The effect of recombinant human intrinsic factor on the uptake of vitamin B12 in patients with evident vitamin B12 deficiency

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We report on the use of recombinant human intrinsic factor (rhIF) in a new vitamin B12 absorption test. Holotranscobalamin (holoTC) was measured before and 24 hours after intake of three 9-µg doses of vitamin B12 (B12) and again 24 hours after intake of the same dose of B12 together with rhIF (rhIF-B12). Nine patients with evident vitamin B12 deficiency had a significantly higher increase in holoTC after intake of rhIF-B12 than after intake of B12. Twenty-eight patients with suspected vitamin B12 deficiency showed no additional increase in holoTC after intake of rhIF-B12. We conclude that rhIF promotes B12 absorption among patients with evident vitamin B12 deficiency.

Key words: intrinsic factor, holotranscobalamin, vitamin B12 deficiency, vitamin B12 absorption.

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The absorption of vitamin B12 is quite complicated requiring intrinsic factor (IF) to ensure efficient uptake.[1,2] After absorption vitamin B12 is bound to transcobalamin; the fraction of transcobalamin saturated with vitamin B12 is referred to as holotranscobalamin (holoTC). Suitable methods for the measurement of holoTC have been introduced,[3,4] and as recently reported, changes in holoTC after intake of a high physiological dose of vitamin B12 may reflect vitamin B12 absorption.[5,6] Usually, serum concentrations of cobalamins and the metabolites methylmalonic acid (MMA) and total homocysteine (tHcy) are employed for the diagnosis of vitamin B12 deficiency. Following the diagnosis, the cause of the deficiency must be determined. For years Shilling's test with labeled vitamin B12 has been used to investigate whether lack of the vitamin is caused by lack of IF.[7] However, Schilling's test is no longer easy accessible because of increasing difficulties in obtaining the required labeled vitamin B12, and difficulties in obtaining the IF used for assessing the absorption of vitamin B12 attached to IF (Schilling's test II). Recently, human IF unsaturated with vitamin B12 has been expressed in the plant *Arabidopsis thaliana*.[8] In the present paper we report on the effect of recombinant human intrinsic factor (rhIF) on the uptake of vitamin B12 in patients with evident vitamin B12 deficiency.

Design and Methods

Protocol

Using the laboratory information system we identified non-hospitalized patients with serum cobalamin levels below the reference range (<200 pmol/L). We examined 37 such patients living in the County of Aarhus or the city of Copenhagen, Denmark, recruited between May 2004 and December 2004. None of the patients received vitamin B12 treatment and none was a vegetarian. Vitamin B12 absorption was evaluated by analysis of holoTC in blood samples obtained before and after oral administration of vitamin B12 and vitamin B12 plus rhIF. The study design was as follows: after a blood sample taken on day 1, the patients were administered three 9-µg oral doses of vitamin B12 with 6 h between each dose (administered at 08:00, 14:00 and 20:00 h). After the blood sample was taken on day 2, the patients were administered three 9-µg oral doses (6.7 nmoL) of vitamin B12 plus 1 g dried leaves of transgenic *Arabidopsis thaliana* corresponding to an IF unsaturated binding capacity of 7.3 nmol (administered at 08:00, 14:00 and 20:00 h). The third blood sample was drawn at 08:00 h on day 3. The patients were allowed to have a light breakfast before blood sampling and were allowed to eat their usual diet. Written informed consent was obtained from all participating patients. The study was approved by the Research Ethics Committee of Aarhus County (project number 2003/0267), the Danish Medicines Agency (project number 2612-2414), and followed the guidelines of Good Clinical Practice.[9]

Laboratory and statistical methods

The rhIF was produced in *Arabidopsis thaliana*[8] and harvested November-December 2002. Tablets, each containing 9 µg vitamin B12 (6.7 nmol) or 9 µg vitamin B12 plus 1 g dried leaves (7.3 nmoL), were

Table 1. **Characteristics of the study population at baseline (day 1).**

Variables	*Whole study population, n=37 Median (Range)*	*Patients with evident vitamin B12 deficiency, n=9 Median (Range)*	*Patients with suspected vitamin B12 deficiency, n=28 Median (Range)*	*Reference range*
Age, years	54 (22-84)	70 (26-83)	53 (22-84)	
Serum holotranscobalamin, pmol/L	35 (5-92)	18 (5-30)	42 (23-92)	≥50*
Serum total transcobalamin, pmol/L	917 (463-1485)	1030 (537-1273)	840 (463-1485)	700-1500*
Serum transcobalamin saturation	0.05 (0.01-0.10)	0.02 (0.01-0.04)	0.05 (0.03-0.10)	≥0.05*
Serum cobalamins, pmol/L	146 (49-219)†	88 (49-120)	151 (93-219)†	200-600
Plasma methylmalonic acid, µmol/L	0.32 (0.08-9.00)	1.80 (0.83-9.00)	0.25 (0.08-0.52)	0.08-0.28‡
Plasma total homocysteine, µmol/L	13.0 (4.9-70.4)	26.7 (17.2-48.9)	10.4 (4.9-70.4)	5.8-11.9‡
Blood hemoglobin, mmol/L				
Females	8.1(6.6-9.1)	7.1(7.0-8.2)	8.1(6.6-9.1)	7.4-9.6
Males	8.8 (7.2-10.6)	8.8 (7.8-10.3)	8.9 (7.2-10.6)	8.4-10.8
Erythrocyte mean cell volume, fL	92 (81-123)	97 (88-115)	92 (81-123)	85-100
Plasma creatinine, µmol/L	83 (54-183)	85 (61-105)	82 (54-183)	44-115

**The reference ranges are based on 161 donors (age range 21-65 years); †all patients were recruited on the basis of serum cobalamins below 200 pmol/L, however, five patients had serum cobalamins just above 200 pmol/L on day 1; ‡Karsten Rasmussen et al.[13]*

produced by Pharma Skan, Skanderborg, Denmark. The vitamin B12 content (measured by the same method as that used for serum cobalamins, see below) and unsaturated rhIF[8] did not decline during the 18 months of storage (*data not shown*). Enzyme-linked immunosorbent assays (ELISA), with an analytical imprecision of <5%, were used to measure serum holoTC[4] and serum total TC.[10] The measurement range of the holoTC assay is 1.6-100 pmol/L and samples were diluted four times prior to analysis, thus the lowest measurable increase that we could measure from an analytical point of view was 6.4 pmol/L. From a previous study we know that the intra-individual variation is 11%.[5] To account both for the measurement range of the assay and for the intra-individual variation we chose to use an increase in holoTC of 10 pmol/L for those having a baseline holoTC below 45 pmol/L (10 pmol/L/0.22=45 pmol/L) to be sure that the increase reflected vitamin B12 absorption. For patients with holoTC at or above 45 pmol/L, holoTC had to increase by 22% (2SD) to be sure that the increase in holoTC reflected vitamin B12 absorption. TC saturation was calculated as holoTC/total TC. Serum cobalamins were measured with an automated chemiluminescence system (ACS: Centaur™, Bayer A/S; Diamond Diagnostics, Holliston, MA, USA) which had an analytic imprecision of <10%. Auto-antibodies against IF were assayed with an in-house ELISA.[11] MMA was evaluated by gas chromatography mass spectrometry[12] and tHcy by Immulite DPC (Los Angeles, CA, USA) with analytic imprecisions of <8% and <5%, respectively. Plasma was separated from the blood cells within less than 2 hours. Hemoglobin and erythrocyte mean cell volume were measured using a Sysmex XE-2100 (Sysmex Corporation; Mundelein, Illinois, USA) (analytic imprecision, <2%). Creatinine was assayed using Jaffe's method on a Cobas Integra 700 analyzer (Roche; Basel, Switzerland) (analytic imprecision, <3%). All reference intervals are indicated in Table 1. Changes (increases or decreases) in biochemical markers as a function of time were analyzed by comparing changes obtained for the same patient relative to day 1 and day 2. Paired t-tests were used to test differences in changes of the biochemical markers. Appropriate logarithmic transformation was applied to correct for skewed distribution of data. The statistical analyses were conducted using Stata 8 software.

Results and Discussion

Initially, we planned to compare rhIF with native IF in a traditional Schilling's test design, however, this approach proved not to be an option. Native IF was no longer available in our country and labeled vitamin B12 for the Schilling's test was not available on a regular basis. We, therefore, chose to test rhIF employing a new vitamin B12 absorption test in patients classified as having vitamin B12 deficiency, as judged from serum cobalamin levels below the lower reference range. In this new absorption test holoTC is measured before and after intake of non-radioactive vitamin B12 with or without rhIF. It has previously been shown that in healthy individuals three 9 µg doses of vitamin B12 result in a median increase in holoTC of 46 pmol/L,[5] while patients with inherited lack of the receptor for IF or lack of IF show no increase in holoTC[6] indicating that this dosage of vitamin B12 does not lead to an increase in holoTC caused by passive vitamin B12 absorption. Among the 37 patients participating in the present study, nine patients had evident signs of vitamin B12 deficiency defined as both serum cobalamins <125 pmol/L and MMA >0.75 µmol/L and tHcy >15 pmol/L. The remaining 28 patients with moderately increased MMA (0.29-0.75 µmol/L) or decreased serum cobalamins combined with MMA within the reference range were categorized as having suspected vitamin B12 deficiency. The biochemical characteristics of the patients are shown in Table 1. One patient with evident vitamin B12 deficiency (serum cobalamins 84 pmol/L and MMA 3.8 µmol/L) had auto-antibodies against IF. The nine patients with evident vitamin B12 deficiency were older than the other patients, though not statistically signifi-

Table 2. **Three distinct absorption patterns.**

	Normal absorption of free vitamin B12; *HoloTC increase ≥10 pmol/L from day 1 to day 2*	*Reduced absorption of both free vitamin B12 and of vitamin B12 bound to rhIF*;* *HoloTC increase < 10 µmol/L both from day 1 to day 2 and from day 2 to day 3*	*Reduced absorption of free vitamin B12 but normal absorption of vitamin B12 bound to rhIF*;* *HoloTC increase <10 pmol/L from day 1 to day 2, but an increase ≥10 pmol/L from day 2 to day 3*
Evident vitamin B12 deficiency, n=9	2	1	6
Suspected vitamin B12 deficiency, n=28	21	5	2
Total	**23**	**6**	**8**

**Recombinant human intrinsic factor.*

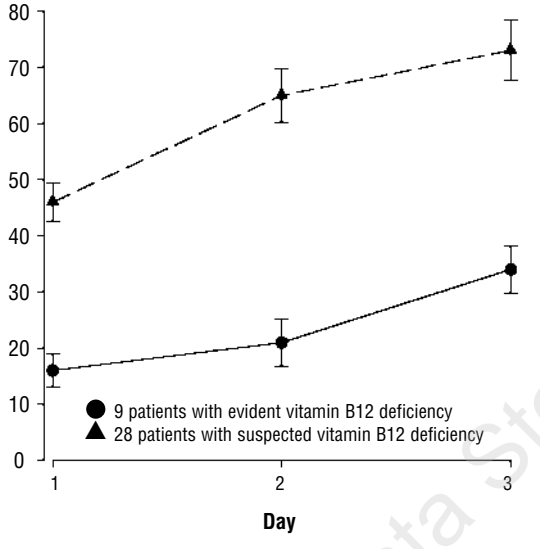

Figure 1. **Changes in mean holotranscobalmin (holoTC) after intake of vitamin B12 (day 1) and vitamin B12 together with recombinant human intrinsic factor (day 2), n =37. Standard errors of the mean are indicated.**

cantly so (p=0.33). We compared the pattern of vitamin B12 absorption between the two groups of patients. As shown in Figure 1, patients with evident vitamin B12 deficiency had a very small increase in holoTC after intake of free vitamin B12 (mean increase; 5 pmol/L from day 1 to day 2) but showed a significantly greater increase in holoTC after intake of vitamin B12 together with rhIF (mean increase; 13 pmol/L from day 2 to day 3) (p=0.02). This finding indicates that rhIF promoted uptake of vitamin B12. In contrast, patients with suspected vitamin B12 deficiency showed an active uptake of free vitamin B12 with a mean increase of holoTC from day 1 to day 2 of 19 pmol/L and a significantly lower increase from day 2 to day 3 (mean increase 9 pmol/L) (p=0.002). Next we looked at the absorption pattern in the individual patients (Table 2) and as expected form a theoretical point of view we identified three different absorption patterns: (i) one group with normal absorption of vitamin B12 reflected by a substantial increase in holoTC after intake of free vitamin B12, indicating vitamin B12 deficiency due to nutritional reasons or malabsorption of vitamin B12 present in food. The majority of the 28 patients with suspected vitamin B12 deficiency belonged to this group (21/28); (ii) the majority of patients (6/9) with evident vitamin B12 deficiency belonged to the second group with limited or no absorption of free vitamin B12 and increased vitamin B12 absorption when the vitamin was given together with IF, indicating lack of IF; (iii) finally, a third group had limited or no absorption of either vitamin B12 alone or vitamin B12 given together with IF, which might indicate malabsorption.

In conclusion our study showed that more than half of patients with low serum cobalamins were capable of absorbing free vitamin B12, and that rhIF increased the vitamin B12 absorption in more than half of the patients unable to absorb free vitamin B12. Based on these results rhIF might be a promising product for development of standardized tests for the evaluation of vitamin B12 absorption.

A-MH, EN, LB, NBL: designed the study. A-MH, TB, BH: included the patients to be performed in the clinical part of the study; A-MH, EN: performed the data analyses; A-MH: wrote the first draft, all authors have contributed actively in the writing process.

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Conflicts of interest. NBL, LB and EN are part of a group of researchers who hold a patent for production of rhIF in plants. Based on this patent the researchers together with investors established the firm Cobento Biotech A/S in 2003, and NBL and LB are now working full time with the company. The rhIF used in this study was produced by the research group as part of their research prior to launching Cobento Biotech A/S.

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