Influence of cobalamin deficiency compared with that of cobalamin absorption on serum holo-transcobalamin II1–3

Xinke Chen, Angel F Remacha, M Pilar Sardà, and Ralph Carmel

ABSTRACT

Background: Cobalamin attached to transcobalamin II (TC II), known as holo-TC II, is the active cobalamin fraction taken up by tissues. Holo-TC II is also the form in which absorbed cobalamin enters the circulation from the ileum. Therefore, holo-TC II has been proposed variously as a marker of cobalamin adequacy, cobalamin absorption, or both, including even its advocacy as a surrogate Schilling test. Such claims carry conflicting diagnostic implications because metabolic adequacy and absorption are not identical.

Objective: The objective was to examine metabolic and absorptive influences on holo-TC II.

Design: Treated patients with pernicious anemia (PA), who have abnormal absorption but a normal metabolic status, were chosen as the model to differentiate between the effects of the 2 cobalamin-related characteristics. Serum holo-TC II and indexes of cobalamin metabolism in 23 treated patients were compared with those of 6 untreated PA patients (abnormal absorption and metabolic status) and 33 control subjects (normal absorption and metabolic status).

Results: Holo-TC II, which correlated directly with cobalamin and inversely with homocysteine, was significantly higher in treated PA patients in metabolic remission than in untreated PA patients (74 ± 59 compared with 9 ± 6 pmol/L) and was significantly lower than in control subjects (105 ± 58 pmol/L), although the latter difference was small and the values overlapped greatly.

Conclusions: Metabolic cobalamin status is a major determinant of serum holo-TC II. Absorption status may have mild influence as well, although other explanations remain possible. Serum holo-TC II cannot be used clinically to diagnose cobalamin malabsorption because of overlap with normal values. The influences on holo-TC II are complex and require careful analysis. Am J Clin Nutr 2005; 81:110–4.

KEY WORDS Holo-transcobalamin II, cobalamin, cobalamin absorption, cobalamin deficiency, homocysteine

INTRODUCTION

The diagnostic approach to patients with suspected cobalamin deficiency requires 2 very distinct determinations: the demonstration that deficiency exists and the identification of what caused the deficiency (1). The first step has usually relied on cobalamin assay, but because deficiency is frequently mild or subclinical and because cobalamin concentrations can be falsely low or falsely normal, metabolic tests, such as the measurement of plasma total homocysteine (tHcy), serum or urine methylmalonic acid (MMA), and deoxyuridine suppression, are often done as well (2). Each of these tests has disadvantages, however.

The second step, defining the cause of the deficiency, usually requires ruling malabsorption in or out, with pernicious anemia (PA; ie, the lack of gastric intrinsic factor) as the classical prototype of cobalamin malabsorption. The Schilling test is commonly used, but its availability is declining. Adequate substitutes have not been found; only about half of the cases of PA can be diagnosed through the demonstration of antibodies to intrinsic factor, and no blood test can identify disorders of cobalamin absorption other than PA (1).

Some investigators have advocated the assay of serum holo-transcobalamin II (holo-TC II)—the small, transient fraction of the total cobalamin that is attached to TC II (3). In theory, holo-TC II, the biologically available cobalamin pool in plasma that all cells take up rapidly via specific receptors for TC II (4, 5), seems a more attractive reflection of cobalamin status than does total cobalamin (3), most of which is attached to TC I.

Much of the persisting uncertainty about holo-TC II has both methodologic and conceptual origins (6). Now that accurate assay methods are available (7, 8), the many conceptual uncertainties can be addressed more reliably. One such difficulty was a claim that holo-TC II could serve as a diagnostic index of cobalamin absorption as well as metabolic cobalamin sufficiency (9, 10). Indeed, the holo-TC II concentration was proposed as a “surrogate” for the Schilling test (11). The hypothesis gained currency because TC II binds absorbed cobalamin abuminally in ileal enterocytes and exits into the portal bloodstream (12, 13). Patients with malabsorption might be unable to generate normal amounts of translocated holo-TC II into the bloodstream. However, the quantitative contribution of ileal holo-TC II to plasma holo-TC II is unknown. Moreover, support for serum holo-TC II as a measure of cobalamin absorption is meager, consisting of a study of a few patients with AIDS (9) and of a comparison of holo-TC II with unreliable markers, such as gastric or even duodenal histology, instead of absorption tests (10). Moreover, many studied patients have had deficiency and malabsorption...
together, which makes clear attributions difficult. The concept that a test can be used to diagnose both deficiency and malabsorption is problematic because the 2 defects are not identical. If holo-TC II truly reflects both processes, holo-TC II changes would perforce lose all diagnostic specificity for either process.

To address these issues of holo-TC II abnormality directly, we studied patients with PA after they were treated with cobalamin. Adequately treated patients still have malabsorption but not deficiency. Normal holo-TC II concentrations in them would favor metabolic status as the chief regulator of serum holo-TC II, whereas low concentrations would favor malabsorption as the major influence; intermediate concentrations would suggest the potential for diagnostic confusion.

SUBJECTS AND METHODS

Subjects

Our 2 laboratories, 1 in the United States and 1 in Spain, used their collections of frozen serum (−20 °C) that were left over after clinically indicated diagnostic tests were conducted in patients with confirmed PA. The samples had been collected over a variable time span of several months to 7 y. The key study group was patients with PA whose cobalamin deficiency was treated for ≥2 mo with cyanocobalamin injections, usually after an initial month of weekly or more frequent injections, and were proven to be metabolically normal, although they still had malabsorption.

In all cases, the diagnosis of cobalamin deficiency had been made previously; all patients had megaloblastic anemia, myeloneuropathy, or both and had subnormal cobalamin concentrations before treatment. The diagnosis of PA, defined as cobalamin malabsorption caused by a loss of gastric intrinsic factor, was established in all patients by one or more of the following tests: a diagnostic Schilling test (low absorption that became normal on retesting with oral intrinsic factor), absence of gastric intrinsic factor in gastric juice collected after pentagastrin stimulation, and presence of antintrinsic factor antibody in a blood specimen obtained at a time remote from cobalamin injection.

The only selection factors were that the clinical and diagnostic information was complete and diagnostically conclusive and that an adequate volume of serum obtained remote in time enough from the previous cobalamin injection was available. The blood was usually obtained just before the next monthly injection, and blood samples with cobalamin concentrations >750 pmol/L were not used. These sampling precautions helped avoid spurious elevation of holo-TC II from the transient saturation of TC II by exogenous cobalamin, which occurs during the first few days after injection. The samples were also assayed for serum cobalamin, plasma tHcy and, if needed, MMA concentrations to ensure that the patients were metabolically cobalamin-replete. Completely normal results from those metabolic tests were obtained in 23 of the 32 serum samples collected from treated patients with PA. In the other 9 samples, however, abnormal metabolic status was identified by a low cobalamin concentration (<180 pmol/L), an elevated tHcy concentration (>14.9 μmol/L in men, and >14.5 μmol/L in women), or an elevated MMA concentration (>279 nmol/L), despite regular monthly cobalamin injections. Holo-TC II concentrations played no role in the metabolic assessment of any patient. To ensure a valid study group of treated patients with PA in complete metabolic remission, the 9 patients were excluded so that their residual biochemical evidence of mild cobalamin deficiency, whatever its precise explanation, would not compromise the interpretation of data in the key study group—the 23 treated patients with PA whose normal metabolic findings satisfactorily indicated cobalamin malabsorption without cobalamin deficiency.

In addition, we tested identically processed and stored samples collected from 6 untreated cobalamin-deficient patients with PA; all 6 had abnormal metabolic findings. Because holo-TC II is known to be low in untreated patients with PA (3, 7, 8), and because holo-TC II concentrations in the 6 samples were all low, additional specimens from untreated patients were not sought. The 23 serum samples from patients with treated PA in metabolic remission also included posttreatment samples from 4 of the 6 untreated PA patients; these 4 pairs of matched samples allowed direct comparisons of holo-TC II to be made before and after treatment.

A final group consisted of 33 samples from apparently healthy persons with no clinical or biochemical evidence for cobalamin deficiency. These persons were slightly but not significantly older than the treated PA patients (59 ± 14 years compared with 53 ± 17 years). An essential requirement was that the samples be obtained over the same time span from the same institutions and were collected and processed in the same way and stored for durations similar to those for the PA patients’ samples. Although 4 of the 33 control subjects had slightly elevated tHcy concentrations, a not unexpected proportion in normal older people, none of the 4 had abnormal cobalamin or MMA (or holo-TC II) concentrations; similarly, the control subject with a low-normal cobalamin concentration of 189 pmol/L had normal tHcy, MMA, and holo-TC II concentrations. On the basis of cobalamin status, the 33 serum samples thus provided a satisfactory control group for holo-TC II concentrations as well as material for retesting in each assay to confirm the reproducibility of the radioimmunoassay for holo-TC II. The collection of all blood samples met the requirements of our Institutional Review Boards.

Methods

Serum holo-TC II was measured by radioimmunoassay (7), which was performed according to the manufacturer’s protocol that accompanies the kit (Holo-TC; Axis-Shield, Oslo). All sera and standards were assayed in duplicate. To avoid possible influences of timing of magnetic separation among the assay tubes, the duplicate samples were spaced apart from each other in the assay’s pipetting sequence. Serum total cobalamin was measured with the Quantaphase II radioassay (Bio-Rad, Hercules, CA), and plasma tHcy was assayed with the IMx fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, IL) in our laboratory. MMA was measured in selected serum samples by gas chromatography–mass spectrometry by Quest Diagnostics (Teterboro, NJ).

Statistical analyses were performed with the assistance of Pavel Kiselev. Analysis of variance was performed by using the general linear model procedure (SAS, version 8.0; SAS Institute, Cary, NC). Because holo-TC II, the main variable of interest, showed a skewed distribution, its values were log transformed to improve the normality characteristics. Analysis of variance was applied with the SAS general linear model procedure to compare log holo-TC II means. Tukey’s method was used to evaluate the
TABLE 1
Serum holo-transcobalamin II (holo-TC II), total cobalamin, and total homocysteine (tHcy) concentrations in all patients with pernicious anemia (PA) and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Holo-TC II (pmol/L)</th>
<th>Total cobalamin (pmol/L)</th>
<th>tHcy (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects (n = 33)</td>
<td>105 ± 58</td>
<td>305 ± 87</td>
<td>11.6 ± 3.1</td>
</tr>
<tr>
<td>Untreated PA patients (n = 6)</td>
<td>9 ± 6</td>
<td>46 ± 31</td>
<td>140.2 ± 53.7</td>
</tr>
<tr>
<td>Treated PA patients in metabolic remission (n = 23)</td>
<td>74 ± 59</td>
<td>319 ± 127</td>
<td>11.2 ± 2.0</td>
</tr>
<tr>
<td>Treated PA patients with residual deficiency (n = 9)</td>
<td>24 ± 8</td>
<td>130 ± 61</td>
<td>12.5 ± 3.1</td>
</tr>
</tbody>
</table>

1 Mean values in a column with different superscript letters are significantly different, P < 0.05 (see Methods for statistical analysis procedures).
2 Total cobalamin values were obtained in only 5 of the serum samples because of a limited sample volume.
3 Methylmalonic acid concentrations in these patients ranged from 270 to 504 nmol/L (normal reference <279 nmol/L).

RESULTS

Metabolic comparisons of the groups
As expected, the untreated PA patients had very abnormal serum total cobalamin and tHcy concentrations (Table 1). The differences in cobalamin and tHcy between the untreated patients and the control subjects were significant (P < 0.0001). Not surprisingly, the 23 treated PA patients in complete metabolic remission had significantly higher cobalamin concentrations and lower tHcy concentrations than did the untreated patients (P < 0.0001 for both comparisons), and these concentrations were not significantly different from those of the control subjects (Table 1).

Holo-TC II comparisons of the groups
The distribution of values was skewed in the patients with treated PA in metabolic remission as well as in the control subjects. Both of these groups had significantly higher holo-TC II concentrations than did the untreated PA patients (74 ± 59 and 105 ± 58 pmol/L, respectively, compared with 9 ± 6 pmol/L, P < 0.0001 (ANOVA), which were significant at the 0.01 concentration using Tukey’s method; Table 1). Holo-TC II concentrations in the treated PA patients in metabolic remission were also lower than those in the control subjects (P = 0.0045, which was significant at the 0.05 level using Tukey’s method). Although the difference was significant, there was nevertheless great overlap of the treated PA values with the control values, which did not exist in comparison with untreated PA patients. Values for the treated PA patients and for the control subjects could not be clearly separated. Moreover, none of the holo-TC II concentrations in the treated PA patients in remission were <30 pmol/L, which placed them all within the published reference interval for the holo-TC II assay (7) and contrasted sharply with the values in the untreated patients with PA.

Not surprisingly, holo-TC II results intermediate between those of the treated PA patients in metabolic remission and the untreated PA patients were seen in the patients with treated PA whose cobalamin deficiency, because of incomplete treatment, incomplete responses, or other reasons, persisted despite treatment (Table 1), as shown by persistently low cobalamin, elevated tHcy, or elevated MMA concentrations or a combination thereof.

Direct comparisons of holo-TC II before and after treatment
Holo-TC II findings in 4 of the 6 patients with treated PA in remission could also be compared directly with their own pretreatment concentrations (Table 2). The initially low holo-TC II concentrations (1–9 pmol/L) became completely normal in all 4 patients (57–130 pmol/L) when measured in sera obtained at a time remote from the last previous cobalamin injection.

Correlations of holo-TC II concentrations with other biochemical markers
Serum holo-TCII concentrations correlated directly with total cobalamin concentrations (r = 0.45, P = 0.0001) and inversely with tHcy concentrations (r = −0.41, P = 0.001).

DISCUSSION
Circulating concentrations of holo-TC II presumably mirror a balance between cobalamin availability and status, holo-TC II...
elaboration and release from the gut and other sources, and up-
take by TC II-receptor–mediated endocytosis. The diagnostic
ramifications of serum holo-TC II are controversial, in part be-
cause the details of holo-TC II regulation and influences on it are
still uncertain and probably vary. Practical issues being debated
are whether holo-TC II assay provides any diagnostic advantages
over the measurement of serum total cobalamin, whether de-
creased holo-TC II represents the earliest sign of cobalamin
depletion, whether less central cobalamin-related phenomena,
such as malabsorption, affect serum holo-TC II concentrations,
and whether phenomena entirely unrelated to cobalamin status
also do so.

The questions are complicated by the variety of possible
sources of circulating holo-TC II and what determines its clear-
ance (6). Besides the gut, which provides holo-TC II carrying
both ingested and enterohepatically recycled cobalamin (12, 13),
the kidney (14), and perhaps other tissues (eg, the liver) may also
contribute a continuous stream of holo-TC II. Kidney and liver
probably play important roles in clearance as well (14–16), and
cobalamin-unrelated changes such as diseases of these or other
tissues may affect holo-TC II homeostasis. For example, kidney
and liver diseases are associated with elevated holo-TC II con-
centrations (17, 18), and decreased holo-TC II has been attributed
to increased uptake by erythroid hyperplasia of the bone marrow
(19). Indeed, low holo-TC II concentrations often have been
noted in patients without cobalamin deficiency (10, 19). Some-
times, the tendency exists to explain isolated low holo-TC II
concentrations as the earliest marker of cobalamin depletion, one
that is even more sensitive than is tHcy or MMA. However, such
claims are inherently unprovable, especially when so little is
known about alternative explanations for low holo-TC II con-
centrations to some extent, creating a subtle, complex inter-
play of influences. Alternative explanations for the tendency for
lower concentrations in these patients than in control subjects.
The difference was small, but the data suggest that cobalamin absorption modifies serum holo-TC II concentrations to some extent, creating a subtle, complex inter-
play of influences. Alternative explanations for the tendency for
lower concentrations in these patients than in control subjects,
other than the effect of malabsorption, are nevertheless possible.
These explanations include incompletely repleted cobalamin
stores despite restored metabolic function, increased holo-TC II
turnover due to an increased demand for cobalamin or to altered
enterohepatic recirculation in PA regardless of treatment, and
the possibility that some of our patients had residual metabolic de-
ficiency that was undetected by current tests.

Despite the apparent influence of malabsorption on serum
holo-TC II, the effect was not as great as was that of poor met-
abolic status, which produces markedly subnormal holo-TC II
concentrations. No concentrations outside the normal range were
apparent in the treated PA patients in metabolic remission, and
overlap with control values was great. These findings, especially
the overlap, rule out a practical diagnostic use for serum holo-TC
II as an index of cobalamin absorption status and, certainly, as a
surrogate Schilling test. Past interpretations of holo-TC II find-
ings and conclusions based on such assumptions require reas-
ssessment.

A new and somewhat related diagnostic question must also be
raised. The now apparent, albeit small, influence of absorption
may compromise the frequently proposed role of holo-TC II as a
reliable measurement of metabolic cobalamin status rivaling that
of MMA or tHcy. The case made for low holo-TC II concentra-
tions as the earliest marker of cobalamin deficiency owes much
to studies of variably cobalamin-deficient patients who often also
had PA and other forms of cobalamin malabsorption (20), in-
cluding food-cobalamin malabsorption which is much more
common than PA (21). It must now be reconsidered whether the
mild early holo-TC II decreases observed in some studies may
have reflected malabsorption rather than the earliest sign of met-
abolic deficiency.

Several potential limitations of our study merit comment. The
number of patients, especially those with untreated PA, was
relatively small because PA is uncommon and collecting highly
selected samples from well-characterized patients is difficult.
Nevertheless, the sample size even for the untreated patients
sufficed in providing clear data resembling those reported in
the literature and providing statistically significant findings. The
patients with untreated PA were also not central to our hypo-
thesis, which rested on treated PA patients. Another potential
limitation was that many of the serum samples had been stored at
−20 °C for periods of up to several years. The effect of such
storage has not been well studied and will require retesting
samples over several years. We addressed this problem in the study
by selecting control sera that were subjected to storage and han-
dling conditions that were nearly identical to those for the PA
sera, thus presumably neutralizing potential but currently un-
known storage and handling effects in the PA sera. Moreover, our
results in control subjects and in PA patients agreed with those in
the fresher samples in our laboratory as well as with existing data
in the literature. Finally, it is clear that cobalamin injection has
immediate, direct, but transient effects on serum holo-TC II (and
total cobalamin) that can distort interpretation. No data exist on
the duration and arc of this artificial inflation of holo-TC II or
the optimum time for sampling, which probably vary among
individuals. However, given the measured half-life of only a few
hours for holo-TC II (22), the therapy-induced artifact is proba-
bly dissipated after several days and almost certainly by 1 or 2
weeks. Sampling in our study was done several weeks after the
last previous cobalamin injection to minimize the risk of such an
artifact. For further assurance, sera with elevated total cobalamin
concentrations were excluded from study to further avoid the risk
of transient injection-induced holo-TC II artifacts.

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XC and RC were involved in the design of the study, the data collection and
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collection and analysis and provided advice. MPS was involved in the data
collection and analysis. None of the authors had a financial interest or other
conflicts of interest related to this work.
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