

COBALAMIN AND FOLATE DEFICIENCY: THE ANTECEDENTS AND ASSOCIATIONS OF ANEMIA

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Introduction

The classical features of florid deficiencies of either of the nutrients cobalamin and folic acid are hematological and include anemia, accompanied by macrocytosis and conspicuous megaloblastic changes in the blood and bone marrow that are the consequence of an underlying defect in DNA synthesis. These features, because of their prominence and for historical reasons, have become enshrined in standard texts and imprinted in the minds of practicing physicians. The clinical diagnosis of folate or cobalamin deficiency, therefore, usually proceeds in three steps: first, the recognition of anemia; second, the observation that the anemia is macrocytic; and third, the suspicion and confirmation that there is underlying cobalamin or folate deficiency to explain the macrocytic anemia.

The diagnosis of cobalamin or folic acid deficiency has generally hinged on the finding of low concentrations of either or both of these vitamins in the blood. The decision to measure cobalamin and folate levels is prompted by clinical grounds to suspect such deficiency, and as noted above, the trigger has traditionally been the finding of a macrocytic anemia. If macrocytic anemia were to be used as the sole criterion for pursuing a diagnosis of these nutrient deficiencies, however, it is clear that a substantial proportion of deficiencies of cobalamin or folate would escape detection, with the potential consequences and costs of continuing morbidity and delayed diagnosis, including impaired well-being and the risks of irreversible organ damage and even death. The neurological sequelae of cobalamin deficiency can be irreversible, as is the case for neural tube defects, and the possible vascular and preneoplastic consequences of folate deficiency can putatively contribute to further morbidity and mortality. Being nutrients, both folate and cobalamin can be replaced if deficient and, with the exceptions noted above, diseases resulting from their deficiency can largely be reversed or even prevented. The opportunity for disease prevention and cure makes it particularly desirable and important to exercise a high level of vigilance for detection of folate and cobalamin deficiencies. Traditionally considered to be the “domain” of the hematologist, folate and cobalamin deficiencies are now recognized as multisystem disorders, the manifestations of which extend across multiple medical specialties. It behooves the hematologist to remain conversant with advances in the understanding of these nutritional deficiency diseases, and to be alert to their many facets.

The Changing Face of Cobalamin and Folate Deficiency

or a number of reasons, “textbook” cases of megaloblastic anemia are seen less frequently than previously, and the clinical pattern of presentation of these nutrient deficiencies appears to be shifting. Possible reasons for this change are summarized in Table 1. Increased physician vigilance and patient awareness are major factors that

prompt earlier presentation and diagnosis. In addition, detection of early, subtle and atypical forms of these nutrient deficiencies has been made possible by the recent development of more sensitive and specific tests. Finally, the clinical spectrum of cobalamin and folate deficiencies has been broadened as a result of several factors. In addition to improved diagnosis, there have been several reports indicating that cobalamin deficiency, in particular, may have inconspicuous or no hematological complications. Such patients present primarily or exclusively with neurological manifestations.⁽¹⁾ In folate deficiency, on the other hand, there are reports of a number of associated risks of comorbidity occurring in patients with suboptimal nutrition for this vitamin, even in the absence of any anemia. Maternal folate deficiency has been implicated as a major causative factor of neural tube defect pregnancies⁽²⁾ and public health measures are underway in certain countries to fortify the diet with folic acid in order to reduce the prevalence of this devastating congenital abnormality. The most prominent of the disease associations with folate deficiency is the apparent increased risk of vascular disease and thrombosis that occurs with elevated levels of homocysteine in the blood. Raised levels of homocysteine is most often caused by folate deficiency.⁽³⁾ There have also been reports linking folate deficiency with preneoplastic conditions of the cervix bronchial epithelium and gastrointestinal tract.⁽⁴⁾ Thus, the classic hematological features of folate and cobalamin deficiencies may be accompanied or even heralded by less commonly recognized hematological or non-hematological manifestations or complications. Indeed, these other hematological and non-hematological features may occur in the total absence of anemia and/or macrocytosis.

Table 1. Reasons for the changing pattern of clinical manifestations of cobalamin and folate deficiencies.

1. Earlier presentation and diagnosis
 - a. physician vigilance
 - b. patient awareness

2. Improved laboratory testing
 - a. greater sensitivity
 - b. better specificity
 - c. redefining “normalcy”

3. Absence or masking of anemia or macrocytosis
 - a. “protective” effect of folate in cobalamin deficiency
 - b. coexistent iron deficiency, thalassemia or anemia of chronic disease
 - c. longevity of red cells
 - d. initial “normal” value

”Atypical” Presentations

Hematological “Variants”

Several atypical presentations may be encountered among patients with cobalamin or folate deficiency.^(5,6) Frequently patients with cobalamin deficiency may have no anemia and may present only with neurological features. There is evidence to suggest that the severity of neurological involvement may be inversely related to the degree of anemia.⁽¹⁾

As methods for the diagnosis of cobalamin and folate deficiencies have become more sophisticated, patients who lack the classical textbook features of these deficiencies have been recognized. Carmel noted absence of anemia in 19% and absence of macrocytosis in 33% of 70 patients with confirmed untreated pernicious anemia who were initially screened on the basis of serum cobalamin levels < 200 pg/ml.⁽⁷⁾ Reasons for masking of the typical hematologic features of cobalamin and folate deficiencies remain to be fully elucidated. Masking of macrocytosis may be due to the presence of a coexisting microcytic process including thalassemia minor, iron deficiency and the anemia of chronic disease.^(8,9)

Delay in the appearance of the typical hematological features of folate or cobalamin deficiencies may be the result of several factors. These are summarized in Table 2. Anemia associated with slight or no macrocytosis is quite frequently present in folate or cobalamin deficiency, particularly but not exclusively during the early stages of the deficiency. During the early stages of an anemia, because of the 120-day life span of red cells, MCV may be normal, and in this situation, laboratory findings consist of a raised red cell distribution width (RDW), reflecting the presence of anisocytosis. Another factor that affects the time-lag between onset of disease and the appearance of macrocytosis in a given patient with anemia is the baseline “normal” MCV for that individual. In someone who starts out with an MCV in the low-normal range, it will take longer for the MCV to attain a level that would be recognized as “abnormal” on an absolute scale. For similar considerations, if the starting MCV in a particular patient is high-normal, then macrocytosis may occur in the absence of anemia. For any given patient, what is perhaps most important is a change in the MCV. So long as the patient’s “normal” MCV is known, an increase of 5 fl or more may be considered to be a directional move toward macrocytosis.

Table 2. Non-hematological complications of cobalamin and folate deficiencies.

	Cobalamin Deficiency	Folate Deficiency
neurological	myeloneuropathy dementia visual disturbances	neural tube defect pregnancies
metabolic	raised homocysteine and related thiols	raised methylmalonic acid
cardiovascular associated with high	homocysteine levels, which occur more commonly in patients with vascular disease	

neoplastic

not known

associated with
preneoplastic changes

The megaloblastic process that occurs in cobalamin or folate deficiency results in an abrogation of DNA synthesis and diminished cell production that is not restricted to the red cell series because the underlying defect in DNA synthesis affects other bone marrow elements as well. Some degree of thrombocytopenia and/or neutropenia is therefore often also present and patients may present with petechiae or purpura resulting from low platelets or may have fever or other signs of infection resulting from neutropenia. In general, the level of thrombocytopenia and neutropenia, when present, appears to parallel the degree of anemia. The number of nuclear lobes in neutrophil polymorphonuclear leukocytes is typically increased and cells with five or more lobes (hypersegmented PMNs) are conspicuous. Performing an actual lobe count is laborious, but the presence of > 5% of PMNs with five lobes (the “rule of fives”) or the presence of even one PMN with more than five lobes represents good presumptive evidence of an underlying megaloblastic process. This feature was previously reported to be the earliest morphological change to appear in the blood in megaloblastic anemia as well as the last to disappear after treatment.⁽¹⁰⁾ However, in a more recent study, in patients with mild cobalamin deficiency, neutrophil lobe counts showed a poor correlation with abnormal results in the deoxyuridine suppression test as well as several other established criteria of cobalamin deficiency.⁽¹¹⁾

Neurological presentations

With respect to cobalamin deficiency, patients with predominantly or exclusively neurological manifestations are being recognized in increasing numbers.^(1,12) The curious absence of anemia in patients with pernicious anemia who present with neurological features was first observed over 100 years ago⁽¹³⁾ and subsequently confirmed by several other authors.^(10,14) There has been renewed interest in this observation; with the advent of improved methods for diagnosis and monitoring of cobalamin deficiency, Lindenbaum et al⁽¹²⁾ observed that 40 of 141 patients with neuropsychiatric disorders attributable to cobalamin deficiency (28.4%) had absence of anemia or macrocytosis, and in 19 of these (13.5%) both the hematocrit and mean cell volume were normal. In a more detailed and extensive study, this group also noted that cobalamin-deficient patients with less anemia tended to have more prominent neurological involvement.⁽¹⁾ The reason for this inverse relationship has not been fully elucidated, although some circumstantial evidence points to a possible role for folate. It has long been known that treatment with folic acid can ameliorate the hematological but not the neurological manifestations of cobalamin deficiency.⁽¹⁵⁾ A recent study on cobalamin-deficient patients from Zimbabwe provides some support for this hypothesis. Patients with normal or increased serum folate levels more commonly displayed neurological signs than those with low serum folate concentrations.⁽¹⁶⁾ It has been estimated that of all patients with confirmed cobalamin deficiency, 90-95% have serum cobalamin levels < 200 pg/ml, 5-10% have levels of 200-300 pg/ml and up to 1% may have values greater than 300 pg/ml.⁽¹⁷⁾

Neurological symptoms are present in 75-90% of patients with cobalamin deficiency. Neurological changes may be the most prominent, and in approximately 25% of patients, are the only clinical manifestations of cobalamin deficiency. The symptoms, usually slow in onset, mainly affect the lower extremities. They include a variety of sensory deficits, most prominent of which are position and vibration sense loss, which are associated with ataxia, Romberg's sign and abnormalities of gait. Vibration sensory loss starts distally and progresses centripetally toward the trunk. These more "typical" neurological complications are related to a pathological process of demyelination described in early reports of patients dying with untreated pernicious anemia, and which appear to predominantly affect the long posterior and lateral columns of the white matter in the spinal cord. Less frequently, there are other sensory deficits, including light touch, pin-prick and deep pain, pressure and temperature. Cranial nerve involvement is rare. The occurrence of motor disturbances in addition to sensory deficits gave rise to the original description of "combined system disease." A variety of cognitive deficits have been reported in cobalamin deficiency, ranging from memory loss and disorientation to frank dementia and disturbances of affect including irritability, mood swings and depression.⁽¹²⁾ Other symptoms, such as insomnia, visual disturbances, impotency and autonomic disturbances, may also occur. On account of the high prevalence of vascular and other neurodegenerative diseases that occur among the elderly, it is inadvisable to attribute impaired mentation to cobalamin deficiency without reliable corroborative evidence in the form of appropriate biochemical tests, or demonstrable improvement in neurological abnormalities following treatment with cobalamin. In folate deficient patients on the other hand, apart from depression, neurological complaints are rare.

"Shift" from Hematological to Neurological Presentations

The possibility that a change has occurred in the clinical spectrum of cobalamin and folate deficiency may perhaps be explained on the basis of changes in the patterns of dietary and other intake of these nutrients. It has long been known that supplemental folic acid can ameliorate the hematological complications of cobalamin deficiency.⁽¹⁵⁾ It is possible that raised folate intake resulting from improved diet, the use of supplements or fortification may be responsible for masking or postponing the hematological manifestations of cobalamin deficiency. The administration of therapeutic doses of folic acid to a patient with cobalamin deficiency may result in a partial or temporary remission of the hematological and other megaloblastic complications of cobalamin deficiency. The administration of folic acid to a patient with cobalamin deficiency therefore carries the potential risk of masking the true underlying cobalamin deficiency. Nervous system deprivation of cobalamin will continue and may therefore result in progressive and irreversible neurological damage. For this reason, it is critically important to exclude cobalamin deficiency before starting treatment with doses of folic acid in excess of 1 mg daily.

Does Folate Mask Hematological Features in Cobalamin Deficiency?

The natural history of pernicious anemia was well documented in the era before cobalamin was identified and specific treatment became available. At that time, it was noted that the progression of the disease was sometimes erratic. Temporary and partial

remissions may have been related to either waxing and waning of the autoimmune process or possibly to dietary intake of food rich in folate that might have intermittently ameliorated the block in DNA synthesis. It is possible that this folate effect may also be responsible for the inverse correlation between neurological and hematological complications in cobalamin deficiency. Patients who have better folate nutrition may manifest fewer or no hematological effects of cobalamin deficiency yet neurological complications may be unaffected. In this regard, the proposal to fortify staple foods with folic acid in order to protect women at risk from having children with neural tube defects may carry with it a potential risk of masking hematological manifestations in patients with pernicious anemia. This may result in progression to irreversibility of neurological complications. Food fortification with folic acid, presently being contemplated in the U.S. to prevent neural tube defect pregnancies, will doubtless also contribute to a lowering of the incidence of nutritional folate deficiency. Although this will carry the risk of masking cobalamin deficiency, there are other potential advantages of improved folate nutrition in the U.S. population including a lowering of the plasma homocysteine concentration. High plasma homocysteine has been linked to premature vascular disease and thrombosis. There is also evidence to suggest that folate deficiency may be associated with premalignant changes in the cervical epithelium and in the colon.

Other Non-Hematological Complications

Gastrointestinal

Gastrointestinal complaints occur quite frequently in patients with either folate or cobalamin deficiency.⁽¹⁰⁾ There may be several possible reasons for this association. To begin with, the mucosal epithelium of the gastrointestinal tract, which is normally undergoing constant replacement and therefore has a high level of DNA synthesis, is, like the bone marrow, subject to conditions of cobalamin or folate deprivation. Judging from the variability in occurrence and severity of gastrointestinal symptoms in patients with known cobalamin or folate deficiency, it seems reasonable to speculate that there is a considerable degree of individual variation in susceptibility of this tissue to vitamin deficiency. Another reason for the frequent occurrence of gastrointestinal symptoms with folate and cobalamin deficiency states is that digestive diseases associated with malabsorption are frequently the cause of the vitamin deficiency. Yet another link with the gastrointestinal tract arises out of autonomic dysfunction that may occur as part of the constellation of neurological complications of cobalamin deficiency. This can give rise to motility disorders, including anorexia, flatulence and disturbances in bowel habits, particularly constipation.

Integumentary

A variety of miscellaneous integumentary and other epithelial changes may occur in cobalamin deficiency.⁽¹⁰⁾ Pernicious anemia patients often show premature graying of the hair and may develop alopecia; about 20% have a smooth tongue caused by papillary atrophy. There is patchy skin hyperpigmentation in about 10% of cobalamin-deficient patients, which is reversible following cobalamin replacement, and patients with pernicious anemia may have hypopigmentation caused by associated vitiligo.

Immunological

There are a number of sporadic reports in the literature of compromised cellular immune status in patients with cobalamin and folate deficiencies affecting neutrophil function⁽¹⁸⁾ and T-cell immunity.⁽¹⁹⁾ It has also been proposed that the high frequency of tuberculosis encountered among Hindu vegetarians may be related to nutritional cobalamin deficiency, which results in impairment of the oxygen burst required for intracellular macrophage killing.⁽²⁰⁾ On the humoral side, hypogammaglobulinemia has been described in association with transcobalamin II deficiency and antibody production in response to antigenic challenge is impaired in cobalamin-deficient infants, which is corrected by treatment of the deficiency.⁽²¹⁾

Reproductive

Infertility has been reported in both sexes in association with cobalamin deficiency⁽¹⁰⁾ and in association with drugs that interfere with folate metabolism, such as methotrexate. In relation to cobalamin, descriptions of male infertility come from observations on strict vegetarians.⁽²²⁾ The peak incidence of pernicious anemia occurs beyond the female reproductive years, yet there are reports of infertility associated with this disease which were corrected by administration of vitamin B₁₂.⁽²³⁾ Also, among patients of Hispanic and African origin, the incidence of pernicious anemia is high among women age 20-40.⁽²⁴⁾

Developmental

Both cobalamin and folate deficiencies during pregnancy are associated with a considerable toll of fetal wastage, congenital malformations, teratogenicity and developmental abnormalities. Following the description of an infant born of a vitamin B₁₂-deficient mother who showed the stigmata of cobalamin deficiency,⁽²⁵⁾ attention was again focussed on the problem by Higginbottom et al.⁽²⁶⁾ This was followed by a series of similar reports, describing a pattern of infants born of mothers who had been strict vegetarians for 5 to 12 years. The infants appeared normal for the first 4 months and then declined with developmental retardation, irritability, weakness, and a panoply of neurological deficits as well as megaloblastic anemia. Their response to treatment with parenteral cobalamin was dramatic. A similar syndrome has been reported in infants born to mothers with pernicious anemia. This literature has been reviewed by Chanarin.⁽²⁷⁾

There is a considerable body of evidence that folate deficiency or the use of antifolate drugs in early pregnancy is teratogenic. This subject has recently gained prominence as a result of the association between neural tube defect pregnancies and marginal maternal folate status in women or fetuses with an underlying genetic defect in folate metabolism. Christensen and Rosenblatt, in an excellent recent review,⁽²⁾ have analyzed the evidence linking this intriguing association of a developmental abnormality with a genetic predisposition that is exacerbated by a nutritional deficiency of folate and prevented by folate supplementation. They also examine the possible mechanisms for this association, including defects in DNA synthesis, the possible role of fragile chromosomal sites, abnormalities of methylation, and the embryotoxic effect of homocysteine. In addition to neural tube defects, other developmental abnormalities have been described in

association with folate deficiency, including craniofacial and other skeletal malformations.

Cardiovascular Complications

There is now a considerable body of evidence that chronic, long-standing elevations in circulating homocysteine level may be responsible for degenerative cardiovascular changes including atherosclerosis and thrombosis.⁽³⁾ Even within the normal reference range for plasma homocysteine, there is an increased odds ratio for coronary artery disease.⁽²⁸⁾ Also, elevated levels of plasma homocysteine are frequently attributable to folate or cobalamin deficiency, and even in apparently healthy normal volunteers, there is evidence of a negative correlation between serum levels of homocysteine and either serum cobalamin, serum folate or red cell folate.^(29,30) This suggests that there may be a high prevalence of subclinical vitamin deficiency, sufficient to cause a significant increase in the levels of serum metabolites, even in persons with apparently normal serum levels of cobalamin and folate. There is considerable evidence to support this view and the topic has been reviewed extensively elsewhere.^(3,31,32)

Neoplasia

Some evidence has been put forward that folate deficiency may be associated with an increased predisposition to preneoplastic and neoplastic change. The concept of localized cellular folate deficiency arose from a report of cytological abnormalities in cervical epithelium of women receiving oral contraceptive agents who had normal circulating levels of folate, but in whom the cytological changes were reversed by oral folic acid. Subsequent studies concluded that nutritional folate deficiency (as judged by low red cell folate levels) increased the odds ratio for cervical dysplasia associated with human papilloma virus infection.⁽³³⁾ However, the same group found no effect of folate supplementation on the course of cervical dysplasia.⁽³⁴⁾ Other studies have been reported suggesting that folate and cobalamin supplements result in a decrease in cellular atypia in smokers with bronchial squamous metaplasia,⁽³⁵⁾ but in view of the variation in sputum cytology, the limited extent of the trial and the large dose of vitamins used, the question of a role for folate in preventing dysplasia must still be considered open. This subject has been reviewed more extensively by Krumdieck.⁽⁴⁾

Laboratory Diagnosis of Folate and Cobalamin Deficiencies

The sequence of development of laboratory findings in patients with cobalamin or folate deficiencies reflects the evolution of progressive changes that are outlined in Table 3. The utility and limitations of various laboratory tests have been reviewed recently.⁽³⁶⁾ Ideally, an accurate diagnosis of folate or cobalamin deficiency should be made with a minimum of laboratory tests. It is frequently possible to do so in straightforward, typical cases. However, it is false economy to skimp on laboratory tests when the diagnosis is not clear. Overutilization of the serum cobalamin test has been criticized as being wasteful for patients with normal MCV levels.⁽³⁷⁾ However, the cost of doing additional tests to establish a diagnosis of cobalamin deficiency should be balanced against the subsequent

cost of protracted care for patients who go on to develop irreversible neurological damage.

Vitamin Assays

Assays for folate and cobalamin still represent the most widely-used screening tests for deficiencies of the vitamins. However, there are problems with the specificity and sensitivity of these assays. For several reasons, accurate direct determinations of cobalamin and folate status are not provided by measurements of serum levels of these vitamins.⁽³⁶⁾ Serum cobalamin and folic acid levels are first-line investigations because they are relatively inexpensive. Assays for serum cobalamin and folate have, for some time, constituted the standard screening test for diagnosis of deficiency of these vitamins. Previously considered to be sensitive screening tests for the detection of cobalamin and folate deficiencies, there is now a mounting body of evidence to indicate that clinically significant deficiencies of these vitamins can occur with only slightly lowered serum levels of the vitamins. Indeed they can occur even in the face of serum levels that are within the normal range. Folate and cobalamin assays in the blood also lack specificity for diagnosis of deficiency of the respective vitamins. This subject has been extensively reviewed elsewhere.⁽³⁶⁾ Since most radioassay kits are designed to measure serum cobalamin and folate simultaneously, it was previously the case that there was no additional expense involved in obtaining both measurements together. However, there has been a recent major trend away from radioassays to non-isotopic methods based on enzyme linked spectrophotometric and chemiluminescent detection techniques. Semiautomated instruments designed for random access are used for these assays so that individual, rather than simultaneous, measurements for serum cobalamin and serum folate are carried out.

Metabolite Assays

In recent years, sensitive and reliable assays have become available for the measurement of compounds that accumulate in the serum or urine during cobalamin or folic acid deficiencies. The two most widely used are methylmalonic acid, which is raised only in cobalamin deficiency, and homocysteine, which can be increased in either cobalamin or folic acid deficiency. The most important application of metabolite measurement has been for detection of early, subtle deficiency states of cobalamin and folate including a predisposition to disease that may be associated with early subclinical deficiency of these vitamins. Clinically significant deficiencies of cobalamin or folate occur when cellular levels are inadequate to satisfy the cofactor or substrate requirements for these vitamins. This interferes with key biochemical pathways and leads ultimately to the disease manifestations found in patients with cobalamin and folate deficiencies. Detection of the metabolic disturbances that accompany vitamin deficiency and that often precede actual disease manifestations offers the possibility of providing an early clue to diagnosis and a means for early recognition of cobalamin and folate deficiency states.

Regarding sensitivity, in a study on 406 patients with cobalamin deficiency, excellent sensitivity was reported using metabolite assays. All but 1 of 406 patients showed an elevation in either serum methylmalonic acid or homocysteine or both metabolites for a combined sensitivity of 99.8%.⁽³⁸⁾ In the same study, 91% of 123

episodes of folate deficiency in 119 patients were associated with an increase in serum homocysteine. It was also noted that methylmalonic acid assay was more useful for non-anemic patients and that homocysteine levels were significantly higher in the group with anemia.

Methylmalonic acid, homocysteine and other metabolites were measured in groups of elderly subjects.⁽³⁹⁾ Elevations in one or more metabolites were found in 63% and 83% of healthy and hospitalized elderly persons, compared with much lower prevalence rates for subnormal serum cobalamin, folate and pyridoxine. From this, and based on the assumption that elevated levels of metabolites in the serum represents evidence of incipient vitamin deficiency, these authors concluded that there is a substantially higher prevalence of tissue deficiencies of these vitamins than is apparent from measurement of serum vitamin concentrations. In support of this are the findings reported on seven apparently healthy individuals with normal serum cobalamin who had serum methylmalonic acid elevated above 300 nmol/L. A single injection of cyanocobalamin resulted in a significant fall in their serum methylmalonic acid levels.⁽⁴⁰⁾ It is uncertain whether such degrees of metabolic impairment are associated with any morbidity or disease risk, although evidence is mounting that higher plasma levels of homocysteine are associated with an increased risk of vascular occlusive disease. The causative relationship between lowered vitamin status and raised metabolite levels in otherwise healthy persons is further substantiated by reports that vitamin supplementation causes a lowering of those levels. This has been demonstrated for homocysteine^(41,42) as well as for methylmalonic acid.⁽⁴³⁾ All these findings point to a need to redefine the normal ranges for serum cobalamin and folate levels in the population, yet this would almost certainly result in a greater number of false low results in normal individuals, further compounding the problem of poor specificity for serum vitamin assays that has been discussed above. Plasma homocysteine level has been used to redefine the lower limit cutoff for plasma folate.⁽⁴⁴⁾ In a study carried out on 101 patients with coronary artery disease and 108 controls, plasma folate was measured using a microbiological assay and compared with plasma homocysteine. Using a defined upper limit for plasma homocysteine, these investigators reasoned that plasma folate concentrations inadequate to prevent elevations in plasma homocysteine were indicative of biochemical deficiency. On this basis, they defined a “lower acceptable” plasma folate at 6.6 ng/ml (15 nmol/L).

In the vast majority of patients who have been studied, raised serum metabolites have been found at a single time point, which coincides with the finding of lowered serum cobalamin or folate levels. It is not ascertainable from this information which occurred first, the raised metabolites or the lowered vitamin levels. Pennypacker and associates⁽⁴³⁾ noted increased serum metabolite levels in three of their subjects, which preceded a fall in serum cobalamin by up to one year. Longitudinal population-based studies can provide further information, such as the study carried out by Lindenbaum and colleagues⁽⁴⁵⁾ on 548 surviving members of the Framingham Study cohort. Of 17 individuals with raised metabolite levels, 13 had serum cobalamin levels between 200-350 pg/ml and five of them had either macrocytosis or anemia. Reports of patients with objective evidence of cobalamin deficiency associated with low serum cobalamin and normal metabolite levels are rare. Savage and colleagues⁽³⁸⁾ described this situation in only one out of a group of 406 patients who were selected on the basis of subnormal

serum cobalamin levels. The finding of a normal homocysteine level in patients with low serum folate and objective evidence of folate deficiency, however, appears to be somewhat more frequent. In the study reported on 119 patients with folate deficiency by Savage and associates,⁽³⁸⁾ eleven had normal serum homocysteine levels. Information on the sequence of changes that occur in developing cobalamin and folate deficiencies has also been derived from studies on patients previously diagnosed and treated for megaloblastic anemia caused by cobalamin or folate deficiency. These patients entered hematological relapse because of discontinued or inadequate treatment.⁽⁴⁶⁾ In all, 42 events were documented in 14 patients and in 13 of those events the serum cobalamin was normal (> 200 pg/ml). In six of these events, both the methylmalonic acid and the homocysteine were raised. On the other hand, both metabolites were normal on only two occasions and only on one of these was the serum cobalamin < 200pg/ml. Elevation of the serum methylmalonic acid level was the single most frequent abnormal test. These observations are consistent with the concept that changes in serum metabolites that are diagnostic of deficiency precede such changes in the serum cobalamin levels.

The clinical usefulness of serum metabolite assays as a sensitive test for diagnosis of cobalamin deficiency is most convincingly demonstrated in patients with atypical presentations such as those who display no anemia or macrocytosis but only neuropsychiatric complications of cobalamin deficiency.^(6,12) It is particularly in patients who have neuropsychiatric symptoms that it becomes important to identify or exclude an underlying and potentially correctable cobalamin deficiency.

The Deoxyuridine Suppression Test

The underlying defect in DNA synthesis that ultimately gives rise to megaloblastic anemia may be detected before there are clear morphologic changes in the blood or bone marrow. Although the molecular basis for megaloblastosis has not been fully elucidated, it is known that in both folate and cobalamin deficiencies, the defect in DNA synthesis is caused by a failure to convert adequate amounts of deoxyuridine to thymidine. This conversion requires a form of folate that is produced in a cobalamin-dependent reaction. Therefore, in cobalamin deficiency there is a state of functional folate deficiency caused by “trapping” of folate in the methyl form. This explains the high serum folate levels frequently encountered in cobalamin deficiency. The biochemical block in DNA synthesis that occurs in either folate or cobalamin deficiency can be demonstrated in short-term bone marrow culture and has been adapted in the form of a test for distinguishing folate from cobalamin deficiency (the deoxyuridine or dU suppression test).⁽⁴⁷⁾ In this test bone marrow is cultured in the presence of 3H-thymidine, which becomes incorporated into DNA. In normal bone marrow, preincubation with dU, which results in the formation of large amounts of deoxythymidine (dT), dilutes the 3H-thymidine incorporation into DNA. However, in either folate or cobalamin deficiencies, the conversion of dU to dT is interdicted, resulting in a failure of suppression of 3H-thymidine incorporation into DNA. This can be corrected in vitro by the addition of cobalamin when the underlying cause is cobalamin deficiency or by folate in the form of 5-methyltetrahydrofolate when the cause is folate deficiency.

Therapeutic Response

If the diagnosis of cobalamin or folic acid deficiency is in doubt, then the response to specific therapy with either cobalamin or folic acid can be determined. The reticulocyte count will rise to reach a peak within 5-8 days of starting daily doses of the appropriate vitamin. Other than the early rise in reticulocyte count, evidence of a response may also be provided by one or more of several changes in blood count after initiation of a course of treatment. An increase in hematocrit of at least 5% or a decrease in MCV of 5 fL or more constitutes objective evidence of a therapeutic response. This is not, however, indicative of a complete response.

Sequence of Development of Abnormal Laboratory Tests

As in any nutrient deficiency anemia, there is a sequence of changes that occurs in deficiencies of cobalamin or folic acid. At progressive stages during the evolution of the deficiency, demonstrable abnormalities in various laboratory tests occur, as do clinical manifestations of the deficiency state. These sequential changes begin to develop after a critical level of depletion of body stores of the vitamin have occurred. Lowering (below the reference range) of serum levels of the vitamin are usually preceded by biochemical effects that can be demonstrated by changes in the levels of metabolites in the blood and urine. Finally, tissue effects occur, such as megaloblastic changes, macrocytosis and anemia. A general outline of the temporal sequence of events is shown in Table 3. In all situations resulting from impairment of absorption, the time to onset of deficiency will depend on several factors, including the size of the body store, the extent of impairment of absorption (partial or complete) and, in diseases like pernicious anemia, the rate of progression of the disease.

Table 3. The sequence of changes during development of cobalamin and folate deficiencies

I	Negative vitamin balance
	⇓
II	Decrease of body vitamin stores (Fall* in serum cobalamin, folate, RBC folate)
	⇓
III	Functional (biochemical) vitamin deficiency (Raised serum methylmalonic acid and/or homocysteine, abnormal dU suppression)
	⇓
IV	Depletion of body vitamin stores (Low** serum cobalamin, folate, RBC folate)
	⇓
V	Structural-morphologic evidence of deficiency (Megaloblastic change, macrocytosis, anemia, demyelination)
	* “fall” indicates a progressive decrease, still within the normal reference range
	** “low” indicates a level below the normal reference range

Little is actually known about the chronological sequence of changes that occur during the development of cobalamin or folate deficiencies. It is known that the rate of onset of clinical and laboratory evidence of deficiency is more rapid for folate than for cobalamin deficiency. This relates, in large measure, to the relative size of the body stores and the daily requirement for the two vitamins. Consequently, the time from cessation of intake to development of the full-blown manifestations of folate deficiency is four to five months.⁽⁴⁸⁾ No similar information is available for cobalamin, but from observations on the time to development of cobalamin deficiency in patients following total gastrectomy, it is known that depletion of normal body stores takes several years.⁽¹⁰⁾ At what point during the course of developing deficiency serum metabolite levels increase above the normal range is unknown. It is probable that the rise above normal in metabolite levels precedes the fall in serum vitamin levels below the reference range.⁽³⁶⁾ This assumption is based, in part, on the description of patients with low normal serum cobalamin concentrations who have raised metabolite levels, which fall following treatment with cobalamin. In some patients, cobalamin treatment also resulted in objective improvement in the clinical picture, such as a fall in MCV.⁽⁴³⁾ Additional evidence that the appearance of abnormal serum metabolite concentrations precedes the finding of deficient serum vitamin levels comes from a report of normal serum cobalamin (> 200pg/ml) in 9 of 173 patients (5.2%) with confirmed cobalamin deficiency and raised serum levels of both methylmalonic acid and homocysteine who had no other known explanation for the abnormal metabolites.⁽⁴⁶⁾ Five of the patients had neurological complaints attributable to the cobalamin deficiency and in 11 there were hematological abnormalities

Combined Use of Vitamin and Metabolite Measurements

The finding of a subnormal cobalamin or folic acid level alone is sufficient to arrive at a presumptive diagnosis of deficiency of the vitamin if this fits with the clinical picture. If it is necessary to confirm the presence of deficiency in a patient with low serum cobalamin, then confirmation can be obtained by measuring serum methylmalonic acid; if folic acid is low, then serum homocysteine is the metabolite measurement of choice; if both vitamins are low, both metabolites should be measured. Methylmalonic acid and homocysteine levels are also helpful when blood levels of folic acid and cobalamin are normal yet there is suspicion of a deficiency of one of these vitamins, or if there has been an incomplete or failed response following treatment with one or both vitamins.

Conclusions

There is mounting evidence that deficiencies of folate or cobalamin may be associated with features other than macrocytic anemia. These other disease manifestations may either precede or accompany the standard hematological features of these vitamin deficiencies. The advent of sensitive serum metabolite measurements has made it possible to identify subtle or atypical forms of vitamin deficiency that may be associated with unusual or previously undiscovered disease manifestations. Thus, in patients who display only neurological manifestations of disease, underlying cobalamin deficiency may be revealed by the finding of raised serum or urine levels of methylmalonic acid. Similarly,

unsuspected folate deficiency may be disclosed by the finding of a raised serum homocysteine. This may have important implications with respect to disease risk, since there is mounting evidence that suboptimal folate nutritional status may be associated with increased risks of vascular disease, neoplasia and birth defects.

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