PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **183**, 333–338 (1986)

Binding of Cobalamin Analogs to Intrinsic Factor–Cobalamin Receptor and Its Prevention by R Binder[1] (42427)

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Abstract. It is now known that nonphysiological cobalamin analogs exist in the gastrointestinal tract, but their metabolic behavior is unclear. In this study, [^{57}Co]cobinamide was used to study its affinity to hog intrinsic factor–cobalamin (IF-Cbl) receptor which has no species specificity against human IF-Cbl receptor, and its relation to human saliva R binder. Cobinamide was prepared from [^{57}Co]cyanocobalamin and separated by paper chromatography. Human IF-Cbl complex was bound to IF-Cbl receptor but free cyanocobalamin was not. Although R binder–cobinamide was not bound to the IF-Cbl receptor, free cobinamide was bound to the IF-Cbl receptor to a significant extent (about one-half of IF–cyanocobalamin binding to the IF-Cbl receptor). We then investigated the binding of cobinamide to R binder and trypsin-treated R binder. Association constant of cobinamide binding to the IF-Cbl receptor was $1.0 \times 10^9\ M^{-1}$ which was much lower than that of cobinamide binding to trypsin-treated R binder and to untreated R binder. Further study indicated that cobinamide binding to the IF-Cbl receptor was blocked by the addition of R binder and also by trypsin-treated R binder. We conclude that one of the roles of R binder is to prevent binding of free cobalamin analogs to the IF-Cbl receptor in the gut.

In 1971, Okuda *et al.* (1) observed an effect of pronase to liberate cobalamin (Cbl) bound to a non-intrinsic factor (IF)–Cbl binder and increase its absorption in the rat small intestine. Their study was soon followed by reports on the effects of trypsin upon Cbl uptake by the intestine (2), degradation of R binder (R) by pancreatic enzyme, and transfer of Cbl from R to IF (3).

Recently, Cbl analogs have been found in human serum (4), animal tissues (5), and vitamin pills containing minerals (6). We have also reported the presence of Cbl analog in human tissues (7, 8) and human bile (9). Brandt *et al.* (10) demonstrated three Cbl analogs in the intestine in man. Cobinamide which is the most prevalent Cbl analog in human intestine (10) has recently been used *in vitro* by us to study its behavior in the alimentary tract (11). In that study, we found that trypsin would act on the R–Cbl complex and transfer Cbl from R to IF, thenceforth to the IF-Cbl receptor (12). It was also found that trypsin would act on the R–cobinamide complex in the presence of free IF and the IF-Cbl receptor (11). However, the role of R is not yet clear, and there is little information on the relationship among Cbl analogs, R, and the IF-Cbl receptor. We therefore examined the role of R in the metabolism and absorption of Cbl and Cbl analogs in an *in vitro* system.

Materials and Methods. *Preparation of cobinamide.* Cobinamide was prepared by the method of Armitage *et al.* (13) Cyanocobalamin ([^{57}Co]CN-Cbl; 200 μCi/μg) (Amersham, Arlington Heights, Ill.) or crystalline nonradioactive cyanocobalamin (CN-Cbl; Nakarai Kagaku, Kyoto, Japan) was treated with concentrated HCl for 60 min at 65°C. Cold water was added to the solution to stop the reaction and HCl was removed by a 2.0 × 30-cm anion-exchange column of AG1-X8 (Bio-Rad Laboratories, Richmond, Calif.). Eluted samples were lyophilized, dissolved with water,and applied to a 0.7 × 4.0-cm *p*-cellulose (Wako Junyaku, Osaka, Japan) column. Crude cobinamide was eluted from the column with 1 *M* NaCl, extracted by phenol, and then separated by paper chromatography (Whatman, Ltd., England) using a solvent which consisted of sec-butanol (800 ml), 7.5 ml of glacial acid, 5.6 μmol of HCN, and a

[1] This work was supported in part by a research grant from the Japanese Ministry of Education.

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0037-9727/86 $1.50

saturating amount of water (386 ml). The purity of cobinamide was analyzed spectrophotometrically, and concentration of cobinamide was determined at 367 nm.

Cbl binding proteins. Human saliva was used as a source of R and was obtained from volunteers, and human gastric juice was also used as a source of IF in this study. R and IF were purified by the method of Allen *et al.* (14) using Cbl–Sepharose.

Anti-IF antibody. Sera from a pernicious anemia patient was evaluated by using the method of Gottlieb *et al.* (15).

Anti-R antibody. To prepare anti(human saliva)-R antibody, 1.0 ml (0.4 mg protein/ml) of purified human saliva R was mixed with an equal volume of Freund's adjuvant and injected to a male New Zealand rabbit. Blood was taken 19 days after the booster injection which was given 24 days after the initial injection (16).

Preparation of the IF-Cbl receptor extract. The method of Katz *et al.* (17) was used for the preparation of the IF-Cbl receptor extract. The hog receptor was used in the study because there is no species specificity between humans and hogs in its binding to IF (18). Immediately after slaughter of a hog, the ileum was taken and the mucosa was scraped. Scrapings weighing approximately 18 g were rinsed with cold 0.15 *M* NaCl and placed in a 30 m*M* sodium phosphate solution of pH 7.4. Samples were homogenized in a mixing blender for 60 sec and centrifuged for 60 min at 18,000*g* three times. Pellets were placed in a 3 m*M* sodium phosphate solution of pH 8.0 and sonicated for four 15-sec bursts. A 1% Triton X-100 (Wako Junyaku) solution was added to the samples to a final concentration of 0.5%, and they were incubated overnight. Supernatants were collected after centrifugation at 100,000*g* for 60 min, and dialyzed exhaustively for 48 hr against distilled water. The dialysates were frozen at −20°C.

Binding of IF-Cbl, free Cbl, and cobinamide to the IF-Cbl receptor. We have earlier demonstrated that 3 pmol of IF-Cbl is sufficient in our *in vitro* system to study IF-Cbl binding to the IF-Cbl receptor (19), and therefore 3 pmol of IF-[^{57}Co]Cbl (or free [^{57}Co]Cbl or [^{57}Co]cobinamide) was used. IF-[^{57}Co]Cbl (or [^{57}Co]Cbl or [^{57}Co]cobinamide) (3 pmol) was incubated with 0.1 ml of 10 m*M* $CaCl_2$ and 1.0 ml of IF-Cbl receptor extract for 60 min at 4°C. The mixture was made up to 5 ml with 30 m*M* Tris–HCl, pH 7.4, containing 0.15 *M* NaCl and 0.02% NaN_3 (buffer A). Nonspecific binding of IF-Cbl to IF-Cbl receptor, which was usually caused by protein aggregation or detergent micelle, was examined by incubating a saturating dose of 10 pmol IF-Cbl instead of IF-[^{57}Co]Cbl with the IF-Cbl receptor in buffer A. Nonspecific binding of cobinamide to the IF-Cbl receptor was also examined using a saturating dose of 10 pmol cobinamide before the addition of [^{57}Co]cobinamide. After incubation, each sample was applied to a column of Sephadex G-200 (2.5 × 70 cm) and gel-filtered with buffer A at a flow rate of 12 ml/hr. Fractions of 3.5 ml were collected in a fraction collector, and the difference of radioactivity in the void volume between total binding and nonspecific binding, which was usually less than 10% of total binding, was calculated as IF-Cbl (or Cbl or cobinamide) binding to the IF-Cbl receptor in the extract. Binding to receptor was expressed in picomoles and association constant (K_a) and maximum binding sites (V_{max}) were calculated (18).

Trypsin-treated R. Trypsin-treated R was made by incubating 6 pmol of R with 1 or 5 mg of trypsin (Sigma, St. Louis, Mo.) for 90 min at 37°C in buffer A. After incubation, trypsin digestion was stopped by adding soybean trypsin inhibitor (STI; Sigma) in an amount threefold in excess of trypsin. Various amounts of [^{57}Co]cobinamide (1.8–18 pmol) were added to the R solution treated by 1 or 5 mg trypsin and volume was adjusted to 5 ml with buffer A. These samples were incubated at room temperature for 30 min. The albumin-coated charcoal method (15) was used for the measurement of cobinamide binding to trypsin-treated R, and K_a of cobinamide for trypsin-treated R was calculated.

Cobinamide binding to the IF-Cbl receptor in the presence of R or trypsin-treated R. A physiological amount (about 6 pmol of unsaturated cobalamin binding capacity (UBBC)) of R or trypsin-treated R was incubated with 3 pmol of [^{57}Co]cobinamide at room temperature for 30 min, 1.0 ml of IF-Cbl receptor extract and 0.1 ml of 10 m*M* $CaCl_2$ were added, and the volume was ad-

justed to 5 ml with buffer A. These samples were applied to the column of Sephadex G-200 and gel-filtered.

Trypsin activity. Trypsin activity was measured by the method of Hummel *et al.* (20). *p*-Toluenesulfonyl-L-arginine methyl ester (TAME; Sigma) was used as a substrate, and the assay showed that 1 mg of trypsin XI (Sigma) contained 7105 *N*α-benzoyl-L-arginine ethyl ester (BAEE) units as trypsin activity.

Results. *Binding of IF-Cbl, Cbl, and cobinamide to the IF-Cbl receptor (Fig. 1).* When free [^{57}Co]Cbl was used in the system, no radioactive peak was detected in the void volume following Sephadex G-200 column gel-filtration, indicating that free Cbl was not bound to the IF-Cbl receptor and also indicating that there was no aggregated R nor another Cbl binding protein in the void volume. When IF-[^{57}Co]Cbl was used, the radioactive peak in the void volume contained about 6–25% of

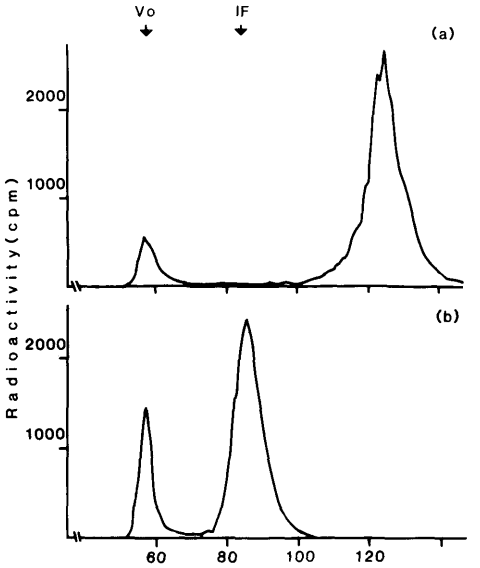

FIG. 1. Elution pattern of intrinsic factor (IF)-cyanocobalamin ([^{57}Co]Cbl) and [^{57}Co]cobinamide after *in vitro* reaction with IF-Cbl receptor. 5.0 ml solution which contained 3 pmol of [^{57}Co]cobinamide (a) or IF-[^{57}Co]Cbl (b) and 1.0 ml of hog IF-Cbl receptor extract were incubated at 4°C and applied to a 2.5 × 70-cm Sephadex G-200 column. Vo, void volume from Sephadex G-200 column; IF, where IF-[^{57}Co]Cbl complex eluted.

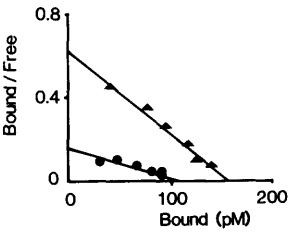

FIG. 2. Scatchard plot of binding of intrinsic factor (IF)-cyanocobalamin ([^{57}Co]Cbl) (▲) or [^{57}Co] labelled cobinamide (●) to IF-Cbl receptor.

the added IF-[^{57}Co]Cbl. Addition of free [^{57}Co]cobinamide to the system resulted in 4–10% [^{57}Co]cobinamide binding to the IF-Cbl receptor, but the size of radioactive peak in the void volume was smaller than that of IF-[^{57}Co]Cbl. The peak in the void volume was confirmed as a complex of IF-[^{57}Co]Cbl and the IF-Cbl receptor by using the saturating dose of 10 pmol cold IF-Cbl prior to addition of IF-[^{57}Co]Cbl or by using 0.1 ml of 100 m*M* EDTA solution (21) which prevented formation of a radioactive peak in the void volume. The radioactive peak that appeared in the region where IF-[^{57}Co]Cbl is known to be eluted was confirmed to be IF-[^{57}Co]Cbl by adding an antibody to IF and observing that the resulting material was eluted in the void volume. [^{57}Co]Cobinamide binding to the IF-Cbl receptor was also confirmed by adding a saturating dose of 10 pmol cold cobinamide to the mixture prior to radioactive cobinamide; this treatment abolished the radioactive peak in the void volume. An excess of cold IF-Cbl also blocked the formation of the complex of [^{57}Co]cobinamide and the IF-Cbl receptor, and an excess of cold cobinamide blocked the binding of IF-[^{57}Co]Cbl to the IF-Cbl receptor.

Association constant (K_a) for binding of IF-Cbl, Cbl, and cobinamide to the IF-Cbl receptor and maximum binding sites (V_{max}). K_a and V_{max} (18) for the binding of IF-[^{57}Co]Cbl, [^{57}Co]Cbl, and [^{57}Co]cobinamide to the IF-Cbl receptor were calculated. Mean values were used for the Scatchard plot (22) which is given in Fig. 2. K_a of IF-Cbl for IF-Cbl receptor was $3.7 \times 10^9\ M^{-1}$ and V_{max} was 161 p*M*. K_a of cobinamide for the IF-Cbl receptor was $1.0 \times 10^9\ M^{-1}$ and V_{max} was 87 p*M*. K_a and V_{max} of Cbl for the IF-Cbl receptor were 0.

K_a of cobinamide for trypsin-treated R. K_a for the binding of [^{57}Co]cobinamide to trypsin-treated R was, with no trypsin, $4.7 \times 10^9\ M^{-1}$; after 30 min digestion with 200 μg/ml trypsin, $3.3 \times 10^9\ M^{-1}$; and with 1 mg/ml trypsin, $1.9 \times 10^9\ M^{-1}$. These K_a values for cobinamide to bind to trypsin-treated R were higher than that of cobinamide for the IF-Cbl receptor.

Inhibitory effect of R on cobinamide binding to the IF-Cbl receptor. The results of the study in which [^{57}Co]cobinamide was incubated with the IF-Cbl receptor in the presence of R or trypsin-treated R are shown in Fig. 3. Cobinamide binding to the IF-Cbl receptor was blocked by R and no radioactivity was detected in the void volume. Trypsin-treated R also blocked a large portion of cobinamide binding to the IF-Cbl receptor in spite of the decrease of cobinamide binding to R after trypsin digestion. The R–[^{57}Co]cobinamide complex peak was confirmed by addition of anti-R antiserum which shifted the radioactive peak to the void volume.

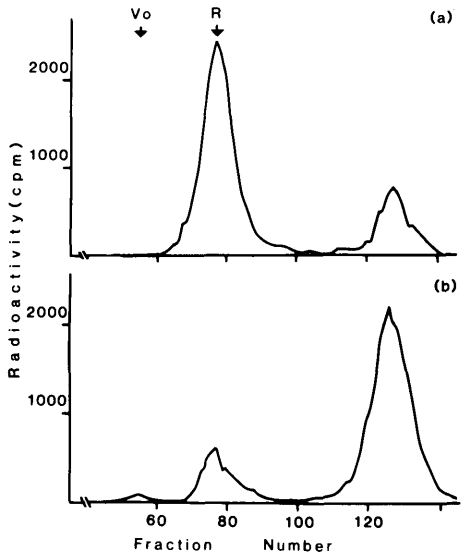

FIG. 3. Elution pattern in a Sephadex G-200 column of a mixture of [^{57}Co]cobinamide and R-binder (R) incubating with intrinsic factor (IF)-cobalamin (Cbl) receptor. Vo, void volume; R, where R-[^{57}Co]cobinamide or R–[^{57}Co]Cbl eluted; (a) elution pattern of a mixture of [^{57}Co]cobinamide and R incubating with IF-Cbl receptor; (b) elution pattern of a mixture of [^{57}Co]cobinamide and trypsin-treated R incubating with IF-Cbl receptor.

Discussion. In the previous studies we demonstrated degradation of R by trypsin, and enterohepatic circulation of Cbl (11, 12). In the present investigation it was found that free cobinamide was bound to the IF-Cbl receptor by 4–10% and that cobinamide binding to the IF-Cbl receptor was blocked by R. Trypsin was used in this study because trypsin was the most effective pancreatic enzyme in degrading R *in vitro* (2, 3). We used human saliva R which is five times more resistant to trypsin than bile R in terms of UBBC (12); it has been suggested that bile R plays a role to discharge Cbl analogs from the body through the liver. Comparison of K_a between cobinamide for trypsin-treated R and cobinamide for the IF-Cbl receptor showed a higher K_a with trypsin-treated R than with the IF-Cbl receptor. These results demonstrated a higher affinity of cobinamide for trypsin-treated R than cobinamide for the IF-Cbl receptor, and also indirectly corroborated the inhibitory effect of R on cobinamide binding to the IF-Cbl receptor. Cobinamide binding to the IF-Cbl receptor was shown by the radioactivity eluted in the void volume that contained a mixture of free [^{57}Co]cobinamide and the IF-Cbl receptor extract (Fig. 1). We ruled out nonspecific cobinamide binding to the IF-Cbl receptor, which is usually caused by detergent micelle or Cbl binder aggregation (21), by using EDTA, acid pH, and an excess amount of cold Cbl in the reaction mixture. Moreover, it has already been reported that holo-R is much more resistant than apo-R to trypsin digestion (23). Therefore we think that the inhibitory effect of R on cobinamide binding to the IF-Cbl receptor is much greater *in vivo* than *in vitro,* and that the difference in K_a between cobinamide for R and cobinamide for trypsin-treated R is smaller in *in vivo* than *in vitro.* In our previous study, free Cbl and Cbl analogs were produced following digestion of bile R by physiological amounts of trypsin. Thus, it seems that Cbl enters the enterohepatic circulation (12), but Cbl analogs do not because they are not bound to IF. The observations that Cbl analogs are bound to saliva R, that the affinity of Cbl analogs to IF is low (24), and that R inhibits binding of Cbl analog to the IF-Cbl receptor suggest that Cbl analog absorption is much smaller than that of Cbl, and perhaps insignificant.

Since guinea pig ileum is not a suitable material for the study of R because the guinea pig mainly has IF as Cbl binder, we used hog ileum. The hog has both IF and R as Cbl binders, and the K_a of human Cbl binders binding to the IF-Cbl receptor of hog ileum is considerably high, and there is no species specificity between hogs and humans for IF-Cbl receptor (18). Mathan *et al.* (25) demonstrated binding of small amounts of free Cbl to the brush border receptor, but under our conditions no Cbl binding to the IF-Cbl receptor was detected. Our study rather showed considerable binding of cobinamide to the IF-Cbl receptor, with the exclusion of the possibility of cobinamide binding to R or other proteins.

These results suggest that Cbl analogs have certain affinity to the IF-Cbl receptor and that R has a capacity to inhibit such binding by sequestering them. It may follow that Cbl analogs are discharged from enterohepatic circulation through such mechanisms. The molecular weight of the IF-Cbl receptor has recently been well characterized (26), and this will permit more precise comparison of the results of these experiments.

If there is no R in the body, free cobinamide or Cbl analogs may compete with IF-Cbl in binding to the IF-Cbl receptor with resultant binding of some cobinamide and Cbl analogs to the IF-Cbl receptor. There has been reported that Cbl analogs are not increased in the serum of R binder deficiency (27, 28), but no report has demonstrated the amount of Cbl analogs in liver, brain, etc. From our experiment, we assume that the Cbl analog level in R-deficient patients may be high in liver, brain, etc. If a high Cbl analog level is detected in R-deficient patients, our data may be supported. However, the Cbl analog turnover depends on absorption, transfer, and excretion, and it may be difficult to predict the Cbl analog level in tissue.

We thank Miss Keiko Matsumaru for her technical assistance in this study.

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Received February 3, 1985. P.S.E.B.M. 1986, Vol. 183.
Accepted July 31, 1986.