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F I Haurani, ... , C A Hall, R Rubin

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Research Article

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Megaloblastic Anemia as a Result of an Abnormal Transcobalamin II (Cardeza)

FARID I. HAURANI, CHARLES A. HALL, and RONALD RUBIN, *Cardeza Foundation for Hematologic Research, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; Hematology Research Laboratory Veterans Administration Hospital, Albany, New York 12208*

ABSTRACT A 34-year-old Black woman had severe megaloblastic anemia in childhood. Initially, and over the years, she responded well to massive doses of parenteral cobalamin (Cbl) or oral folic acid. Metabolic reactions involving Cbl and folate enzymes were normal during both relapse and remission except for the absence of thymidylate synthetase in relapse. Amino acid analyses of urine and plasma showed no significant abnormalities. Neither cystathionine, homocystine, formiminoglutamic acid, nor methylmalonic acid was detected in the urine. The serum Cbl level was repeatedly elevated even when the patient was receiving only folic acid therapy. The elevation of the vitamin in the serum was found to be a result of markedly increased levels of transcobalamin II (TC II), as identified by several physicochemical techniques. The patient's TC II-Cbl shared immunologic properties with normal TC II but did not facilitate or impede the uptake of Cbl or Cbl bound to normal TC II, respectively, by human cells.

INTRODUCTION

A patient, a Black woman born in 1944 and described previously (1), presented herself at the age of 12 yr with multiple superficial mouth ulcers, pancytopenia, and megaloblastic anemia. Two sisters allegedly died of similar illness at ages 5 and 12, respectively. The patient had free acid in the gastric juice, a normal Schilling test, performed twice, 20 yr apart, and an increased serum level of cobalamin (Cbl)¹ and a normal serum level of folate during relapse. She responded to phar-

macologic doses of either Cbl or folic acid. For a 10-yr period she received folic acid without developing evidence of posterior and lateral column degeneration of the spinal cord.

Because of repeatedly elevated levels of serum Cbl during relapse, studies of serum R binders, transcobalamin II (TC II) and cellular uptake of Cbl bound to TC II were undertaken. This paper reports the results of these studies, which demonstrate in this patient the presence of a TC II of poor function. We will refer to this variant Cbl binding protein as "TC II Cardeza."

METHODS

Assay of folate enzymes and related analyses. Serum folate and Cbl were determined by microbiologic (2, 3) and radioassay (4) methods, respectively. Amino acid analyses of plasma or urine, determination of urinary methylmalonic acid, and assay of plasma or urinary cystathionine and homocystine were performed according to published methods (5) during relapse, as confirmed by a megaloblastic bone marrow. Urinary formiminoglutamic acid was determined during relapse by the cellulose-acetate electrophoresis method (6). The following folate enzyme levels were determined during remission and relapse: dihydrofolic acid reductase (7), formate-activating enzyme (8), cyclohydrolase (9), 5,10 methylene tetrahydrofolic acid reductase (10), serine hydroxymethyl transferase (11), and thymidylate synthetase (12). Peripheral leukocytes were used for the assay of all these enzymes except thymidylate synthetase, for which phytohemagglutinin (PHA)-stimulated lymphocytes were used. Protein was measured by the method of Lowry et al. (13).

Measurements of serum Cbl binding proteins. Serum collected from the patient was frozen once and then thawed without raising the temperature above 4°C, immediately separated into small aliquots, and then frozen. It was kept at -20°C until use. Total serum TC II and R binders were measured by radioimmunoassay (14, 15). Total serum Cbl and its division between TC II and R binders were measured by an isotope dilution assay (16). The TC II-Cbl was separated before assay by fractionation with 1.96 M (NH₄)₂SO₄ (15, 16). The unsaturated Cbl binding capacity was determined by saturating the apoprotein (apo) TC II and serum R with radioactive Cbl and then separating the TC II from the serum R binders by both Sephadex G-200 (Pharmacia Fine Chemicals,

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¹Abbreviations used in this paper: apo, apoprotein; Cbl, cobalamin; PHA, phytohemagglutinin; TC II, transcobalamin II.

Inc., Piscataway, N. J.) gel filtration (17) and $(\text{NH}_4)_2\text{SO}_4$. Gel filtration was used to evaluate the patient's TC II and to detect any potential TC II-antibody complex. 1 ml of the patient's serum was labeled with 2.565 ng of CN^{57}Co Cbl, 20% over unsaturated Cbl binding capacity, and separated on Sephadex G-200. The void volume was marked with blue dextran (Pharmacia Fine Chemicals, Inc.). The column had been previously calibrated by fractionation of labeled normal serum R-type binders and TC II. An aliquot of the TC II-Cbl obtained by gel filtration was concentrated (Amicon PM-10 ultrafiltration membrane, Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and allowed to react with a 10-fold excess of rabbit antihuman TC II (30 min at 37°C, and then 17 h at 4°C) and then filtered through a second Sephadex G-200 column. A shift of the labeled TC II to the void volume was indicative of coupling with anti-TC II.

Cellular uptake of Cbl bound to TC II. The activity of the patient's TC II in the promotion of Cbl uptake by HeLa cells was studied in two ways. The first was the standard form of such studies in which uptake of CN^{57}Co Cbl is detected (18). Before the study, the holo TC II and apo TC II of both the patients and control sera were measured as above. For each experiment, all apo TC II was converted to holo TC II with CN^{57}Co Cbl of appropriate specific activity. 3×10^6 cells were exposed during culture to amounts of whole serum that contained 2.0 ng of holo TC II, part of which was radioactive. Any R-Cbl in the serum can be ignored because HeLa cells do not take up R-Cbl. After a 3-h culture at 37°C in medium M-199 (Microbiological Associates, Inc., Walkersville, Md.), the cells were harvested, washed three times, and the radioactivity was counted. Uptake, while detected by the radioactivity, was calculated on the basis of the total TC II-Cbl taken up, which was in part TC II- CN^{57}Co Cbl, and the remainder Cbl of various forms bound naturally to the serum TC II. There were several variations of this basic technique in a search for inhibitors of TC II-Cbl in the patient's serum. Experiments were conducted in duplicate.

In the unlikely event that CN^{57}Co Cbl bound to TC II reacts differently than Cbl carried naturally, a second approach was applied for confirmation. The principle of the experiment was the uptake of the Cbl naturally bound to TC II, the end point being bioassay of the lysed HeLa cells by *Euglena gracilis* (16). One control serum and TC II-Cbl partially purified from Cohn fraction III were handled the same way as the patient's. Before the study, the amounts of holo and apo TC II were measured for each source of TC II. $1.0\text{--}1.3 \times 10^6$ HeLa cells were exposed to 1.0 ng of TC II-Cbl for 3 h at 37°C. The cells were harvested, washed, and assayed for total Cbl content. Included were cells not exposed to added Cbl of any sort; their content was subtracted from the experimental values to obtain the amount of TC II-Cbl uptake. Variants of the basic technique are given below. Experiments were conducted in duplicate.

The uptake of CN^{57}Co Cbl bound to normal or patient's TC II, by PHA-stimulated human lymphocytes was studied by using slightly modified published techniques (19). TC II from normal serum and from the patient's serum was prepared by ammonium sulfate precipitation and dialysis. Holo, apo, and total TC II were measured as described above. Because the concentration of the added radioactive Cbl was known, the amount of total Cbl uptake by the lymphocytes could be determined. Human lymphocytes from normal individuals and once from the patient were separated and cultured with PHA for 3 d. The incubation mixture in duplicate consisted of 1 ml of stimulated lymphocytes (5×10^6 cells) in medium M-199 and 0.5 ml of the patient's TC II or normal TC II, each containing CN^{57}Co Cbl. The final volume was adjusted to 3 ml with the same medium, and the incubation was carried

out for 1 or 3 h at 4°C or 37°C. The concentration of Cbl in the incubation mixture varied from 0.25 to 1.0 ng. The lymphocytes were washed three times with saline, lysed with water, and their radioactivity measured. A saturation study using various concentrations of the patient's TC II and of a normal TC II were done to determine whether or not the patient's defective TC II might not facilitate transport into cells when used at higher concentrations. The concentration of TC II used varied from 0.25 to 5.0 ng/3 ml vol that contained 5×10^6 stimulated lymphocytes. The cells were incubated at 37°C for 3 h. In the mixing experiments, for detection of inhibitors, the patient's isolated nonradioactive TC II was incubated with the stimulated lymphocytes for 0.5 h before the addition of normal TC II- CN^{57}Co Cbl. After an incubation period of 1 h at 37°C, the cells were washed three times, lysed, and then their radioactivity was measured.

Clearance of TC II in the rabbit (20). The TC II of normal serum or the patient's serum after saturation with radioactive Cbl was isolated by ammonium sulfate precipitation, dialysed, and subjected to Sephadex G-200 gel filtration. The TC II radioactive fractions were pooled and then concentrated to a volume of 3–5 ml by Amicon PM-10 ultrafiltration membranes. Each rabbit was anesthetized with 75 mg of Nembutal (Abbott Laboratories, North Chicago, Ill.), given intravenously with 500 U of heparin. This was followed by an injection of 10–15 ng of TC II-Cbl, partly radioactive, in a volume of 2–4 ml. 4 ml of blood specimens (four to six in number) were collected by cardiac puncture over 60–100 min. (It was not always possible to keep the schedule of 10, 20, 30, 45, 60, and 100 min.) Plasma was separated, the radioactivity measured, and the data plotted on a semilog paper to determine the $t_{1/2}$ of TC II-Cbl clearance.

RESULTS

Assay of folate enzymes and related analysis. During relapse, the serum levels of folic acid varied between 6.0 and 11.5 ng/ml (normal: 1.9–14.0), whereas the serum levels of Cbl varied between 2,500 and 5,000 pg/ml (normal: 200–1,100). Over the years, the serum Cbl levels were determined by the bioassay method as well as by the radioimmunoassay method, and the results were not significantly different. As the patient went into relapse after cessation of folic acid therapy, serum Cbl levels decreased, albeit still remaining high.

The folate-related enzymes of the patient's peripheral blood leukocytes were normal except for thymidylate synthetase. Dihydrofolic acid reductase was normal in four determinations, $2.4 \pm 1.5 \text{ m}\mu\text{M/h}$ per mg protein, compared with 3.7 ± 3.3 in eight determinations from separate normal individuals; the level of formate-activating enzyme was $0.17 \pm 0.04 \text{ nM/h}$ per mg protein, compared with 0.17 ± 0.05 in 22 determinations from nine normal individuals. The cyclohydrolase was $0.25 \pm 0.11 \Delta \text{ OD/h}$ per mg protein in four determinations, compared with $0.30 \pm 0.15 \Delta \text{ OD/h}$ per mg in nine determinations of normal persons. Serine hydroxymethyl transferase level was 0.0–1.0 $\mu\text{M/h}$ per mg protein in four determinations, compared with 0.0–1.18 in four normal persons. 5,10-methylenetetrahydrofolic

TABLE I
Binder Levels and Capacities (Radioimmunoassay)

	Total levels		Endogenous serum Cbl			Serum unsaturated Cbl binding capacity		
	TC II	Serum R	Total	On TC II	On R	Total	By TC II	By R
	ng/liter		ng/liter			ng/liter		
10 Normals*	917±91	550±61	560±143	47±40	407±79	1092±211	969±204	167±48
Patient	6,400	920	4,384	3,824	268	2,139	1,874	274

* Mean±SD.

reductase level was 0.17 ± 0.12 U/min per mg protein in four determinations, compared with 0.36 ± 0.07 in six determinations of normal people. Thymidylate synthetase activity of the patient's PHA-stimulated lymphocytes was absent in two determinations during relapse and became normal, 0.6 and 0.7 nmol/h per 10^7 cells during remission. This observation led to the definition of the role of Cbl in the megaloblastic development (21).

Urinary formiminoglutamic acid, methylmalonic acid, and cystathionine were not present in urine during relapse. Similarly, there was no cystathionine or homocystine in plasma. Analysis for 20 amino acids in plasma revealed normal values except for the branched-chain amino acids, which were approximately one-third less than the lower range of normal values. Methionine in the plasma was 0.22 mg/dl (normal: 0.42 ± 0.08).

Measurement of serum Cbl binding proteins. The serum for these studies was collected while the patient was in complete remission and receiving 15 mg of folic acid daily but no Cbl. Table I depicts the concentrations of Cbl binders in the patient's serum compared with values from sera from 10 normal people. The patient's total TC II, as measured by radioimmunoassay, was seven times the normal mean. The somewhat high serum R binders may have been the consequence of cell-serum contact before separation. The total serum Cbl was increased eightfold, and 90% was carried by TC II. The methods used tend to underestimate the Cbl bound to TC II when levels are normal (16) but probably give close to the true value with the amounts observed here. The patient's serum TC II was much increased and 60% saturated with Cbl in contrast to $\approx 10\%$ saturation of normal sera. Fig. 1a shows that the labeled apo TC II had the same gel filtration properties as normal TC II, and there was no evidence of any unusual Cbl binding protein or of a circulating complex that contained normal binders. Fig. 1b shows that the patient's TC II-Cbl reacted with antibody against normal human TC II in a normal fashion.

The TC II of the patient while increased was able to bind Cbl in vivo and in vitro. It behaved like normal TC II by gel filtration and in $(\text{NH}_4)_2\text{SO}_4$ solubility and

was immunologically competent by two systems of evaluation.

Uptake of TC II-Cbl by two systems. Table II shows the uptake of TC II-Cbl by HeLa cells "detected" on the basis of TC II-CN[^{57}Co]Cbl but "expressed" as uptake of total TC II-Cbl. The TC II of the patient's serum did not promote uptake. In two sets of experiments serum from the patient did not inhibit the uptake of normal TC II-Cbl. In a final experiment, cells were preexposed to the patient's serum, which again failed to inhibit.

Table III shows the uptake by HeLa cells of Cbl, regardless of its form, detected by bioassay. For the first three experiments (all controls), CN[^{57}Co]Cbl was added to give 1.0 ng of free or TC II-bound Cbl. The Cbl, which had been checked for chemical purity and

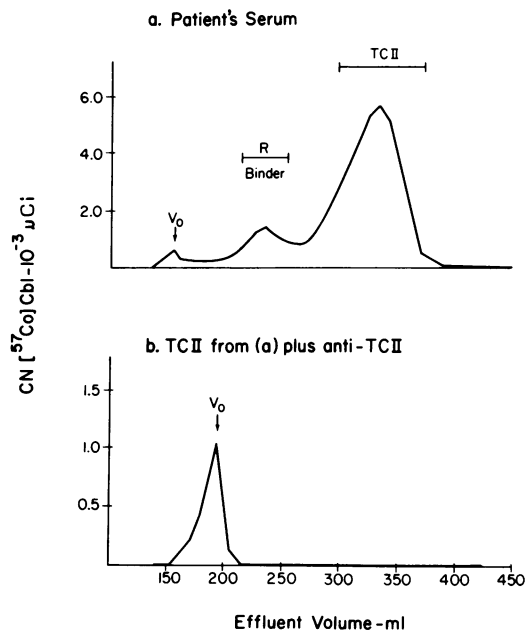


FIGURE 1. Serum Cbl binding proteins of patient. (a) Gel filtration of patient's labeled Cbl serum binders, and (b) after interaction of patient's TC II with rabbit antibodies against normal human TC II. Pattern (a) coincides with that of normal serum Cbl binding proteins as does pattern (b) after treatment of normal TC II with specific antibodies. V_0 , void volume.

TABLE II
Uptake by HeLa Cells (Radioactive Method)

Binding substance	Amount of Cbl per incubation mixture		Uptake*
	ng	pg Cbl/mg cell protein	
None (free Cbl)	2.0		3
Patient serum	2.0		6
Normal serum	2.0		199
Patient serum plus normal serum	2.0	+	164
Patient serum plus normal serum	2.0	+	127
Cells plus patient serum for 1 h, washed and then added	1.0		
CN ^[57Co] Cbl bound to normal serum	1.0		
	2.0		175

3 × 10⁶ HeLa cells were incubated with normal or patient serum at 37°C for 3 h.

* Calculated on the basis of the total TC II-Cbl taken up.

biological activity, was measured by bioassay. The normal TC II promoted uptake. For the first three studies of the patient's serum, the cells were exposed to 1.0 ng of TC II-Cbl that was either (a) a mixture of native TC II-Cbl and TC II-CN^[57Co]Cbl, (b) a mixture of native TC II-Cbl and CN Cbl, or (c) all native TC II-Cbl. In a final study, the TC II-Cbl was increased to 2.5 ng. The patient's TC II did not promote Cbl uptake in any of these experiments.

With a second system, namely the PHA-stimulated lymphocytes, we obtained similar results. These results are expressed per cells rather than per cell protein (22) and are depicted in Table IV. The results are expressed in geometric mean with the coefficient of variation in

parenthesis. The latter was high in one subgroup (patient at 37°C) because it included three low values, each 0.3. Two-way analysis of the variance (with correction for disproportionate numbers in the subgroups) and with log transformation to help equalize intra-group variances, draws the following conclusions: No significant interaction between temperature and disease status, no significant difference between 1 and 3 h of incubation, highly significant difference between control and patient (control, 7.5 times as big as patient, $P < 0.001$), and highly significant difference between 4° and 37°C when control and patient values are combined (37°C, 2.2 times as big as 4°C, $P < 0.01$). Again, in this system, the patient's TC II did not interfere with the function of normal TC II in facilitating the uptake of Cbl by the PHA-stimulated lymphocytes. In addition, the patient's TC II did not interfere with the function of normal TC II in facilitating the uptake of Cbl by these cells, even though larger amounts of the patient's TC II were used than in the previous study (HeLa cells). In this experiment, the uptake of normal radioactive TC II-Cbl after incubation of the stimulated lymphocytes (5 × 10⁶ cells) with patient's nonradioactive TC II-Cbl for 0.5 h was 9.8 and 10.8 pg (compared with a control of 10.0 and 10.5). Fig. 2 depicts the effect of increasing concentration of patient or normal TC II on uptake. At a concentration of 5.0 ng of TC II-Cbl, in the incubation mixture, the uptake of Cbl by the lymphocytes passed the maximum for both patient or control.

When the patient's own stimulated lymphocytes (5 × 10⁶ cells) were the cells of the system, the uptake of normal TC II-Cbl was 10 pg, compared with 0.75 of the patient's TC II-Cbl.

The t_{1/2} clearance of TC II-CN^[57Co]Cbl prepared from two different batches of patient's serum in four different rabbits was 40, 51, 62, and 66 min. The t_{1/2} clearance of TC II prepared from two normal individuals was 36 and 64 min in two different rabbits.

TABLE III
Uptake by HeLa Cells (Bioassay Method)

Binding substance	Endogenous	CN Cbl	CN ^[57Co] Cbl	Uptake of
	holo TC II	added	added	TC II-Cbl
TC II and Cbl as Pg Cbl				pg/10 ⁶ cells
None [Cbl free]	0	0	1,000	1.7
Normal serum	580	0	420	60.2
Partially pure normal TC II	80	0	920	86.2
Patient serum	606	0	401	Below basal
Patient serum	606	401	0	Below basal
Patient serum	1,000	0	0	Below basal
Patient serum	1,515	0	1,000	Below basal

TABLE IV
Cbl Uptake by PHA-Stimulated Lymphocytes
(5×10^6 cells)

	4°C	37°C
Patient TC II-Cbl, μg	0.9 (61%)	2.3 (257%)
No. of experiments	13	16
Normal TC II-Cbl, μg	8.2 (69%)	15.3 (56%)
No. of experiments	13	19

Values expressed in geometric mean with the coefficient of variations in parentheses. Highly significant difference between patient and normal TC II-Cbl uptake.

DISCUSSION

Human Cbl binding proteins can be divided into three classes (23): (a) gastric intrinsic factor, which facilitates the absorption of Cbl from the terminal ileum; (b) R protein binders (TC I and III), found in granulocytes and body fluids (including plasma), which bind Cbl but do not have a clearly defined function (absence of these binders does not lead to megaloblastic marrow in spite of low plasma levels of Cbl [24]); and (c) plasma TC II, which enhances the cellular uptake of Cbl by many tissues and seems to be the primary plasma Cbl transport protein. The transport process involves binding of TC II-Cbl to a specific cell surface receptor, which, *in vitro*, can be detected at 4°C, the internalization via endocytosis of the TC II-Cbl complex, and, lastly, the degradation of TC II and release of Cbl (25).

Congenital deficiency of TC II has been associated with mouth ulcers, pancytopenia, normal plasma levels of Cbl, and severe neonatal megaloblastic anemia that

responds to large and frequent injections of Cbl (26–28). In addition, these patients have been prone to infection (decreased production of immunoglobulin, in one case), have had poor intestinal absorption of Cbl, and have had a rapid rate of relapse after discontinuation of Cbl therapy. Our patient had similar, but not identical, clinical and hematologic findings. The onset of anemia in our patient was delayed, perhaps suggesting that in time the TC II-Cbl receptors of the bone marrow cells changed in configuration and became more specific and discriminatory to the structure of TC II. The family history of the patient suggested a familial rather than a mutational abnormality. The fact that the patient's TC II could bind Cbl could explain the normal intestinal absorption of the vitamin. In addition, the patient was not prone to infection, and the rate of relapse after cessation of Cbl but not folate therapy was fast.

Our patient suffered the lack of Cbl only in tissues of rapidly dividing cells (bone marrow and gastrointestinal). The two well-known reactions in which Cbl acts as coenzyme were not affected in our patient, which suggested that these reactions could be divorced from the megaloblastic development. In these two reactions, methyl Cbl is a cofactor in the methylation of homocysteine to methionine (29) and adenosyl Cbl is a cofactor in the isomerization of methylmalonic acid CoA (30). Consequently, in Cbl deficiency there is homocystinemia, cystathioninuria, severe hypomethioninemia (0.07 mg/dl) and methylmalonic aciduria (4, 31, 32). During relapse, neither cystathionine nor homocysteine was present in patient's plasma or urine. Neither was methylmalonic aciduria detected, which indicated that the patient's TC II-Cbl could convert homocysteine and methylmalonic acid. Her slightly

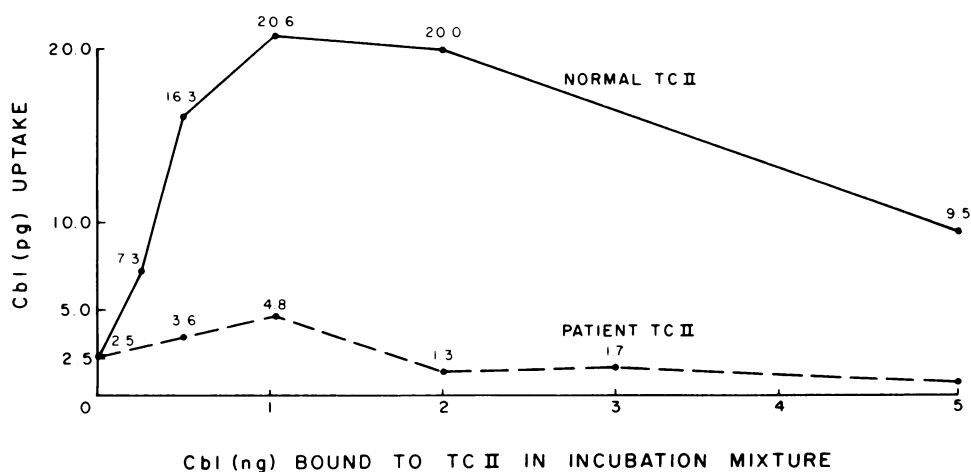


FIGURE 2 Concentration curves of the patient and of normal TC II-Cbl. 5×10^6 PHA-stimulated lymphocytes were incubated with normal or patient's TC II-CN⁵⁷Co]Cbl at 37°C for 3 h. Each point represents the mean duplicate uptakes.

diminished level of methionine (0.22 mg/dl) and other branched amino acids could have been a reflection of anorexia and diminished food intake during relapse. These findings suggest that the two known Cbl-dependent reactions occur primarily or predominantly in tissues in which the mechanism for the uptake of Cbl may be different from that of the bone marrow.

The increased amounts of TC II in our patient could bind Cbl and react with specific antibody but could not facilitate the uptake of the vitamin, which is analogous to the abnormal human intrinsic factor that could bind but could not facilitate the intestinal absorption of the vitamin (33). The uptake of TC II-Cbl was tested in two systems: HeLa cells and stimulated lymphocytes. The patient's TC II did not facilitate the uptake of Cbl, nor did it interfere with the function of normal TC II-Cbl. The observation that the uptake of patient's TC II-Cbl was also low at 4°C suggests that the abnormal TC II-Cbl could not bind to the specific cell receptors (25). The patient's own stimulated lymphocytes took up normal TC II-Cbl but not that of the patient's, which indicates that the defect was not at the level of the patient's uptake mechanism. The clearance of the patient's TC II-Cbl from the rabbit's circulation was similar to that of normal human TC II-Cbl. Apparently, the structural difference between the patient's TC II and normal TC II was not recognized by the rabbit tissues. However, more studies are needed to confirm this view.

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