REVIEW ARTICLE

Laboratory Diagnosis of Vitamin B_{12} and Folate Deficiency

A Guide for the Primary Care Physician

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At one time, the diagnosis of a deficiency of vitamin B_{12} or folate was considered to be relatively straightforward. As knowledge has accumulated, the limitations of such tests as serum vitamin level measurements and the Schilling test have become apparent. With the development of newer tests, atypical and subclinical deficiency states have been recognized. In this review, available tests used in the diagnosis of vitamin B_{12} and folate deficiency are discussed, and a rational approach to the diagnosis of these deficiency states is presented. *Arch Intern Med. 1999;159:1289-1298*

VITAMIN B_{12} (COBALAMIN)

Strictly speaking, vitamin B_{12} refers to only cyanocobalamin, but several other Cobalamins have identical nutritional properties, and in the hematology literature, the terms cobalamin (Cbl) and vitamin B_{12} are used interchangeably. Cobalamin is synthesized by bacteria and is found in soil and in contaminated water. Foods of animal origin (meat, eggs, and milk) are the primary dietary sources. The amount of Cbl in the average Western diet (5-15 µg/d) is more than sufficient to meet the recommended dietary allowance of 2 µg/d. Therefore, except in strict vegetarians, the presence of Cbl deficiency implies the presence of an absorptive problem. The body stores a large amount of Cbl (2-5 mg) relative to daily requirements. Therefore, it takes 2 to 5 years to develop Cbl deficiency even in the presence of severe malabsorption. **Table 1** summarizes the steps in Cbl absorption and transport.

FOLIC ACID (FOLATE)

The term folic acid can designate a specific compound, pteroylglutamic acid, but more commonly it is used as a general term for a class of related compounds (also called folates) with similar nutritional activity. Folates are synthesized by microorganisms and by plants and are widely distributed in the diet. Vegetables, fruits, dairy products, and cereals are the most important sources. Americans ingest an average of 200 to 300 µg/d, which is close to the recommended dietary allowance. Unlike Cbl, the body stores of folate (5-10 mg) are small relative to daily requirements. **Table 2** summarizes the steps in folate absorption and distribution.

BIOCHEMICAL ASPECTS OF Cbl AND FOLATE DEFICIENCIES

In mammals, only 2 Cbl-dependent enzymatic reactions have been identified. One involves the conversion of methylmalonyl–coenzyme A (CoA) to succinyl-CoA using adenosyl-Cbl (Ado-Cbl) as a cofactor (**Figure 1**). It can be seen that a deficiency of Cbl will lead to an increase in methylmalonyl-CoA and in its hydrolysis product methylmalonic acid (MMA).

The other Cbl-dependent reaction involves the synthesis of methionine from homocysteine (Hcy) using methyl-Cbl as a cofactor (**Figure 2**). This reaction is believed to be of primary importance in explaining the pathophysiological aspects of Cbl and folate deficiencies. The following points should be noted:

1. A deficiency of either Cbl or folate will impair the production of tetrahydrofolate. In patients with megaloblastic anemia, this causes a defect in DNA synthesis that prevents cell division in the marrow, whereas RNA synthesis and the synthesis of cytoplasmic components remain unaffected. The result is the production of large erythrocytes. In addi-

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Table 1. Cobalamin (Cbl) Absorption and Transport

Step	Clinical Significance
In many foods, Cbl is bound to proteins. When food enters the stomach, acid facilitates the release of Cbl. The presence of food also stimulates the secretion of intrinsic factor (IF), a glycoprotein produced by gastric parietal cells. However, in conditions of low pH, unbound Cbl preferentially attaches to salivary haptocorrin (formerly referred to as R binder) rather than to IF.	If gastric production of acid is reduced, Cbl absorption from food may be impaired, even when IF is present. However, unbound Cbl in supplemental vitamin preparations will still be absorbed normally. A decrease in serum Cbl levels has been demonstrated after 3-4 y of omeprazole therapy, and it has been suggested that serum Cbl levels be monitored in patients undergoing prolonged treatment with proton pump inhibitors.[1]
In the neutral pH of the duodenum, IF displaces the salivary haptocorrin with the aid of pancreatic enzymes.	Patients with pancreatic insufficiency may have reduced Cbl absorption, although they rarely become Cbl deficient.[2]
The IF-Cbl complex passes through the small intestine and is taken up by receptor-mediated endocytosis in the terminal ileum.	Bacterial overgrowth or fish tapeworm infestation may cause Cbl deficiency because of competition for Cbl in the intestine before the IF-Cbl complex reaches the terminal ileum.
Cbl is released from the complex and binds either to the transcobalamin (TC) II protein or to serum haptocorrins (formerly referred to as TC I and TC III). TC II, which accounts for only 10%-30% of the measurable serum Cbl level, is the physiologically important transport protein.	Congenital TC II deficiency may cause megaloblastic anemia despite normal serum Cbl levels. In some patients with myeloproliferative disorders or liver disease, levels of serum haptocorrins may increase, leading to increases in the measured serum Cbl level.
An active enterohepatic circulation conserves the vitamin. Once Cbl reenters the gut in the bile, IF is again necessary for reabsorption.	Compared with patients with pernicious anemia, strict vegetarians (who produce IF normally) retain more Cbl from the enterohepatic circulation and therefore take much longer (10-20 y) to become Cbl deficient.

Table 2. Folate Absorption and Transport

Step	Clinical Significance
Dietary folates may be susceptible to oxidation when heated or may leach into cooking water.	50% or more of the vitamin may be lost during cooking, especially if food is boiled.[3]
Most folates in food are polyglutamate derivatives and must be deconjugated to monoglutamate forms in the gut before absorption.	Some drugs (eg, sulfasalazine) may interfere with deconjugation of dietary folates and inhibit absorption. Also, inhibition of the brush border conjugase enzyme by food components may contibute to the observed variability and unpredictability of dietary folate absorption.[4]
Absorption of folate occurs in the jejunum. After extensive jejunal resection, the transport system can be induced in the ileum.	Malabsorption as the primary cause of folate deficiency is unusual, unless extensive small bowel disease (eg, sprue) is present.
After absorption, the principal storage site is the liver. Distribution of folate to other tissues depends mostly on an enterohepatic recirculation, in which folate in a methylated form is reabsorbed from bile into the serum.	Interruption of bile flow results in an immediate decrease in serum folate levels. Similarly, alcohol ingestion interferes with the release of folate from the liver into the bile, causing a rapid decrease in the serum folate level.[5]
After folate enters most tissues, including erythrocytes, it remains throughout the life span of the cell.	Erythrocyte (red blood cell) folate levels reflect the state of folate nutrition at the time the cell is first formed and are slow to decrease after folate deprivation.

tion, a deficiency of either vitamin will result in accumulation of the substrate Hcy.

2. The production of S-adenosylmethionine (SAM) from methionine is thought to be critical to nervous system function, which probably explains the neuropathic effects of Cbl deficiency. Although it would seem that folate should be required for the production of SAM, neurologic complications are unusual in folate deficiency, probably because the cell has developed alternate mechanisms that preserve the supply of SAM in times of folate deprivation.[6]

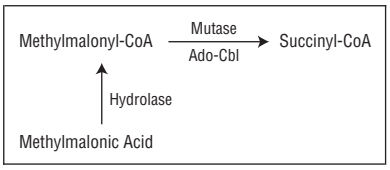

Figure 1. *Conversion of methylmalonyl–coenzyme A (CoA) to succinyl-CoA. Ado-Cbl indicates adenosyl-cobalamin.*

3. Unlike dietary folate, synthetic folic acid (pteroylglutamic acid) is reduced directly to tetrahydrofolate without the need for Cbl as a cofactor. For this reason, the administration of supplemental amounts of folic acid to a Cbl-deficient patient may correct megaloblastic hematologic changes and still allow neurologic disease to progress.

CLINICAL ASPECTS OF Cbl AND FOLATE DEFICIENCY

Cobalamin deficiency is usually suspected because of the presence of unexplained anemia, macrocytosis, or neurologic disease. The more common neurologic symptoms are paresthesias, numbness, and ataxia, although dementia and psychosis may occur.[7] Although hematologic abnormalities frequently develop before the onset of neurologic disease, more than one quarter of patients with neurologic manifestations of Cbl deficiency have either a normal hematocrit or a normal mean corpuscular volume (MCV); sometimes both values are normal.[8]

Causes of Cbl deficiency are listed in **Table 3**. Although pernicious anemia has been considered to be the most common disorder leading to the development of Cbl deficiency, many patients with suspected deficiency in recent series have normal Schilling test results. Controversy exists as to whether these patients represent cases of protein-bound Cbl malabsorption caused by relative achlorhydria (Table 1) or in fact are being incorrectly diagnosed as Cbl deficient in the first place.[9,10]

Folate deficiency is usually suspected because of the presence of unexplained anemia or macrocytosis. When neuropsychiatric disorders are encountered in folate-deficient patients, they are perhaps more likely to be caused by coexisting Cbl deficiency or other disorders.[11] How-

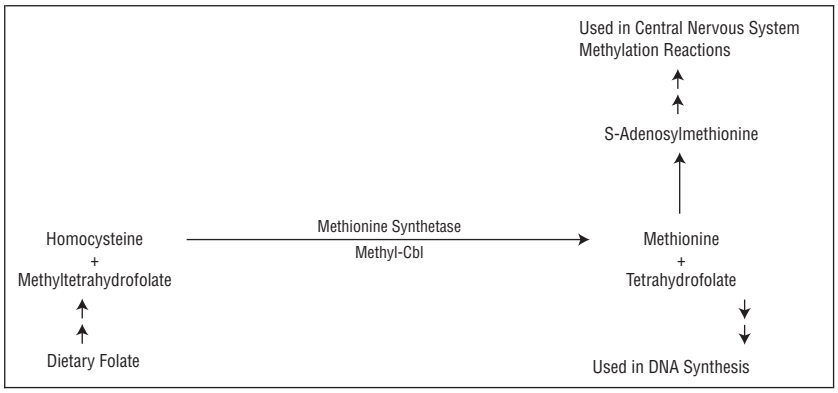

Figure 2. *Synthesis of methionine from homocysteine. Cbl indicates cobalamin.*

ever, there is some evidence that, on occasion, depression, dementia, and other neurologic syndromes may be caused by folate deficiency.[12] Causes of folate deficiency are listed in **Table 4**.

SPECIFIC TESTS USED IN THE DIAGNOSIS OF Cbl AND FOLATE DEFICIENCIES

Mean Corpuscular Volume

Macrocytosis (mean corpuscular volume [MCV] >100 fl) with or without anemia is a common finding in adults undergoing automated complete blood cell counts. Patients with an elevated MCV may have a megaloblastic disorder, defined by the presence of morphologic changes in the bone marrow that reflect defective DNA synthesis, or a nonmegaloblastic disorder. The presence of megaloblastic changes in the marrow usually implies a diagnosis of Cbl or folate deficiency. However, some myelodysplastic disorders are associated with similar morphologic changes, and the administration of zidovudine or of certain chemotherapeutic agents that affect DNA synthesis can induce a megaloblastic marrow. Nonmegaloblastic causes of macrocytosis are listed in **Table 5**.

In patients with either Cbl or folate deficiency, the MCV tends to increase before the hemoglobin level decreases significantly.[13,14] However, even when there is biochemical evidence of vitamin deficiency, the MCV often remains within the reference range,[15] especially if concurrent iron deficiency or thalassemia is present. The MCV also lacks specificity for the diagnosis of Cbl or folate deficiency. In a study of 100 patients with an MCV greater than 115 fl (much higher than the usual upper limit of the reference range), only 50% had subnormal values of serum Cbl, erythrocyte folate, or both; only an MCV of 130 fl or higher was found to reliably predict the presence of low vitamin levels.[16]

Peripheral Blood Smear

In discussions concerning the evaluation of macrocytic anemia, the importance of examining the peripheral smear is often emphasized.[11,17] However, compliance with this recommendation is poor among physicians other than hematologists. To the skilled examiner, a variety of smear findings may provide diagnostic clues. For example, the presence of oval macrocytes suggests a megaloblastic disorder, whereas the presence of stomatocytes is significantly more common among patients with macrocytosis caused by alcoholism than among Cbl-deficient patients.[18] The presence of hypersegmented neutrophils has been said to be highly sensitive and specific for the diagnosis of megaloblastic anemia.[11,19] For example, in one study[20] in which hypersegmentation was defined as the presence of at least 1 neutrophil with 6 lobes or more, the sensitivity of this finding for the diagnosis of megaloblastic anemia was 98%. However, patients in this study had unequivocal megaloblastic changes on bone marrow examination and low serum vitamin concentrations. In more recent studies[21,22] of patients with mild Cbl deficiency, the finding of neutrophil hypersegmentation was neither sensitive nor specific. It is doubtful that examination of the smear, especially by physicians other than hematologists, will avoid the need for use of less observer-dependent tests in the evaluation of macrocytic anemia.

Table 3. Causes of Cobalamin (Cbl) Deficiency

Malabsorption
Pernicious anemia
Gastrectomy or gastric bypass
Protein-bound Cbl malabsorption
Ileal disease or resection
Pancreatic insufficiency
Drug induced (colchicine, neomycin, *p*-aminosalicylic acid, or omeprazole)
Congenital absence or dysfunction of intrinsic factor
Biologic competition for dietary Cbl
Bacterial overgrowth syndromes
Fish tapeworm infestation
Dietary lack
Strict vegetarianism
Impaired utilization
Congenital transcobalamin II deficiency

Table 4. Causes of Folate Deficiency

Inadequate intake
Increased demands
Pregnancy
Infancy
Diseases associated with rapid cellular proliferation (hemolysis, leukemia, exfoliative dermatitis, and others)
Malabsorption
Jejunal diseases
Short-bowel syndrome
Biologic competition for dietary folate
Bacterial overgrowth
Drug induced (mechanisms often unclear)
Anticonvulsants (phenytoin, primidone, phenobarbital)
Oral contraceptives
Sulfasalazine
Methotrexate
Triamterene
Alcohol

Serum Cbl Measurement

Depending on the technique used in measurement, the lower limit of normal for serum Cbl is variable; usually it is set at about 148 pmol/L (200 pg/mL). Published estimates of the sensitivity and specificity of the test in the diagnosis of Cbl deficiency vary widely. This relates in part to the lack of a consistently defined

Table 5. Nonmegaloblastic Causes of Macrocytosis With or Without Anemia

Disorder	Cause of Macrocytosis	Comments
Alcoholism	Direct effect of alcohol on the bone marrow	Concurrent folate deficiency or liver disease may contribute to the macrocytosis
Liver disease	Defective DNA synthesis or accelerated erythropoiesis	Concurrent folate deficiency or alcoholism may contribute to the macrocytosis
Hemolysis	Accelerated erythropoiesis with reticulocytosis; hemolysis may also increase folate requirements	The presence of associated folate deficiency may be masked by an increase in the serum folate level caused by red blood cell lysis
Posthemorrhagic anemia	Accelerated erythropoiesis with reticulocytosis	. . .
Hypothyroidism	Uncertain	Mean corpuscular volume is usually normal or only slightly elevated; pernicious anemia is more prevalent in patients with autoimmune thyroid disease
Cold agglutinin disease	Artifactual: clumping of red blood cells	. . .
Severe hyperglycemia	Artifactual: swelling of red blood cells	. . .

Table 6. Causes of Misleading Serum Cobalamin Levels

- Falsely normal
  - Myeloproliferative disorders
    - Polycythemia vera
    - Chronic myelogenous leukemia
    - Others
  - Liver disease
  - Congenital transcobalamin II deficiency
  - Intestinal bacterial overgrowth
  - Antecedent administration of cobalamin
- Falsely low
  - Folate deficiency
  - Pregnancy
  - Use of oral contraceptives
  - Congenital deficiency of serum haptocorrins
  - Multiple myeloma

gold standard for the diagnosis and in part to varying study designs.

For example, in considering the sensitivity of the test, one study[23] estimated that 90% to 95% of patients with Cbl deficiency had levels of less than 148 pmol/L (200 pg/mL), 5% to 10% had levels of 148 to 221 pmol/L (200-300 pg/mL), and less than 1% had levels greater than 221 pmol/L (300 pg/mL). Patients were considered to be Cbl deficient if they had characteristic clinical features that responded to Cbl therapy, although for some patients follow-up data after therapy were lacking. Because the authors did not systematically evaluate most patients with normal serum Cbl values, they recognized that the 90% to 95% figure for test sensitivity was probably an overestimate. At the other end of the spectrum are prospective studies of elderly outpatients in which elevations of serum and urine metabolite levels (see below) are used as evidence of Cbl deficiency. With this design, only about half of ostensibly Cbl-deficient patients have serum levels below 148 pmol/L (200 pg/mL),[24,25] with 14% having levels above 258 pmol/L (350 pg/mL).[25] However, there has been concern that the use of metabolite assays as a gold standard for the diagnosis of Cbl deficiency may lead to overdiagnosis and overtreatment.[10,26] Causes of falsely normal serum Cbl levels are listed in **Table 6**. Note that intestinal bacterial overgrowth, a cause of Cbl deficiency, may also cause falsely normal serum Cbl levels because of the production of biologically inactive Cbl analogues by the bacteria.[27]

The reported specificity of a low serum Cbl level in predicting Cbl deficiency is also variable.[28,29] In a study of unselected patients with suspected Cbl deficiency and levels below 148 pmol/L (200 pg/mL), 60% of evaluable patients had a hematologic or neurologic response to parenteral Cbl administration.[28] The specificity of a serum Cbl level below 74 pmol/L (100 pg/mL) was 90% in this study. In most cases, the reason for a falsely low serum Cbl is not apparent, although there are recognized causes (Table 6). It is important to note that as many as one third of patients with folate deficiency will have low serum Cbl levels (some with levels <74 pmol/L [100 pg/mL]) that return to normal with folate therapy.[14] The mechanism for reduced serum Cbl levels in some folate-deficient patients is poorly understood; this finding is less frequent in alcoholic populations because of the tendency of liver disease to increase serum Cbl levels. If after administration of folate low serum Cbl levels do not return to normal, concurrent Cbl deficiency may be present.[30]

Serum Folate and Erythrocyte (Red Blood Cell) Folate

The lower limit of the reference range for serum folate varies depending on technical factors but is usually set at about 6.8 nmol/L (3 ng/mL). Serum folate levels decrease within a few days of dietary folate restriction,[31] although tissue stores may be normal. This is perhaps why serum folate levels are low in about one third of hospitalized patients.[32] Serum folate levels increase with feeding, and the use of fasting determinations has been recommended.[33] However, the contention that a brief period of adequate feeding will normalize levels in deficient patients has been questioned.[34,35] Folate concentrations within erythrocytes are much higher than in serum, and even a slight degree of hemolysis will cause a falsely elevated serum folate level.

The sensitivity of serum folate measurement for the diagnosis of clinically significant folate deficiency is uncertain; it is known that in some patients with clear-cut megaloblastic anemia caused by folate deficiency, the serum folate level is normal or only slightly low.[35,36] Serum folate measurement is also nonspecific; low levels are often seen in patients without other evidence of deficiency. Alcohol intake in particular may cause a short-term decrease in serum levels in patients with adequate tissue stores (Table 2).

Serum folate levels tend to increase in patients with Cbl deficiency, presumably because impairment of the methionine synthetase pathway leads to the accumulation of methyltetrahydrofolate, the principal form of folate in the serum. Serum folate levels are above normal in 20% of patients with pernicious

Table 7. Causes of Alterations in Serum Levels of Methylmalonic Acid and Homocysteine

May elevate methylmalonic acid
Cobalamin deficiency
Renal insufficiency
Hypovolemia
Inherited metabolic defects
May reduce methylmalonic acid
Antibiotic-related reductions in bowel flora
May elevate homocysteine
Cobalamin deficiency
Folate deficiency
Pyridoxine deficiency
Renal insufficiency
Hypovolemia
Hypothyroidism
Psoriasis
Inherited metabolic defects

Table 8. Performance of Serum Metabolite Assays in Patients With Clinically Defined Cobalamin (Cbl) or Folate Deficiency*

	Prevalence in Patients With, %	
Metabolic Levels	Cbl Deficiency	Folate Deficiency
Elevated serum MMA	98	12
Elevated serum Hcy	96	91
Serum MMA elevated, Hcy normal	4	2
Serum Hcy elevated, MMA normal	1	80
Serum MMA and Hcy both normal	0.2	7

**Data from Savage et al.[36] Elevations were defined as >3 SDs above the mean. MMA indicates methymalonic acid; Hcy, homocysteine.*

anemia but inexplicably are low in up to 10% of affected patients.[32]

Because of the limitations of the assay for serum folate, the determination of red blood cell (RBC) folate levels has been advocated as a better measure of folate tissue stores. As noted previously, erythrocytes incorporate folate as they are formed, and levels remain constant throughout the life span of the cell; RBC folate levels are less sensitive to short-term dietary effects than are serum folate levels. In addition, the RBC folate level has been shown to correlate more strongly than the serum folate level with the presence of megaloblastic changes in the peripheral blood smear and bone marrow.[37] However, as with serum folate testing, limitations of sensitivity and specificity reduce the value of RBC folate measurement. For example, only 76% of pregnant women[38] and 69% of alcoholic persons[35] with megaloblastic erythropoiesis believed to be caused by folate deficiency had low RBC folate levels (<340 nmol/L [150 ng/mL]). Most falsely normal values were in the borderline range of 340 to 567 nmol/L (150-250 ng/mL), but values in many patients with normal erythropoiesis also fall within this range. The test also lacked specificity in these studies; 19% of pregnant women and 31% of alcoholics had RBC folate levels less than 340 nmol/L without evidence of megaloblastic erythropoiesis. Although it can be argued that such patients have early folate deficiency, it is difficult to be certain in the absence of a well-defined gold standard for the diagnosis. An important cause of falsely low RBC folate levels is Cbl deficiency; 60% of patients with pernicious anemia have low RBC folate levels,[32] presumably because Cbl is required for the normal transfer of methyltetrahydrofolate from plasma to cells.[39]

Most studies of the performance of RBC folate measurement have used microbiologic assays that are no longer available in clinical laboratories today; instead, the same radioassays used for serum Cbl and serum folate measurement are used to determine RBC folate concentration. Serious questions have been raised about the reliability of these isotopic methods for determination of RBC folate, and one recent review recommended against their use in diagnostic testing.[14]

Methylmalonic Acid and Homocysteine

The use of metabolite measurements for the diagnosis, confirmation, and differentiation of Cbl and folate deficiencies has been made possible by the development of accurate assays for serum and urine MMA and serum Hcy. As discussed previously, elevations of both metabolite levels are anticipated in patients with Cbl deficiency, whereas only Hcy levels would be expected to increase in patients with folate deficiency. Factors other than Cbl or folate status can increase serum MMA levels (**Table 7**). Renal insufficiency causes serum levels of both metabolites to increase, although the elevations are typically modest compared with those caused by Cbl deficiency.[36] Antibiotic drug therapy–related reductions in bowel flora may decrease the serum MMA level. Measurement of urinary MMA levels adjusted for creatinine excretion may be a useful alternative to serum MMA measurement, especially for patients with renal disease.[24] Serum MMA and Hcy levels also increase with age, although this may be caused by an increased prevalence of subclinical vitamin deficiency in the elderly.[40] The reference range for these tests is variable depending on the laboratory.

Serum MMA and Hcy measurement seems to be sensitive for the diagnosis of Cbl deficiency. In a study[28] using a response to Cbl therapy as evidence of deficiency, 86% of Cbl-responsive patients had elevated levels of MMA and 85% had elevated levels of Hcy; 94% had elevated levels of at least 1 serum metabolite. Data from another study[36] that looked at the sensitivity of metabolite tests in the diagnosis of Cbl and folate deficiencies are summarized in **Table 8**. Patients had either low serum Cbl or low serum folate levels and clinical evidence of deficiency based on hematologic findings or a hematologic response to replacement therapy. Serum MMA and Hcy levels were highly sensitive for the diagnosis of Cbl deficiency, although some patients had elevated levels of only 1 metabolite (usually MMA). The sensitivity of the serum Hcy level for the diagnosis of folate deficiency was somewhat lower. Also, in 15 of 123 patients with folate deficiency, the serum MMA level was elevated. However, 11 of these patients had renal insufficiency and 3 had severe volume depletion, which are known causes of elevated levels of serum MMA.

The sensitivity of metabolite measurements for milder deficiency states is uncertain. In Cbl deficiency, it seems likely that elevated levels of serum MMA and Hcy usually precede the development of hematologic abnormalities and reductions in the serum Cbl level.[34] One screening study of geriatric outpatients found that 14.5% had serum Cbl levels of 221 pmol/L (300 pg/mL) or less and elevated levels of at least 1 serum metabolite.[25] These patients were believed to be Cbl deficient despite the relative absence of hematologic or neurologic findings based on their response to Cbl therapy: all treated patients had a marked decrease in metabolite levels, and a significant number had a decrease of 2 fl or more in their MCV.

It is difficult to estimate the specificity of metabolite tests for the diagnosis of Cbl or folate deficiency. There are several known causes of false-positive results, especially for serum Hcy (Table 7). Particularly in the elderly, the prevalence of an elevated serum MMA or Hcy level is much higher than the prevalence of low serum vitamin levels or of clinically evident vitamin deficiency.[25,41] Is this because the tests are nonspecific or because subclinical deficiencies of Cbl, folate, and pyridoxine are highly prevalent in older patients? The answer is uncertain, but caution has been urged in the interpretation of elevated metabolite levels in the absence of other evidence of deficiency.[10,26]

In patients with Cbl or folate deficiency, metabolite levels tend to normalize 7 to 14 days after the onset of replacement therapy.[34] As noted previously, treatment-related reductions in levels of serum MMA and Hcy have been equated with evidence of deficiency.[25] In addition, it has also been reported that in folate-deficient patients who are inappropriately treated with Cbl, the serum Hcy remains elevated, whereas in Cbl-deficient patients who are inappropriately treated with folate, the serum MMA level remains elevated.[42] Therefore, measurement of metabolite levels before and after initiation of treatment has been proposed as a means of distinguishing Cbl deficiency from folate deficiency in cases in which both serum vitamin levels are low. However, other reports[43,44] suggest that normalization of metabolite levels after initiation of folate and Cbl supplement use is a nonspecific response that may occur in nondeficient subjects.

Table 9. Interpretation of Schilling Test Results in Cobalamin (Cbl)-Deficient Patients

Result*	Possible Interpretations
Stage 1 normal	Dietary deficiency
	Protein-bound Cbl malabsorption
	Hypochlorhydria
	Partial gastrectomy
	Congenital transcobalamin II deficiency
Stage 1 abnormal; stage 2 normal	Pernicious anemia
	Previous gastrectomy or gastric bypass
	Congenital absence or dysfunction of intrinsic factor
	Inadequate urine collection during stage 1
Stages 1 and 2 both abnormal	Ileal disease or resection
	Pernicious anemia with ileal dysfunction secondary to prolonged Cbl deficiency
	Pernicious anemia with inadequate urine collection during stage 2
	Renal insufficiency
	Inadequate urine collection during both stages
	Bacterial overgrowth syndromes
	Fish tapeworm infestation
	Pancreatic insufficiency

**Some results (particularly those in the 7%-10% range) may be reported as indeterminate.*

Antiparietal Cell and Anti–Intrinsic Factor Antibodies

A variety of autoantibodies can be detected in serum samples from patients with pernicious anemia, including antibodies to gastric parietal cells and to intrinsic factor (IF). Antiparietal cell antibodies may have a causative role in the autoimmune gastritis of pernicious anemia[45] and are present in about 85% of affected patients.[32,46] Antiparietal cell antibodies are nonspecific; they are often present in patients with autoimmune endocrinopathies and are present in 3% to 10% of healthy persons, depending on age.[47]

Compared with the antiparietal cell assay, the anti–IF antibody test is relatively insensitive; only about half of the patients with pernicious anemia have detectable anti-IF antibody.[32,48] However, anti-IF antibody is highly specific; it is rarely present in healthy patients or in patients with other autoimmune disorders,[11] although it may be detected in some patients with Graves disease. In such cases, a nonprogressive atrophic gastritis (with normal production of IF) has been described.[49]

Schilling Test

The Schilling test is used in patients with Cbl deficiency to document defects in absorption of the orally administered vitamin. In the stage 1 test, a dose of crystalline Cbl labeled with radioactive cobalt is given by mouth, and the subsequent percentage of ingested radiolabeled Cbl excreted during 24-hour urine collection is measured. If the results of the first stage of the Schilling test are abnormal, a second stage is performed 3 to 7 days later, using oral administration of labeled Cbl and IF. Possible interpretations of the results of the Schilling test are listed in **Table 9**. It is important to note the following:

1. With a partial deficiency of IF or gastric hypochlorhydria, there may be sufficient absorption of the crystalline Cbl administered in stage 1 to yield a normal result, although malabsorption of dietary Cbl may be present.

2. A significant percentage of patients with pernicious anemia may malabsorb the Cbl-IF complex (resulting in an abnormal stage 2 test result) because of ileal dysfunction, which is induced by Cbl deficiency and reversible with prolonged Cbl administration.[50] However, in this set-

ting it is likely that the stage 2 result would increase compared with the stage 1 result, even if still abnormal.

3. Inadequate urine collection is a significant cause of falsely abnormal Schilling test results,[51] particularly if the urine aliquot produced 8 to 12 hours after administration of the radiolabeled Cbl is lost.

4. Renal insufficiency may delay excretion of the labeled Cbl and cause falsely abnormal results; extending urine collection for an additional 24 hours or measuring radiolabeled Cbl in plasma samples may be required to document normal absorption of Cbl.[11,52]

A variation of the standard Schilling test is the dual isotope (or "single-stage") Schilling test, which combines the stage 1 and stage 2 tests. However, use of this test has been discouraged because of a high rate of indeterminate results compared with the standard protocol.[50]

Because of its many limitations, the Schilling test is probably overused in the evaluation of Cbl deficiency; often the result does not alter clinical management.[9] Arguments in favor of performing the Schilling test (especially if relevant antibody tests are negative) include the ability to confirm a diagnosis of pernicious anemia, which may affect the risk of the development of other autoimmune disorders and some cancers, and the occasional ability to diagnose occult intestinal or pancreatic disease. Also, a normal Schilling test result, which may indicate dietary deficiency or protein-bound Cbl malabsorption, increases the likelihood of successful oral Cbl replacement therapy.

Therapeutic Trials

If a trial of therapy is elected as a diagnostic tool, a control period of 7 to 10 days before therapy is started is recommended to ensure stability of the RBC and reticulocyte counts because apparently "spontaneous" hematologic responses may occur without specific therapy.[53] In patients with megaloblastic anemia, replacement therapy with the appropriate vitamin will result in an increase in the reticulocyte count in 2 to 3 days, with a peak reticulocyte count in 5 to 8 days.[32] The RBC count will increase appreciably within 1 week and will normalize within 4 to 8 weeks.[11] Typically, the MCV increases for the first 3 to 4 days (presumably because of reticulocytosis) then begins to decrease, reaching the reference range in 25 to 78 days.[54] Several points of caution should be noted:

1. In patients with Cbl or folate deficiency, the response to appropriate therapy may be absent or incomplete if concurrent iron deficiency is present or in the presence of other acute or chronic diseases that impair reticulocyte response.

2. Results of the therapeutic trial may be ambiguous in patients who are only mildly anemic because the degree of the response is proportional to the severity of the anemia. For example, the reticulocyte count peaks at 50% to 70% in patients with an initial RBC count of less than 1×10^{12}/L but only increases to 4% to 9% in patients with an initial RBC count of 3.0 to 3.6×10^{12}/L.[32]

3. As discussed earlier, administration of therapeutic doses of folate may correct the hematologic abnormalities of Cbl deficiency and still allow neurologic damage to progress. Although administration of therapeutic doses of Cbl to folate-deficient patients does not usually result in hematologic improvement, it has been reported to do so on occasion.[55] Because of this potential for "cross-reactivity" of responses, a therapeutic trial may be of most value if the patient does not respond.[14] It has been proposed that the use of small ("physiologic") intramuscular doses of Cbl (3 µg/d) or folate (0.1-0.2 mg/d) can improve the specificity of the therapeutic trial.[56] However, low-dose trials are difficult to perform because often the vitamins are not available at the required dosage levels, and concurrent dietary restriction is necessary.

In nonanemic patients, a response to therapy of nonhematologic clinical parameters is sometimes cited as evidence of Cbl or folate deficiency despite the often subjective nature of the response. Neurologic disease related to Cbl deficiency may improve within weeks, especially if the symptoms are of short duration.[32] In some patients, however, neurologic manifestations will not improve despite appropriate Cbl replacement, although in all cases progression of disease is halted. As discussed previously, in patients suspected of Cbl or folate deficiency who are not anemic (or who have only a mild anemia), measurement of metabolite levels (MMA and Hcy) before and during the therapeutic trial may be useful.

Other Tests

The fasting serum gastrin level is elevated in 80% to 90% of patients with pernicious anemia.[32,57] Levels also tend to be elevated in patients with lesser degrees of gastric atrophy who may have malabsorption of protein-bound Cbl despite a normal Schilling test result. Therefore, the presence of an elevated serum gastrin level may be used as indirect evidence of protein-bound Cbl malabsorption. However, the test has limited sensitivity and specificity when used to detect subclinical gastric abnormalities.[58,59]

Bone marrow examination is rarely necessary or useful in the diagnosis of Cbl or folate deficiency. In mild deficiency states, it may be hard to identify unequivocal megaloblasts in the marrow.[11] For example, in a study[8] of nonanemic patients with neurologic abnormalities caused by Cbl deficiency, 2 of 10 marrow smears with evidence of megaloblastic changes were initially read as normoblastic by a consulting hematologist. The test also lacks specificity; as discussed above, "megaloblastoid" changes can be seen in patients with myelodysplastic disorders.

APPROACH TO DIAGNOSIS IN PATIENTS WITH HEMATOLOGIC ABNORMALITIES

In patients with hematologic findings suggestive of Cbl or folate deficiency, it is reasonable to start the evaluation by measuring serum Cbl and fasting serum folate levels. In patients with at least several days of poor dietary folate intake, one may choose to measure the erythrocyte folate level, although the limitations of radioassays for RBC folate should be kept in mind. **Table 10** summarizes the initial diagnostic approach based on serum vitamin levels.

Patients with low serum Cbl levels (<74 pmol/L [100 pg/mL]) usually have Cbl deficiency. **Figure 3** summarizes the diagnostic approach. Because the serum folate level is more likely to increase than to decrease in patients with Cbl deficiency, patients with low levels of both vitamins are likely to have a mixed deficiency. However, if RBC folate level rather than serum folate level is measured, the finding of low levels of both vitamins may be explained by isolated Cbl deficiency because of the propensity of the RBC folate level to decrease in Cbl-deficient patients.

Patients with slightly low or borderline serum Cbl levels (74-221 pmol/L [100-300 pg/mL]) may have Cbl deficiency, but a higher standard of proof is required than for patients with a serum Cbl level of less than 74 pmol/L (100 pg/mL). It is reasonable to order serum MMA and Hcy determinations in this setting and to use the results of the serum folate and metabolite measurements to determine the course of action. **Table 11** summarizes the diagnostic approach. If the serum folate level is also low, isolated folate deficiency is a diagnostic consideration because serum Cbl levels tend to decrease in folate-deficient patients.

Patients with serum Cbl levels greater than 221 pmol/L (300 pg/mL) are unlikely to be Cbl deficient. If the serum folate level is low, it should be replaced. If the serum folate level is normal or if replacement therapy in a patient with a low serum folate level does not result in hematologic improvement, alternative diagnoses should be sought. If an alternative explanation for the hematologic findings cannot be found, one may consider the measurement of metabolite levels (see previously). If serum MMA or Hcy levels are elevated, a therapeutic trial of vitamin replacement therapy may be undertaken.

Table 10. Approach to Initial Evaluation of Patients With Hematologic Abnormalities Suggesting Possible Cobalamin (Cbl) or Folate Deficiency

Results of Serum Vitamin Assays	Interpretation	Approach
Cbl level <74 pmol/L and folate level normal	Probable Cbl deficiency	Go to Figure 3
Cbl level <74 pmol/L and folate level low	Probable mixed deficiency of Cbl and folate	Go to Figure 3
Cbl level 74-221 pmol/L and folate level normal	Possible Cbl deficiency	Go to Table 9
Cbl level 74-221 pmol/L and folate level low	Indeterminate	Go to Table 9
Cbl level >221 pmol/L and folate level normal	Cbl and folate deficiencies unlikely	Consider other causes of hematologic findings (see Table 5)
Cbl level >221 pmol/L and folate level low	Probable folate deficiency	Replace folate; if complete blood cell count abnormalities do not correct completely, consider other causes of hematologic findings (see Table 5)

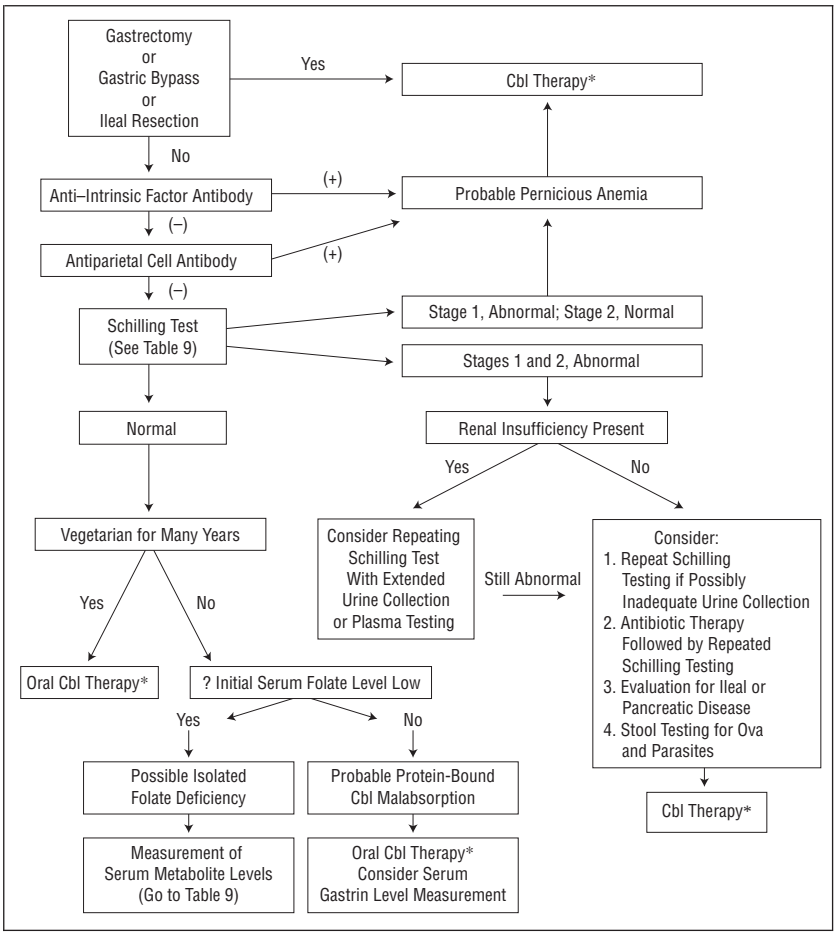

Figure 3. *Approach to the patient with hematologic abnormalities and a serum cobalamin (Cbl) level less than 74 pmol/L (100 pg/mL). The asterisk indicates that the complete blood cell count of patients treated with oral or intramuscular Cbl should be monitored until the hematologic abnormalities are corrected completely; if the initial serum folate level is low, it is prudent to administer folic acid in addition to Cbl.*

APPROACH TO DIAGNOSIS IN PATIENTS WITH ISOLATED NEUROLOGIC FINDINGS

As discussed above, patients with Cbl deficiency may have overt neurologic disease in the absence of hematologic findings. Patients with neurologic symptoms and signs and a normal complete blood cell count require a modified diagnostic approach because of several considerations. First, folate deficiency is an unlikely cause of neurologic disease. Second, the neurologic disease of Cbl deficiency may be irreversible if treatment is withheld or delayed; because Cbl therapy is non-

Table 11. Evaluation of Patients With Hematologic Abnormalities and a Serum Cobalamin (Cbl) Level of 74 to 221 pmol/L Using Metabolite Assays

Serum Folate Level	Serum MMA Level*	Serum Hcy Level*	Interpretation	Approach
Normal	Normal	Normal	Cbl and folate deficiencies unlikely	Consider other causes of macrocytosis (Table 5) or anemia
Normal	Normal	Elevated	Possible Cbl deficiency	Follow algorithm in Figure 3
Normal	Elevated	Normal	Possible Cbl deficiency	Follow algorithm in Figure 3
Normal	Elevated	Elevated	Probable Cbl deficiency	Follow algorithm in Figure 3
Low	Normal	Normal	Possible folate deficiency	Trial of folate; monitor complete blood cell count
Low	Normal	Elevated	Probable folate deficiency	Trial of folate; monitor complete blood cell count
Low	Elevated	Normal	Possible Cbl deficiency or mixed deficiency	Follow algorithm in Figure 3
Low	Elevated	Elevated	Indeterminate	Follow algorithm in Figure 3; consider therapeutic trials

**Caution must be observed in interpreting modest elevations of serum methylmalonic acid (MMA) or serum homocysteine (Hcy) levels in patients with renal insufficiency or hypovolemia. See also the "MMA and Hcy" section in the text.*

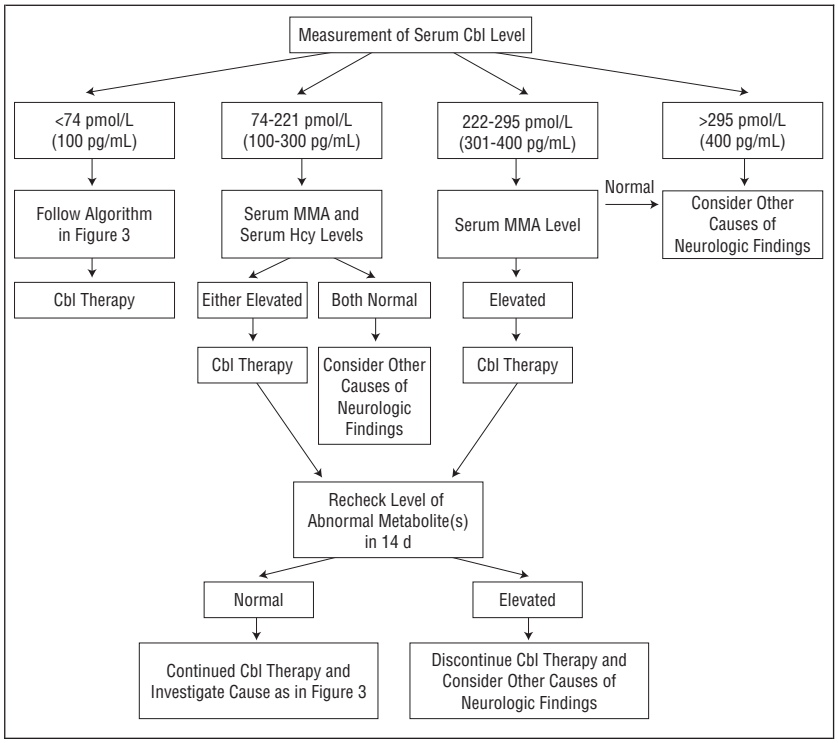

Figure 4. *Evaluation of neurologic symptoms and signs suggestive of possible cobalamin (Cbl) deficiency in patients with normal complete blood cell counts. Symptoms include paresthesias, ataxia, mental status changes, and vibratory and position sense impairment. MMA indicates methylmalonic acid; Hcy, homocysteine.*

toxic, the risk-benefit ratio favors treatment in questionable cases. Finally, an apparent response to therapy (or lack of response to therapy) is less definitive in ruling in or ruling out Cbl deficiency than is the serial measurement of abnormal initial hematologic parameters. Even in patients with a normal complete blood cell count, it may be worthwhile to monitor the MCV after treatment because a significant decline within the normal range provides additional evidence of Cbl deficiency.

An approach to the diagnosis of Cbl deficiency in patients with isolated neurologic findings is outlined in **Figure 4**. Relevant to the development of this algorithm is a study[23] of 419 patients with Cbl deficiency, 12 of whom had serum Cbl levels greater than 148 pmol/L (200 pg/mL). All 12 had elevated levels of serum Hcy and serum MMA. Five patients with normal serum Cbl levels had neurologic disease, and 1 of the 5 had a level greater than 221 pmol/L (300 pg/mL). All 5 patients had a clinical neurologic response to Cbl therapy and normalization of metabolite levels.

CONCLUSIONS

The accurate diagnosis of Cbl and folate deficiency is a complex task. No easily performed test can reliably serve as a diagnostic gold standard. Consequently, the performance characteristics of the available laboratory tests are difficult to ascertain. In each case, the primary physician must integrate clinical information, laboratory test results, and response to specific treatment and keep in mind the relative risks and benefits of administering or withholding vitamin replacement therapy. The algorithms in this article should not be considered rigid guides; if diagnostic uncertainty exists, consultation with a hematologist is advisable.

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