbl D mutants) (26, 27), and an inability to form N^5-methyltetrahydrofolate due to 5,10-methylenetetrahydrofolate reductase deficiency (28, 29). However, homocystinuria was not present in patients with transcobalamin II deficiency (30), a patient with a lysosomal block in Cbl transport (31), or in a patient with congenital folate malabsorption (32), all of whom had hematologic abnormalities that would be associated with a decrease in methionine synthetase activity.

We have developed a new capillary gas chromatographic–

Address correspondence to Dr. Stabler, Div. of Hematology, Campus Box B170, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262.

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1. *Abbreviations used in this paper:* Cbl, cobalamin; CN-Cbl, cyano-Cbl; Hct, hematocrit; LDH, lactate dehydrogenase; MCV, mean corpuscular volume.

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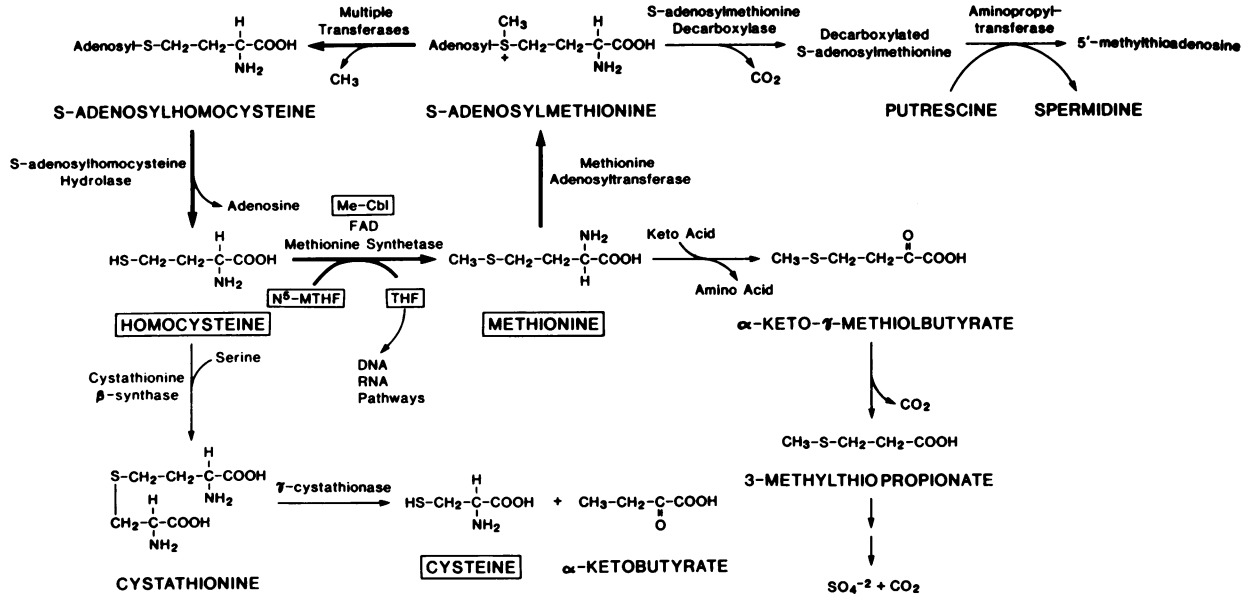

Figure 1. Various pathways involved in the metabolism of methionine including the methylcobalamin-dependent methylation of homocysteine to form methionine by the enzyme, methionine synthetase.

mass spectrometric assay for serum methylmalonic acid and have shown that 69 out of 73 patients with clinically confirmed Cbl deficiency have elevated values (33). We have recently developed similar assays (24) for the quantitation of total homocysteine, methionine, and total cysteine in serum and now report on the measurement of these amino acids in a large number of patients with clinically confirmed deficiencies of Cbl and folate.

Methods

Assay of total homocysteine,[2] methionine, and total cysteine.[2] Serum total homocysteine, methionine, and total cysteine were assayed using capillary gas chromatography–mass spectrometry as previously described in detail (34). Briefly, 50 μl of H_2O containing 5 nmol of D,L-[3,3,3′,3′4,4,4′4′-$^2H_8$]homocystine (98.4%), 15 nmol of L-[methyl-$^2H_3$]methionine (98%), and 25 nmol of D,L-[3,3,3′,3′-$^2H_4$]cystine (98%), is added to 100 μl of human serum. The sample is then heated with 2-mercaptoethanol in order to reduce and release endogenous homocysteine and cysteine from proteins and other disulfides and to equilibrate them with their stable isotope internal standards, which are also reduced during this procedure. Protein is then precipitated with sulfosalicylic acid and the supernatant is partially purified by sequential cation exchange and anion exchange chromatography. The *t*-butyldimethylsilyl derivatives of the amino acids are formed with *N*-methyl-(*t*-butyldimethylsilyl)trifluoroacetamide, followed by their extraction into hexane and volume reduction using a stream of nitrogen. The samples are analyzed on a Durabond DB-1 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific, Inc. (Rancho Cordova, CA) and a Hewlett-Packard Co. (Palo Alto, CA) 5992B gas chromatograph–mass spectrometer equipped with a falling needle injector. Quantitation is based on the ratio of the areas of the base peak ion 420.2 for homocysteine, 320.2 for methionine, and 406.2 for cysteine, each of which elutes at a different time, to the areas of the base peak ions of 424.2, 323.2, and 408.2 for the derivatives of their respective stable isotope internal standards. Total homocysteine, methionine, and total cysteine are very stable in collected serum since no change or trend in values for these amino acids was noted in a sample of pooled normal human serum that was frozen, thawed, and assayed > 30 times over a 12-mo period.

Values of serum total homocysteine obtained with blood samples that were drawn and immediately centrifuged at 4°C were the same (< 10% difference) as those obtained with portions of the same blood samples that were incubated at room temperature for 1 h before centrifugation, but increased by ~ 35 and 75% when the incubation was prolonged for 4 and 24 h, respectively, before centrifugation. Values for serum methionine were unchanged at 1 h and increased by 10 and 25% at 4 and 24 h, respectively. Values for serum total cysteine were unchanged over the 24-h incubation period. Values for urine total homocysteine, total cysteine, and total methionine were unchanged when urine samples were incubated at room temperature for 0–24 h. Serum samples and the internal standards of homocysteine, methionine, and cysteine were stable for > 1 yr, based on their gas chromatographic–mass spectrometric behavior, when stored at −20°C in between numerous freezings and thawings over this time period.

Subjects and patients. Serum samples from 50 normal blood donors, 25 males and 25 females, ranging in age from 18 to 65 yr were obtained as described previously (35). Patient samples were selected by Dr. Lindenbaum from an extensive serum collection that has been assembled over the past 15 yr. Both the normal and the patient samples were allowed to clot for ~ 1–4 h at room temperature before the serum was removed and stored at −20°C. Thus, postcollection increases for total homocysteine and methionine should have been modest and similar in both the normal and patient groups (see above). The diagnosis of Cbl deficiency was based on low serum Cbl levels, megaloblastic bone marrow morphology, appropriate hematologic or neurologic abnormalities, and a significant response to treatment with parenteral Cbl. The diagnosis of pernicious anemia was based on an abnormal

2. The term "total homocysteine" as applied to biologic samples such as serum and urine refers to the sum of homocysteine and the homocysteine that is linked via disulfide bond formation in a variety of compounds that include homocystine (homocysteine–homocysteine disulfide), homocysteine–cysteine mixed disulfide, proteins via their cysteine moieties, and peptides such as glutathione via their cysteine moieties. The term "total cysteine" is used in the same way and refers to the sum of cysteine and the cysteine linked via disulfide bond formation in compounds such as cystine (cysteine–cysteine disulfide), homocysteine–cysteine mixed disulfide, and proteins and peptides via their cysteine moieties.

Schilling test that corrected with exogenous intrinsic factor and/or the presence of anti-intrinsic factor-blocking antibodies in the serum. The diagnosis of folate deficiency was based on low serum folate values, normal or elevated serum Cbl values, megaloblastic bone marrow morphology, appropriate hematologic abnormalities, and responses to folic acid therapy. In 17 of the patients there was a history of alcoholism and poor diet; one had history of poor diet alone, and one had tropical sprue. The samples in the Cbl-deficient infrequently treated group were from patients with pernicious anemia who were previously diagnosed as Cbl deficient as described above, but who received only intermittent treatment with parenteral Cbl at intervals of 6–9 mo due to poor compliance or as part of studies of Cbl requirements to be reported elsewhere. They had low, borderline, or normal levels for serum Cbl, lacked hematologic and neurologic abnormalities, and were asymptomatic at the time the samples were collected. Serum Cbl levels were assayed using the *Lactobacillus leichmannii* method or a number of radiodilution assays utilizing purified intrinsic factor or gastric juice with > 95% of Cbl binding activity due to intrinsic factor. These assays were performed in Dr. Lindenbaum's laboratory where it has been found that very similar patient values and normal ranges are obtained with all of the various Cbl assays. Serum folate was assayed with the *Lactobacillus casei* method or by milk binder radiodilution assay. Most of the patients' samples were coded in a manner such that the categories to which they belonged and the number of patients in each category were not known by the personnel involved in the performance of the total homocysteine, methionine, and total cysteine assays, until after the results were reported to Dr. Lindenbaum. A large number of Dr. Lindenbaum's samples from patients without Cbl or folate deficiency have been assayed and found to have normal values for these amino acids, thus ruling out the possibility that elevated values might arise due to storage in the freezers used in Dr. Lindenbaum's laboratory.

Statistical methods. A number of factors were examined individually for possible relationships with serum total homocysteine, methionine, and total cysteine. For factors that were discreet, such as sex, race, and diagnosis, the Wilcoxon two-sample test was used to determine the significance of the relation. For assessing possible relationships with neurologic severity, groups 0, 1, and 2 (as defined in Table I) were combined and compared with combined groups 3 and 4. Factors that were continuous, such as age or mean corpuscular volume (MCV), were examined using Spearman correlation coefficients.

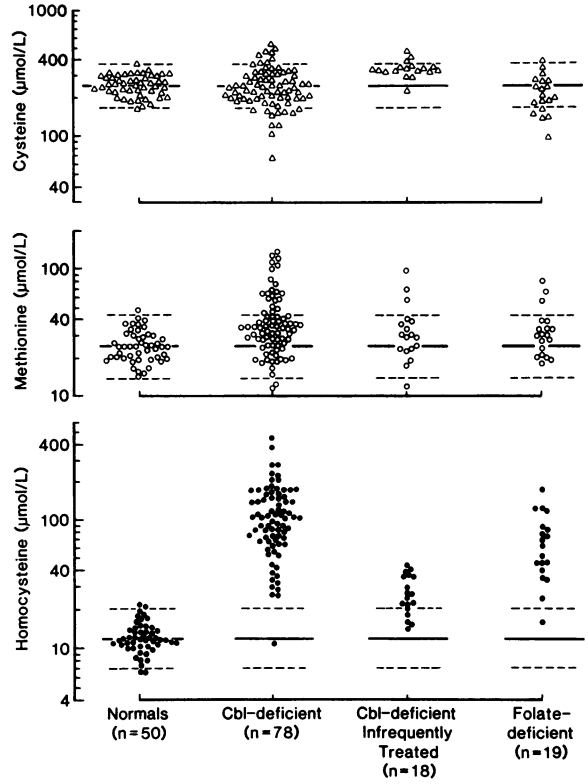

Figure 2. Levels of serum total homocysteine (*bottom*), serum methionine (*middle*), and serum total cysteine (*top*) in patients with clinically confirmed Cbl deficiency, folate deficiency, and Cbl-deficient infrequently treated patients who had no hematologic or neurologic abnormalities, using capillary gas chromatography–mass spectrometry. The normal range for total homocysteine is (7–22 μmol/liter), for methionine (14–44 μmol/liter), and for cysteine (173–378 μmol/liter). These ranges were calculated as the mean±2 SD after log transformation to correct for skewness towards higher values.

Results

The values obtained for serum total homocysteine, methionine, and serum total cysteine for the normal subjects and patients in the various categories are shown in Fig. 2. In the Cbl-deficient group, 77 of the 78 patients had values for serum total homocysteine above the normal range of 7–22 μmol/liter. The highest value was 476 μmol/liter and the median value was 113 μmol/liter. In the folate-deficient group, 18 of the 19 patients had serum total homocysteine levels above the normal range. The highest value in this group was 185 μmol/liter and the median value was 67 μmol/liter. In the Cbl-deficient infrequently treated group, 13 of the 18 patients had elevated values that ranged as high as 47 μmol/liter at a time when they did not have hematologic or neurologic abnormalities. Of the thirteen patients with elevated total homocysteine values, serum *L. leichmannii* Cbl concentrations were low (88–180 pg/ml) in nine, two others were assayed by radiodilution and were both low at 115 pg/ml, and two were normal by the *L. leichmannii* method at 205 and 275 pg/ml, respectively. Of the five patients with normal total homocysteine values, two had low Cbl values by radioassay and one by the *L. leichmannii* method.

To determine if the elevations of serum total homocysteine were due to Cbl or folate deficiency and not due to illness in general, we studied 25 consecutive patients (13 hospitalized, 12 outpatients; age, 25–86 yr) who had sera submitted for serum Cbl and serum folate assays and in whom the Cbl values were > 500 pg/ml (normal, 200–1,000 pg/ml) and the folate values were > 5 ng/ml (normal, 3–20 ng/ml). Of the 25 patients, 21 had values within the normal range of 7–22 μmol/liter (mean for the 21 patients, 13.7 μmol/liter) and 1 had a very slightly elevated value of 22.5 μmol/liter. Significantly elevated values of 32.6 and 27.4 μmol/liter were observed in two patients with chronic renal failure who had serum creatinine values of 5.6 and 13.3 mg/dl, respectively. Various forms of homocysteine have been previously reported to be elevated in the plasma of patients with renal failure (36, 37). We are currently studying the incidence of elevated total homocysteine values in chronic renal failure. Preliminary results indicate that total homocysteine is elevated in many but not all such patients. A significantly elevated value of 34.4 μmol/liter was also observed in one patient with Cbl deficiency since 1984, who had been intermittently treated with Cbl. The patient had not been treated for several months but had a serum Cbl of > 2,000 pg/ml because a Cbl injection was given 10–15 min before the blood was drawn for the Cbl assay. Taken

together, these results indicate that serum total homocysteine is not elevated in most ill patients.

In the Cbl-deficient group values for serum methionine were below the normal range of 14–44 μmol/liter in only 2 of the 78 patients and above the normal range in 20 patients. Of the 19 folate-deficient patients, 3 had values above the normal range as did 3 of the 18 Cbl-deficient infrequently treated patients. None of the folate-deficient patients and only one of the Cbl-deficient infrequently treated patients had a value for serum methionine below the normal range.

Total serum cysteine was below the normal range in 11 of 78 Cbl-deficient patients, 4 of 19 folate-deficient patients, and none of the Cbl-deficient infrequently treated patients. It was above the normal range in 7 of 78 Cbl-deficient patients, 1 of the 19 folate-deficient patients, and 3 of the 18 Cbl-deficient infrequently treated patients.

The clinical data for the 78 Cbl-deficient patients in addition to their serum total homocysteine, methionine, total cysteine, and methylmalonic acid values are shown in Table I. They are arranged in descending order of their serum total homocysteine values. There was a significant positive correlation between serum total homocysteine and serum folate ($r = 0.27$, $P < 0.05$), MCV ($r = 0.23$, $P < 0.05$), serum lactate dehydrogenase (LDH) ($r = 0.34$, $P < 0.01$), and serum methylmalonic acid ($r = 0.74$, $P < 0.0001$). There was a significant negative correlation between serum total homocysteine and platelets ($r = -0.26$, $P < 0.05$), and hematocrit (Hct) ($r = -0.26$, $P < 0.05$). Patients with more severe neurologic abnormalities (groups 3 and 4) had higher serum total homocysteine levels (166±98 mean±SD, median 145 μmol/liter) than those with no or less severe abnormalities (groups 0–2) (105±65 mean±SEM, median 89 μmol/liter) ($P < 0.01$). Serum total homocysteine was not significantly correlated with serum Cbl ($r = -0.20$, $P < 0.08$). There was no significant correlation between serum total homocysteine and any of the following: serum methionine, serum total cysteine, sex, race, etiology, presence of anti-intrinsic factor antibody, and presence of glossitis.

Serum methionine was not significantly correlated with any of the parameters mentioned above and significant differences were not observed between any of the various subgroups.

The hematologic data presented in Table I on the 77 Cbl-deficient patients with elevated levels of total homocysteine, demonstrates wide variations in their degree of anemia, macrocytosis, and other abnormalities. For instance, only 32 (42%) had a severe anemia (Hct < 25%), while 27 (35%) had only a moderate degree of anemia (Hct, 25–34% for females, 25–39% for males), and 18 (23%) were not anemic at all. Only 45 (58%) of the patients had a marked elevation in MCV (> 110 fl), while 23 (30%) had only a moderate elevation of MCV (101–110 fl), and 9 (12%) had a normal MCV. There was a wide range of serum Cbl levels in these patients also; only 47 (61%) had markedly decreased levels to < 100 pg/ml, while the other 30 (39%) had levels between 100 and 200 pg/ml.

The clinical data on the 19 patients with folate deficiency along with their serum total homocysteine, methionine, total cysteine, and methylmalonic acid values are also shown in Table I. Because of the small number of patients tested, correlations between their clinical and laboratory abnormalities were not evaluated.

We have previously reported that serum methylmalonic acid is increased in 95% of patients with Cbl deficiency (33). As shown in Table I, 74 of the 78 Cbl-deficient patients had methylmalonic acid levels above the normal range of 19–76 ng/ml (median, 1,240 ng/ml, range, 78 to 2,300 ng/ml). The one Cbl-deficient patient (No. 78) with a serum total homocysteine level within the normal range also had a normal serum methylmalonic acid. His diagnosis was tropical sprue. The other three patients with normal serum methylmalonic acid levels (Nos. 49, 61, and 73) all had elevated serum total homocysteine levels ranging from 34 to 92 μmol/liter. Their diagnoses consisted of tropical sprue, postgastrectomy syndrome, and pernicious anemia.

As we have reported previously (33), most folate-deficient patients have normal levels of serum methylmalonic acid, although some have mild elevations. In the current study, as shown in Table I, 13 of the 19 folate-deficient patients had normal values for serum methylmalonic acid, and the other 6 had mild elevations (median for the 6 patients, 132 ng/ml, range, 79 to 195 ng/ml).

Fig. 3 shows the serum and urine homocysteine levels both before and after treatment in a patient with classic pernicious anemia and in another patient with alcoholism and nutritional folate deficiency. The Cbl-deficient patient had markedly elevated levels of total homocysteine both in serum and urine, which fell into the normal range within 3 d of treatment with parenteral cyano-Cbl (CN-Cbl). This same patient has been previously shown (33) to have a similar rapid fall in serum and urine methylmalonic acid in response to this course of CN-Cbl treatment. The patient with folate deficiency had an elevated serum total homocysteine which over several days decreased to the upper border of the normal range after several doses of oral folic acid. The urine total homocysteine which was at the upper border of the normal range also fell markedly after treatment. This data suggests that serum and urine homocysteine levels correlate with each other in patients with Cbl or folate deficiency and could be used to monitor the response of treatment, although more patients would need to be evaluated before this could be concluded with certainty. Initial serum and urine methionine levels were normal in both patients and did not change significantly after CN-Cbl or folate treatment (data not shown).

Discussion

Our studies demonstrate that serum total homocysteine levels are likely to be clinically useful, since they were above the normal range in 77 of 78 Cbl-deficient patients and 18 of 19 folate-deficient patients. The serum total homocysteine appears to be similar in sensitivity to the serum methylmalonic acid in the Cbl-deficient patients, since the latter was elevated in 74 of the same 78 Cbl-deficient patients. Using the serum total homocysteine level in combination with the serum methylmalonic acid level will often be helpful in distinguishing patients with Cbl deficiency from those with folate deficiency, since most patients with folate deficiency have normal levels of serum methylmalonic acid and the rest have only mild elevations.

Additional clinical studies using measurements of serum total homocysteine and serum methylmalonic acid in addition to measurements of serum Cbl and serum folate should make it possible to further define the clinical spectra of Cbl and folate deficiency, and to define the proper diagnostic approach

Table I. Clinical Data for 78 Patients with Cbl Deficiency and 19 Patients with Folate Deficiency

Patient	Age	Sex*	Race‡	Serum Cbl	Serum folate	Hct	MCV	White blood cells	Platelets	Serum LDH	Neuroabnormalities§	Glossitis‖	Intrinsic factor antibody	Diagnosis¶	Serum methylmalonic acid	Serum methionine	Serum total cysteine	Serum total homocysteine
				pg/ml	*ng/ml*	*%*	*fl*	*No./μl*	*No./μl*	*U/ml*					*ng/ml*	*μmol/liter*	*μmol/liter*	*μmol/liter*
Normals				200–1,000	3–25	M 40–52 F 35–47	80–100	4,000–10,000	150,000–450,000	<225	0	0	0	0	19–76	14–44	174–378	7–22
Cbl deficient																		
1	61	F	B	68	26.0	30	108	3,000	149,000		3		0	PA	22,200	43	216	476
2	64	F	B	122	50.0	18	130	3,200	138,000	>600	3		+	PA	7,410	24	321	399
3	68	M	H	81	3.8	20	135	3,700	140,000	956	0	+	0	TS	1,160	30	190	299
4	71	F	W	145	10.0	40	119	6,800	Adequate	327	3	0	0	PA	1,711	60	489	289
5	76	F	B	26	3.6	30	115	5,200	381,000	215	1	0	+	PA	3,900	18	316	258
6	17	F	H	10	28.5	12	105	1,300	260,000	3,250	0	0	+	PA	2,660	28	178	251
7	61	M	W	10	15.0	25	120	3,000	180,000	800	3	+	+	PA	4,580	18	262	228
8	88	F	B	150	10.0	19	120	1,600	Decrease	>600	4	0	+	PA	7,449	41	318	223
9	52	F	B	10	28.0	18	108	4,800	20,000	3,925	1	0	+	PA	1,320	35	228	205
10	65	M	B	78	13.0	16	122	14,300	71,000	9,500	2	0	+	PA	5,560	41	571	196
11	67	M	B	125	29.0	35	109	6,000	234,000		0	0	+	PA	3,350	116	127	196
12	78	F	B	70	8.6	11	83	12,800	65,000	5,640	3	+	+	PA	2,740	27	190	187
13	83	F	W	50	10.7	39	115	5,500	Adequate	247	4	+	0	PA	2,220	34	291	187
14	56	M	H	64	8.7	18	126	3,700	110,000	3,900	3	0	0	PA	637	35	206	184
15	54	F	B	50	25.0	33	117	4,500	117,000	350	3	+	+	PA	2,790	21	229	183
16	56	F	H	25	15.0	20	106	4,000	100,000	3,205	2	+	+	PA	9,500	24	255	182
17	62	F	B	75	8.8	38	110	4,400	255,000	255	3		+	PA	4,800	67	210	174
18	42	F	B	20	4.8	25	126	5,000	548,000	786	0	0	0	PA	546	102	220	173
19	72	M	B	110	3.4	45	101	8,000	212,000	237	3	+	+	PA	4,800	29	330	170
20	69	M	B	85	48.0	28	111	2,600	170,000	262	3	+	+	PA	5,790	28	151	163
21	65	F	H	50	5.5	45	123	9,100	625,000				+	PA	7,470	41	348	160
22	69	F	H	44	19.0	20	113	6,200	150,000	4,450	2	+	+	PA	1,380	32	300	157
23	53	M	H	115	20.0	21	107	4,600	185,000	1,525	1	+	0	TS	1,990	38	236	153
24	56	M	W	132	4.7	22	98	5,200	115,000	1,550	0	0	+	PA	3,400	27	286	151
25	81	F	H	115	19.3	26	135	5,900	50,000	805		0	0	PA	22,300	33	235	150
26	68	F	W	145	50.0	38	104	5,600	Adequate	205	3	0	+	PA	9,090	18	208	149
27	86	F	W	10	38.0	11	112	3,000	112,000	1,525	0	+	+	PA	8,300	38	280	146
28	54	F	W	62	>29.0	19	118	4,400	Adequate	2,125	3	+	0	PA	1,700	34	200	140
29	65	F	B	140	9.2	15	115	2,800	136,000	3,860	2	0	+	PA	1,530	26	250	130
30	88	F	B	45	28.0	22	137	4,800	98,000	425	3	+	0	PA	1,100	28	126	128
31	54	F	B	45	3.5	21	111	7,200	380,000	2,125	4		+	PA	1,770	24	241	128
32	22	M	H	44	3.3	16	120	4,000	101,000	1,872	1	0	+	PA	1,120	42	264	126
33	65	M	H	36	5.7	17	132	7,900	250,000	4,100	3	0	0	PA	6,200	129	209	120
34	72	F	H	58	>29.0	29	121	3,500	260,000	800	0	+	0	TS	2,950	18	159	118
35	64	F	B	48	28.0	19	118	6,400	99,000	5,000	0	+	+	PA	4,180	120	160	117
36	28	M	B	120	32.0	31	99	10,700	315,000	200	0	0	+	PA	2,735	32	158	117
37	66	F	H	120	2.4	15	119	8,600	96,000	>600			+	PA	518	30	351	114
38	47	F	W	150	11.0	31	109	4,800	160,000	280	3	0	+	PA	2,080	66	295	113
39	61	F	H	150	5.7	18	99	2,200	140,000	2,073	0	+	+	PA	1,040	38	208	113
40	51	M	W	125	14.3	38	106	5,200	Adequate	106	3	+	0	PA	5,640	139	67	111
41	66	F	H	150	50.0	36	117	6,900	307,000	232	0	+	+	PA	751	18	329	109
42	92	F	B	73	4.1	41	114	11,500	Adequate	206			0	PA	1,870	51	509	106
43	84	F	W	68	15.8	26	118	3,300	Adequate	201	4	+	+	PA	386	19	348	99
44	80	F	W	50	6.3	38	115	8,800	Adequate	350	2	0	0	PA	453	76	505	97
45	40	F	B	10	7.4	22	109	5,200	99,000	5,170	3	+	+	PA	280	37	304	97
46	88	F	B	130	7.0	34	115	12,100	530,000	143	2	0	+	PA	1,530	64	360	95
47	47	M	B	135	11.7	14	109	1,400	53,000	680	2	0	+	PA	7,605	11	168	95

48	84	F	B	130		39	99	6,100	Adequate	222	4	0	0	PA	2,620	23	305	93
49	70	F	B	62	3.1	27	121	6,600	350,000	2,600	0	0	+	PA	59	37	230	92
50	85	M	H	43	2.8	15	125	4,200	84,000	862	1	0	0	TS	231	45	218	89
51	17	M	W	78	50.0**	39	109	3,600	Adequate		4		0	IR	21,900	37	207	88
52	41	M	B	110	7.3	29	132	6,600	140,000	>600	0	0	0	PG	137	36	106	86
53	83	M	W	150	4.3	35	113	7,500	990,000	165	0	0	0	PA	1,160	64	366	83
54	72	F	B	95	32.0	27	115	2,700	191,000	230	0	+	+	PA	352	34	279	80
55	73	M	B	60	5.5	44	97	5,100	174,000		3	+	+	PA	335	20	279	78
56	70	M	H	120	3.8	38	103	9,300	208,000	359	1		+	PA	399	28	426	76
57	35	F	B	50	11.0	13	110	5,200	223,000	5,522	0	+	+	PA	300	12	157	75
58	81	F	B	16	12.0	34	82	10,100	245,000		0	0	+	PA	149	14	398	73
59	62	F	H	10	5.2	20	112	4,200	120,000	2,900	0	+	+	PA	132	57	264	73
60	59	M	H	90	1.2	14	100	4,900	45,000	2,000	0	+	0	TS	272	64	272	73
61	65	M	H	96	7.5	16	114	2,500	Adequate	1,550	2	0	0	TS	73	17	215	69
62	70	F	W	130	12.4	14	115	2,200	Adequate	1,875	2	+	+	PA, IR	154	34	206	68
63	20	F	B	10	12.0	35	129	5,300	300,000	200	0	0	+	PA	1,656	48	210	64
64	69	F	W	98	5.2	36	119	6,200	Adequate	157	4	0	+	PA	273	30	393	63
65	50	F	H	67	6.8	31	116	4,600	380,000	336	0	0	0	PA	170	32	261	60
66	70	F	B	130	8.5	22	126	5,000	107,000		1	+	+	PA	270	21	187	58
67	51	F	H	35	8.5	22	118	4,700	465,000	1,350	0	+	0	TS	78	45	198	55
68	72	M	H	45	41.0	42	84	9,300	Adequate	168	4	0	+	PA	169	35	390	47
69	71	F	H	170	7.8	23	114	5,000	275,000	800	2	0	+	PA	333	16	232	45
70	70	F	H	136	3.0	33	118	7,100	Adequate	179	0	+	+	PA	536	106	440	42
71	82	F	B	135	17.0**	35	105	4,000	Adequate	246	0	0	+	PA	526	59	304	38
72	52	F	W	93	9.9	45	109	8,000	276,000		0	0	0	PA	139	24	273	36
73	73	F	B	150	2.9	28	105	7,600	141,000	545	0	+	0	PG	17	28	211	34
74	80	M	H	129	3.8	35	110	6,600	189,000	201	0	0	0	PA	340	84	360	32
75	29	F	H	76	5.7	30	108	3,000	510,000	680	0	0	+	PA	111	33	248	30
76	76	F	H	74	11.5	39	102	4,400	261,000	219	0	0	+	PA	116	23	224	28
77	83	F	H	125	10.5	43	103	5,800	205,000	201		0	+	PA	194	19	276	27
78	50	M	H	87	5.9	38	105	6,400	283,000	422	0	0	0	TS	55	26	163	11
Folate deficient																		
79	42	M		790	0.5	11		9,100	64,000	>600	0	+	0	ALC, P	47	27	210	185
80	41	M		420	0.5	12		2,100	55,000	540	PN	+	0	ALC, P	15	39	193	135
81	59	M		520	0.6	32	114	5,600	153,000	170	0	0	0	ALC, P	79	30	238	133
82	52	M		3,150	0.5	8	100	1,500	7,000	1,800	0	+	0	ALC, P	37	81	252	132
83	78	F		375	2.0	18	110	3,400	105,000	180			0	ALC, P	92	21	273	95
84	64	F		1,070	1.2	25	142	9,600	240,000	304	0		0	ALC, P	53	29	326	93
85	31	M		425	2.3	33	114	5,600	110,000	230	PN	0	0	ALC, P	36	27	91	83
86	47	M		1,160	1.1	15	108	7,900	161,000	250	PN	+	0	ALC, P	47	61	193	80
87	22	M		1,170	0.4	23	113	13,000	250,000	385	0	0	0	ALC, P	32	30	277	74
88	38	M		780	1.5	13		11,400	89,000	575	0	0	0	ALC, P	130	19	395	67
89	77	F		340	0.8	28	104	8,600	306,000	425		0	0	ALC, P	195	39	352	56
90	53	M		750	0.9	18	114	14,400	39,000		PN		0	ALC, P	152	33	281	50
91	36	M		1,340	1.3	8	116	6,800	166,000	1,460	0	0	0	ALC, P	49	32	196	49
92	87	F		420	0.8	15	83	2,900	130,000	2,340	0		0	PD	27	20	164	49
93	37	M		170	0.1	13	123	3,800	78,000	3,465	0	0	0	ALC, P	108	19	137	43
94	53	M		370	1.0	27	112	4,500	190,000			0	0	ALC, P	73	33	179	37
95	47	F		200	0.1	12	122	5,500	320,000		0		0	ALC, P	15	56	248	36
96	46	F		1,110	0.5	33	114	6,600		488	0	0	0	TS	22	18	144	26
97	26	F		1,950	1.9	27	123	19,200	290,000	364	0	0	0	ALC, P	32	23	152	17

* F, Female; M, male. ‡ B, Black; H, Hispanic; W, white. § 4, Severe advanced combined systems disease; 3, combined systems disease or severe cerebral dysfunction responsive to Cbl treatment; 2, no neurological symptoms but impairment of position and/or vibration sense on exam; 1, paresthesias with negative neurological exam; PN, peripheral neuropathy associated with alcoholism. ‖ Sore tongue, glossal atrophy, or both. ¶ IR, ileal resection; MJD, multiple jejunal diverticula; PA, pernicious anemia; PG, postgastrectomy; TS, tropical sprue; ALC, P, alcoholism with poor diet; PD, poor diet. ** Receiving folic acid.

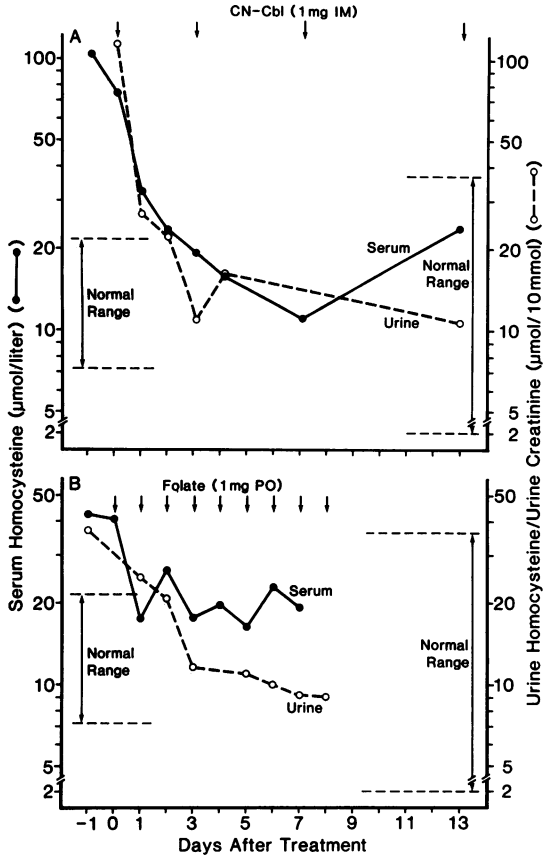

Figure 3. Levels of serum (●) and urine (○) total homocysteine in (*A*) a patient with pernicious anemia before and after treatment with CN-Cbl, and (*B*) a patient with nutritional folate deficiency and alcoholism using capillary gas chromatography–mass spectrometry. Patient A was a 32-yr-old white male with pancytopenia, megaloblastic bone marrow findings, a serum Cbl value of 43 pg/ml, serum antiintrinsic factor blocking antibodies, and an abnormal Schilling test that corrected with exogenous intrinsic factor. Patient B was a 52-yr-old black male with alcoholism and a poor diet who had severe anemia, megaloblastic bone marrow findings, a plasma folate value of 2.0 ng/ml, a red cell folate value of 41 ng/ml, and a serum Cbl value of 690 pg/ml. The Cbl was given by intramuscular (IM) injection and the folate was given by mouth. PO, per os.

to these patients. The importance of these studies is suggested by the fact that many of the Cbl-deficient patients in the current study had normal or only mildly abnormal values for various individual hematologic parameters such as Hct and MCV that are thought to be markedly or at least moderately abnormal in the vast majority of Cbl-deficient patients. Additional studies concerning the specificity of measurements of serum total homocysteine will also be of interest.

The serum total homocysteine and methylmalonic acid levels both have one advantage that is not shared by serum Cbl and serum folate levels in that one can treat a patient suspected of being Cbl-deficient with Cbl and then observe the effect on the serum total homocysteine and methylmalonic acid levels or treat a patient suspected of being folate-deficient with folate and then observe the effect on the serum total homocysteine level. If such treatment results in a decrease in the appropriate metabolite from the elevated to the normal range, this is strong presumptive evidence that the patient was Cbl deficient, or folate deficient, as was the case with the two patients described in detail in this report. This advantage is not shared by the serum Cbl and serum folate levels, since these values are always elevated or at least normal after treatment with Cbl or folate, respectively, regardless of whether a patient is Cbl- or folate-deficient or not.

That levels of serum total homocysteine are almost always elevated in deficiencies of Cbl and folate, whereas levels of serum methionine are almost never decreased in these conditions, indicates that the pathways shown in Fig. 1 are regulated to a considerable degree. The maintenance of methionine levels within a relatively narrow range appears reasonable, since methionine is an essential amino acid that plays an important role in protein synthesis, in various methylations, and in a large number of other metabolic reactions. The enzymatic reaction or reactions that represent the site or sites of regulation in Cbl deficiency and in folate deficiency are not known. Cystathione β-synthase (see Fig. 1) could play a role, since regulation of this enzyme is important in the maintenance of methionine homeostasis during changes in dietary methionine (38–40).

The fact that methionine levels are maintained in Cbl and folate deficiency at the expense of elevated levels of total homocysteine might be harmful, since elevations in homocysteine due to homozygosity or heterozygosity for cystathionine synthetase deficiency have been associated with an increase in vascular disease and thrombosis (41–43). That these problems have not been reported as being present in increased incidence in Cbl deficiency or in folate deficiency may be due to the shorter duration of Cbl and folate deficiencies or to possible differences in other regulatory factors that may exist in these conditions.

Our finding that serum total homocysteine is negatively correlated with platelet count and Hct and positively correlated with MCV and LDH in the Cbl-deficient patients supports the concept that a block in methionine synthetase activity occurs in Cbl deficiency and that this block is responsible for the megaloblastic anemia and other hematologic abnormalities that are seen in these patients (12), and which are indistinguishable from those seen in patients with folate deficiency. We have previously reported (33) and have found in this study that serum methylmalonic acid is not significantly correlated with Hct, MCV, or LDH in Cbl-deficient patients. This supports the concept that the second Cbl-dependent enzyme, L-methylmalonyl-CoA mutase, does not play a role in the megaloblastic anemia and other hematologic abnormalities seen in Cbl deficiency.

The cause of the neuropsychiatric abnormalities that are seen in Cbl deficiency but not in folate deficiency has long been of interest. Some investigators have favored a defect in L-methylmalonyl-CoA mutase as the cause and have suggested that the neuropsychiatric abnormalities are due to a buildup in propionyl-CoA and a resultant increase in odd number carbon fatty acids in peripheral nerves and the central nervous system (44–46). Other investigators have favored a defect in methionine synthetase as the cause and have suggested that the neuropsychiatric abnormalities are due to a lack of methionine and a resultant decrease in various methylation reactions in peripheral nerves and the central nervous system (47–49). Our studies are not helpful in distinguishing between these two possibilities since we have found that serum methylmalonic acid and serum total homocysteine are both strongly positively correlated with the presence and severity of

neuropsychiatric abnormalities in patients with Cbl deficiency. Our finding that serum methionine is not decreased in patients with Cbl deficiency would appear to argue against a mechanism involving a lack of methionine as the cause of the neuropsychiatric abnormalities, but it should be emphasized that levels of methionine in serum may not reflect levels of methionine in peripheral nerves and the central nervous system. Measurements of methylmalonic acid, total homocysteine, and methionine in cerebral spinal fluid and various tissues would be of interest in this regard.

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