Limited value of serum holo-transcobalamin II measurements in the differential diagnosis of macrocytosis

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Abstract

Aim—To study the value of serum holo-transcobalamin II (holo-TCII) measurements in the differential diagnosis of macrocytosis.

Methods—Holo-TCII concentrations were measured in serum samples from 50 healthy non-vegetarian subjects and 30 patients with macrocytosis, using a technique based on the adsorption of holo-TCII with amorphous, precipitated silica. Deoxyuridine (dU) suppression tests were performed on the bone marrow cells of all the patients. Haematological diagnoses were made using standard criteria.

Results—The causes of macrocytosis were cobalamin (Cbl) deficiency due to pernicious anaemia or following partial gastrectomy (10 patients), dietary folate deficiency with/without Cbl deficiency (four patients), chronic alcoholism (four patients), myelodysplastic syndrome (five patients), treatment with methotrexate or azathioprine (three patients), and congenital dyserythropoietic anaemia (CDA) (four patients). Undetectable or low holo-TCII concentrations were found in all patients with Cbl deficiency and in some or all patients from each of the other diagnostic categories. There was also no correlation between the dU suppressed value and the holo-TCII concentration: all 15 patients with high dU suppressed values and nine of 15 with normal dU suppressed values, including four patients with CDA, had low holo-TCII concentrations.

Conclusions—Measurements of serum holo-TCII concentrations by the silica adsorption method are not of value in the differential diagnosis of macrocytosis. The finding of low serum holo-TCII concentrations in patients with macrocytosis due to causes other than Cbl deficiency may result not only from a state of negative Cbl balance but also from other factors, such as increased utilisation of holo-TCII as a consequence of erythroid hyperplasia.

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the Cbl binding serum protein involved in delivering Cbl to cells.¹⁻³ Normally, only some of the TCII molecules contain bound Cbl and such molecules are termed holo-TCII. It has been proposed that a reduction in the serum holo-TCII concentration may occur as an early sign of negative Cbl balance in the absence of other biochemical, haematological or neurological manifestations of Cbl deficiency.^{4,5} In a recent study none of 13 patients with normal total serum Cbl and low holo-TCII concentrations and only nine of 30 patients with a low total serum Cbl and a low holo-TCII concentration were considered to show the effects of tissue Cbl deficiency.⁶ The present study was undertaken to determine the value of holo-TCII measurements in the differential diagnosis of macrocytosis.

Methods

Thirty patients with macrocytosis who underwent bone marrow aspiration during the course of their investigation were studied. Macrocytosis was diagnosed on the basis of mean corpuscular volume values determined using a Technicon H2 analyser (Bayer Diagnostics, Basingstoke, UK). Red cell folate concentrations were determined using the Becton Dickinson folate (¹²⁵I) radioassay kit. In addition to other standard laboratory investigations the following special investigations were carried out.

DEOXYURIDINE SUPPRESSION TEST

An aliquot of freshly aspirated bone marrow was mixed with heparinised Hanks' solution and used to determine the deoxyuridine suppressed value using the method described by Matthews and Wickramasinghe.⁷

HOLO-TCII MEASUREMENTS

Serum holo-TCII concentrations were measured in 50 healthy, non-vegetarian adults and in the 30 patients. A modification of the method of Das *et al*⁸ based on the adsorption of holo-TCII by silica was used, except that total and holo-TCII depleted serum Cbl concentrations were determined by the IM_x Cbl assay using the IM_x system (Abbot Diagnostics Division, Maidenhead, Berks, UK). The IM_x Cbl assay is a microparticle enzyme immunoassay (MEIA) incorporating microparticles coated with porcine intrinsic factor to bind the Cbl.

Preliminary studies were carried out to determine the extent of removal of holo-TCI and holo-TCII from serum by synthetic amor-

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About 6-20% of the cobalamin (Cbl, vitamin B₁₂) in serum is bound to transcobalamin II,

phous precipitated silica (Sipernat 283 LS) (PQ Corporation, Valley Forge, Philadelphia, USA). Normal serum was incubated with 2.5 ng ^{57}Co -cyanocobalamin per ml (^{57}Co -CNCbl; specific activity 10 $\mu\text{Ci}/0.05\text{ }\mu\text{g}$; Amersham International) at 37°C for one hour and dialysed for 24 hours against phosphate buffered saline (^{57}Co -CNCbl treated serum). Either 30 mg silica powder or 300 μl of an aqueous slurry containing 30 mg silica was added to 1 ml of ^{57}Co -CNCbl treated serum. The mixtures were vortexed, left at room temperature for 10 minutes, centrifuged at $5000 \times g$ for 10 minutes and the supernatants removed. Either 800 μl of these supernatants or of silica unadsorbed ^{57}Co -CNCbl treated serum was run on a Sephadex G-200 column as described by Bloomfield and Scott,⁹ collecting 4 ml fractions at a flow rate of 12 ml/hour, and the radioactivity in 1 ml of each fraction determined using a Wallac 80000 gamma counter.

To determine holo-TCII concentrations in serum samples, holo-TCII was removed from serum using a slurry containing 6 g silica (Sipernat 283 LS) in 100 ml distilled water; 250 μl of the slurry containing 15 mg silica was mixed with 500 μl serum, vortexed and left at room temperature for 10 minutes. The mixture was then centrifuged at $5000 \times g$ for 10 minutes. The supernatant fluid was assayed for Cbl (that is, for holo-TCI and holo-TCIII concentrations) using the IM_x method, allowing for the dilution caused by mixing with the silica slurry (multiplication factor for dilution, $\times 1.49$). Total serum Cbl concentrations were also measured simultaneously using the IM_x method and the holo-TCII concentration calculated by subtracting the holo-TCI plus holo-TCIII concentration from the total serum Cbl concentration.

Results

Figure 1 shows the distribution of radioactivity in the fractions collected by Sephadex G-200 chromatography; the data for the serum treated with silica slurry were corrected for dilution. It is evident that silica did not remove significant amounts of holo-TCI and holo-TCIII (that is, holo-haptocorrin) and removed most of the holo-TCII. In two experiments with silica powder, the percentage removal of holo-TCI was 0.9 and 0.5 and the percentage removal of holo-TCII was 89 and 88, respectively. In one experiment with silica slurry, the removal of holo-TCI and holo-TCII was 0% and 87.5%, respectively.

The precision of total serum Cbl measured by the IM_x method was good, both when assayed in a single run and in different runs, with a coefficient of variation of 3.8% (serum Cbl 355–670 ng/l) and 3.4% (serum Cbl 195–785 ng/l), respectively. The coefficient of variation of Cbl concentrations in aliquots of a single serum sample that were treated separately with silica and assayed in a single run was 4.3%. However, when aliquots of the same serum sample were treated separately with silica and both the untreated and silica treated serum samples were assayed in different runs,

the precision for holo-TCII measurements was less satisfactory, with a coefficient of variation of 17.7%; the total Cbl concentration in the serum sample studied in this way was 289 ng/l and its holo-TCII concentration was 43.9 ng/l.

The serum holo-TCII concentrations in the 50 healthy adults were log normally distributed, with a median value of 87 ng/l and 95% reference limits of 12.9–544.7 ng/l. The serum holohaptocorrin concentrations were also log normally distributed, with a median value of 381 ng/l and 95% reference limits of 188–771 ng/l.

The eventual diagnoses in the 30 patients studied were: pernicious anaemia (nine patients); post-gastrectomy Cbl deficiency (one patient); dietary deficiency of folate or folate and Cbl (four patients); chronic alcoholism (four patients); myelodysplastic syndrome (MDS) (five patients); macrocytosis secondary to treatment with methotrexate for rheumatoid arthritis (two patients) or to treatment with azathioprine (one patient); and congenital dyserythropoietic anaemia (CDA) (four patients). In three of the patients with CDA, Cbl and folate independent megaloblastic erythropoiesis was the major abnormality present; the fourth patient had CDA type I.

Fifteen patients had low total serum Cbl concentrations (that is, $< 211\text{ ng/l}$ by the IM_x method) and a combination of a low total serum Cbl and a low holo-TCII concentration was found in all patients with pernicious anaemia, all but one of the patients with nutritional deficiency, one patient each with chronic alcoholism or MDS, and one patient with CDA. The combination of a normal total serum Cbl and a low holo TCII concentration was found in three patients with CDA, two patients with chronic alcoholism, one patient each with dietary folate deficiency or MDS, and one of the patients on methotrexate. The combination of a normal total serum Cbl and a normal holo

Figure 1 Radioactivity per ml of fractions obtained by Sephadex G-200 chromatography of ^{57}Co -CNCbl treated serum before and after reaction with silica powder or silica slurry.

Table 1 Distribution of normal and high deoxyuridine suppressed values (dU-sv) and normal and low holo-TCII concentrations in the various categories of patients studied

Cause of macrocytosis	Number of cases			
	dU-sv		Holo-TCII	
	Normal	High	Normal	Low
Pernicious anaemia	—	9	—	9
Post-gastrectomy Cbl deficiency	—	1	—	1
Dietary deficiency of folate ± Cbl	—	4	—	4
Chronic alcoholism	4	—	1	3
Myelodysplastic syndromes	5	—	3	2
Methotrexate treatment	1	1	1	1
Azathioprine treatment	1	—	1	—
CDA	4	—	—	4

Figure 2 Relation between total serum Cbl and holo-TCII concentrations in 50 healthy volunteers, in 15 patients with normal deoxyuridine suppressed values (dU-sv) and in 15 patients with high deoxyuridine suppressed values.

TCII concentration was found in three patients with MDS, one chronic alcoholic and one patient each on azathioprine or methotrexate.

All of the patients with pernicious anaemia or dietary folate deficiency, the patient with post-gastrectomy Cbl deficiency and one of the two patients on methotrexate had high deoxyuridine suppressed values—that is, they had impaired methylation of deoxyuridylate to thymidylate in their bone marrow cells. The remainder had normal values—that is, had no evidence of impairment of this Cbl and folate dependent reaction. The relation between the deoxyuridine suppressed values and serum holo-TCII concentrations is presented in table 1. It is evident that all 15 patients with high deoxyuridine suppressed values and nine of 15 with normal deoxyuridine suppressed values, including the four patients with CDA, had low holo-TCII concentrations. All but four of the patients with normal deoxyuridine suppressed values and low holo-TCII concentrations had normocellular marrow fragments; the four exceptions were the patients with CDA who had very hypercellular marrow fragments as a result of erythroid hyperplasia.

Figure 2 illustrates the relation between total serum Cbl and holo-TCII concentrations in patients and controls. It is evident that there is a considerable overlap between the results in healthy subjects and patients and between the results in patients with high and normal deoxyuridine suppressed values.

Discussion

Three methodological difficulties were identified in the measurement of serum holo-TCII concentrations by the silica adsorption method. Firstly, the lower limit for the sensitivity of most currently used methods for the measurement of Cbl, including the IM_x Cbl assay, is around 60 ng/l. Holo-TCII assays on serum samples giving a Cbl value for silica adsorbed serum below 60 ng/l will be unreliable. Secondly, treatment with silica does not result in the complete removal of holo-TCII; however, the extent of removal is high, being about 88%. Thirdly, the reproducibility of holo-TCII measurements was relatively poor, partly because holo-TCII measurements are calculated as a difference between two assay results, each with its own coefficient of variation. More accurate holo-TCII measurements on serum samples with a low total Cbl concentration would require the use of the *Euglena gracilis* microbiological assay, which can measure Cbl concentrations accurately down to 20 ng/l.¹⁰

In the present study, all 10 patients with Cbl deficiency had undetectable concentrations of holo-TCII. However, undetectable or low concentrations were also found in patients with dietary folate deficiency, in one of the two patients on methotrexate and in a mixed group of nine patients with normal deoxyuridine suppressed values in which the diagnoses were chronic alcoholism, MDS or CDA. Thus, whereas a low holo-TCII concentration seems to be a common feature of a degree of Cbl deficiency which gives rise to macrocytosis and a high deoxyuridine suppressed value, it does not help in distinguishing between Cbl deficiency and other causes of macrocytosis.

TCII is synthesised by several cell types including hepatocytes, endothelial cells and ileal enterocytes.³ As the initial plasma half-life of human holo-TCII injected into humans and rabbits is short (about 60 minutes^{11 12}), holo-TCII must be in a very dynamic state. The concentration of holo-TCII in plasma would depend on a complex balance between the rate of removal of Cbl from plasma holo-TCII by various cell types and the rates of entry into the plasma of new pre-formed holo-TCII molecules or generation in the plasma of holo-TCII by the reaction of newly released Cbl with plasma apo-TCII. Newly absorbed Cbl seems to combine with apo-TCII within the enterocyte and enter the circulation as holo-TCII.³ A low holo-TCII concentration may therefore result from decreased absorption of Cbl in the terminal ileum, a reduced rate of release of Cbl from the liver and other tissues for binding to apo-TCII or an increased rate of clearance of holo-TCII by binding to specific receptors on haemopoietic and other cells, or

by some other mechanism. Previous investigators have only considered the first of these possibilities. Consequently, they have interpreted low holo-TCII concentrations in patients without haematological or biochemical changes attributable to Cbl deficiency as evidence of negative Cbl balance of recent onset due to reduce intake or absorption of this vitamin.^{4 5} Our finding of low holo-TCII concentrations in as many as 60% of patients with normal deoxyuridine suppressed values suggests that one or more of the other mechanisms mentioned above may also be important. In particular, the finding of low holo-TCII concentrations in all four patients with erythroid hyperplasia due to CDA, indicates that noticeably increased erythropoietic activity may be an important cause of low holo-TCII concentrations. The poor precision of serum holo-TCII concentrations when measured in different assay runs by current methods must also contribute to the high prevalence of apparently low holo-TCII concentrations.

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