Original Article

High prevalence of vitamin B-12 insufficiency in patients with Crohn's disease

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Background and Objectives: In Crohn's disease (CD), belonging to inflammatory bowel disease, the small intestine is involved in most cases. Most frequently affected is the distal ileum, where vitamin B-12 is specifically absorbed. Therefore, malabsorption of vitamin B-12 is quite likely to occur in patients with CD. In this study, we have studied the vitamin B-12 status in CD patients. **Methods and Study Design:** Forty eight patients with CD were evaluated for their food intake, and circulating concentrations of vitamin B-12, folic acid, and homocysteine (Hcy) as a sensitive marker for the insufficiency of these vitamins and a risk factor of atherosclerosis. **Results:** Plasma Hcy concentration was significantly correlated with serum vitamin B-12 concentration alone, and 60.4 % of the subjects had hyperhomocysteinemia. Receiver Operating Characteristics (ROC) analysis showed that serum concentration of vitamin B-12, but not folic acid, predicted hyperhomocysteinemia. Their intake of vitamin B-12 was much higher than the Japanese RDA, but not correlated with blood concentrations of vitamin B-12 or Hcy, probably due to malabsorption. **Conclusions:** Vitamin B-12 insufficiency and hyperhomocysteinemia were highly prevalent in CD patients. Recently, the significance of extra-intestinal complications of CD has been increasingly recognized, and our finding is likely to be of clinical importance.

Key Words: vitamin B-12, homocysteine, Crohn's disease, malabsorption, vitamin insufficiency

INTRODUCTION

Inflammatory bowel disease (IBD) consists of Crohn's disease (CD) and ulcerative colitis (UC). As its name implies, the involvement of UC is basically limited to the large intestine. In contrast, any part of the digestive tract can be affected in CD,[1,2] but small intestinal involvement is found in most cases.[3] Since most nutrients are absorbed in the small intestine, malnutrition is a more serious problem in patients with CD.[4]

Unlike other water-soluble vitamins, the gastrointestinal absorption of vitamin B-12 has some unique features. Firstly, after its release from foods by the action of gastric acid, vitamin B-12 binds to intrinsic factor (IF) secreted from the gastric parietal cells. Vitamin B-12-IF complex thus formed is specifically absorbed from the distal ileum.[5] Of possible clinical importance is the fact that distal ileum is most frequently involved in CD.[6] Then one can easily imagine that specific absorption of vitamin B-12-IF complex from the distal ileum would be impaired in CD. Secondly, since vitamin B-12 is reabsorbed from the digestive tract via enterohepatic circulation,[5] inflammation in the small intestine in CD will impair the enterohepatic circulation of vitamin B-12, and further contribute to its malabsorption.

Thus, malabsorption of vitamin B-12 is quite likely to occur in CD. In this paper, we have studied the vitamin B-12 status in patients with CD by evaluating their dietary intake, and measuring circulating concentrations of vitamin B-12, folic acid, and homocysteine (Hcy) which is an index representing the vitamin B-12 or folic acid insufficiency.

MATERIALS AND METHODS

Subjects

Forty eight outpatients with CD attending the Gastroenterology Clinic at Kyoto University Hospital participated in the study. Written consent to participate in this study was obtained after explanation of the objectives and protocols. The study's protocol was approved by the Ethical Committee of Kyoto University Hospital (Ethics Approv-

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Table 1. Background profiles and results from blood tests in patients with CD

	All subjects (n=48)	Those taking vitamin B-12 excluded (n=37)
Age (years old)	40.1±9.0 (39.5)	39.8±8.7 (39.0)
Age of onset (years old)	23.3±8.3 (21.0)	23.3±8.3 (21.0)
Disease duration (years)	16.8±9.0 (15.0)	16.4±8.1 (15.0)
Sex (male/female)	33/15	23/14
BMI (kg/m^2)	20.8±2.9 (20.2)	20.8±3.2 (19.9)
Albumin (g/dL)	4.6±5.1 (3.9)	4.9±5.8 (4.0)
Total cholesterol (mg/dL)	152±34 (147)	155±34(151)
C-reactive protein(mg/dL)	0.32±0.61 (0.10)	0.27±0.53 (0.10)
Serum vitamin B-12 (pg/mL)	447±242 (386)	393±210 (334)
Serum folic acid (ng/mL)	8.2±7.1 (6.4)	8.5±7.7 (6.5)
Plasma homocysteine (nmol/mL)	17.2±9.0 (15.0)	17.9±8.9 (15.2)

Data are expressed as mean±SD with values in parentheses showing the median.

al number; E2315) . Eleven patients were under oral vitamin B-12 administration.

Methods

Biochemical measurements

After centrifugation, serum or plasma was stored at -70˚C until measurement, serum concentrations of vitamin B-12 and folic acid were measured by chemiluminescent enzyme immunoassay (CLEIA). Plasma concentration of Hcy was measured by HPLC as the marker for the insufficiency of these vitamins.

Dietary intake

Dietary information was obtained by FFQ using a brief-type self-administered diet history questionnaire (BDHQ).[7]

Statistical analyses

Statistical analyses were performed using SPSS 21.0 for Windows (IBM Japan, Tokyo, Japan). The difference between two independent groups was analyzed by an unpaired t test. Correlations between two independent variables were analyzed by Pearson's correlation. Receiver Operating Characteristics (ROC) analysis was performed to determine the diagnostic value of serum vitamin B-12 and folic acid concentrations for hyperhomocysteinemia. The adequate cut-off value was calculated based on Youden's index as the value giving the maximal value of "sensitivity + specificity - 1".[8]

RESULTS

The baseline characteristics including data from blood examination are shown in Table 1. Although the patients' mean age was 40.1 years old, they had developed CD at age 23.3 as the average. Therefore, the mean disease duration was quite long.

Their BMI and serum concentrations of albumin and cholesterol were within the reference range, suggesting that these patients were not generally malnourished. The average serum vitamin B-12 concentration was higher than the reference value of 203 pg/mL and 170 to 250 pg/mL suggested by WHO[9] and DRIs for vitamin B-12 in USA/Canada,[10] respectively. Serum folic acid was also higher than the reference value of 4ng/mL suggested by WHO.[9] The mean plasma Hcy was higher than the reference value of 14 nmol/mL, a cut-off value adopted in DRIs for Japanese 2015.[11-13] Analyses excluding those under vitamin B-12 treatment have yielded similar results. Controversy exists however, on the cut-off value for these parameters, and more detailed consideration on a reference value of vitamin B-12 will be made in the "Discussion".

Correlation of serum vitamin B-12, folic acid, and plasma Hcy concentrations

Plasma Hcy was significantly correlated with serum vitaminB-12, but not with serum folic acid. Serum vitamin B-12 and folic acid concentrations were not correlated (data not shown). Again, the results were the same after excluding the subjects under medication. (Table 2)

ROC analysis

The diagnostic value and cut-off concentrations of serum vitamin B-12 and folic acid concentration for hyperhomocysteinemia were studied by ROC analysis. Hyperhomocysteinemia was defined as plasma Hcy concentration above 14 nmol/mL.[11-13] In this analysis, a curve away from the diagonal line, or in other words, the area under the curve (AUC) greater than 0.5 indicates a good diagnostic value.[8]

AUC for vitamin B-12 was 0.753 (95% CI; 0.606-0.900), significantly higher than 0.5. (Figure 1) The adequate cut-off value was calculated to be 503 pg/mL based on Youden's index.[8] Serum vitamin B-12 concentration was less than this cut-off value in 68.8% of the subjects. In contrast, serum folic acid concentration did not significantly predict hyperhomocysteinemia (data not shown).

Analysis of food intake

Food intake could be evaluated in 45 patients, of whom16 subjects were on partially enteral nutrition; their intake

Table 2. Correlation analysis for blood tests

	All subjects (n=48)	Those taking vitamin B-12 excluded (n=37)
Vitamin B-12, homocysteine	-0.380*	-0.435*
Folic acid, homocysteine	-0.122	-0.169

The asterisk denotes the significant correlation ($^*p< 0.01$) by Pearson's correlation.

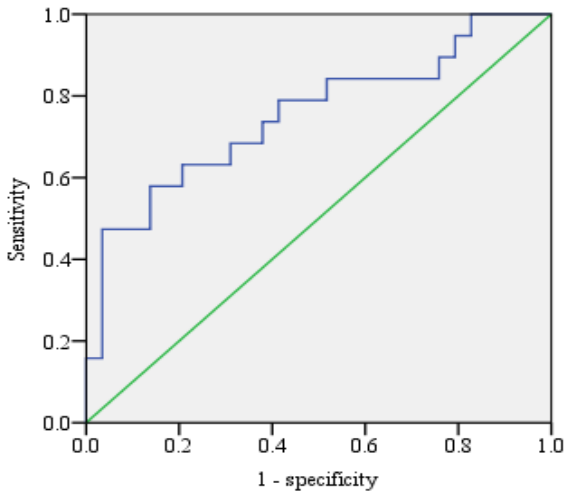

Figure 1. Receiver Operating Characteristics (ROC) curve for the predictive value of serum vitamin B-12 concentration for hyperhomocysteinemia. A curve distant from the diagonal line; in other words, area under the curve (AUC) significantly higher than 0.5 indicates a significant predictability. In this figure AUC was 0.753 (95% CI; 0.606-0.900).

Table 3. Total intakes

	n=45
Energy (kcal)	1916±660 (1746)
Protein (g)	73.3±28.1 (66.4)
Fat (g)	45.3±23.6 (42.4)
Vitamin B-12 (μg)	9.0±4.5 (8.4)

Values represent the mean±SD with values in parentheses showing the median. "Total intake" denotes intake from dietary source and enteral feeding.

being both from oral and enteral sources. The average intake of vitamin B-12 was 8.2 μg/day from the dietary source, and the total intake, including that from the enteral feeding, was 9.0 μg/day. It was much higher than the RDA of 2.4 μg/day in DRIs for Japanese 2015.[11] (Table 3) Total intake of vitamin B-12 was not correlated with either blood concentrations of vitamin B-12 or Hcy (data not shown).

Comparison of subjects with small intestinal resection and those without it

Patients with small intestinal resection had lower serum vitamin B-12 concentration (412±248 pg/mL) than those without it (485±257 pg/mL), although not statistically significant.

DISCUSSION

In this paper, we have reported the vitamin B-12 status in patients with CD by measuring circulating concentrations of vitamin B-12, folic acid, and Hcy, in addition to their dietary intake. The average serum vitamin B-12 and folic acid levels were higher than the reference values proposed by WHO[9] or in the DRIs in Japan and the USA/Canada.[10,11] In addition, total intake of vitamin B-12 was much higher than RDA.[11] In order to evaluate the current data, the basis for these reference values must be first considered.

Vitamin deficiency causes classical deficiency diseases such as beriberi due to vitamin B-1 deficiency[14] and rickets due to vitamin D deficiency.[15] Recently, there has been an increasing concern regarding the significance of vitamin insufficiency. Vitamin insufficiency, milder than vitamin deficiency, does not cause classical deficiency disease, but renders one at an increased risk for diseases. For example, vitamin D insufficiency does not cause rickets, but increases the risk of osteoporotic fracture.[15] In the case of vitamin B-12, although pernicious anemia is well known to be its classical deficiency disease,[16] the significance of its insufficiency has received far less attention.

In mammals, vitamin B-12 is involved in two metabolic pathways.[16] Firstly, it is involved in the methionine metabolism. Methionine is metabolized to S-adenosylmethionine, S-adenosyl-homocysteine, then to Hcy, which has two metabolic fates. One is its conversion to cysteine, which is vitamin B-6 dependent. The other pathway is its metabolism to methionine with vitamin B-12 and folic acid as the coenzyme[17] and methyl group donor, respectively.[18] Thus, the insufficiency of these vitamins results in hyperhomocysteinemia, which can be a marker for their insufficiency.[12,19] Secondly, vitamin B-12 is a coenzyme of methylmalonyl-CoA mutase in fatty acid metabolism. Impaired conversion of methylmalonyl CoA to succinyl CoA due to vitamin B-12 deficiency results in excessive production of methylmalonic acid (MMA).[20] Thus, vitamin B-12 status can be assessed by measuring serum vitamin B-12 concentration and circulating concentration of markers representing vitamin B-12 status; Hcy or MMA. Indeed, measurement of two parameters; one representing circulating vitamin B-12 concentration and another representing its insufficiency is recommended by experts engaged in NHANES.[21]

In the current study, although we have preliminarily measured circulating MMA concentration, it was undetectable, probably reflecting that MMA is mostly excreted in urine.[22] Unfortunately, urine samples were not preplanned to be obtained. Therefore, in the current study, vitamin B-12 status was evaluated by serum vitamin B-12 and plasma Hcy. Since plasma Hcy is influenced by serum folic acid,[23] it was also measured. Plasma Hcy was defined as less than 14 nmol/mL, according to the DRIs for Japanese 2015.[11] Similar values are also defined in the DRIs in the USA/Canada.[10] In the current subjects, although serum vitamin B-12 and folic acid exceeded the reference value for these vitamins, the average plasma Hcy was much higher than 14 nmol/mL, and hyperhomocysteinemia thus defined was observed in 60.4% of CD patients.

In "Conclusions of a WHO Technical Consultation on folate and vitamin B-12 deficiencies", reference values for serum vitamin B-12 and folic acid were decided to be as higher than 203 pg/mL, and 4 ng/mL, respectively. These values were thus determined as two or three SDs away from a reference range's mean.[21] Although this decision process seems apparently reasonable, it can be valid only when the majority of subjects in the population are sufficient with regard to the nutrient status. When the prevalence of deficiency or insufficiency is high, it would yield an inappropriately low value. Low reference values are also shown in the DRIs in Japan and the USA/Canada,

136 pg/mL in the former and 170 to 250 pg/mL in the latter.[10,11] These DRIs are, however, determined based on data from treatment of patients with pernicious anemia by intramuscular injection of vitamin B-12.[10,11] Thus, hitherto published reference values were considered inappropriate when the insufficiency is taken into account.

Elevated plasma Hcy concentration is known to be a risk factor for atherosclerosis independent of dyslipidemia.[24] Since insufficiency of vitamin B-12 and folic acid leads to increased plasma Hcy, the insufficiency of these vitamins must be avoided. Plasma Hcy is a parameter reflecting the insufficiency.[12] Serum concentrations of vitamin B-12 or folic acid required to avoid their insufficiency would be much higher than that to prevent their deficiency. In the current study, serum concentrations of vitamin B-12 and folic acid exceeded the deficiency-based reference value, but was considered to be unsatisfactory for the prevention of insufficiency.

Then, it was considered necessary to decide the serum vitamin B-12 concentration based on the prevention of hyperhomocysteinemia. Plasma Hcy concentration was significantly correlated with serum vitamin B-12 concentration, but not with serum folic acid concentration. When the predictive ability of serum vitamin B-12 for hyperhomocysteinemia was analyzed with ROC analysis, AUC was 0.753 (95% CI; 0.606-0.900), significantly higher than 0.5, which indicated that serum vitamin B-12 predicted hyperhomocysteinemia. The adequate cut-off value of serum vitamin B-12 to prevent hyperhomocysteinemia was calculated to be 503 pg/mL. Serum vitamin B-12 concentration was less than this cut-off value in 68.8% of the subjects. Serum folic acid had no predictive value for hyperhomocysteinemia. From the above results, it was considered that serum vitamin B-12, rather than folic acid, is the major determinant of hyperhomocysteinemia in the current subjects, and the significant percentage of patients with CD had their serum vitamin B-12 concentration below the cut-off value based on the prevention of hyperhomocysteinemia. Thus, a substantial percentage of the current patients were considered vitamin B-12 insufficient, even if not vitamin B-12 deficient.

The next issue to be considered is the cause for vitamin B-12 insufficiency. A lack of correlation between vitamin B-12 intake and circulating concentration of vitamin B-12 or Hcy raises the possibility of malabsorption. Although some patients received small intestinal resection, there were no statistically significant differences in vitamin B-12 related parameters between patients with and those without it. There are also possible alternative or additional mechanisms responsible for malabsorption. Besides its specific absorption from distal ileum, vitamin B-12 can also be non-specifically absorbed through the entire small intestine,[25] which may also be disturbed in CD patients. Additionally, after its excretion into the bile, vitamin B-12 is reabsorbed through enterohepatic circulation,[5] which is also likely to be disturbed in CD patients. Thus, although the precise underlying mechanism is currently unknown, malabsorption of vitamin B-12 was considered likely to be involved.

We believe that our results have two implications; one clinical, and the other related to DRIs. Needless to say, the most important signs and symptoms of CD are the gastrointestinal ones. Recently, however, the significance of the extra-gastrointestinal manifestations of CD, including thromboembolism, has been increasingly recognized.[26] Thus, vitamin B-12 insufficiency in CD patients would be of clinical relevance. In face of impaired specific absorption from distal ileum, vitamin B-12 could be widely non-specifically absorbed.[25] Therefore, a large dose of vitamin B-12 administration can be effective even in patients with impaired absorption.[27] In the current study, 11 out of 48 patients, given a large dose of methylcobalamin, had significantly higher serum vitamin B-12 concentration than those without it.

The other possible implication is on the decision of vitamin B-12 requirement in DRIs. Determination of Estimated Average Requirement (EAR) is currently made based on the clinical data in patients with pernicious anemia both in the USA/Canada and Japan.[10,11] This decision method has been criticized based on various reasons, including only a small number of subjects studied and ignorance of neurological involvement that may precede the occurrence of anemia.[11] Recently released DRIs for vitamin D in the USA/Canada have defined EAR for vitamin D based on the amount required to prevent increased risk of fracture.[28] In other words, it was decided taking the insufficiency - fracture risk into account, rather than solely based on avoiding the classical deficiency - rickets and osteomalacia. In addition to its classical deficiency disease; pernicious anemia,[16] vitamin B-12 insufficiency is known to be related to various morbidities.[12] Our data clearly show that serum of vitamin B-12 needed to avoid hyperhomocysteinemia is much higher than that in the current reference values decided based on the prevention of classical deficiency. Our data would be of value in the future revisions of DRIs.

Subclinical vitamin B-12 deficiency, which corresponds to insufficiency in this paper, is defined as low serum vitamin B-12 and confirmatory biomarker elevation such as Hcy or MMA, and diagnosis based on these measurements is recommended by experts engaged in NHANES.[21] In a recently published systematic review on vitamin B-12 status of IBD patients, Battat et al selected 42 papers including 3,732 patents. They have pointed out that in none of the studies they selected, were recommendations by NHANES adopted, and diagnosis was made only by circulating vitamin B-12 concentration.[29] The strength of our data includes that serum vitamin B-12, plasma Hcy concentrations, and vitamin B-12 intake were simultaneously evaluated, and vitamin B-12 insufficiency was diagnosed based on the avoidance of hyperhomocysteinemia.

Our study, however, is not free from limitations. Firstly, although plasma Hcy is also influenced by vitamin B-6 status,[30] its serum concentration could not be measured due to technical reasons. All three vitamins- vitamin B-12, B-6, and, folic acid- can be measured using bioassay based on these vitamins' effects on bacterial growth.[31] Bioassay was considered to be inappropriate in the current study, since substantial numbers of subjects were under long-term antibiotics treatment, which may possibly affect the bioassay. Based on these considerations, we have employed immunoassay for the measurement of vitamin B-12 and folic acid. However, chromatographic

analysis is needed for vitamin B-6 measurement. In this study, required amounts of blood could not be obtained for the vitamin B-6 measurement. Secondly, folic acid status was evaluated only by its serum concentration. Folic acid concentration in the serum and blood cells reflects the short-term and long-term folic acid status, respectively.[32] Although measurement of both concentrations is favorable, it was not possible in the clinical settings. We believe, however, that patients in the current study were in a stable status for a long period, and lack of data on the folic acid concentration in the blood cells does not largely affect the results. Thirdly, only Hcy was used as the marker for vitamin B-12 insufficiency. The fact that plasma Hcy can be influenced by folic acid and vitamin B-6 status in addition to vitamin B-12 status is the major drawback for its use as the marker for vitamin B-12 insufficiency. MMA is advantageous in that it is influenced by vitamin B-12 status alone, but can be measured only by GC/MS.[12] In the current study, serum MMA concentration was unmeasurable, probably because it is excreted in the urine.[20,22] As previously stated, urine sample collection was not preplanned in the present study. An additional study, with urinary MMA measurement included, is required. Fourthly, SNP in methylenetetrahydrofolate reductase- an enzyme in folic acid metabolism known to significantly affect circulating Hcy concentration[33] could not be analyzed.

In conclusion, we have reported that hyperhomocysteinemia was highly prevalent in CD patients, probably due to the malabsorption of vitamin B-12.

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AUTHOR DISCLOSURES

None of the authors have any conflicts of interest.

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