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Characterization of Monoclonal Antibodies to Epitopes of Human Transcobalamin II

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Cellular uptake of cobalamin (Cbl) is mediated by transcobalamin II (TCII), a Cbl binding protein in the plasma. The TCII-Cbl complex binds to a cell surface receptor and is internalized by endocytosis. We have generated monoclonal antibodies (mAbs) to human TCII that can be distinguished into three functional types on the basis of interaction with three different regions of the protein. Type 1: Receptor blocking. This mAb binds holo-TCII and inhibits the cellular uptake of Cbl. Type 2: Cbl blocking. This mAb binds apo-TCII at or near the Cbl binding domain and inhibits the formation of holo-TCII. Type 3: Precipitating. This mAb binds both holo-TCII and apo-TCII but does not interfere with Cbl binding. Whereas type 1 and type 2 mAb, following incubation with TCII-[^{57}Co]Cbl or apo-TCII, respectively, inhibit the uptake of radio-labeled Cbl by K562 cells, type 3 mAb has no such activity with either form of TCII. These properties of type 1 and type 2 mAb that inhibit the cellular uptake of Cbl, may serve to induce rapid Cbl deficiency and provide a model to study the effect of selective Cbl depletion on cell division and differentiation as well as on the pathways dependent on the two Cbl cofactors, methyl-Cbl and 5′-deoxyadenosyl-Cbl.

Transcobalamin II (TCII) is a 45 kDa plasma protein that binds cobalamin (Cbl) during assimilation in the distal ileum (1) and then transports and promotes the cellular uptake of the vitamin by endocytosis via receptors on the plasma membrane for TCII-Cbl. TCII is synthesized primarily by vascular endothelium (2) and circulates predominantly as apo-TCII because holo-TCII is cleared rapidly from the plasma with a half-life of ~90 minutes (3).

Cellular deficiency of Cbl impairs two metabolic pathways: 1. molecular rearrangement of methylmalonyl-CoA to succinyl-CoA which requires 5′-deoxyadenosyl-Cbl as the cofactor (4); and 2. methylation of homocysteine to form methionine and this requires both the methyl-Cbl cofactor and the N^5-methyltetrahydrofolate substrate that provides the methyl group for homocysteine (5). This transfer of the methyl group also regenerates the tetrahydrofolate pool. In Cbl deficiency, methyl-Cbl is depleted "trapping" N^5-methyltetrahydrofolate which, in turn, reduces the pool tetrahydrofolate from which the folate cofactors required for nucleic acid synthesis are derived (6). The clinical consequence of the perturbation of these pathways in humans is megaloblastic hematopoiesis often accompanied by functional and structural disorders of the nervous system (7).

There have been a number of reports of antimetabolites which impair the intracellular function of Cbl cofactors (8). The deleterious effect of nitrous oxide on methionine synthase (9) and on the synthesis of methyl Cbl (10) has been established experimentally and observed clinically with the development of megaloblastic erythropoiesis following exposure to this anesthetic (11). These observations establish the essential role of Cbl in normal cell replication and provide a rational basis for inducing Cbl depletion as a strategy to impair cell replication. Toward that end, we have generated monoclonal antibodies (mAb) to TCII which block the binding of TCII-Cbl to the TCII receptor on the plasma membrane and impair the cellular uptake of this essential vitamin. In this report, we describe the immunoreactive and functional properties of these antibodies.

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METHODS

TCII was purified from Cohn fraction III of human plasma as previously described (12) and apo-TCII was prepared by denaturation and dialysis of holo-TCII (13). BALB/c female mice were immunized with 10 μg of a mixture of holo and apo-TCII followed by 2 booster injections of 10 μg at two week intervals and the splenocytes were fused with mouse myeloma NS-1 cells. The hybridomas generated were screened for antibody to holo-and apo-TCII using the ELISA plate assay. Positive supernates in the ELISA assay were further screened for epitope specificity.

Preparation of TCII saturated with [^{57}Co]Cbl. Partially purified TCII (12) was incubated at room temperature in 1 ml of Tris buffered saline (TBS) with sufficient [^{57}Co]Cbl to saturate the binding capacity. After ~30 min incubation, free [^{57}Co]Cbl was removed by the addition of 0.5 ml of a suspension of hemoglobin coated charcoal (14). The mixture was vortexed and the charcoal pelleted by centrifugation. The supernatant fraction was collected and used as the [^{57}Co]Cbl labeled TCII in the assay.

Immunoprecipitation of holo-TCII. The immunoreactivity of [^{57}Co]Cbl-TCII was determined by adding 1 μl of hybridoma medium to ~5,000 cpm (15 pg Cbl) of TCII-bound [^{57}Co]Cbl in 500 μl TBS followed by incubation for 2 hr at 4°C. Antibody bound TCII-[^{57}Co]Cbl was determined by the addition of 5 μl of rabbit anti-mouse antiserum followed in 60 min by the addition of 50 μl of a 10% suspension of Omnisorb membranes (Calbiochem). After mixing for 10 min at 4°C, the membranes were pelleted at 15,000 ×g for 15 min, washed once with 1 ml TBS /1% Triton X-100 and the radioactivity in the pellet determined.

Indentification of mAb which blocks the binding of [^{57}Co]Cbl by TCII. Partially purified TCII was titrated to bind approximately 7–8pg of [^{57}Co]Cbl. This diluted apo-TCII was incubated with 1 μl of hybridoma medium in 500 μl of TBS for 2 hr at 4°C followed by the addition of 15 pg of [^{57}Co]Cbl and the incubation continued for an additional 30 min. Free [^{57}Co]Cbl was removed by adsorption to hemoglobin coated charcoal as described above. The radioactivity in the supernatant fraction, representing TCII-[^{57}Co]Cbl, was determined in a gamma counter. A control contained either no mAb or unrelated hybridoma medium. The decrease in the binding of [^{57}Co]Cbl to TCII in samples containing mAb compared to the control was computed as percent blocking.

Identification of the mAb which blocks the binding of TCII-[^{57}Co]Cbl to the TCII receptor on the plasma membrane. K562 cells from a 48–77 hr suspension culture in Dulbecco's minimal essential medium (DMEM) and 10% fetal bovine serum (FBS) were washed in Hank's balanced salt solution containing 1% bovine serum albumin (HBSS/1% BSA) and resuspended at a density of 2×10^6/ml. A 0.5 ml aliquot of this suspension was added to 0.5 ml of HBSS/1% BSA containing 15 pg [^{57}Co]Cbl-TCII that was preincubated for 2 hr with 2 μl of hybridoma medium. This cell suspension was incubated at 37°C for 60 min, pelleted at 1000 ×g for 5 min, washed once with 1 ml of cold HBSS/1% BSA and the radioactivity in the pelleted cells determined. Control samples contained no mAb or contained medium from an unrelated hybridoma. The blocking effect of the mAb on the binding of TCII-[^{57}Co]Cbl to the cell membrane was computed as the percent decrease in membrane bound radioactivity compared to the control samples.

Effect of the mAb on the uptake of [^{57}Co]Cbl by K562 cells. K562 cells (0.2×10^6) were seeded into 2 ml of DMEM/10% FBS contained in each well of a 12 well plate. The effect of the mAb was determined using two Cbl concentrations: 1. the addition of the cell suspension to wells containing 880 pM of [^{57}Co]Cbl bound to TCII and preincubated with the mAb overnight at 4°C with 200 μl of hybridoma medium; 2. the addition of the cell suspension to apo-TCII that was preincubated overnight at 4°C with 100 ml hybridoma medium followed by the addition of [^{57}Co]Cbl for a final concentration of 370 pM. The plates were then incubated at 37°C and cell viability determined by trypan blue exclusion, and cell number, enumerated using a hemocytometer, were assessed at 24, 48 and 72 hr. The cells were then pelleted by centrifugation at 1000 ×g for 5 min, washed twice with 2 ml of cold HBSS/1% BSA and the radioactivity determined in the cell button. Control samples lacked mAb or contained unrelated hybridoma medium.

RESULTS

Primary screening of the medium from hybridomas using the ELISA plate assay identified ~100 clones that reacted with apo- and/or holo-TCII. The epitope specificity and functional properties of 12 clones with an apparently high titer of mAb were then further characterized. As shown in Figure 1, all 12 mAb(s) characterized immunoprecipitated TCII-[^{57}Co]Cbl. However, only 6 of these blocked the binding of [^{57}Co]Cbl to TCII (Figure 2, clones 5–18, 1–6, 1–9, 2–6, 3–5, and 3–9). Of the remaining six mAb(s), three blocked virtually all of the TCII-[^{57}Co]Cbl binding to the TCII receptor on K562 cells (Figure 3, clones 2–2, 3–11, and 4–7). Five of the mAb(s) that blocked the binding of [^{57}Co]Cbl to TCII (shown in Figure 2) also decreased to some extent the binding of TCII-[^{57}Co]CBl to TCII (shown in Figure 2) also decreased to some extent the binding of TCII-[^{57}Co]Cbl to the TCII receptor when preincubated with holo TCII (Figure 3, clones 5–18, 1–6, 1–9, 2–6, and 3–9). The three mAb(s) that immunoprecipitated TCII-[^{57}Co]Cbl (clones 1–2, 1–12, and

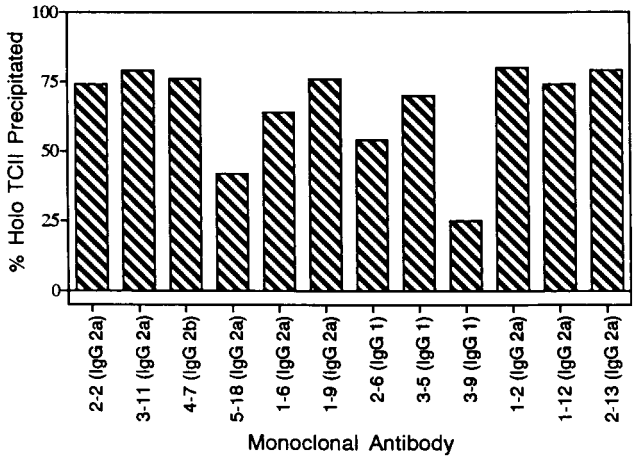

FIG. 1. Immunoprecipitation of TCII-[^{57}Co]Cbl by the mAbs. Each mAb is identified by the designated clone number and the specific immunoglobulin subtype is shown in parentheses.

2–13 shown in Figure 1) neither blocked the binding of Cbl to TCII (Figure 2) nor the binding of TCII-[^{57}Co]Cbl to the TCII receptor (Figure 3).

These 12 mAb(s) are grouped into three types based on their epitope specificity and effect on the functional properties of TCII (Table 1). All three types will immunoprecipitate TCII-[^{57}Co]Cbl but the epitope on TCII for type 1 is in apparent close proximity to the TCII receptor binding site and that for the type 2 is located close to the Cbl binding site. The epitope for type 3 is apparently located at a distance from these two functional regions of TCII because this mAb will not block either the binding of Cbl to apo-TCII or the binding of the complex to the TCII membrane receptor. Since the type 1 and type 2 mAb do not have overlapping immunoreactive specificity, it is likely that the two functional domains of TCII (i.e. Cbl binding and receptor binding) are well separated.

The type 1 and type 2 mAb, in addition to their common functional property of inhibiting TCII-Cbl from binding to the TCII receptor on the cell membrane, also inhibit the cellular uptake of Cbl. Figure 4 shows uptake of [^{57}Co]Cbl-TCII by K562 cells during 72 hr in culture. Four of the Cbl-blocking antibodies tested inhibited Cbl uptake by 75–90% during the culture period. A

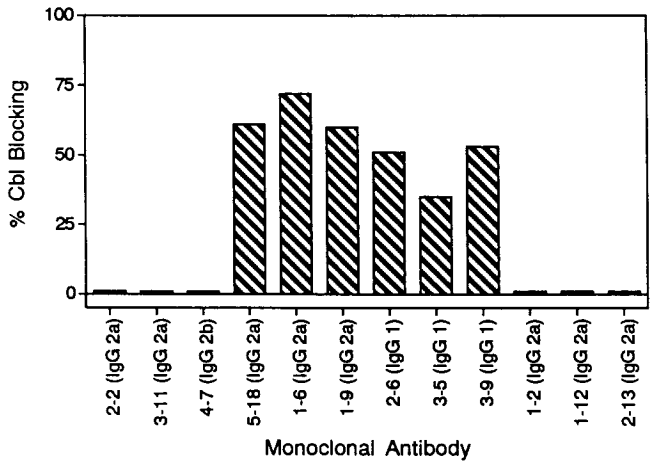

FIG. 2. The blocking effect of the mAb(s) on the binding of [^{57}Co]Cbl by TCII. Apo-TCII was preincubated with the mAb before the addition of [^{57}Co]Cbl. The percentage blocking was calculated from the decrease in the binding of the [^{57}Co]Cbl observed with the mAb as compared to the binding observed in the control lacking the anti-TCII mAb.

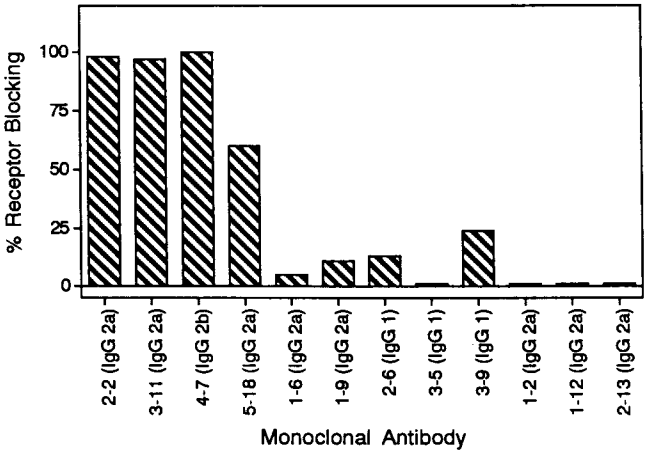

FIG. 3. Blocking of TCII-[^{57}Co]Cbl binding to the TCII receptors on K562 cells by the mAb. TCII-[^{57}Co]Cbl was incubated at 37°C with K562 cells in HBSS containing Ca^{++}. Duplicate tubes containing 10 mM EDTA were used to determine non-specific binding. The decrease in TCII-[^{57}Co]Cbl binding to the TCII receptor is expressed as a percentage of the TCII-[^{57}Co]Cbl bound to cells in the control that did not contain the mAb.

type 3 mAb had no such effect though it did bind apo and holo-TCII. The remaining two type 2 mAbs similarly blocked the uptake of [^{57}Co]Cbl-TCII over 72 and 96 hr (data not shown). The three receptor blocking (type 1) mAbs also inhibited by 25–50% the cellular uptake of [^{57}Co]Cbl (Figure 5). It should be noted that in both experiments the inhibition by type 1 and type 2 mAb of Cbl uptake (Figures 4 and 5) persisted throughout the 72 hr culture period.

DISCUSSION

In this report we describe the immunoreactive and functional properties of three types of mAb(s) generated against human TCII. The type 1 mAb blocks the binding of the TCII-Cbl complex to the TCII receptor on the plasma membrane of K562 cells and the type 2 mAb blocks the binding of Cbl to TCII. The type 3 mAb neither blocks the binding of Cbl to TCII nor the binding of the TCII-Cbl to the TCII membrane receptor although it immunoprecipitates the TCII-Cbl complex.

A significant property of the type 1 and type 2 mAb is that they both block the cellular uptake

TABLE 1
Epitope Specificity and Functional Property of the Monoclonal Antibodies to TCII

mAb	Designation	Epitope	Functional property
Type 1	2–2 3–11 4–7	In proximity to receptor binding site	Inhibits cellular uptake of TCII-Cbl
Type 2	1–6 1–9 2–6 3–5 3–9 5–18	In proximity to Cbl binding site	Blocks binding of Cbl and Inhibits cellular uptake of TCII-Cbl
Type 3	1–2 1–12 2–13	Distant from receptor or Cbl binding site	Does not inhibit cellular uptake of TCII-Cbl

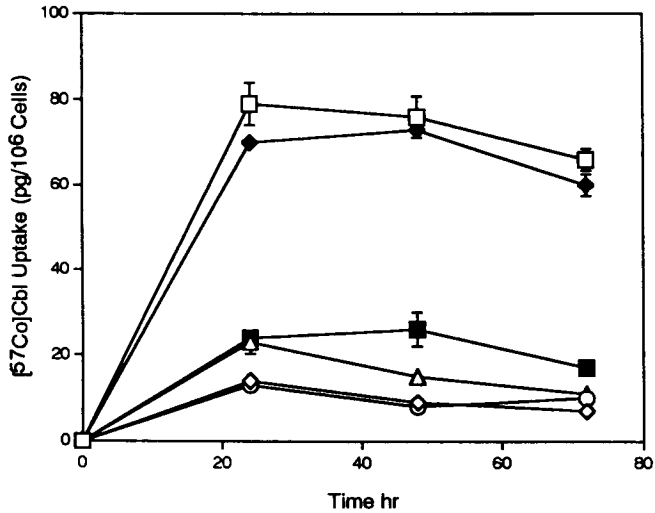

FIG. 4. Inhibition of Cbl uptake in K562 cells by type 2 (Cbl blocking) mAb. Apo-TCII was incubated with the mAb overnight and the mixture added to the cells in culture with sufficient [^{57}Co]Cbl to saturate the binding capacity of TCII. The uptake of [^{57}Co]Cbl by the cells was determined at 24, 48 and 72 hr as described in Methods. The four type 2 mAbs shown are 1-9 (-■-); 2-6 (-○-); 3-5 (-◇-) and 3-9 (-△-). Type 3 mAb is 2-13 (-◆-); control uptake (-□-). Each point is the mean of duplicate samples; the vertical bars represent the range.

of Cbl. For these experiments we used a physiologic concentration of Cbl (370 pM) to study the effect of the Cbl-blocking mAb on the cellular uptake of Cbl. We also observed substantial blocking of the uptake of Cbl even when the concentration of Cbl in the culture medium (880 pM) exceeded that found in normal plasma (150–600 pM). Carmel and Linker-Israeli (15) have previously reported the generation of two different mAbs against TCII. Neither mAb inhibited the binding of Cbl to TCII but one of the two inhibited by 60% the uptake of Cbl by K562 cells.

Cbl deficiency in humans perturbs a number of essential metabolic pathways. The consequent

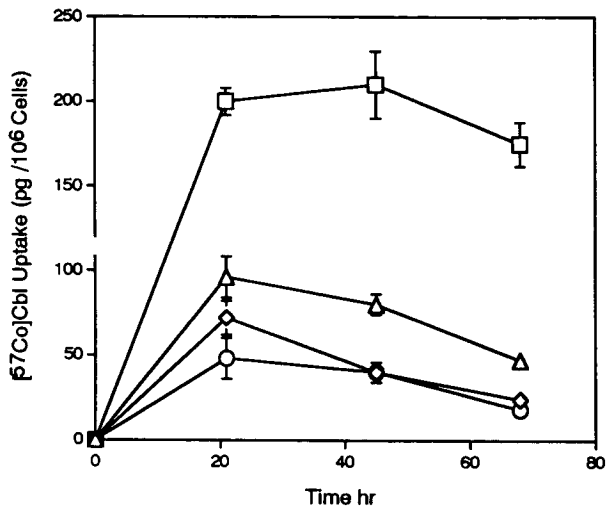

FIG. 5. The effect of type 1 (receptor blocking) mAb on the uptake of TCII-Cbl by K562 cells in culture. The mAb was incubated with TCII-[^{57}Co]Cbl overnight and the mixture was added to the cells in culture. The uptake of [^{57}Co]Cbl by the cells was determined at 24, 48 and 72 h as described in Methods. The three type 1 mAbs are 2-2 (-△-); 3-11 (-○-) and 4-7 (-◇-); control uptake, (-■-). Each point is the mean of triplicate samples; the vertical bars represent the range.

deficiency of methyl-Cbl impairs the methylation of homocysteine resulting in a decrease in methionine and its important metabolite, S-adenosylmethionine (16). A second consequence of methyl-Cbl deficiency is the trapping of N^5-methyltetrahydrofolate, the source of the carbon unit for methylation of homocysteine, and this also reduces the pool of folate cofactors that are essential for nucleic acid synthesis (6). The hematologic sequela of this deficiency is megaloblastic hematopoiesis with intramedullary cell death that is apparently due to apoptosis (17). For this reason, perturbation of Cbl metabolism has been sought as a means to control proliferation of neoplastic cells.

The mAb(s) we generated against TCII may be an alternative to anti-metabolites that target an intracellular pathway(s) and that have secondary and often deleterious effects. In preliminary studies on the biological effect of blocking Cbl uptake (18), we have also observed that K562 cells cultured for 12–15 days with type 1 mAb induces morphological characteristics of apoptotic cell death thus duplicating an effect of folate deficiency (17).

A clinical observation that has never been satisfactorily clarified is that patients with severe Cbl deficiency who have folate trapped as N^5methyltetrahydrofolate may have a hematologic response to daily administration of folic acid in excess of 400 μg (19). The induction of isolated Cbl depletion of sufficient magnitude to cause apoptosis in cultured cells by blocking the uptake of TCII-Cbl could provide the experimental model to precisely quantify the level of folate required to bypass this "methyl trap" and provide sufficient folate cofactors to restore nucleic acid synthesis. In addition, the inhibitory effect of the mAbs on the uptake of TCII-Cbl by neuronal cells and especially, the Schwann cells that deposit myelin around neuronal axons, may provide the biochemical basis for the demyelinization that occurs in Cbl deficiency and rarely seen in folate deficiency.

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