Transcobalamin II Mediated Delivery of Albumin-Bound Hydroxocobalamin to Human Liver Cells (43654)

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Abstract. **We show that hydroxocobalamin bound to human serum albumin can dissociate and bind to transcobalamin II present in serum. Human liver cells in culture exposed to hydroxocobalamin bound to albumin incorporated less of the vitamin than when similar amounts of unbound hydroxocobalamin or cyanocobalamin were present. In the presence of transcobalamin II, a 4.5-fold increase in cellular uptake occurred, but this amount was less than when hydroxocobalamin or cyanocobalamin were added to transcobalamin II. These results indicate that albumin, by binding hydroxocobalamin, can alter the dynamics of binding to transcobalamin II and the subsequent cellular incorporation of this form of the vitamin.** [P.S.E.B.M. 1993, Vol 204]

Approximately 75% of cobalamin (Cbl, Vitamin B12) in human plasma is bound to R-type binders whose physiological function is not yet clear. Less than 3% is bound to protein which elutes in the excluded volume of a G-200 gel filtration column (Vo binder). The remaining Cbl is bound to transcobalamin II (TCII), which binds to high affinity receptors on a number of cells, and is internalized and the Cbl is made available for cell metabolism. The physiological properties of these proteins have been amply reviewed (1).

Cbl deficiency is treated by im injection of cyanocobalamin (CN-Cbl) or hydroxocobalamin (OH-Cbl). The latter may be the preferred Cbl, since a more sustained and higher plasma level of Cbl is obtained due to its slower rate of disappearance from the site of injection (2), as well as the ability of OH-Cbl to associate nonspecifically with plasma proteins (3), particularly albumin (4, 5).

The purpose of the present study was to extend our previous observations on the binding of OH-Cbl to plasma proteins (6) by determining to what extent the OH-Cbl bound to albumin may participate in the delivery of Cbl to cells. We have observed that OH-Cbl bound to human albumin is not readily available to human liver cells in culture. However, in the presence of apoTCII, the albumin-bound OH-Cbl can dissociate and bind to TCII, which facilitates its entry into the cell.

Material and Methods

Cobalamin Solutions. CN-[^{57}Co]Cbl (approximately 200 μCi/μg; Amersham Corp.) was either used as purchased or diluted with nonradioactive CN-Cbl (Sigma Chemical Co). OH-[^{57}Co]Cbl was prepared by acid photolysis of CN-[^{57}Co]Cbl as described previously (7). All radioactive solutions were sterile filtered, stored at 4°C in the dark, and assayed periodically for Cbl content and purity as described (8, 9). Pharmaceutical grade OH-Cbl (Eli Lilly and Co.) and CN-Cbl (Wyeth Laboratories Inc) were stored at 4°C in the dark. Analysis of each by thin layer chromatography (10) revealed only one spot by visual inspection.

Protein Solutions. Human serum albumin, (HSA; Fraction V; Sigma) was dissolved in 0.05 *M* sodium phosphate buffer (pH 7.4) containing 0.15 *M* NaCl and stored at -20°C. Labeling of the HSA with excess CN-[^{57}Co]Cbl followed by G-200 chromatography showed it to bind 5 fmol/mg of protein. This radioactive peak eluted in the vicinity of R-binder and was considered to be an impurity in the albumin. No CN-[^{57}Co]Cbl binding was detected in the Vo peak or bound to TCII.

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[^{57}Co]Cbl Binding Ability. The capacity of a substance to bind CN-[^{57}Co]Cbl or OH-[^{57}Co]Cbl was determined by incubation with the respective [^{57}Co]Cbl form for 30 min at 37°C, followed by separation of bound from free [^{57}Co]Cbl using coated charcoal (11) or gel filtration on either Sephadex G-50 (12) or G-200 (13).

Preparation of OH-[^{57}Co]Cbl- and OH-Cbl-Labeled HSA. HSA was incubated with OH-[^{57}Co]Cbl (65 fmol/mg protein) or nonradioactive OH-Cbl (2398 fmol/mg protein) for 30 min at 37°C and the albumin-bound OH-Cbl was separated from unbound OH-Cbl by chromatography on Sephadex G-50 at 4°C (12). The material eluting in the excluded volume of the column was pooled and the Cbl content was determined by either bioassay (8) or counting of radioactivity. The resulting material contained 31 and 1362 fmol of Cbl bound/mg protein, respectively. The material was frozen at -20°C in suitable aliquots that were used only once. The above material was used in the studies to determine the transfer of OH-Cbl from albumin to human serum Cbl binders. Additional albumin-bound OH-[^{57}Co]Cbl (OH-[^{57}Co]Cbl-albumin) was prepared for use in cell culture. HSA was labeled with OH-[^{57}Co]Cbl (32 fmol/mg protein), incubated as above, and then fractionated on Sephadex G-200. The resulting albumin peak was pooled and concentrated in an Amicon Diaflow Ultrafilter fitted with a PM-10 membrane. The resulting material contained 15 fmol of OH-[^{57}Co]Cbl bound/mg protein. OH-Cbl-Albumin was also isolated by G-200 gel filtration of serum from a patient who was receiving injections of OH-Cbl for treatment of Cbl deficiency. This material did not react with antibody to human TCII, eluted in the albumin region on DEAE chromatography and precipitated with rabbit anti-human albumin serum (data not shown).

Determination of the Ability of OH-[^{57}Co]Cbl Bound to Albumin to Dissociate and Bind to Specific Cbl Binding Proteins. The ability of the OH-Cbl bound to HSA to dissociate and bind to specific Cbl binding proteins (R and TCII) was tested by incubation of nonradioactive OH-Cbl-albumin or OH-[^{57}Co]Cbl-albumin with normal human serum at 37°C. At various intervals, aliquots of serum were removed and analyzed for TCII-Cbl by either gel filtration on Sephadex G-200 (13) or by adsorption of the TCII to Quso (14). Experiments that were done with the nonradioactive OH-Cbl-albumin entailed analysis of the serum aliquot for its ability to bind CN-[^{57}Co]Cbl during a subsequent 10-min incubation. Dissociation of OH-Cbl from the albumin and subsequent rebinding to specific Cbl binders will be detected as a percentage of inhibition of CN-[^{57}Co]Cbl binding during the second incubation. Experiments using OH-[^{57}Co]Cbl-albumin were performed by direct analysis of the serum for the presence of OH-[^{57}Co]Cbl-labeled TCII or R-binder.

Effect of ApoTCII on the Ability of Human Liver Cells to Incorporate OH-[^{57}Co]Cbl Bound to Albumin. The ability of human cells to utilize the OH-[^{57}Co]Cbl bound to HSA was tested in a human liver cell carcinoma (HepG2) in tissue culture (15). Cells (5×10^5) were plated in 35-mm petri dishes for 4 days in 3 ml of Eagle's minimal essential media containing 10% fetal calf serum. Before the addition of the specific substance to be tested, the cells were washed three times with 3 ml of Dulbecco's phosphate-buffered saline. Duplicate dishes received either 369 fmol of OH-[^{57}Co]Cbl bound to 18 mg of albumin, 369 fmol of CN-[^{57}Co]Cbl and 18 mg of albumin, 369 fmol of OH-[^{57}Co]Cbl without albumin, or 369 fmol of CN-[^{57}Co]Cbl without albumin. An additional four sets of duplicate dishes contained the same as above, but also 863 fmol of apoTCII. All dishes contained 1 ml of test sample in minimal essential media without fetal calf serum and were incubated for 6 hr at 37°C. The cells were then processed as described (15) and the intracellular radioactivity was determined in a gamma spectrometer. Protein was measured in each cell lysate by the method of Lowry (16). All data were analyzed by Student's *t* test (17).

Results

Figure 1 shows the effect of incubating OH-Cbl-albumin (nonradioactive) with normal human serum on the subsequent binding of CN-[^{57}Co]Cbl to the serum Cbl binders as determined by coated charcoal. The OH-Cbl-labeled albumins were obtained either by G-200 gel filtration of serum from a patient being treated with OH-Cbl (Fig. 1A) or by labeling HSA (Sigma) with nonradioactive OH-Cbl and isolating the albumin from free OH-Cbl by G-50 gel filtration (Fig. 1B). Incubation of either source of OH-Cbl-albumin with normal human serum resulted in a gradual increase in inhibition of binding of subsequently added CN-[^{57}Co]Cbl. This inhibition was not due to breakdown of the Cbl binders in the serum, because serum alone maintained its CN-[^{57}Co]Cbl binding ability through most of the study time points. Addition of an equivalent amount of either unbound CN-Cbl or OH-Cbl to serum produced within 30 min an expected rapid inhibition of binding of the subsequently added CN-[^{57}Co]Cbl. This data show that OH-Cbl that is bound to albumin can dissociate and bind to specific Cbl binding proteins in serum.

In order to determine the extent of binding to TCII, a similar study was performed utilizing OH-[^{57}Co]Cbl-HSA that had been isolated by G-50 to remove any unbound OH-[^{57}Co]Cbl. Serum was incubated for various intervals with OH-[^{57}Co]Cbl-HSA and then the TCII adsorbed onto Quso. Figure 2 shows that with the CN-[^{57}Co]Cbl plus albumin, binding to serum TCII

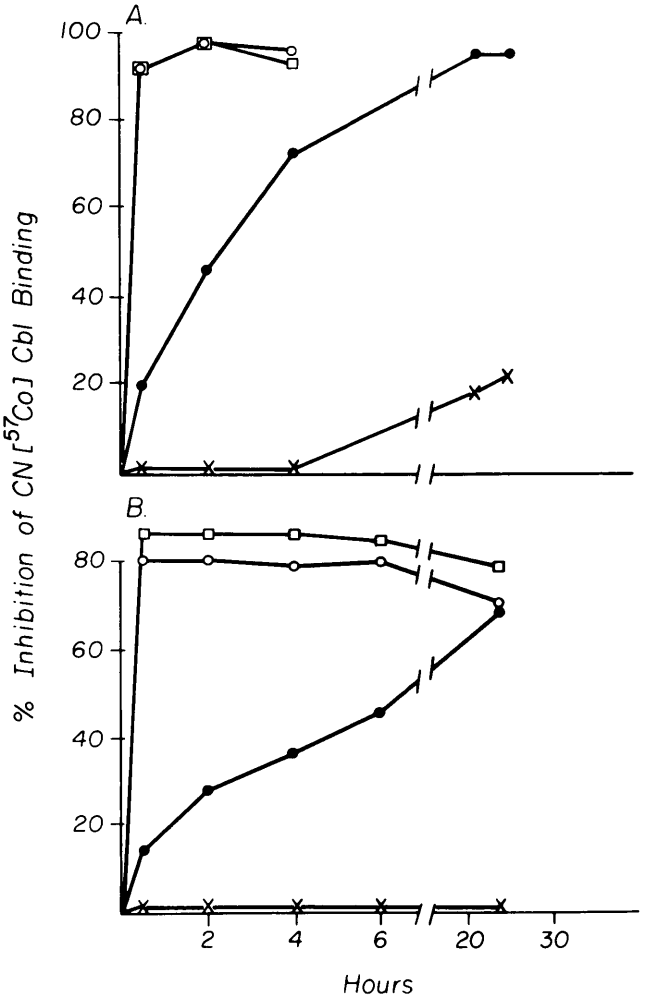

Figure 1. Effect of albumin-bound OH-Cbl on the subsequent binding of CN-[^{57}Co]Cbl to human serum Cbl binding proteins. (A) Normal human serum (100 μl) was incubated at 37°C with either 221 fmol of OH-Cbl bound to albumin isolated from a patients serum by G-200 gel filtration (●), 221 fmol of CN-Cbl (○), 221 fmol of OH-Cbl (□), or without any addition (X). Samples were adjusted to a final volume of 2 ml with phosphate-buffered saline before incubation. At the indicated intervals, 200 μl (148 fmol) of CN-[^{57}Co]Cbl were added for 10 min and the free radioactivity was removed by addition of 2 ml of coated charcoal. (B) Two-milliliter aliquots of normal human serum were added to either 2213 fmol of OH-Cbl-labeled HSA (1.53 mg of protein) that had been isolated by G-50 gel filtration (●), 2213 fmol of free OH-Cbl (□), 2213 fmol of CN-Cbl in the presence of 1.53 mg of G-50-isolated HSA (○), or no addition (X). Samples were in a final volume of 2.5 ml and after incubation for the specified interval at 37°C, duplicate 100-μl aliquots were assayed for CN-[^{57}Co]Cbl binding ability by the coated charcoal assay. Each point represents the average of duplicate determinations.

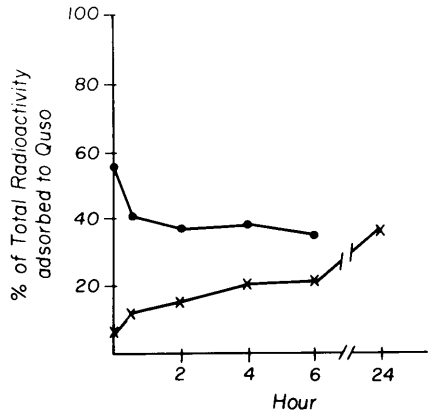

Figure 2. Transfer of OH-[^{57}Co]Cbl from human albumin to serum TCII as detected by an increase in the Quso adsorbable radioactivity. Normal human serum (1.8 ml) was incubated at 37°C with either HSA-OH-[^{57}Co]Cbl (59 fmol Cbl and 1.8 mg protein) that had been isolated by G-50 gel filtration (X) or HSA + CN-[^{57}Co]Cbl (●; 59 fmol Cbl and 1.8 mg of protein). At the indicated intervals, duplicate 100-μl aliquots were treated with 3 mg of Quso in a final volume of 2.0 ml. The Quso was collected by centrifugation and the radioactivity was determined in a gamma spectrometer. Each point represents the average of duplicate determinations.

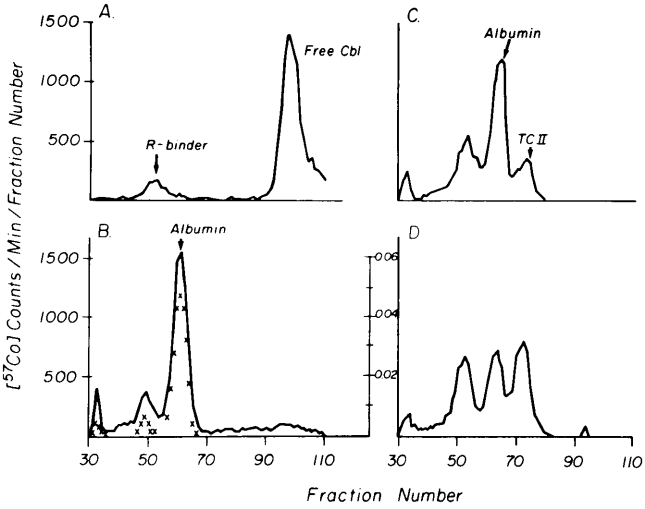

Figure 3. Transfer of OH-[^{57}Co]Cbl from HSA to TCII as determined by G-200 gel filtration. (A) 1.8 mg of HSA labeled with 59 fmol of CN-[^{57}Co]Cbl for 30 min; no serum was present. (B) HSA-OH-[^{57}Co]Cbl (59 fmol of Cbl; 1.8 mg protein) without serum or incubation. Protein was determined by absorbance at 280 nm (X). (C) Normal human serum (1.8 ml) incubated at 37°C with HSA-OH-[^{57}Co]Cbl (59 fmol of Cbl; 1.8 mg protein) for 30 min or (D) 6 hr.

occurred within the first 30 min (40% of the total counts) and did not change significantly after that time. Part of the 60% that adsorbed to Quso at the zero time interval is due to unbound CN-[^{57}Co]Cbl that is present before binding to specific Cbl binding proteins. Incubation of serum with the OH-[^{57}Co]Cbl-albumin resulted in a gradual increase from 6% to 36% of the total counts adsorbed to Quso over a 24-hr period. This shows that OH-Cbl associated with albumin is gradually bound to serum TCII as detected by adsorbance to Quso.

To confirm the above observation, OH-[^{57}Co]Cbl-HSA was incubated with normal human serum for 30 min and 6 hr followed by gel filtration on G-200 (Fig. 3). The HSA bound no CN-[^{57} Co]Cbl to a substance the size of TCII, with 91% eluting as unbound Cbl and the remainder bound to R-binder (Fig. 3A). Figure 3B shows the binding of the same amount of OH-[^{57}Co] Cbl almost exclusively to the albumin protein (68%),

with 7% and 17% eluting in the V_0 and R-binder regions of the chromatograph respectively. Over the time studied, progressively more of the radioactivity was found bound to TCII (14% at 30 min and 30% at 6 hr) and less to the albumin (51% at 30 min and 35% at 6 hr) (Fig. 3, C and D).

Figure 4 shows the effect of apoTCII on the incorporation of the OH-[^{57}Co]Cbl bound to HSA into liver cells. In the absence of apoTCII, the OH-[^{57}Co]Cbl associated with albumin was taken up less than either free OH-[^{57}Co]Cbl ($P < 0.01$) or CN-[^{57}Co]Cbl, although the latter did not reach statistical significance. Addition of apoTCII to those cultures containing OH-[^{57}Co]Cbl or CN-[^{57}CO]Cbl resulted in a 4.8- and 6.8-fold increase in uptake, respectively ($P < 0.01$). This enhanced uptake occurred most likely from the binding of the Cbl to the apoTCII in the media with subsequent facilitation of incorporation. Addition of apoTCII to those cultures containing OH-[^{57}Co]Cbl-albumin resulted in a 4.6-fold increase in uptake ($P < 0.01$), but the absolute amount was less than when apoTCII was added to unbound CN-[^{57}Co]Cbl or OH-[^{57}Co]Cbl. These data show that human liver cells do not appreciably incorporate the OH-Cbl bound to albumin unless a source of apoTCII is available.

Discussion

In addition to its role in maintaining normal oncotic pressure and acid-base balance, albumin binds a number of endogenous and exogenous substances. In so doing, it also serves as a transport protein and defines the pharmacodynamics of those molecules bound to it.

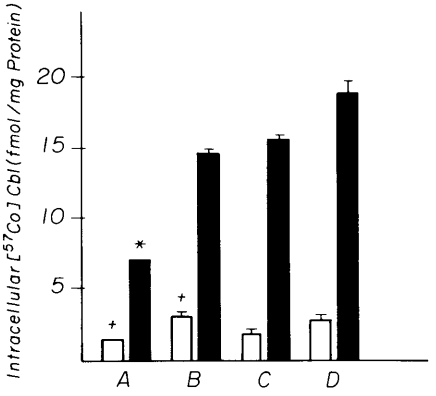

Figure 4. Effect of apoTCII on the incorporation of the OH-[^{57}Co]Cbl bound to albumin (Sigma; Fraction V) into human liver cells. Quadruplicate cultures were incubated with either (A) 369 fmol of OH-[^{57}Co]Cbl bound to 18 mg of albumin that was isolated by G-200 gel filtration; (B) 369 fmol of OH-[^{57}Co]Cbl without albumin; (C) 18 mg of albumin obtained by G-200 but labeled with 369 fmol of CN-[^{57}Co]Cbl after chromatography; or (D) 369 fmol of CN-[^{57}Co]Cbl without albumin. Duplicate cultures from each group contained either no apoTCII (□) or 863 fmol of apoTCII (■). Each bar represents the mean of duplicate determinations. Asterisk indicates values significantly different from all other filled bar values, $P < 0.01$. Plus sign indicates opened bar values significantly different from each other, $P < 0.01$. The presence of apoTCII significantly increased the Cbl internalized in all four studies ($P < 0.01$).

Albumin in human serum may contain small amounts of endogenous Cbl, but it is difficult to accurately quantify in the presence of the Cbl bound to R and TCII. It is more easily identified in patients who lack R or TCII and after treatment with pharmacological doses of OH-Cbl (18–20). R-binder, TCII, and albumin differ greatly in their affinities for OH-Cbl (4, 21). Therefore, exogenous OH-Cbl would be expected to first bind to R and TCII that have high affinity but low capacity and then to albumin that has a low affinity but higher capacity. CN-Cbl, which does not bind to albumin, binds to R and TCII only. Due to these differences, there is a different distribution of Cbl on serum proteins after pharmacological doses of OH-Cbl and CN-Cbl. Higher and more sustained serum levels of Cbl are obtained after OH-Cbl treatment than with CN-Cbl, less is excreted in the urine, and liver concentrations are higher (2, 22, 23).

Our previous studies have shown that OH-[^{57}Co]Cbl bound to TCII is internalized and converted to coenzyme forms by HeLa cells and fibroblasts better than equivalent amounts of CN-[^{57}Co]Cbl-TCII (6, 24). Serum labeled with excess OH-[^{57}Co]Cbl, where much of the excess is bound to albumin, did not enhance nor inhibit the uptake of Cbl, which was determined solely by the amount bound to TCII. The present results (Fig. 4) confirm these observations because OH-[^{57}Co]Cbl bound to albumin was taken up less than unbound OH-[^{57}Co]Cbl. More importantly, we extend these previous observations to show that in the presence of apoTCII, albumin can play a role in OH-Cbl transport by acting as a pool of bound Cbl that is made available to TCII for subsequent delivery to cells.

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