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Monoclonal Antibodies to Different Sites on Human Transcobalamin II (42709)

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Abstract. Two IgG_1K monoclonal antibodies to human transcobalamin II (TC II) were generated. These antibodies, 16.1 and 16.6, did not cross-react with the other two types of human cobalamin-binding proteins, intrinsic factor and R binder (TC I). Both antibodies cross-reacted with orangutan and simiang TC II but not with TC II from cynomolgus and howler monkeys, who are less closely related to humans. This finding suggests close structural similarity of human to ape TC II. The antibodies also did not react with TC II of lower mammals which included the horse, dog, guinea pig, and mouse; in particular, reaction did not occur with rabbit TC II, which has been considered structurally close to human TC II. Neither of the two antibodies was directed at the cobalamin-binding site of TC II. However, antibody 16.6 hindered TC II binding to cell receptor. This reactivity with the receptor-binding site should prove particularly useful in studies of that region of the TC II molecule.

Transcobalamin (TC) II is a critical trace protein, without which cobalamin (vitamin B_{12}) cannot gain entry into cells. TC II is a single-chain polypeptide of molecular size 32,000–43,000 Da (depending on the method used) that is present primarily in serum, seminal fluid, and cerebrospinal fluid. As reviewed elsewhere (1–3), TC II binds cobalamin, attaches to specific receptors for TC II on the membranes of most cells, and thus promotes cellular uptake of the TC II–cobalamin complex by pinocytosis. Children lacking this transport protein develop life-threatening cellular cobalamin deficiency.

Many gaps remain in our knowledge about this essential transport protein. One of the limiting factors for precise studies is the minute concentration of TC II in the human body. Most studies to date have depended on the indirect approach of tagging TC II with radioactive cobalamin as a label. However, this limits the ability to study TC II in its original apoprotein form. We prepared monoclonal antibodies against TC II, and report here on two such antibodies that are directed against different sites of the molecule.

Methods and Materials. Purified TC II (4) was provided by Drs. Lindemans and van Kapel. Approximately 70 μg cobalamin-saturated TC II (980 μg/ml) in 0.1 ml complete Freund's adjuvant (Difco, Detroit, MI) was injected subcutaneously into a 3-week old Balb-C mouse. A subcutaneous booster injection of approximately 100 μg TC II in Freund's adjuvant was given 2 weeks later. At 6 weeks, an intraperitoneal injection of approximately 50 μg TC II was given. Four days later, the mouse was killed and a sterile splenic cell suspension was prepared and fused following the protocol described by Goding (5). Briefly, the murine splenic cells were mixed with M-5 myeloma cells in a 2.5:1 ratio and fused in the presence of 34% (v/v) polyethylene glycol (1.5 kDa, Aldrich, Milwaukee, WI). The fused cells were mixed with unimmunized Balb-C mouse splenic feeder cells and cultured in HAT selection medium. Cultures were screened daily for clone growth. Viable colonies developed in more than 60% of the fusions.

At 12 days, cultures containing growing colonies were screened for anti-TC II activity by enzyme-linked immunosorbent assay (ELISA). Selected supernates positive by ELISA were then also tested for reaction with serum containing TC II labeled with [^{57}Co]-cyanocobalamin, using Sephadex G-200 gel chromatography with 0.1 *M* sodium phosphate–1 *M* NaCl buffer, pH 7.4. Shift of the elution peak of the TC II–[^{57}Co]cyanocobalamin peak upon incubation with a supernate indicated positive reaction (6). The

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Sephadex gel column was calibrated with the two [^{57}Co]cyanocobalamin-labeled transcobalamins of serum, TC II and R binder, whose molecular sizes by Sephadex gel chromatography are well established (1–3), and with aldolase, molecular weight 158,000 (Pharmacia, Piscataway, NJ). Cultures that were positive by both ELISA and chromatography were cloned by limiting dilutions. Cultures where growth of only one clone was evident were retested for anti-TC II activity and recloned by limiting dilutions. Positive clones were expanded *in vitro.*

The ELISA for anti-TC II antibody was done in 96-well polystyrene plates. The wells were coated at 4°C for 18 hr with 100 μl TC II (0.1 μg) in 0.05 *M* carbonate buffer, pH 9.6, and blocked with 100 μl of 2% purified globulin-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 90 min at 37°C. After thorough washing with 0.9% NaCl containing 0.05% Tween 20, 100 μl of optimally diluted monoclonal anti-TC II antiserum (in this case, 1:1000), test material, control serum, or nonfused myeloma supernatant control was incubated in the appropriate wells for 90 min at 37°C. After washing, goat anti-mouse immunoglobulin (Ig) antiserum linked to horseradish peroxidase and diluted 1:5000 was used as a second antibody. After 90 min of incubation at 37°C and washing, freshly prepared substrate and developer (*O*-phenylene diamine in citrate buffer, pH 5, and hydrogen peroxide) were added for 7 min. The reaction was stopped with 100 μl 6 *M* sulfuric acid, and color development in the wells was read in a Titertek Multiskan (Flow Laboratories). All assays were done in triplicate. Positive reactions had readings more than twice those of control wells.

Ig isotyping was done by the ELISA technique with a mouse Ig subtype identification kit (Boehringer–Mannheim Biochemicals, Indianapolis, IN) and was confirmed by observing shifts of the monoclonal antibody-TC II–[^{57}Co]cyanocobalamin peaks from their normal elution position on Sephadex G-200 gel chromatography following incubation with the various anti-Ig antibodies. The anti-Ig antibodies were shown not to react with TC II itself.

Sources of human TC II were from sera from three individuals and from a pool of human serum in use in our laboratory for a decade. Human seminal fluid was obtained from a healthy volunteer (7). Mouse (Balb-C), rabbit (New Zealand White), and guinea pig sera were obtained from animals during previous studies. Serum specimens from the orangutan (*Pongo pygmaeus*), siamang (*Symphalangus syndactylus*), and howler monkey (*Alouatta seniculus*) were kindly provided by the Los Angeles County Zoo Health Center. Lyophilized dog and horse sera were purchased from Sigma Chemical Co. and reconstituted with water. Cynomolgus monkey (*Macaca philippinensis*) serum was kindly provided by Dixie Fisher. All sera were stored at −20°C until their use in the studies.

The effect of monoclonal antibody on cell uptake of TC II was studied, using the K562 human leukemia cell line. Various mixtures of serum TC II labeled with [^{57}Co]-cyanocobalamin and monoclonal antibody were incubated for 1 hr at 37°C in triplicate wells in a 24-well plate. After addition of K562 cells in RPMI medium, incubation was carried out at 37°C in 5% CO_2 for 18 hr. The reaction was terminated by adding cold 0.9% NaCl, followed by transfer of the cells to centrifuge tubes and several washings with cold NaCl. The radioactivity of the cell buttons was then counted in a well-type gamma scintillation counter. Cell counts, using trypan blue exclusion, demonstrated that the antibodies did not have any direct toxic effect themselves on the K562 cells.

Results. Two monoclonal antibodies, 16.1 and 16.6, reacted with serum TC II by the ELISA method up to a dilution of 1:5000. Both antibodies also shifted the TC II–[^{57}Co]cyanocobalamin peak from its normal elution position on Sephadex G-200 gel chromatography. Antibody 16.6 shifted the TC II to a peak corresponding to an approximate molecular size of 180,000 Da (Fig. 1). Antibody 16.1 produced a slightly smaller complex of 160,000 Da. Neither antibody cross-reacted with intrinsic factor obtained from gastric juice of an R-binder deficient subject or R binder (TC I) obtained from normal human saliva. The two antibody supernates contained negligible cobalamin-binding capacities themselves.

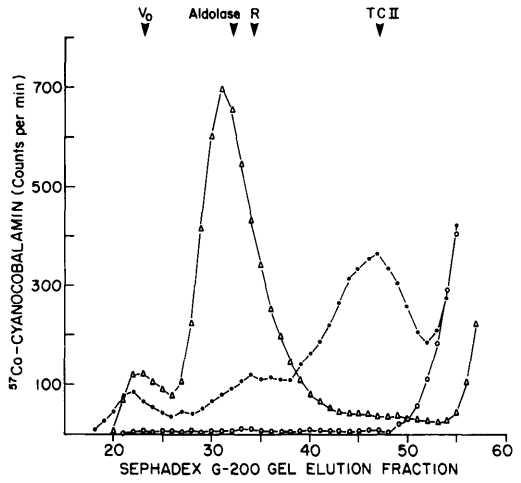

FIG. 1. Reaction of antibody 16.6 with normal pooled serum TC II, shown by Sephadex G-200 gel chromatography. (●) Normal serum alone, labeled with [^{57}Co]-cyanocobalamin; (△) labeled normal serum incubated with antibody 16.6; (○) antibody 16.6 alone, incubated with [^{57}Co]cyanocobalamin. Vo, void-volume position; R, position where R binder (molecular size 130,000 Da by Sephadex gel chromatography) elutes; TC II, position where free TC II elutes. Aldolase has a molecular size of 158,000 Da. The increasing radioactivity eluting beyond fraction 50 represents free [^{57}Co]cyanocobalamin.

Using anti-mouse specific subtype antisera, both antibodies 16.1 and 16.6 were characterized as IgG_1K by ELISA. The reaction with anti-IgG_1 and anti-K, and failure to react with antibody to any other IgG subtype or with anti-IgA, anti-IgM, or anti-λ antisera, was confirmed by Sephadex gel chromatography. Incubation of the antibody-TC II complex peak only with anti-mouse IgG_1 or anti-K antisera shifted its elution to the void volume on rechromatography.

Cross-reactivity with TC II from various sources. Antibody 16.6 reacted with human TC II from three different sera and a serum pool, as well as with seminal fluid TC II, when tested by Sephadex gel chromatography. Cross-reaction was shown by the chromatographic technique with orangutan and simiang serum TC II, but not with TC II from any other mammals tested (Table I). Antibody 16.1 behaved similarly. All of these animals' sera were shown to contain a cobalamin-binding protein with a Sephadex gel elution pattern identical to that of TC II; this protein constituted 33–98% of the unsaturated cobalamin-binding capacity of the animals' sera.

Site of TC II molecule reacting with monoclonal antibody. Experiments using TC II to which [^{57}Co]cyanocobalamin was not added until 1 hr after reaction with either antibody 16.6 or 16.1, instead of labeling the TC II with [^{57}Co]cyanocobalamin before incubating with antibody, showed that both antibodies reacted with apo-TC II (TC II not carrying cobalamin) as well as they did with holo-TC II (Table II). Moreover, the antibodies did not react with the cobalamin-binding site on the TC II molecule, since the TC II was able to bind virtually as much of the [^{57}Co]-cyanocobalamin after incubation with antibody as before such incubation.

Next, we examined whether either antibody was directed against the receptor-binding site of TC II. Their effect on the normal ability of TC II to attach to receptors on K562 leukemia cells was therefore tested. Antibody 16.6 interfered with the ability of TC II to normally attach to K562 cell receptors and promote uptake of [^{57}Co]-cyanocobalamin (Table III). Inhibition of TC II–cobalamin uptake was apparent even when antibody 16.6 was diluted 1:10. In contrast, antibody 16.1 did not inhibit the ability of TC II to promote cobalamin uptake by these cells, even when the antibody-to-TC II ratio was increased to 50:1.

Discussion. Polyclonal antibodies to

TABLE I. CROSS-REACTION OF MONOCLONAL ANTI-HUMAN TC II ANTIBODIES WITH TC II FROM OTHER MAMMALS

Source of TC II	Reactivity with monoclonal antibody	
	16.6	16.1
Orangutan	+	+
Simiang	+	+
Cynomolgus monkey	–	–
Howler monkey	–	–
Horse	–	NT[a]
Dog	–	NT
Rabbit	–	–
Guinea pig	–	NT
Mouse	–	NT

[a] NT, not tested.

TABLE II. EFFECT OF ANTIBODIES ON HOLO-TRANSCOBALAMIN II AND APO-TRANSCOBALAMIN II

	Serum 1 +16.6	Serum 2 +16.6	Serum 3 +16.1	Serum 4 +16.1
	Holo-TC II reaction			
Antibody–TC II complex[a]	6440	26910	6325	16124
Total serum[b]	12933	39968	9049	20473
	Apo-TC II reaction			
Antibody-TC II complex[a]	6214	24089	5330	16049
Total serum[b]	13051	36915	8048	19931

Note. In the holo-TC II reaction, the four sera had their TC II labeled with [^{57}Co]cyanocobalamin before being exposed to the monoclonal anti-TC II antibody; in the apo-TC II reaction, the serum TC II was exposed unlabeled to the antibody and was later incubated with [^{57}Co]cyanocobalamin. After Sephadex gel chromatography, the distribution of [^{57}Co]cyanocobalamin labeling was determined and the amount of binding that had occurred was quantitated.

[a] The results are expressed in counts per minute [^{57}Co]cyanocobalamin label of the TC II that has reacted with antibody and been shifted to a new position on Sephadex gel chromatography.

[b] The results are expressed in counts per minute [^{57}Co]cyanocobalamin label of all TC II in the serum (whether it has reacted with antibody and been shifted on Sephadex gel chromatography or has remained unreacted and eluted in the original TC II position).

TABLE III. EFFECT OF ANTIBODIES ON UPTAKE OF TC II–COBALAMIN BY K562 LEUKEMIA CELLS

	Percentage uptake	*N*
TC II	100	5
TC II + negative hybridoma supernate control	111.0 ± 19.1	4
TC II + antibody 16.6	39.1 ± 8.9	5
TC II + antibody 16.1	102.7 ± 12.2	5
TC II + antibody 16.6 + antibody 16.1	40.7	1

Note. Percentage uptake is presented in each experiment as percentage of the uptake that was obtained with TC II alone (which was arbitrarily designated as 100% uptake). The results are given as mean ± 1 standard deviation. *N* is the number of experiments (done in triplicate). The number of K562 leukemia cells ranged from 10^5 to 6×10^5 cells per well in different experiments, but the number of cells within each experiment was constant for all wells. Antibody or negative hybridoma supernate (0.2 ml) was used in four of the five experimens; 0.1 ml was used in the fifth experiment. The TC II was provided by a normal serum pool labeled with [^{57}Co]cyanocobalamin; in one of the experiments, the serum was not labeled with the [^{57}Co]cyanocobalamin until after the TC II had been incubated with the antibody (the results were similar to those in the other experiments). Each antibody itself mediated negligible uptake of [^{57}Co]cyanocobalamin in the absence of TC II, barely more than the uptake of free [^{57}Co]cyanocobalamin in buffer alone. The antibodies were not toxic to the cells either.

human TC II have been available from two sources. One is the serum of patients who acquire the antibody either spontaneously (6, 8) or after therapy for pernicious anemia with long-acting cobalamin preparations (9). The second source has been polyclonal antibody produced in animals immunized with human TC II. Since TC II is difficult to purify to homogeneity (10), the specificity of the latter antibodies is uncertain although most investigators have described monospecificity.

We have now produced antibodies 16.1 and 16.6, two monoclonal IgG antibodies to human TC II which each appear to recognize different sites on the TC II molecule. Antibody 16.6 inhibited TC II-mediated uptake of cobalamin by K562 cells, suggesting that this antibody binds to or near that portion of TC II which attaches to the cell receptor for TC II. The latter possibility seems more likely because inhibition of cellular TC II uptake by antibody 16.6 was not total. The site for antibody 16.1 attachment to TC II is not yet apparent. However, it was obviously neither the site that binds cobalamin, as evidenced by the antibody's equal affinity for apo-TC II and holo-TC II and its failure to inhibit binding of [^{57}Co]cyanocobalamin by

TC II, nor the site that attaches to the cell receptor, as evidenced by its failure to inhibit TC II uptake by K562 cells.

Both antibodies were specific for TC II and failed to react with the other known cobalamin-binding proteins in humans. Cross-species reactivity seemed to extend to some primate TC II but not to TC II from nonprimate mammals. Polyclonal antibodies to human TC II, usually prepared in rabbits, have been reported to cross-react to a limited degree with TC II of rabbit, guinea pig, rat, and dog (11–13), and polyclonal anti-rabbit TC II antibody cross-reacts with human TC II (14). Considerable structural homology of human with rabbit TC II has been reported (10, 13, 15). In contrast, our monoclonal antibodies are directed at sites of human TC II apparently shared only by primates more closely related to man. The immunologic cross-reactivity was apparent in the orangutan, which is an ape (Pongidae family) and thus belongs to the same superfamily as man (16), and in the simiang, which some (17) but not all (16) taxonomists also classify with the Pongidae. There was no cross-reactivity with TC II from the cynomolgus monkey, an Old World monkey, or the howler monkey, a New World monkey, both of which are much less closely related to man (16).

The different epitope specificities of the two antibodies suggest their value in future studies dissecting the TC II molecule. Antibody 16.6, which seems directed at or near the site that attaches to cell receptors, should prove particularly useful in the characterization of that part of the TC II molecule.

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