

# ***In Vitro* and *in Vivo* Inactivation of Transcobalamin II Receptor by Its Antiserum\***

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**Rabbits injected with pure human placental transcobalamin II-receptor (TC II-R) failed to thrive with no apparent tissue or organ damage, but a 2-fold elevation of the metabolites, homocysteine, methylmalonic acid, and the ligand, transcobalamin II, in their plasma. Exogenously added transcobalamin II-[<sup>57</sup>Co]cyanocobalamin bound very poorly (2–5%) to the affected rabbit liver, kidney, and intestinal total or intestinal basolateral membrane extracts relative to the binding by membrane extracts from normal rabbit tissues. The activity was restored to normal values following a wash of affected rabbit tissue membranes with pH 3 buffer containing 200 mM potassium thiocyanate. Immunoblot analysis of normal and affected rabbit kidney and liver total membranes revealed similar amounts of 124-kDa TC II-R dimer protein. The neutralized and dialyzed extract from the affected rabbit membranes inhibited the binding of the ligand to pure TC II-R and the harvested affected rabbit serum inhibited the uptake of TC II-[<sup>57</sup>Co]cobalamin (Cbl) from the basolateral side of human intestinal epithelial (Caco-2) cells and decreased the utilization of [<sup>57</sup>Co]Cbl as coenzymes by the Cbl-dependent enzymes. The loss of exogenously added ligand binding or the binding of [<sup>125</sup>I]-protein A occurred with the intestinal basolateral, but not the apical membranes. Based on these results, we suggest that circulating antibodies to TC II-R cause its *in vivo* functional inactivation, suppress Cbl uptake by multiple tissues, and thus cause severe Cbl deficiency and the noted failure to thrive.**

The plasma transport of absorbed dietary and biliary cobalamin (Cbl: vitamin B<sub>12</sub>)<sup>1</sup> bound to plasma transporter, transcobalamin II (TC II) occurs by receptor mediated endocytosis (1) via TC II-receptor (2) (TC II-R), which is expressed as a non-covalent dimer of molecular mass of 124 kDa in all tissue plasma membranes (3). Disruption in the cellular uptake of TC II-Cbl will ultimately result in intracellular Cbl deficiency and decreased synthesis of coenzyme forms of Cbl, methyl-Cbl, and adenosyl-Cbl (4). This in turn will affect the enzymatic conver-

sion of homocysteine to methionine by methionine synthase and methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase, respectively. Thus, intracellular Cbl deficiency cause increased plasma levels of homocysteine (HC) and methylmalonic acid (MMA), and their measurements in plasma are indicative of intracellular functional deficiency of Cbl (5, 6).

Many acquired or inherited causes of defective Cbl absorption and transport (7) and an autoimmune disorder, pernicious anemia, lead to the development of Cbl deficiency. Although some aspects of pathophysiology of development of Cbl deficiency due to acquired and inherited disorders are known (7), how altered immunity causes pernicious anemia is not fully understood. Many patients with pernicious anemia have circulating antibodies to gastric intrinsic factor or other parietal cell surface and cytoplasmic antigens (8). Due to gastric mucosal atrophy, these patients fail to produce intrinsic factor, a secretory glycoprotein essential for the absorption of Cbl and thus develop Cbl deficiency due to malabsorption of the vitamin. In contrast, no known autoimmune disorders leading to defective plasma transport of Cbl involving functional loss of either transcobalamin II or its cell surface receptor has been reported to date. However, inherited disorders involving lack or defective expression of transcobalamin II are known (9), and these children generally develop Cbl deficiency faster than those with absorption defects (4). Thus, the consequence of defective uptake of plasma TC II-Cbl due to functional loss of TC II-R should also result in the faster development of Cbl deficiency, since TC II-R-mediated uptake of TC II-Cbl is the only mode of delivery of physiological amounts of Cbl to all the tissues (10). This hypothesis was validated in rabbits that were injected with human TC II-R for the purposes of raising polyclonal antibodies to TC II-R. The results of the current study show that human TC II-R antibodies inhibit both *in vivo* and *in vitro* the binding of TC II-Cbl in effect creating functional loss of TC II-R activity, thus suppressing Cbl transport, development of intracellular Cbl deficiency, and the noted failure to thrive.

## MATERIALS AND METHODS

The following chemicals were purchased as indicated: [<sup>57</sup>Co]Cbl (15 µCi/ml, Amersham Corp.), [<sup>125</sup>I]-protein A (>30 µCi/µg, ICN Radiochemicals, Irvine, CA), cellulose nitrate membranes (Schleicher and Schull). Transcobalamin II used in TC II-R activity measurements was partially purified from human plasma according to Lindemans *et al.* (11). Intrinsic factor used in intrinsic factor-cobalamin receptor assays was purified from rat gastric mucosa by affinity chromatography of gastric mucosal extracts on Cbl-Sepharose column as described earlier (12). Antiserum to rabbit transcobalamin II raised in goat was a gift from Dr. Robert H. Allen (University of Colorado Health Science Center, Denver, CO).

**Injections of TC II-R**—New Zealand White rabbits weighing 3 kg were injected subcutaneously at multiple sites with pure human placental TC II-R (3) (30 µg/rabbit) emulsified in Freund's complete adjuvant. Ten days later, the rabbits received a booster dose of TC II-R (30

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<sup>1</sup> The abbreviations used are: Cbl, cobalamin; TC II, transcobalamin II; TC II-R, transcobalamin II-receptor; IFCR, intrinsic factor-cobalamin receptor; KSCN, potassium thiocyanate; HC, homocysteine; MMA, methylmalonic acid.



FIG. 1. The photograph illustrates the relative sizes of an affected (A) and a normal (N) rabbit maintained for 6 weeks. The rabbits were weighed, anesthetized, and photographed. The initial weight of rabbits were 2.8 kg (normal) and 3.0 kg (affected). After 6 weeks, the affected rabbit weighed 1.65 kg and the normal rabbit 3.6 kg.

$\mu\text{g}/\text{rabbit}$ ) emulsified in incomplete adjuvant. The rabbits were bled through the ear vein, seven days later. The rabbits were bled once more the following week. Twenty-four days following the initial injections, the rabbits were pale and looked listless. In the next 3–4 weeks, rabbits injected with TC II-R started wasting and became progressively sick and were sacrificed at the end of 6 weeks. Kidney, liver, and intestine were harvested and stored at  $-70^\circ\text{C}$ .

**Serum Analysis**—The serum obtained from the rabbits was tested for the presence of antibodies to TC II-R by immunoblotting human placental total membranes and for its ability to inhibit, *in vitro*, the binding of TC II- $^{57}\text{Co}$ Cbl to pure placental TC II-R (3). The serum from the affected and normal rabbits was subjected to the following analysis: total  $^{57}\text{Co}$ Cbl binding ability was assessed by the charcoal adsorption method (13). The radioactive Cbl bound to serum of normal and affected rabbits was immunoprecipitated with antiserum to rabbit TC II to assess the serum TC II levels as described previously (14). Normal and affected animal serum MMA and HC levels were determined by a single gas chromatography/mass spectrometry run analysis (15).

**Cbl Receptor Activity and Protein Determination**—TC II-R activity in the Triton X-100 extracts of normal and affected rabbit tissue total membranes or isolated intestinal apical and basolateral membranes was determined as described earlier (3). In some experiments, prior to detergent extraction and ligand binding assay, the total membranes were treated as follows. Total membrane prepared was suspended and homogenized in 2 ml of (a) 10 mM Tris-HCl buffer, pH 7.5 or (b) 10 mM Tris-HCl adjusted to pH 5 and containing 5 mM EDTA or (c) 0.1 M glycine HCl buffer, pH 3, containing 200 mM KSCN and incubated for 1 h at room temperature. The membranes were pelleted down by centrifugation, washed once with the same respective buffers, re-pelleted down, and finally suspended and homogenized in 10 mM Tris-HCl buffer, pH 7.5, and extracted with Triton X-100 for 6 h. The intrinsic factor-cobalamin receptor activities in the total or in the isolated apical and basolateral membranes were determined using rat IF- $^{57}\text{Co}$ Cbl (2.5 pmol) as described earlier (16). Protein concentration in membrane and membrane extracts was determined by the method of Bradford (17).

The ability of membrane extracts (0.1 M glycine HCl/KSCN and pH 5/EDTA extracts) and the harvested rabbit serum to inhibit *in vitro*, the binding of TC II- $^{57}\text{Co}$ Cbl to pure human TC II-R was carried out as described earlier (3). The extracts were neutralized to pH 7.4 and dialyzed against 4 liters of 10 mM Tris-HCl buffer, pH 7.4, for 24 h, with one 2-liter exchange of the dialysis buffer at the end of 12 h, prior to use.

Affected and normal rabbit tissue total membranes from kidney (5  $\mu\text{g}$  of protein) and liver (150  $\mu\text{g}$  of protein) were subjected to nonreducing SDS-polyacrylamide gel electrophoresis (7.5%), separated proteins transferred to nitrocellulose membranes (90-min transfer time) and probed with 1000-fold diluted antiserum to TC II-R and  $^{125}\text{I}$ -protein A. The bands were visualized by autoradiography and quantitated by the AMBIS radioimaging system.

**Isolation of Apical and Basolateral Membranes from Normal and Affected Rabbit Intestine**—The intestine from normal and affected rabbits was harvested and flushed with 100 ml of ice-cold saline, and the mucosa was scraped from the entire gut. The apical membranes were isolated from rabbit intestine by the  $\text{Ca}^{2+}$  aggregation method of Mal-

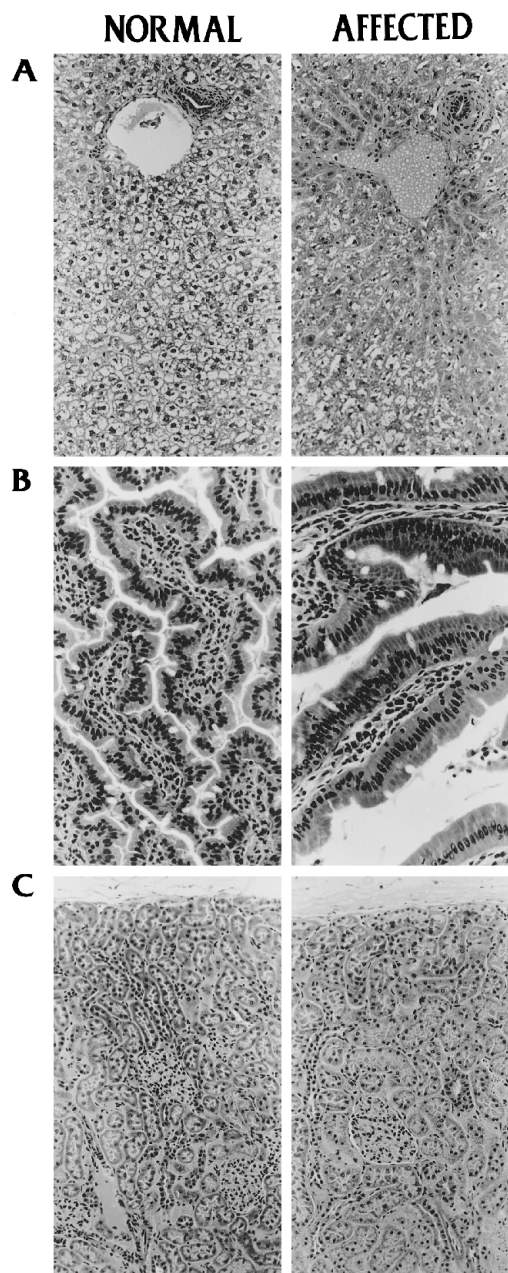


FIG. 2. Light micrographs of liver (A)  $\times 50$ , intestine (B)  $\times 100$  and kidney (C)  $\times 50$  sections (6  $\mu\text{m}$ ) stained with hematoxylin and eosin.

athi *et al.* (18) as described earlier (19). The apical brush-border membrane was enriched for the following apical markers, IFCR (10-fold, 15% recovery),  $\gamma$ -glutamyl transferase (12-fold, 10% recovery) and contained  $<0.5$ –1% of NADPH-cytochrome *c* reductase and  $\beta$ -glucuronidase. The basolateral membranes from rabbit intestine and kidney were isolated by the sucrose gradient centrifugation method of Molitoris and Simon (20). TC II-R activity was enriched about 7-fold and  $\text{Na}^+/\text{K}^+$ -ATPase was enriched 10-fold with 15–20% yield, and the activity recovery of apical markers, maltase and alkaline phosphatase, was  $<1$ –2%.

The binding of  $^{125}\text{I}$ -protein A was carried out as follows. The isolated intestinal basolateral and apical membranes (250  $\mu\text{g}$  of protein) from affected and normal rabbits were incubated with 50–2000 pg of  $^{125}\text{I}$ -Protein A (116  $\mu\text{Ci}/\mu\text{g}$ ) in a volume of 500  $\mu\text{l}$  containing 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride for 1 h at  $22^\circ\text{C}$ . The reaction mixture was microcentrifuged, and the resulting pellet was washed twice with 2 ml of incubation buffer containing 1 mg of bovine serum albumin/ml, and the resulting pellet was counted in a  $\gamma$  counter.

**TC II- $^{57}\text{Co}$ Cbl Transport and Cbl Utilization by Filter-grown**

**Caco-2 Cells**—Post-confluent Caco-2 cells were grown on culture inserts as described before (21). Transepithelial resistance was measured according to Fuller *et al.* (22), and filter-grown cells that demonstrated electrical resistance of 250–300 ohms/cm<sup>2</sup> were used. The ligand, human TC II-[<sup>57</sup>Co]Cbl (2 pmol), was presented on the basolateral side, and surface binding was determined at 5 °C. Specific binding to TC II-R was determined by subtracting the amount of ligand bound at the same temperature to the basolateral side of cells that were incubated with TC II-R antiserum (25 µl in 2 ml of basolateral medium) for 30 min, prior to the addition of the ligand. Uptake and transport of Cbl was carried out at 37 °C for 6 h by allowing the bound ligand to internalize. After 6 h, the cells were scraped, washed with phosphate-buffered saline, and extracted in the same buffer containing Triton X-100 (0.5%). The extract (1 ml) was subjected to size exclusion chromatography on Sephadex G-150 to separate Cbl incorporated into Cbl-dependent enzymes and that which was still bound to TC II according to Rosenblatt *et al.* (23).

**Light Microscopy of Tissues from Normal and Affected Rabbits**—Tissues were fixed in neutral buffered formalin and cut 6 µm thick and stained with hematoxylin and eosin prior to light microscopy.

## RESULTS AND DISCUSSION

**Development of Cbl Deficiency in Rabbits Injected with Transcobalamin II-Receptor**—Following 3–4 weeks of subcutaneous injection of human placental TC II-R, the affected rabbits were listless, failed to thrive, and were wasting despite doubling of the diet dose. By 6 weeks, the animals were sick and had lost nearly 50% of their initial body weight of 3 kg. The smaller size due to weight loss of an affected rabbit and a normal rabbit maintained on the same diet for 6 weeks is shown in Fig. 1. Despite the loss of body weight, the affected rabbit tissues such as liver, intestine, and the kidney showed normal morphology (Fig. 2) under light microscopy. Liver sections (Fig. 2, panel A), including portal triad, showed no consistent differences in the hepatic histology between the affected and normal animals. Intestine of both normal and affected animals showed normal morphology with long villi lined by tall columnar cells with predominant brush borders (Fig. 2, panel B). Renal cortex of both normal and affected rabbits showed normal morphology with no differences in the glomeruli, tubule, and interstitial tissue (Fig. 2, panel C). Since the animals were extremely pale, they were suspected to be anemic and thus they were tested for Cbl deficiency by measuring plasma levels of HC and MMA. Compared with normal rabbit serum,

MMA, HC, and unsaturated Cbl binding due to TC II were elevated 2-fold in the affected rabbits (Table I). The increase of both HC and MMA by 2-fold clearly indicated that the rabbits had developed intracellular deficiency of Cbl, and the increase in plasma TC II levels further indicated that the development of Cbl deficiency could be due to decreased uptake of plasma TC II-Cbl. These initial observations suggested that the noted failure to thrive of these animals was not due to antibody induced organ or tissue damage but instead could be due to the noted Cbl deficiency. Recent studies have shown that *in vitro*, antiserum to TC II-R inhibits the binding of TC II-[<sup>57</sup>Co]Cbl to pure TC II-R (3), suggesting the possibility that circulating TC II-R antiserum may also be functional, *in vivo*, in blocking the binding of TC II-Cbl and hence its tissue uptake.

**Reversible Loss of TC II-R Activity in Affected Rabbit Tissues**—TC II-R activity was determined in the Triton X-100 extracts of the tissue total membranes of the normal and affected rabbits (Table II). Relative to normal rabbit tissue TC II-R activity, there was dramatic decline (97–98%) of TC II-R activity in affected rabbit tissues, such as kidney, intestine, and liver. In contrast, the intrinsic factor-cobalamin receptor activity in either intact membranes or the Triton X-100 extracts of both the normal and affected kidney and intestine were the same. These results indicated that the loss of exogenously added TC II-[<sup>57</sup>Co]Cbl binding activity is due specifically to loss of functional TC II-R and may be due to occupancy of ligand binding sites by either the endogenous ligand TC II-Cbl, known to be in excess in circulation (Table I) or the circulating TC II-R antiserum, which is known to inhibit, *in vitro*, the binding of exogenously added ligand (3) or to the loss of receptor protein from the cell surface. In order to test these possibilities, the following experiments were carried out.

When the tissue membranes from affected rabbits were treated with pH 5/EDTA buffer, the activity in all the three tissue membranes rose only by a very modest amount of <5–10%, indicating that the loss of binding sites was not due to occupancy by the endogenous ligand (data not shown). The binding of TC II-Cbl to TC II-R requires Ca<sup>2+</sup> and neutral pH and pH 5/EDTA treatment dissociates the bound ligand from the receptor. However, when the membranes were treated with glycine HCl buffer, pH 3, containing 200 mM KSCN, there was a dramatic increase in the binding of TC II-[<sup>57</sup>Co]Cbl and 100% of the binding was recovered in all the tissues tested (Table II). These results indicated that the recovery of ligand binding was not due to the release of endogenous TC II-Cbl, but due to the release from the membrane surface, the antibodies to TC II-R. Acidic pH buffers containing chaotropic salt such as KSCN are known to dissociate the immune complexes. However, in order to prove directly that the recovery of receptor activity was actually due to the removal of TC II-R antibody, the neutralized

TABLE I

Serum levels of methylmalonic acid, homocysteine, and transcobalamin II in normal and affected rabbits

The values reported are average values for two rabbits from each group. Other details are provided in the method section.

Animals	Methylmalonic acid	Homocysteine	Transcobalamin II
	µmol/liter		ng/dl
Normal	1.10	12.50	1000.00
Affected	2.15	27.50	2400.00

TABLE II

TC II-R and IFCR activities in tissue membranes of normal and affected rabbits

Indicated tissue total membranes from normal and affected rabbits were incubated for 60 min at room temperature with 0.1 M glycine buffer, pH 3.0, containing 200 mM KSCN, centrifuged, and the resulting membrane pellet was suspended in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride and homogenized. Both the treated and untreated membranes were extracted with Triton X-100 (1%) and centrifuged. The supernatant was assayed for TC II-[<sup>57</sup>Co]Cbl binding. In the case of binding of IF-[<sup>57</sup>Co]Cbl, the total membranes were used directly. The values reported are an average of duplicate assays performed using extracts from each of the indicated tissue obtained from two normal and affected rabbits. Other details of membrane treatment, extraction and assay are provided in methods section.

	Normal rabbit <sup>a</sup>				Affected rabbit <sup>a</sup>			
	TC II-[ <sup>57</sup> Co]Cbl		IF-[ <sup>57</sup> Co]Cbl		TC II-[ <sup>57</sup> Co]Cbl		IF-[ <sup>57</sup> Co]Cbl	
	–	+	–	+	–	+	–	+
	pmol/mg of protein				pmol/mg of protein			
Kidney	40.00	39.63	1.20	1.18	0.83	42.53	1.25	0.13
Intestine	4.92	5.15	0.12	0.11	0.15	5.20	0.11	0.13
Liver	1.46	1.51			0.02	1.52		

<sup>a</sup> With (+) or without (–) ligand pH 3/KSCN treatment.

FIG. 3. *In vitro* inhibition of TC II-[ $^{57}\text{Co}$ ]Cbl binding to pure TC II-R. Indicated amounts of antiserum to TC II-R (●), or the neutralized and dialyzed extracts, 0.1 M glycine HCl buffer pH 3.0/KSCN (■) or the pH 5/EDTA (▲) treated were first incubated with diluted pure receptor for 30 min at room temperature and then assayed for ligand binding. The values reported represent an average of triplicate assays performed at each concentration of the extracts or antiserum.

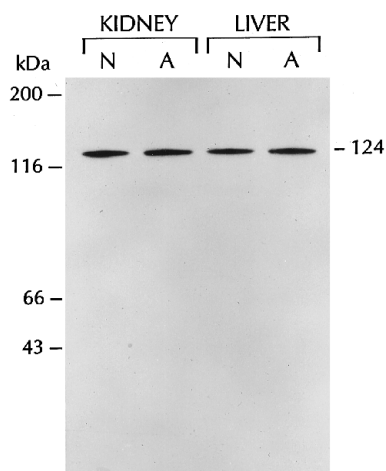
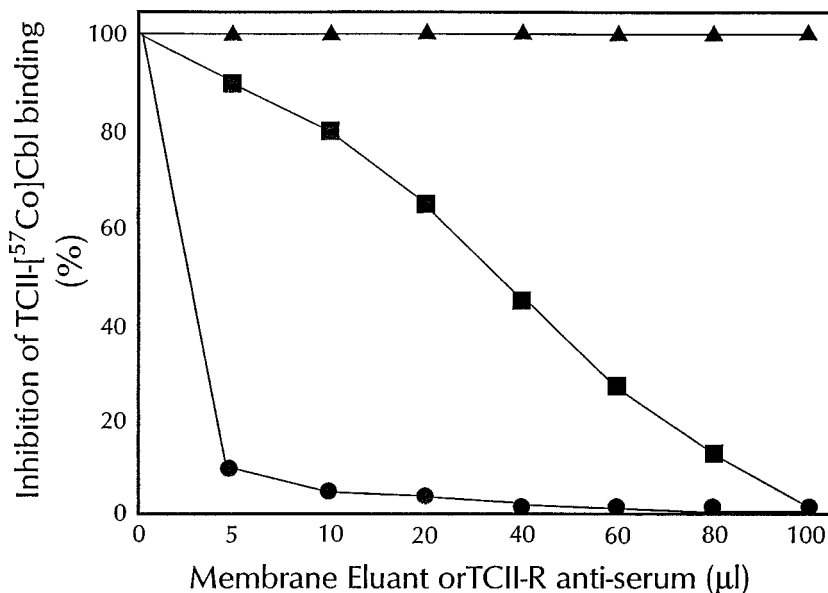


FIG. 4. Immunoblots of normal (N) and affected (A) rabbit kidney and liver membranes. Indicated tissue membranes from normal and affected rabbits were separated on nonreducing SDS-polyacrylamide gel electrophoresis (7.5%) and subjected to immunoblotting. Other details are provided under "Materials and Methods."

and dialyzed membrane eluant was titrated for its ability to inhibit, *in vitro*, the binding of TC II-[ $^{57}\text{Co}$ ]Cbl to pure human TC II-R (Fig. 3). The neutralized and dialyzed pH 3/KSCN extract was able to inhibit ligand binding, and 50% inhibition was noted with 35  $\mu\text{l}$  of the eluant compared with similar amount of inhibition of ligand binding by only 2.5  $\mu\text{l}$  of directly harvested TC II-R antiserum from rabbit blood. This difference was due to the dilution of the antibody in the membrane extract. In contrast, there was no inhibition of binding with pH 5/EDTA membrane extract. These results clearly indicate that the loss of TC II-R activity in the affected rabbits was due to occupancy of the receptor ligand binding sites by TC II-R antibody. Furthermore, immunoblot analysis (Fig. 4) of liver and renal total membranes from normal and affected rabbits revealed similar amounts of 124-kDa TC II-R, demonstrating that in affected rabbit membranes, TC II-R was present, but was inactive in ligand binding. It is interesting to note that the size of TC II-R revealed in rabbit membranes is 124 kDa, the exact size of TC II-R dimer revealed using the same antiserum against human (3) and rat tissue (24) membranes. The recognition of a single protein band of 124 kDa in tissue membranes across species demonstrated that the antiserum contained an-

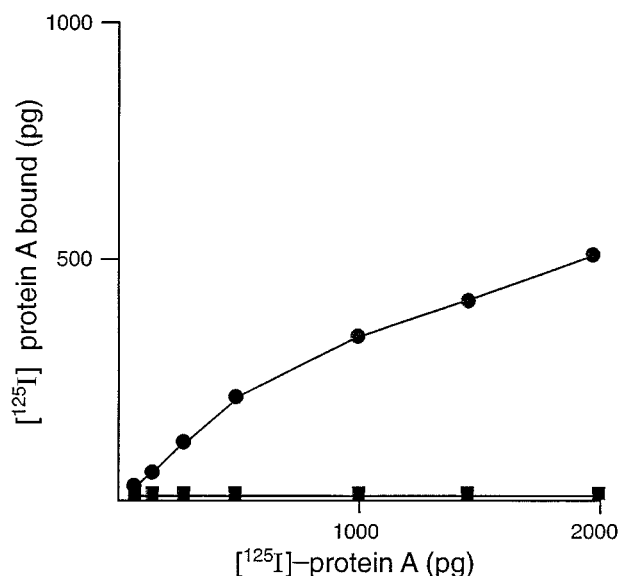


FIG. 5. Binding of  $^{125}\text{I}$ -protein A to the apical and basolateral membranes of the affected rabbits. Isolated intestinal basolateral (●) and apical (■) membranes (250  $\mu\text{g}$  of protein) from affected rabbits were incubated with different concentrations of  $^{125}\text{I}$ -protein A (50–2000 pg) for 1 h at 22 °C. Other details are provided under "Materials and Methods."

tibody to a single membrane antigen and that the observed effects of Cbl deficiency are due to functional loss of a single membrane component, TC II-R.

In contrast to this observation, when intrinsic factor-cobalamin receptor (25, 26) is injected into rabbits, the rabbits do not become Cbl-deficient, although they produced antibodies to IFCR, and this antiserum inhibited, *in vitro*, the binding of IF-[ $^{57}\text{Co}$ ]Cbl to IFCR (25, 26). However, the lack of effect of circulatory antibodies to IFCR in causing Cbl deficiency may be due to the fact that the receptor which is functionally active in the intestinal luminal or apical membranes will not be in direct contact with the circulating antibodies. If this hypothesis is true then the antiserum binding to TC II-R and its subsequent functional inactivation in the affected rabbit intestine should be a property of TC II-R expressed in the basolateral membranes where it will be exposed to the circulation.

*In Vivo Inactivation of the Basolaterally Expressed TC II-R*

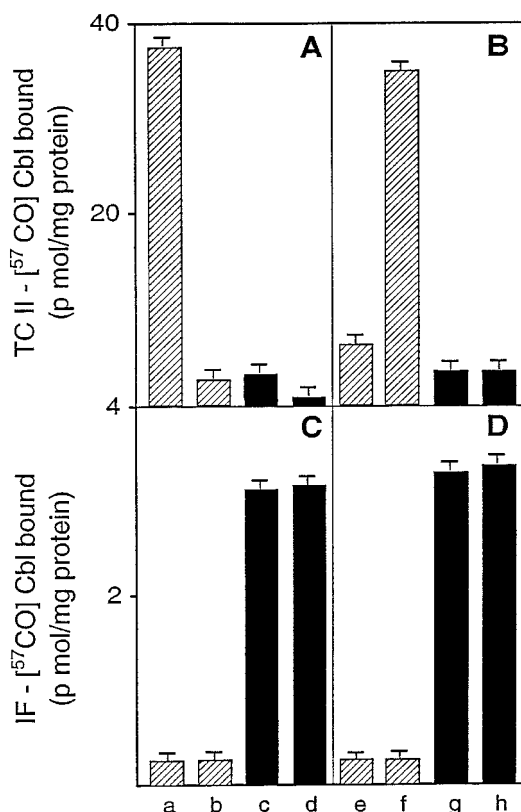


FIG. 6. *In vitro* (panels A and C) and *in vivo* (panels B and D) effects of TC II-R antiserum on TC II-R (panels A and B) and IF-CR (panels C and D) activities in the intestinal apical and basolateral membranes. Panel A, isolated basolateral membranes (250  $\mu$ g) and apical membranes (250  $\mu$ g) from normal rabbits were incubated with (columns b and d) or without (columns a and c) TC II-R antiserum (20  $\mu$ l). Following 1-h incubation with TC II-R antiserum at 5 °C, the membranes were pelleted down and washed with phosphate-buffered saline. The pellet was then solubilized with Triton X-100 (1%), and the detergent extract was used for TC II-R assay. Panel B, isolated basolateral membranes from normal (column f) and affected (column e) rabbits and apical membranes from normal (column h) and affected (column g) rabbits were assayed for TC II-<sup>[57Co]</sup>Cbl binding. The membranes were extracted with Triton X-100 (1%), and the extract was assayed for TC II-R activity. Panel C, isolated basolateral membranes (250  $\mu$ g) and apical membranes (250  $\mu$ g) from normal rabbits were incubated with (columns a and c) or without (columns b and d) TC II-R antiserum (20  $\mu$ l). The TC II-R antiserum treated and untreated membranes were then assayed for IF-<sup>[57Co]</sup>Cbl binding. Panel D, basolateral membranes from normal (column f) and affected (column e) and apical membranes from normal (column h) and affected (column g) rabbits were assayed for IF-CR activity. The values reported are mean  $\pm$  S.D. of duplicate assays performed using five separate isolated apical and basolateral membrane preparations from two normal and affected rabbits.

and *in Vitro* Inhibition of TC II-<sup>[57Co]</sup>Cbl Uptake and <sup>[57Co]</sup>Cbl Utilization from the Basolateral Domain of Polarized Human Intestinal Epithelial Cells—Endogenous presence of TC II-R antibody bound to the basolateral and apical surface membranes of the intestinal mucosa was measured by determining the amount of <sup>125</sup>I-protein A binding to these membranes (Fig. 5). The results show that saturable binding of protein A occurred with the isolated mucosal basolateral but not the apical membranes of the affected rabbit. No binding of <sup>125</sup>I-protein A occurred with either apical or basolateral membranes of normal rabbit mucosa (data not shown). In addition, TC II-R activity in the isolated apical and the basolateral membranes from the normal and affected rabbit intestinal mucosa was also determined. The results show (Fig. 6, panel A) that TC II-R antiserum added *in vitro* inhibited the TC II-<sup>[57Co]</sup>Cbl binding to both the apical (column d) and basolateral membranes (col-

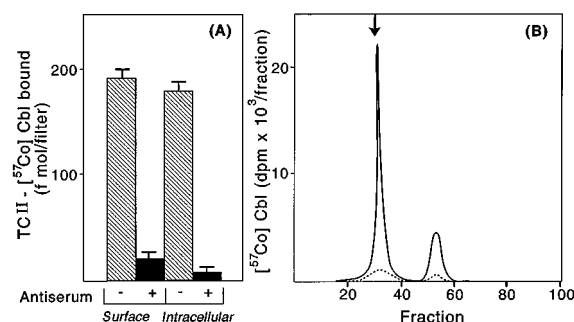


FIG. 7. Panel A, inhibition of surface binding of TC II-<sup>[57Co]</sup>Cbl and transport of <sup>[57Co]</sup>Cbl in filter-grown Caco-2 cells treated on the basolateral side with antiserum to human TC II-R. Panel B, chromatography of cellular extract on Sephadex G-150 (1  $\times$  60 cm). Solid lines indicate distribution of <sup>[57Co]</sup>Cbl in untreated (solid lines) and in antiserum-treated (broken lines) cells.

umn b) isolated from normal rabbit intestinal mucosa. The activity was inhibited by >75–80% compared with the untreated apical (column c) and basolateral membranes (column a). On the other hand, when the TC II-<sup>[57Co]</sup>Cbl binding to the apical and basolateral membranes of affected rabbit intestine was measured (Fig. 6, panel B), the ligand binding to the basolateral (column e) but not the apical membrane (column g) was affected. The loss of ligand binding to the basolateral membrane was completely restored following treatment of basolateral (column f) membranes with pH 3/KSCN buffer. Similar washing of the apical membranes from the affected rabbit had no effect on the receptor activity (column h). The specificity of both the *in vitro* and *in vivo* antibody effect on the TC II-R activity was borne out by the observation that the antiserum *in vitro* (Fig. 6, panel C) or *in vivo* (Fig. 6, panel D) had no effect on the binding of ligand, IF-<sup>[57Co]</sup>Cbl, to the apical or the basolateral membranes. It is interesting to note that similar results were noted with the isolated apical and basolateral membranes from the normal and affected rabbit kidney (data not shown). In support of these observations, direct evidence that antiserum to TC II-R by blocking ligand binding sites also suppressed TC II-mediated Cbl transport was obtained using polarized Caco-2 cells (Fig. 7). In these cells, like in the intact intestinal mucosa (Fig. 6) and in the kidney cortex (24), TC II-R is expressed 8-fold higher in the basolateral membranes (Fig. 7), and >90% of the surface binding is due to TC II-R (panel A). TC II-R antiserum inhibited not only the surface binding of TC II-<sup>[57Co]</sup>Cbl, but also intracellular Cbl levels and the utilization of Cbl by the Cbl-dependent enzymes (Fig. 7, panel B). Previously we have shown (23) that following uptake of TC II-Cbl, Cbl liberated from within the lysosomes is incorporated into intracellular Cbl-dependent enzymes, methionine synthase and MMA CoA mutase, and that these enzymes eluted near void volume on size exclusion chromatography earlier ( $V_e/V_0$ , 1.1) well separated from Cbl still bound to TC II ( $V_e/V_0$ , 2.0). These results have convincingly shown that cells exposed to TC II-R antiserum fail to transport Cbl and thus become Cbl-deficient.

In conclusion, our studies have shown that intracellular deficiency of Cbl can be induced by preventing tissue uptake of TC II-Cbl by antiserum to TC II-R. This experimental approach can be used for creating intracellular Cbl deficiency in cells in culture to study the role of Cbl on cellular proliferation and differentiation or in animals to study the pathophysiology of hematological and/or neurological complications known to occur in Cbl deficiency.

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