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Recombinant Human Intrinsic Factor Expressed in Plants Is Suitable for Use in Measurement of Vitamin B_{12},

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Competitive binding approaches with use of specific binding proteins are the most commonly used methods to measure vitamin B_{12} in laboratory medicine. Various binding proteins have been used in these methods, including intrinsic factor (IF), pooled human or chicken serum, transcobalamin (TC), and saliva (1, 2). The most widely used of these are non-human IF preparations, usually obtained from hog gastric mucosa. Numerous problems have been reported with their application, however, including difficulty of purification, instability on storage, and variation in the binding capacity connected with changes in serum protein and vitamin B_{12} concentrations (1, 2). If IF is not highly purified, it may contain haptocorrins (also called R proteins), which bind not only vitamin B_{12} but also related metabolically inactive compounds that may be present in the sample, thereby causing artificially increased vitamin B_{12} results (1, 3).

To circumvent the problems associated with nonhuman IF, we recently expressed human IF in plants and obtained a product free of endogenous vitamin B_{12} and contaminating vitamin B_{12}-binding proteins (4). In the current study, we examined the feasibility of using this recombinant human IF for measurement of vitamin B_{12} bound to TC.

Human IF was expressed in the recombinant plant *Arabidopsis thaliana* and purified as described previously (4). As a first step, recombinant human IF was coupled to magnetic beads (Dynabeads, M-280 Tosylactivated; DYNAL) according to the protocol for ligand-binding applications as recommended by the manufacturer. The beads from 1 mL of the standard suspension were washed three times in 0.2 mol/L phosphate-buffered saline, pH 7.4. The washed beads were mixed with 1 mL of recombinant human IF (0.5 g/L) and incubated for 24 h at 37 °C with gentle and continuous agitation. The beads were precipitated, 1 mol/L Tris (pH 7.5) was added to the pellet, and the incubation was then continued for 4 h. The treated beads were then washed five times with 2 mL of 0.05 mol/L Tris (pH 7.5)–0.5 mol/L NaCl and suspended in this buffer.

Recombinant human IF coupled to magnetic beads was used for measurement of vitamin B_{12} in the last step of the Axis-Shield Holo-TC assay in place of the binding protein supplied with the assay. This step measures the vitamin B_{12} bound to TC trapped by insolubilized antibodies (5). The Axis-Shield Holo-TC assay was used to demonstrate

the feasibility of using recombinant human IF for measurement of vitamin B_{12} because it was the only commercial assay that allowed us to use our binder instead of the one supplied by with the assay. The vitamin B_{12} content of the sequestered holo-TC is released under reducing and alkaline conditions, converted to the stable cyano form with potassium cyanide, and quantified in a competitive binding assay with $[^{57}Co]$-cobalamin as tracer and immobilized IF as the vitamin B_{12}-binding protein. In this last step, we modified the assay and used recombinant human IF coupled to Dynabeads in place of the hog IF provided. The recombinant human IF was dissolved in the buffer supplied with the assay (borate buffer, pH 9.6) in a stock solution of ~0.1 μmol/L. This stock solution was stored at −4 °C until application. The stock solution was further diluted (50 μL of stock solution in 9 mL of borate buffer) to obtain "zero" binding of ~75% of the total count, which was comparable to the binding recommended in the assay insert. Fig. 1A shows a calibration curve generated using the manufacturer-supplied calibrators at 0, 10, 20, 40, 80, and 160 pmol/L vitamin B_{12}.

The performance of the vitamin B_{12} assay using human recombinant IF was evaluated by analyzing three serum samples with low, intermediate, and high holo-TC concentrations in five consecutive analytical runs (interassay variation) with each sample analyzed in four replicates (intraassay variation). This enabled us to determine intraassay and total variation for the assay. The imprecision (intraassay and total variation) and mean values (in parentheses) were 8% and 8% (41 pmol/L), 3% and 6% (63 pmol/L), and 3% and 6% (123 pmol/L). These results indicate that the imprecision of the assay using human recombinant IF was comparable to that for the original assay, which was reported to be <10% for the three different sera (5).

In a buffer matrix stored at −4 °C, recombinant human IF coupled to Dynabeads was stable for 2 months as determined by the analysis of the controls for the holo-TC assay in three concentrations (data not shown).

We compared the vitamin B_{12} assay for measurement of holo-TC using recombinant human IF as binding protein with the original Axis-Shield assay on a total of 96 samples ranging from 10 to 151 pmol/L [71 human serum samples and 25 controls (3 in-house controls and 2 control materials supplied by the manufacturer)]. For all samples and controls (Fig. 1B), the regression equation relating the new assay (y) with the original method (x) was: $y = 1.04x + 2.2$ pmol/L ($S_{y|x} = 6.91$ pmol/L; $r = 0.98$). The 95% confidence intervals for the slope and intercept were 1.002–1.008 and −0.88 to 5.2 pmol/L, respectively.

The recent availability of recombinant human IF expressed in plants allowed us to use this product for diagnostic purposes for the first time. Recombinant human IF was first coupled to magnetic beads and then used as a binder in the last step of the Axis-Shield Holo-TC assay for the measurement of the vitamin B_{12} bound to TC. Previously, our human recombinant IF was shown to be stable during storage and to have properties comparable to those obtained for human gastric IF in terms of the

IF-aquocobalamin spectrum, the relative affinity for cobalamin or the analog cobinamide, and binding to the IF receptor cubilin (4). In the current study, use of the Axis-Shield assay allowed us to search for weaknesses in use of the recombinant IF because this assay was designed to measure low concentrations of vitamin B_{12}.

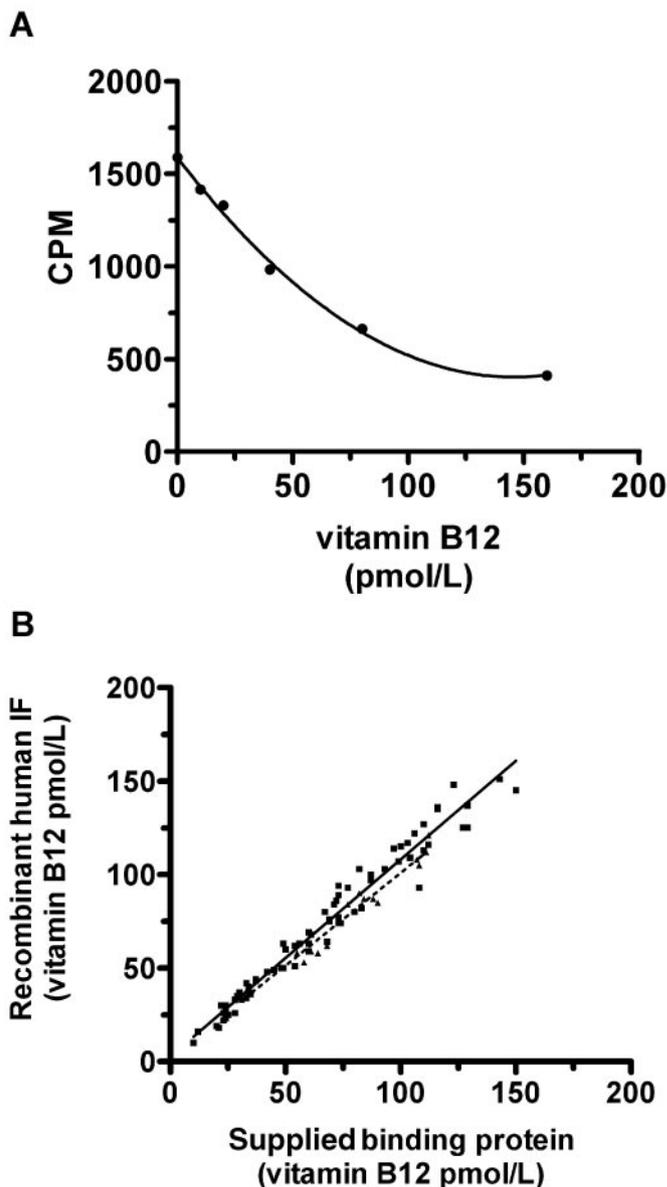We conclude that recombinant human IF produced in

Fig. 1. Calibration curve for vitamin B_{12} used for measurement of holo-TC (Axis-Shield) with use of recombinant human IF (A), and comparison of the performance of recombinant human IF and binding protein supplied by Axis-Shield in the holo-TC analysis (B).

(A), the vitamin B_{12} bound to TC is quantified by a competitive-binding radioassay. The calibration curve covers vitamin B_{12} concentrations of 0–160 pmol/L. (B), 96 samples [71 human serum samples (■) and 25 controls (▲)] ranging from 10 to 151 pmol/L holo-TC were analyzed by the holo-TC assay using recombinant human IF as binding protein and the original Axis-Shield assay. The regression lines obtained were as follows: for human serum samples (n = 71; solid line), $y = 1.05x + 2.8$ pmol/L ($r = 0.98$); for controls (n = 25; dashed line), $y = 0.99x + 1.7$ pmol/L ($r = 0.99$).

plants may well be an optimal binder for use in assays of vitamin B_{12} in plasma and/or serum.

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Effect of Anticoagulants and Storage Temperature on the Stability of Receptor Activator for Nuclear Factor-κB Ligand and Osteoprotegerin in Plasma and Serum, *Benjamin Y.Y. Chan,[1] Katherine A. Buckley,[2] Brian H. Durham,[1] James A. Gallagher,[2] and William D. Fraser[1]** ([1] Department of Clinical Chemistry, Royal Liverpool University Hospital, The University of Liverpool, and [2] Human Bone Cell Research Group, Department of Human Anatomy and Cell Biology, The University of Liverpool, Liverpool, L69 3GA, UK; * address correspondence to this author at: Department of Clinical Chemistry, 4th Floor Duncan Bldg., Liverpool L69 3GA, UK; fax 44-151-706-5813, e-mail w.d.fraser@liverpool.ac.uk)

The discovery of receptor activator for nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) as the fundamental factors controlling osteoclast formation and activation has advanced the understanding of the processes involved in osteoclastogenesis and bone remodeling *(1–3)*. RANKL is important for osteoclast survival, differentiation, maturation, and activation, whereas OPG opposes these actions. Expression of RANKL and OPG is altered in many bone remodeling disorders, suggesting that determination of the role of these proteins in bone diseases is of value in understanding their etiology *(4)*.

Accurate quantification of sRANKL and OPG concentrations in serum samples is paramount in research involving metabolic bone disease. The availability of ELISAs for both sRANKL and OPG has led to investigation of the concentrations of these proteins in human samples collected from patients with several disorders and has allowed monitoring of the effect of treatment in bone-related diseases *(4–9)*. Previous reports of OPG

concentrations in postmenopausal women with osteoporosis have produced discordant results in relation to bone turnover *(5, 8)*. Other reports using the sRANKL:OPG ratio to estimate the extent of Paget disease of bone and the effect of bisphosphonate treatment have also shown inconclusive and inconsistent results *(9–11)*. These data suggest a possible variability in sRANKL and OPG measurements in human serum or plasma. In this study we aimed to clarify whether this variability is attributable to genuine differences among the groups of patients studied or whether it reflects inaccuracies resulting from the sampling process. We investigated several factors that may influence the concentrations of sRANKL and OPG in human blood samples by (*a*) collecting blood into different anticoagulants; (*b*) varying the time between blood collection and plasma/serum separation; (*c*) altering the length of storage of samples; and (*d*) altering the temperature at which samples were stored.

The study population consisted of 10 human volunteers with different disease activities, including 4 healthy individuals, 2 newly diagnosed patients with Paget disease of bone, and 4 Pagetic patients receiving treatment with bisphosphonate. Ethical permission for the study was obtained from the local research ethics committee.

Blood was drawn into tubes containing lithium heparin, clot activator, or EDTA, or into protease inhibitor tubes containing aprotinin, leupeptin, pepstatin, and EDTA. All sample collection tubes were purchased from SARSTEDT-MONOVETTE®, except for the protease inhibitor tubes, which were obtained from Nichols Institute Diagnostics.

To investigate the effectiveness of different anticoagulants on stabilizing sRANKL and OPG activity, plasma/serum was separated at 15, 30, and 60 min after venesection by centrifugation at 1814g for 10 min at 4 °C. Separated serum/plasma was stored at 4 and −70 °C for up to 24 h before assay. Samples were then thawed at room temperature before determination of sRANKL and OPG by ELISAs (obtained from Biomedica).

To assess the effect of storage of samples on sRANKL and OPG stability, blood was drawn into lithium-heparin and EDTA collection tubes, allowed to stand at room temperature for 30 min, and centrifuged at 1814g for 10 min at 4 °C before the plasma was collected. A portion of each lithium-heparin- and EDTA-plasma sample was assayed immediately for sRANKL/OPG, and the remainder of each sample was stored at −20 and −70 °C for 6 weeks (lithium heparin and EDTA) and 6 months (EDTA). At the end of these time periods, samples were thawed at room temperature before determination of sRANKL and OPG concentrations.

Statistical analysis was carried out by two-way ANOVA with Tukey's hypothesis. P values <0.05 and a difference of ± 10% were considered significant. Data are presented as the mean (SE).

Interassay imprecision for sRANKL and OPG was established by measuring quality-control material provided in the assays (n = 16 assays). The sRANKL assay had an interassay CV of 13% across the range 3.3–5.4