

## BRIEF REVIEW

## Status of Laboratory Testing in the Diagnosis of Megaloblastic Anemia

By John Lindenbaum

**W**HAT WAS ONCE relatively straightforward, seems lately to have become more confusing. In the past decade, new opportunities and new problems in the diagnostic approach to the patient with a possible megaloblastic anemia have appeared with the development and nearly ubiquitous use of new technologies—electronic determination of the mean corpuscular volume (MCV) and an apparent myriad of “easy-to-do” radioisotope dilution assays (RIDs) of serum cobalamin and folate levels. Hopefully, the following brief summary of the state of the art may serve as a temporary guide to the perplexed.

### ERYTHROCYTE MEAN CORPUSCULAR VOLUME

The accurate measurement of the MCV by electronic counters and the wide availability of the test as part of the routine CBC has had a number of consequences for diagnosis. It is apparent that mild macrocytosis, easily missed on blood smear by the technician or hematologist, is frequently detected by the Coulter Counter,<sup>1,2</sup> facilitating early discovery of megaloblastic states, in which the MCV elevation typically precedes the development of anemia, often by months or years.<sup>3,4</sup> Abnormally high MCVs occur in 2%–4% of patients seeking general medical care, most commonly in the absence of anemia.<sup>2,5,6</sup> Only a minority of patients with MCV levels above 100 fl are deficient in vitamin B<sub>12</sub> (Cbl) or folate.<sup>1,2,5</sup> Alcoholism in the absence of folate deficiency is probably the most common cause of an MCV elevation.<sup>1,2,5,7</sup> Others are listed in Table 1. No cause is apparent in a sizeable minority of patients.<sup>1,2,5,6</sup> Inspection of the blood smear for the presence of macroovalocytes and hypersegmented neutrophils remains necessary in the interpretation of MCV elevations. Although neither morphological finding is specific,<sup>8</sup> their combination is highly suggestive of Cbl or folate deficiency. Both often remain present for many days or weeks after therapy.<sup>9</sup>

On occasion, the MCV may be normal in patients with megaloblastic anemias, even in the absence of concomitant iron deficiency or thalassemia.<sup>10</sup>

### SERUM COBALAMIN LEVELS

In the 1950s and 1960s, many investigators established that microbiologic assays of serum Cbl concentrations were highly sensitive indicators of the presence of deficiency.<sup>11</sup> Low values are invariably seen in the presence of megaloblastic change due to lack of Cbl, with the rare exceptions of patients with chronic myelocytic leukemia, transcobalamin II deficiency, and nitrous oxide anesthesia. These assays are rather cumbersome and time-consuming for routine use by hospital laboratories, however, and are affected by the presence of antibiotics in serum. Over the past 2 decades, many RIDs were devised and promoted commercially in the form of kits, and despite many technical problems associated with their use, rapidly became widely employed, virtually replacing the microbiologic procedures before adequate validation of their diagnostic reliability.<sup>11</sup> Serum vitamin levels often were noted to be higher by radioassay. In 1978, two groups reported that serum Cbl concentrations fell within the normal range in 10%–20% of patients with pernicious anemia (PA), leading in some cases to dangerous delays or failure to diagnose and treat Cbl deficiency.<sup>12,13</sup> These errors were attributed to the presence of “R-proteins” in the intrinsic factor (IF) preparations used to bind radioactive Cbl in the assays. R-binders are less specific for active forms of Cbl than IF, and evidence was presented that these RIDs measured inactive Cbl analogues as well as Cbl.<sup>13</sup> Although some investigators have failed to demonstrate these analogues in serum,<sup>14</sup> if the assays are modified so as to eliminate the binding activity of the R-proteins (either by adding large amounts of analogues in vitro or by using purified IF) serum levels of Cbl show a much better correlation with those found by microbial assay as well as with the clinical picture.<sup>13,15,16</sup> However, some of the modified kits have not been found to be completely reliable, and some of the earlier RIDs in which the stated lower limit of normal will include some patients with Cbl deficiency continue to be marketed as of this writing. It has also been argued that measurement of “total corrinoid” levels, i.e., Cbl

*From the Departments of Medicine, Harlem Hospital Center, and the College of Physicians and Surgeons, Columbia University, New York, N.Y.*

*Submitted July 30, 1982; accepted November 14, 1982.*

*Address reprint requests to J. Lindenbaum, Department of Medicine, Harlem Hospital Center, New York, N.Y. 10037.*

*© 1983 by Grune & Stratton, Inc.*

*0006-4971/83/6104-0002\$01.00/0*

**Table 1. ↑MCV (>100 fl) in the Absence of Cobalamin or Folate Deficiency**

Common causes
Alcoholism
Liver disease
Cancer chemotherapy
Bone marrow malignancies:
Myeloma, leukemias, lymphomas, metastatic carcinoma
No cause apparent
Reticulocytosis
Relatively uncommon causes
Hypothyroidism
Bone marrow disorders:
Aplastic anemia, pure red cell aplasia, refractory anemia, sideroblastic anemia, myelofibrosis
Hereditary hydrocytosis
Laboratory artefacts
Cold agglutinins
Marked hyperglycemia
Marked leukocytosis

and its analogues, using mixtures of IF and R-binders may give an equally good or even better separation between normal and deficient patients,<sup>17</sup> providing the lower limit of normal is adjusted upward. The case for measuring “total” corrinoids, rather than “true” cobalamins, as assayed in the absence of active R-binders, remains to be established, however. At this time it remains imperative that any clinical laboratory determine independently the normal range of the RID it is using, as well as its sensitivity in detecting Cbl deficiency (which should be 100%).

The serum Cbl level, when performed by a reliable assay, is an early indicator of Cbl deficiency, falling to low values before macrocytosis, megaloblastic change, or neuropathy occurs, as in patients with atrophic gastritis or following gastrectomy. However, certain other conditions may occasionally cause low serum concentrations in the absence of an underlying disorder capable of causing deficiency (Table 2). In addition, with several of the “modified” RID kits currently in

**Table 2. Low Serum Cobalamin in the Absence of Cobalamin Deficiency**

Folic acid deficiency
Normal pregnancy; oral contraceptive agents
Multiple myeloma
Transcobalamin I deficiency
No apparent cause
?? Iron deficiency anemia
Interfering agents in serum
Isotopes, e.g., gallium-67 (RIDs)*
Antibiotics (MAs)†
Chlorpromazine (some MAs)
Antifols (some MAs)

\*Radioisotope dilution assays.

†Microbiologic assays.

use, we and others<sup>16, 18</sup> have found unaccountably low values in certain normal or nondeficient individuals. The cause for this artefact, which may lead to unnecessary diagnostic testing, has not been established.

#### FOLATE ASSAYS

The serum folate concentration, as measured microbiologically with *Lactobacillus casei*, although typically decreased in patients with megaloblastic anemia due to lack of the vitamin, is highly affected by clinically unimportant changes in folate intake and balance.<sup>19</sup> It is thus frequently low in the absence of megaloblastic anemia in patients with anorexia, alcoholism, normal pregnancy, anticonvulsant therapy, and a variety of systemic illnesses. Its main use is in association with the serum Cbl level in the patient with proven megaloblastic change. As with the Cbl assay, a host of recently developed RIDs using varying methodologies have become widely used without satisfactory documentation of clinical validity,<sup>15,20</sup> although technical simplicity and lack of interference by antibiotics and antineoplastic drugs are points in favor of the isotopic procedures. It is uncertain whether any of these radioassays is superior or inferior to the microbiologic method.<sup>15,20</sup> Significant overlap between normals and deficient patients has been noted with both types of technique.

Erythrocyte folate concentrations, as measured microbiologically, show a much better correlation with the presence of megaloblastic change than serum levels<sup>21</sup> and are unaffected by dietary changes during the first few days in hospital. RBC folate levels, however, are low in one-half of patients with primary Cbl deficiency, may be normal in megaloblastic states due to folate lack when anemia is slight or absent, and are often obscured by blood transfusion. Meaningful documentation of the validity of currently marketed RIDs for RBC folate is nonexistent.

#### SERUM ANTIBODIES TO INTRINSIC FACTOR

A highly useful but relatively neglected test detects serum antibodies to IF of the “blocking” type.<sup>22</sup> Such antibodies are demonstrable in 50%–60% of patients with PA. Only rare false-positives are encountered, usually in patients with underlying chronic gastritis in whom Cbl absorption is normal.<sup>22</sup> The combination of megaloblastic anemia, low serum Cbl, and serum antibodies to IF is essentially diagnostic of PA and obviates further investigation, including Schilling tests. The procedure is thus extremely useful and cost-effective, greatly simplifying the work-up of the majority of patients with PA. The test is rapid and easy to perform and well within the reach of routine labora-

ories, and its wider application should be encouraged. In contrast, tests for parietal cell antibodies are more sensitive for PA but much less specific and are rarely useful.<sup>23</sup>

#### SCHILLING TESTS

Standard serial Schilling tests remain a diagnostic cornerstone despite certain limitations,<sup>23</sup> including frequent incomplete urine collections and dependence on adequate renal function, and appear superior to modified variants, such as single testing with two isotopes. Commercially supplied IF capsules are best dissolved in water and mixed in vitro with radioactive cyanocobalamin before administration to the patient.<sup>24</sup> Combined plasma and urine testing for radioactivity may increase diagnostic accuracy.

Schilling tests are indicated in patients with Cbl deficiency who lack antibodies to IF and have not undergone gastrectomy or substantial ileal resection. In many patients, Cbl lack induces a period of transient ileal dysfunction,<sup>25</sup> so that we prefer to postpone testing until after 1 wk of vitamin therapy. In the patient with proven Cbl deficiency in the United States, the Schilling test done without added IF is almost always low. We recommend that "part II" (i.e., with exogenous IF) be done first, since there is usually little point in performing the test without IF in a patient who cannot absorb Cbl with IF. Schilling tests also often help resolve the diagnostic puzzle when serum levels of both vitamins are subnormal.

#### OTHER TESTS

The ability of deoxyuridine to suppress the incorporation of thymidine into thymidylic acid and DNA is dependent on the supply of metabolically active folates at the cellular level and is impaired in megaloblastic anemias due to either deficiency state. This can be tested in incubated marrow cells in the "dU suppres-

sion test," and the in vitro ability of added folate or cobalamin compounds to correct an abnormal result used to define the underlying vitamin deficiency.<sup>26</sup> This highly sensitive test may detect deficiency in patients with borderline or even absent hematologic changes and is also useful in megaloblastic anemia when both serum folate and Cbl are low (or both normal) or inborn errors in Cbl and folate metabolism are suspected. A modified version of the test employing peripheral blood lymphocytes may even provide useful information in previously treated patients.<sup>27</sup> Another test of diagnostic value is the measurement of urinary methylmalonic acid (MMA) excretion,<sup>28</sup> which is elevated in almost all cases of Cbl lack; MMA does not accumulate in folate deficiency. Although simplified versions of these procedures have been developed, at the moment they are too demanding of time or equipment to be performed in most nonresearch laboratories. Gastric acidity testing usually provides relatively nonspecific information, and when done, would better be combined with assay of gastric IF itself.<sup>23</sup>

#### CONCLUSIONS

Commercial enthusiasm and hospital laboratory acceptance has led to the widespread use of incompletely validated RIDs for Cbl and folate levels. Although many kits for measurement of serum Cbl have recently been improved by elimination of R-binders and/or redefinition of the lower limits of normal, our current advice is: *caveat emptor*. The patient with macrocytosis and neurologic changes compatible with Cbl deficiency should receive further diagnostic attention, such as tests of Cbl absorption and serum antibodies to IF, as well as a course of vitamin B<sub>12</sub> treatment, even if the serum Cbl is normal. The clinician still needs to put together the clinical and morphological observations, and at times be willing to view the laboratory data with a tinge of icterus.

#### REFERENCES

- Hattersley PG: Macrocytosis of the erythrocytes. A preliminary report. *JAMA* 189:997-999, 1964
- Davidson RJL, Hamilton PJ: High mean red cell volume: Its incidence and significance in routine haematology. *J Clin Pathol* 31:493-498, 1978
- Carmel R: Macrocytosis, mild anemia, and delay in the diagnosis of pernicious anemia. *Arch Intern Med* 139:47-50, 1979
- Hall CA: Vitamin B<sub>12</sub> deficiency and early rise in mean corpuscular volume. *JAMA* 245:1144-1146, 1981
- Breedveld FC, Bieger R, van Wermeskerken RKA: The clinical significance of macrocytosis. *Acta Med Scand* 209:319-322, 1981
- McPhedran P, Barnes MG, Weinstein JS, Robertson JS: Interpretation of electronically determined macrocytosis. *Ann Intern Med* 78:677-683, 1973
- Lindenbaum J: Folate and vitamin B<sub>12</sub> deficiencies in alcoholism. *Semin Hematol* 17:119-129, 1980
- Lindenbaum J: Pernicious anemia and other megaloblastic anemias, in Conn HF, Conn RB (eds): *Current Diagnosis-6*. Philadelphia, Saunders, 1980, pp 411-416
- Nath BJ, Lindenbaum J: Persistence of neutrophil hypersegmentation during recovery from megaloblastic granulopoiesis. *Ann Intern Med* 90:757-760, 1979
- Prentice AG, Evans IL: Megaloblastic anaemia with normal mean cell volume. *Lancet* 1:606-607, 1979
- Mollin DL, Anderson BB, Burman JF: The serum vitamin B<sub>12</sub> level: Its assay and significance. *Clin Haematol* 5:521-546, 1976
- Cooper BA, Whitehead VM: Evidence that some patients with pernicious anemia are not recognized by radiodilution assay for cobalamin in serum. *N Engl J Med* 299:816-818, 1978
- Kolhouse JF, Kondo H, Allen NC, Podell E, Allen RH: Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays

are not specific for true cobalamin. *N Engl J Med* 299:785–792, 1978

14. Begley JA, Hall CA: Forms of vitamin B<sub>12</sub> in radioisotope dilution assays. *J Clin Pathol* 34:630–636, 1981
15. Dawson DW, Delamore IW, Fish DI, Flaherty TA, Gowenlock AH, Hunt LP, Hyde K, MacIver JE, Thornton JA, Waters HM: An evaluation of commercial radioisotope methods for the determination of folate and vitamin B<sub>12</sub>. *J Clin Pathol* 33:234–242, 1980
16. Kubasik NP, Ricotta M, Sine HE: Commercially-supplied binders for plasma cobalamin (vitamin B<sub>12</sub>), analysis—“Purified” intrinsic factor, “cobinamide”-blocked R-protein binder, and non-purified intrinsic factor-R protein binder—compared to microbiological assay. *Clin Chem* 26:598–600, 1980
17. Herbert V, Colman N: Evidence humans may use some analogues of B<sub>12</sub> as cobalamins (B<sub>12</sub>): Pure intrinsic factor (IF) radioassay may “diagnose” B<sub>12</sub> deficiency where it does not exist. *Clin Res* 29:571A, 1981
18. Altz-Smith M, Miller RK, Cornwell PE, Butterworth CE: Low plasma levels of vitamin B<sub>12</sub> in patients with rheumatoid arthritis. *Clin Res* 29:858A, 1981
19. Herbert V: Experimental nutritional folate deficiency in man. *Trans Assoc Am Physicians* 75:307–320, 1962
20. Baril L, Carmel R: Comparison of radioassay and microbiological assay for serum folate, with clinical assessment of discrepant results. *Clin Chem* 24:2192–2196, 1978
21. Hoffbrand AV, Newcombe BFA, Mollin DL: Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. *J Clin Pathol* 19:17–28, 1966
22. Chanarin I: *The Megaloblastic Anaemias* (ed 2). Oxford, Blackwell, 1979, pp 362–364
23. Carmel R: The laboratory diagnosis of megaloblastic anemias. *West J Med* 128:294–304, 1978
24. McDonald JWD, Barr RM, Barton WB: Spurious Schilling test results obtained with intrinsic factor enclosed in capsules. *Ann Intern Med* 83:827–829, 1975
25. Lindenbaum J, Pezzimenti JF, Shea N: Small-intestinal function in vitamin B<sub>12</sub> deficiency. *Ann Intern Med* 80:326–331, 1974
26. Herbert V, Tisman G, Go LT, Brenner L: The dU suppression test using <sup>125</sup>I-UdR to define biochemical megaloblastosis. *Br J Haematol* 24:713–723, 1973
27. Das KC, Herbert V: The lymphocyte as a marker of past nutritional status: persistence of abnormal lymphocyte deoxyuridine suppression test and chromosomes in patients with past deficiency of folate and vitamin B<sub>12</sub>. *Br J Haematol* 38:219–233, 1978
28. Norman EJ, Martelo OJ, Denton MD: Cobalamin (vitamin B<sub>12</sub>) deficiency detection by urinary methylmalonic acid quantitation. *Blood* 59:1128–1132, 1982