

Laboratory testing for cobalamin deficiency in megaloblastic anemia

Matthew J. Oberley, and David T. Yang*

Cobalamin (vitamin B12) deficiency is a common cause of megaloblastic anemia in Western populations. Laboratory evaluation of megaloblastic anemia frequently includes the assessment of patient cobalamin and folate status. Current total serum cobalamin measurements are performed in the clinical laboratory with competitive binding luminescence assays, whose results may not always accurately reflect actual cobalamin stores. Surrogate markers of cobalamin deficiency such as methylmalonic acid and homocysteine have been utilized to improve diagnostic accuracy; however, the specificity of these tests by themselves is rather low. Measurement of the biologically active fraction of cobalamin, holotranscobalamin, has been proposed as a replacement for current total cobalamin assays. Although holotranscobalamin measurements appear to have slightly better sensitivity, the specificity of this assay remains to be determined. The relative merits and demerits of commonly available methods to assess cobalamin deficiency in patients with suspected megaloblastic anemia are discussed. Am. J. Hematol. 00:000–000, 2013. © 2013 Wiley Periodicals, Inc.

Introduction

Megaloblastic anemia is characterized by distinctive hematopoietic cell morphology, ineffective hematopoiesis, and is frequently mediated by underlying biochemical deficiencies in cobalamin and/or folate. Megaloblastic anemia can also result from congenital disorders (e.g., orotic aciduria, Lesch–Nyhan syndrome, and congenital dyserythropoietic anemia), as a consequence of myelodysplastic syndrome, or from acquired disorders of DNA synthesis seen in the settings of chemotherapy. It is important to note that megaloblastic anemia is a morphologic diagnosis based on the cytologic and histologic features seen on the peripheral smear, bone marrow aspirate, and bone marrow core biopsy. However, as cobalamin deficiency is a common cause of megaloblastic anemia in Western populations, a biochemical diagnosis of megaloblastic anemia owing to cobalamin deficiency based on the results of clinical chemistry assays, without correlative bone marrow evaluation, frequently dictates therapy choices. This article will review the various clinical laboratory assays utilized to evaluate cobalamin deficiency and present their potential pitfalls in the assessment of megaloblastic anemia.

Megaloblastic Anemia

Ineffective DNA synthesis in hematopoietic progenitor cells is the underlying mechanism that leads to megaloblastic anemia. The consequent dyssynchrony between nuclear and cytoplasmic development is most apparent in Wright-stained hematopoietic precursors from bone marrow aspirates. Despite peripheral cytopenias, the bone marrow is hypercellular, frequently with a relative erythroid hyperplasia. Erythroid precursors have nuclei that are larger than normal and appear immature relative to cytoplasmic development with open sieve-like nuclear chromatin patterns. In the neutrophil lineage, giant band nuclei are characteristic. The development of megakaryocytes is also affected and reflected by peripheral thrombocytopenia. Morphologically characteristic findings in the peripheral blood include macrocytic anemia with anisopoikilocytosis and hypersegmented neutrophils.

Biochemical Consequences of Cobalamin Deficiency

Cobalamin is an obligate cofactor for two specific intracellular metabolic reactions required to produce the basic building blocks of DNA, RNA, and protein. In the

cytoplasm, cobalamin is a cofactor for methionine synthetase, which catalyzes the reduction of homocysteine (HCY) to methionine. Deficiency of cobalamin results in inhibition of methionine synthetase activity and an increase HCY levels. In mitochondria, cobalamin is a required cofactor in the methylmalonyl CoA mutase-catalyzed production of succinyl CoA from methylmalonyl CoA. Deficiencies of cobalamin will lead to increased levels of methylmalonic acid (MMA) owing to a block at this step. As serum levels of MMA and HCY are increased with cobalamin deficiency, these metabolites are utilized clinically as surrogate markers for cobalamin deficiency.

Ultimately, cobalamin deficiency leads to inhibited conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. The resulting elevated deoxyuridine triphosphate (dUTP) levels lead to misincorporation of dUTP into nascent DNA. Normally, DNA uracil glycosylase excises dUTP residues from nascent DNA strands, but because there is no deoxythymidine triphosphate available for replacement, DNA strand breaks occur and there can be significant DNA fragmentation. Presumably, this is the biochemical underpinning of the morphologic features seen in the nuclei of hematopoietic precursors that are diagnostic of megaloblastic anemia.

Clinical Consequences of Cobalamin Deficiency

Cobalamin deficiency results in both neuropsychiatric and hematological deficits (for review, see [1,2]). A classic and specific finding in patients with advanced cobalamin deficiency is subacute combined degeneration of the dorsal and lateral spinal columns owing to myelinopathy of these neural tracts. These changes are irreversible. Other

Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI

*Correspondence to: David T. Yang, Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Box 8550 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792. E-mail: dtyang@wisc.edu

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affected neurological functions can include cerebellar changes that result in ataxia and cognitive decline. Neurological manifestations of cobalamin deficiency may occur in the absence of hematological findings. As cobalamin deficiency progresses, peripheral cytopenias in all three lineages become increasingly pronounced and manifest the unique morphologic features that are described above.

Cobalamin Metabolism

As cobalamin is not synthesized by plants, humans depend chiefly on foods of animal origin to maintain adequate cobalamin stores. To ensure efficient absorption of cobalamin, our gut has a complex process that includes (1) freeing cobalamin from food by proteases and acids, (2) binding free cobalamin with salivary haptocorrin in the stomach, (3) digestion of haptocorrin in the proximal small bowel and transfer of cobalamin to intrinsic factor (IF) secreted by gastric parietal cells, (4) attachment of cobalamin-IF to specific receptors in the ileum, and (5) endocytosis of the cobalamin-IF complex followed by release from IF.

Once absorbed in the ileum, cobalamin is largely bound to two proteins in the serum. In total, 70–90% is biologically inactive and bound to haptocorrin (transcobalamin I), whereas the remainder is bound to transcobalamin II and termed holotranscobalamin. Transcobalamin II is required for B12 transport to cells and congenital deficiency of transcobalamin II results in the typical neurologic and hematologic findings seen in cobalamin deficiency [3]. Haptocorrin deficiency does not appear to result in clinically apparent cobalamin deficiencies [4]. Transcobalamin II–cobalamin complexes are endocytosed after binding to transcobalamin II receptors on target cells. Once in the cell, cobalamin is released from transcobalamin II, reduced, and bound as a cofactor for methylmalonyl-CoA mutase (mitochondria) or methionine synthase (cytoplasm).

Etiology of Cobalamin Deficiency

Interference of any of the requirements for ingestion, absorption, distribution, or utilization can result in clinically evident cobalamin deficiency. Diets lacking in an exogenous source of cobalamin will, over time, result in deficiency although it may take 2–5 years for cobalamin deficiency to manifest clinically [5]. Cobalamin deficiency can be precipitated by any malabsorption syndrome or surgical alteration to the gastrointestinal tract such as ileal bypass. Pancreatic exocrine insufficiency can also result in malabsorption owing to the failure of enzymatic degradation of cobalophilin–cobalamin complexes and release of cobalamin for subsequent binding to IF.

Pernicious anemia is an autoimmune disease characterized by megaloblastic anemia that is a direct consequence of autoantibody production which targets gastric parietal cells or IF, leading to malabsorption of cobalamin. Medications may also affect the release of IF (H₂ blockers) or block absorption of cobalamin-IF complexes (neomycin and metformin). In some instances, cobalamin deficiency may result from infection with the fish tapeworm, *Diphyllobothrium latum*, which establishes itself in the small intestine and can out-compete the host for cobalamin absorption.

Finally, as mentioned above, inherited defects in factors required for absorption or processing of cobalamin have been shown to result in the characteristic neurologic and hematologic findings seen in B12 dietary deficiency, which has contributed to the delineation of the physiological molecules responsible for in vivo cobalamin transport and metabolism.

Clinical Methods for Measuring Cobalamin

There is no universally agreed upon gold standard assay for determining cobalamin levels in humans. In fact, surrogate biomarkers (MMA and HCY) of cobalamin deficiency are widely utilized in clinical medicine to improve diagnostic sensitivity, despite poor specificity. Elevated levels of MMA are often utilized as a gold standard with which to compare new cobalamin testing platforms despite published data which show that increased levels of MMA by themselves do not necessarily correlate with clinically evident cobalamin deficiency [6].

Historically, the first widely used clinical assay for cobalamin was a microbiologic assay. This assay utilized strains *Lactobacillus leichmannii* or *Euglena gracilis* that depended on exogenously added cobalamin for growth. Cobalamin from a patient serum sample was extracted and incubated with the bacterium and growth was proportional to the amount of cobalamin present. These assays suffered from several drawbacks including an extended incubation time of several days, bacterial growth that could be affected by a number of interferences such as antibiotics [7], and the fact that microbiological assays are difficult to standardize across laboratories.

Subsequently, a radiodilution assay was developed and widely adopted in the 1970s. Here, cobalamin was extracted from patient serum, converted to cyanocobalamin, and then mixed with radiolabeled⁵⁷cyanocobalamin. The level of radiolabeled cyanocobalamin binding to purified IF was measured, and from this, the amount of patient cyanocobalamin was calculated. This test also suffered several limitations, not the least of which was the use of radiolabeled isotopes in the clinical laboratory. In addition, it had been noted by the late 1970s that this assay may give falsely normal values of serum cobalamin levels in patients with pernicious anemia [8]. The failure of the assay was ascribed to cobalamin analogues present in affected patients and the use of an impure form of IF which bound these analogues.

Another cobalamin-related test of historical note is the Schilling test, first introduced in 1954, but not utilized today in part because of the need for radiolabeled isotopes. The Schilling test is a multistep assay that is capable of assessing the etiology of cobalamin malabsorption, but because pernicious anemia is the most common cause of cobalamin deficiency in Western populations, it has largely been replaced by assays for the IF-blocking antibodies and anti-parietal cell antibodies that are associated with pernicious anemia.

Competitive Binding Luminescence Assay

Modern laboratory testing for total plasma cobalamin levels commonly involves a competitive binding chemiluminescence assay which has the advantage of easy scalability to a high-throughput automated procedure. The sensitivity of these tests for the detection of frank cobalamin deficiency (<200 pg/mL) in patients with clinical manifestations, such as megaloblastic anemia, is estimated to exceed 90–95%, with some notable exceptions discussed below. The specificity of cobalamin measurements relating to clinical deficiency have not been formally determined but have been estimated to be <80%. The sensitivity of detection of sub-clinical cobalamin deficiency is notably less, with estimates ranging from 40 to 80% [9]. Each automated platform offers a proprietary variation on the following theme which results in a chemiluminescence output measurement:

1. Total vitamin B12 is liberated from protein binders in the serum to a free form.

- The free B12 is allowed to then compete with exogenously added labeled-B12 for binding to a limited amount of purified IF.
- IF is bound to a solid phase (or IF is initially coated on paramagnetic beads), unbound ligand is washed away, and a conjugate to the labeled-B12 is added. After addition of substrate, the conjugate creates chemiluminescence that is proportional to the amount of labeled-B12 present. Thus, there is an inverse relationship between the quantity of patient B12 present in the serum and the amount of luminescence generated.

Despite multiple variations on this theme, a common factor to all the competitive binding luminescence assays (CBLAs) is the use of purified IF as a means to specifically bind vitamin B12. In contrast to the previous use of impure forms of IF that were implicated in spuriously elevated cobalamin levels owing to nonspecific binding to cobalamin analogs in the serum, current CBLAs utilize a highly purified IF that has low affinity for cobalamin analogues [10]. However, the use of purified IF may render the CBLA-based tests particularly susceptible to interfering anti-IF antibodies.

Technical Causes of Spuriously Measured Cobalamin Levels

Because of the inverse relationship between patient cobalamin levels and assay output of chemiluminescence, any substance that interferes with the chemiluminescence production will spuriously elevate cobalamin levels. This is especially problematic in patients with pernicious anemia who have IF-blocking antibodies that may bind the test IF reagent. This assay failure has been reported by multiple institutions over the last decade with multiple CBLA-based testing platforms [11–17]. The largest of these studies utilized patient serum from patients with documented anti-IF antibodies, who all had clinically expressed cobalamin deficiency. Total cobalamin levels were determined by radioimmunoassay. Three different CBLA platforms showed significant diagnostic error [15]. All of these errors were false-negative results that reported normal cobalamin levels when, in fact, the patients were significantly deficient.

CBLAs commonly have an antibody denaturation step intended to denature IF-blocking antibodies and failure in this step has been implicated in spurious elevation of cobalamin levels [11]. In addition, it has been argued that CBLA assays are imprecise upon repeated testing [18] although whether this is owing to temporal physiologic fluctuations of cobalamin, or CBLA assay imprecision is subjected to debate [19].

Finally, cutoff values to define cobalamin deficiency continue to be controversial. It has been estimated that defining cobalamin deficiency based on the common cutoff of 200 pg/mL may falsely identify 30% of elderly patients who have no clinical or metabolic signs of cobalamin deficiency as cobalamin deficient [5]. On the other hand, subclinical cobalamin deficiency is also a genuine concern in the same elderly population and increasing the cutoff value may exacerbate this problem. Readers are referred to a review of this area [20]. Supplementation of cobalamin testing with MMA and HCY will increase the specificity of testing, and borderline cases often warrant a trial of cobalamin therapy.

Physiologic Causes of Spuriously Measured Cobalamin Levels

Physiologic conditions that have been reported to cause low-measured cobalamin levels without associated clinical signs of cobalamin deficiency include mild to severe

haptocorrin deficiency [21] and folate deficiency [22]. In cases of folate deficiency, cobalamin levels can normalize with folate therapy. Others have reported spuriously low-measured cobalamin levels associated with multiple myeloma [23], HIV [24], pregnancy, and oral contraceptive use [25]. In these cases, decreased production of serum cobalamin-binding proteins is thought to be responsible (Table I).

A measurement of cobalamin levels above the upper limit of the reference range has been associated with occult malignancy including myeloproliferative neoplasms that cause increased haptocorrin levels. In addition, a recent study found associations between high levels of measured cobalamin and alcoholic liver disease, solid malignancies, and renal disease [26]. In part owing to these variables contributing to significant intraindividual temporal variation in measured cobalamin levels, some authors have suggested that cobalamin testing is an unreliable indicator of response to cobalamin therapy [18].

Measurement and Clinical Utility of MMA and HCY Levels

Elevated levels of MMA and HCY are commonly used as adjuvant diagnostics to confirm a suspected diagnosis of cobalamin deficiency. Although many studies have used serum MMA as a “gold standard” for evaluating cobalamin assays, this practice has been subjected to controversy. The sensitivity of elevated serum MMA measurements in detecting patients with overt cobalamin deficiency is reported to be >95%; however, the specificity of this test has not been determined [9,27]. For example, Hvas et al.

TABLE I. Performance Characteristics of Clinical Assays Used for Detecting Cobalamin Deficiency

Criteria	Sensitivity	Specificity	Pitfalls
Serum total cobalamin (<200 pg/mL)	95–97%[9]	Uncertain, possibly <80%[9]	<i>Elevated levels seen with:</i> Assay technical failure Occult malignancy Alcoholic liver disease Renal disease <i>Decreased levels also seen with:</i> Haptocorrin deficiency Folate deficiency Plasma cell myeloma HIV Pregnancy
Elevated serum methylmalonic acid	>95%[9,27]	Uncertain[6]	<i>Elevated levels seen with:</i> Renal insufficiency Hypovolemia Congenital metabolic defects Amyotrophic lateral sclerosis
Elevated serum homocysteine	>95%[9,27]	Uncertain, less specific than methylmalonic acid	<i>Elevated levels seen with:</i> Folate or pyridoxine deficiency Renal insufficiency Hypovolemia Hypothyroidism Psoriasis Congenital metabolic defects Neurodegenerative disease Malignancy Medications
Decreased serum holotranscobalamin	Similar to total cobalamin [32–37]	Uncertain [32–37]	<i>Levels may be affected by:</i> Liver disease Macrophage activation Autoantibodies

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[6] did not find any correlation between elevated plasma levels of MMA and clinical manifestations of cobalamin deficiency even after controlling for patients with renal disease. Moreover, this study of 432 untreated patients with modestly elevated MMA levels, who did not exhibit signs or symptoms of cobalamin deficiency showed MMA levels that spontaneously declined in 44% and increased in 16%, demonstrating considerable biological and temporal variability. Thus, caution is required when interpreting MMA levels that are near commonly used cutoff points, and MMA should never be a sole laboratory criterion for determining cobalamin deficiency. Measurement of MMA is largely restricted to larger reference laboratories because it is most commonly quantified by isotope dilution technology with gas chromatography-mass spectrometry.

In addition to cobalamin deficiency, there are multiple factors that can affect MMA levels. Patients who are hypovolemic or who have renal insufficiency may have elevated levels of MMA unrelated to cobalamin status. Patients with congenital metabolic defects such as methylmalonic aciduria have elevated levels of MMA. In addition, it has been reported that MMA (and HCY) levels are elevated in patients with neurodegenerative disorders such as amyotrophic lateral sclerosis [28].

HCY levels are also elevated in cases of cobalamin deficiency with a similar sensitivity to that of MMA [27]; however, elevated HCY is less specific for cobalamin deficiency than MMA. Elevated HCY levels are also associated with folate deficiency, renal insufficiency, hypovolemia, hypothyroidism, psoriasis, congenital metabolic defects, and neurodegenerative disease (Table I). Medications such as methotrexate, theophylline, phenytoin, hydrochlorothiazide, and levodopa are also established causes of HCY elevation.

Measurement and Clinical Utility of Holotranscobalamin Levels

It has long been suggested that serum holotranscobalamin may be a better indicator of B12-deficiency states than serum cobalamin because it represents the biologically active fraction of cobalamin in humans and may be depleted first in subclinical cobalamin deficiency. However, there is concern that the noncobalamin-related determinants of physiologic and pathologic holotranscobalamin variations have yet to be fully elucidated [29]. For example, there is some limited data, suggesting that transcobalamin levels are affected by liver disease, macrophage activation, and transcobalamin autoantibody generation [30].

Traditionally, holotranscobalamin has been measured through a modified radioimmunoassay. More recently, reliable monoclonal antibody-based assays for determining serum levels of holotranscobalamin have become available [31]. Several studies utilizing this assay suggest that serum holotranscobalamin measurements are more sensitive in detecting cobalamin deficiency than traditional total serum cobalamin measurements.

Miller et al. [32] compared total serum cobalamin and serum holotranscobalamin levels in a population of patients who were 60 years old, utilizing MMA and HCY levels as gold standards for cobalamin deficiency. They found that cobalamin and holotranscobalamin measurements were essentially equivalent in their ability to identify cobalamin-deficient patients. Significantly, only about half of the patients with elevated MMA and HCY levels were identified as cobalamin deficient, implying a poor sensitivity for either method in this population when cobalamin deficiency is defined as elevated MMA and HCY levels. They also found that patients identified with both low total cobalamin and

holotranscobalamin levels were more likely to have higher concentrations of MMA. The conclusion was that utilization of both tests would be superior than either one alone. Unfortunately, they were unable to control for potential confounding variables other than renal insufficiency.

Two other studies examined the relative ability of holotranscobalamin and total cobalamin to identify patients with elevated MMA levels and found that holotranscobalamin performed more robustly than total cobalamin [33,34]. Similarly, serum holotranscobalamin was superior to total cobalamin and MMA levels in predicting red cell cobalamin levels although the significance of red cell cobalamin levels with regards to cobalamin deficiency remains to be determined [35].

Conversely, when holotranscobalamin, MMA, or HCY levels were used to assess which was a better predictor of response to vitamin B12 therapy in clinically cobalamin-deficient patients, none of the tested metrics performed better than low total cobalamin in predicting who would benefit from therapy [36].

Although initial studies of the sensitivity of holotranscobalamin for detecting cobalamin deficiency appears to slightly improve on that of direct total cobalamin measurements, studies have suggested that the specificity of holotranscobalamin measurements to detect cobalamin deficiencies remains low [33,37]. Problems remain in defining a gold standard test and definition of cobalamin deficiency with which to compare new assays to. As pointed out by Carmel [29], factors unrelated to cobalamin levels that affect holotranscobalamin levels remain to be elucidated. In addition, in vivo transient variation patterns of holotranscobalamin levels in cobalamin-replete individuals remain to be determined. For example, can a brief reduction in dietary cobalamin transiently reduce holotranscobalamin levels and lead to erroneous conclusions regarding cobalamin status?

Although preliminary data suggest that the measurement of holotranscobalamin may provide a modest improvement over total cobalamin for the detection of cobalamin deficiency, there is insufficient evidence to support the wholesale adoption of holotranscobalamin testing in routine clinical practice.

Conclusions

Advanced cobalamin deficiency can result in hematologic and neurologic manifestations where diagnosis commonly revolves around laboratory testing of total cobalamin levels. Current testing for total cobalamin levels is based on CBLAs which provide good sensitivity and reasonable specificity in clinically overt cobalamin deficiency. Adjuvant assessment of MMA and HCY levels can further improve the specificity of testing in patients with adequate renal function. However, it is important to be aware that failure of CBLA assays to detect cobalamin deficiency can occur, especially in cases of pernicious anemia where IF-blocking antibodies have been implicated in causing assay interference. In addition, early or subclinical cases of cobalamin deficiency may not be detected with current testing platforms and standard reference ranges. Given these caveats, when laboratory measurements are incongruous with the clinical impression, alternative evaluations such as review of the peripheral blood smear, can provide valuable and reliable information to support the clinical impression. Finally, holotranscobalamin testing appears to show promise as a superior assay for detecting cobalamin deficiency; however, more work needs to be done to identify physiologic and pathologic variations of holotranscobalamin levels at the population level before its potential incorporation into routine testing.

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