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The Role of Transcobalamin II in the Methionine Dependency of Human Lymphocytes[1] (42801)

CHARLES A. HALL, RICHARD C. CHU, AND JAMES A. BEGLEY

Nutrition Laboratory for Clinical Assessment and Research, Veterans Administration Medical Center, and the Department of Medicine, Albany Medical College, Albany, New York 12208

Abstract. Neither normal human B lymphoblasts (RPMI 6410) transformed by the EB virus nor human peripheral blood lymphocytes (PBL) stimulated by a mitogen replicated well when the methionine (Met) of the medium was replaced with homocysteine (Hcy). Cbl bound to human transcobalamin II (TC II) substantially increased cell division over that observed when the Cbl of the medium was in the free form. Although, as expected, the TC II enhanced the cell entry of Cbl 1000-fold, this was not the basis of the TC II effect. Through adjustment of the respective concentrations of free Cbl and TC II-Cbl in the medium, equal amounts of Cbl entered the cell, yet the TC II effect persisted. TC II-Cbl did not restore cell division in the absence of Met by virus-transformed lymphoblasts from a child with defective Met synthesis from Hcy. The TC II did not act by enhanced induction of the Cbl-dependent methionine synthase activity of cell extracts but the ability of intact cells to produce Met from Hcy by the Cbl-dependent process appeared to have a role in the TC II effect.

Previously we demonstrated that neither stimulated peripheral blood lymphocytes (PBL) nor virus-transformed human B lymphoblasts (RPMI 6410) would replicate normally in the absence of methionine (Met) from the culture medium (1). The cells were unable to use the combination of homocysteine (Hcy), 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) plus free cyano- or hydroxocobalamin (CN-Cbl or OH-Cbl) to compensate for the deleted Met but near control levels of growth occurred when the Cbl was bound to transcobalamin II (TC II). The effect of TC II could have been related to the known property of TC II in the promotion of the entry of Cbl into cells. The present studies were, therefore, conducted in order to determine the basis for the effect of TC II. The active principle in overriding the auxotrophy for Met was found to be the TC II per se, functioning in some process in addition to the promotion of cell entry of Cbl.

Methods. The cell processing, culture techniques, assay for the activity of lysed cell 5-CH_3-H_4PteGlu:Hcy methyltransferase [methionine synthase (MS)] (EC 2.1.1.13), statistical methods, and most materials were as described before (1–4). The assay for MS activity was the standard, widely used measurement of the ability of cell extracts to promote the transfer of the methyl of 5-CH_3-H_4PteGlu to Hcy to form Met (3). By convention, activity in the absence of exogenous Cbl is referred to as holo activity and that measured with the inclusion of large amounts of free Cbl, here 52 μM CN-Cbl, to the reaction mixture as total activity. Except as noted, L-homocysteine thiolactone (Sigma Chemical Co.) was used in the culture medium in place of the DL-Hcy (Sigma) of the earlier studies.

The principal experimental model was the RPMI 6410 human lymphoblast line, derived from a normal peripheral B lymphocyte immortalized by the Epstein–Barr virus (EBV). Its Cbl metabolism has been well evaluated (1–4). Experiments were also conducted with mitogen-stimulated human PBL, and B lymphoblasts from a child with the Cbl G mutation also transformed by the EBV. The latter were included because they have an isolated defect in an enzyme of interest, the Cbl-dependent MS (5, 6). Essentially the defect is expressed biochemically as impaired synthesis of Met from Hcy and 5-CH_3-H_4PteGlu by intact cells owing to impaired formation of methyl Cbl (MeCbl) (5,

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6). Cbl G lymphoblasts have receptors for TC II-Cbl, internalize it as well as control cells, attach it to the respective apoenzymes but fail in the final step of formation of the active coenzyme on the MS.[2]

The purification of the TC II was carried one step further than before (1) because of the presence of "nonspecific" factors that promoted cell division. The starting material of human Cohn fraction III was processed through the step of CM Sephadex as before. It was then saturated with CN [^{57}Co] Cbl (sp act = 0.2 μCi/μg) and further purified by gel filtration chromatography (7). The TC II-Cbl peak was concentrated and equilibrated by dialysis with Dulbecco's PBS (7). For several experiments the TC II-Cbl was removed from the final preparation by immunoabsorption (1, 7).

Internalization of Cbl by the RPMI 6410 cells was measured by first culturing the cells in medium containing either TC II-Cbl or free Cbl. The cells were harvested, washed, and sonicated as applied to other types of cells (8). The supernatant obtained by centrifugation (8) was assayed for Cbl content with *Euglena gracilis* as applied previously to lymphoblasts (2). Only the solubilized Cbl was considered to be internalized.

The rate of Met synthesis by intact cells was measured by the 18 hr incorporation of the [^{14}C] of 5-[^{14}C]CH_3-H_4PteGlu into both TCA-precipitable material and the supernatant (9). The incorporation medium contained the labeled folate Hcy but no Met. This widely applied approach measures methylated products other than Met but is an indirect measure of Met synthesis because the cells of the present study can transfer the methyl label only through Met.

For the experiments with the RPMI 6410 lymphoblast, the counting and harvesting for MS activity and the initiation of the 18 hr incubation for intact cell Met synthesis were performed on 48 hr cultures in the log phase of growth.

The minimum requirements of the key constituents of the cultures had been determined in preparation for the previous studies (1, 2), but were reestablished for the change in Hcy from DL Hcy to L Hcy thiolactone. Each substance was manipulated individually in the presence of adequate amounts of all other key constituents. Cell division was the endpoint. For Met, Hcy, 5-CH_3-H_4PteGlu, and Cbl three concentrations were identified: (i) that increasing cell division when compared to division in the absence of the substance, (ii) the lowest that produced maximum division, and (iii) that depressing division. Hcy requirements were derived in the absence of Met. Cbl requirements were determined in cells both replete in and depleted of Cbl. 5-CH_3-H_4PteGlu requirements were determined in the presence of either Met or Hcy in cells both repleted in or depleted of folate. When cultures were continued beyond 48 hr the methylfolate was renewed every third day. The basic concentrations selected for routine use were slightly greater than those required for optimal cell division, but not approaching the range of toxicity. The ratio of free Cbl:TC II-Cbl of 220 n*M*: 0.22 n*M* was derived from previous studies (1, 2).

Results. *Entry of Cbl into RPMI 6410 lymphoblasts.* This set of experiments formed a bridge, validating the earlier studies (1) and enabling the present studies. OH-Cbl was the form of Cbl for this set of experiments because it was the form used in the original studies of auxotrophy for Met (1). In the context of both the earlier and present studies, OH-Cbl and CN-Cbl could be interchanged (1, 2). Free OH-Cbl at 220 n*M* put at least as much Cbl into the soluble material within the cells as 0.22 n*M* TC II-OH Cbl, Table I. The free Cbl, as earlier (2), induced a greater activity of MS, an enzyme of the cytosol, and converted more apo enzyme to total enzyme than the TC II-Cbl, Table I. These data together demonstrate that at the ratios used, as much or more free Cbl was internalized than was TC II-Cbl. This observation is fundamental in the interpretation of all of the experiments that follow.

The possibility that Cbl bound to TC II entered the cells faster than when in the free form was explored in seven experiments either in the presence or absence of Met. The analyses were conducted at 6 and 24 hr of

[2] After acceptance of the manuscript the following article which describes the defect in Cbl G appeared: Watkins D and Rosenblatt DS, J Clin Invest **81**:1690–1694, 1988.

TABLE I. 48 hr INCORPORATION OF Cbl INTO THE SOLUBLE INTRACELLULAR MATERIAL OF RPMI 6410 CELLS (Mean ± SEM)

Form and amount of Cbl in medium	Soluble cell Cbl pg/10^6 (n = 5)	Lysed cell MS activity nmole Met/hr/mg protein (n = 6)	
		Holo	Total
Free OH-Cbl, 220 n*M*	43.8 ± 4.1	1.94 ± 0.71	6.04 ± 1.88
TC II-Cbl, 0.22 n*M*	32.0 ± 6.0	0.77 ± 0.39	3.64 ± 1.22
	$P < 0.1$	$P < 0.05$	$P < 0.05$

Note. The cells were grown in medium containing 10% FCS, Hcy at 130 μM, and 5-CH_3-H_4PteGlu at 1.5 μM. They were harvested, washed, and lysed by sonication and the Cbl in the supernatant only was assayed by *E. gracilis*.

culture as well as at 48 hr. The rate of entry was the same for both free Cbl at 220 n*M* and TC II-Cbl at 0.22 n*M*. The Cbl for this series was CN-Cbl, the form used in the present study, and not the OH-Cbl of Table I and that used previously (1). The intracellular Cbl 48 hr after exposure to CN-Cbl or TC II-CN-Cbl were equal (not shown).

Suitability of the preparation of TC II. The additional step of purification described under Methods increased the specific activity of the product more than 20-fold over that of the earlier preparation (1). The new preparation was stable under the conditions of culture, in contrast to the completely pure preparation (1), and preserved the one known property of TC II, the binding of the complex of TC II-Cbl to specific receptors (data not shown). As recorded in the first column of data in Tables II–III, the intact preparation also promoted cell division. Upon removal of all TC II and Cbl, the preparation became inactive, Tables II–III. Thus the additional step of purification removed the previous "nonspecific" growth factor(s) (1). CN-Cbl was the form used in all Met auxotrophy experiments since it much simplified the making of the purer TC II-Cbl.

Met dependency of RPMI 6410 lymphoblasts. The statistical analysis of these and subsequent data was by the paired t test, applying two-tailed criteria. The data are given in Table II. Replacement of the Met with Hcy and deletion of Cbl impaired the growth of the cultures. Restoration of the Cbl in the free form had a small, but significant, effect

TABLE II. THE EFFECTS OF Cbl ON RPMI 6410 CELLS AT 48 hr OF CULTURE (Mean ± SEM, n = 5)

Cultural conditions		Cell number × 10^6/ml, Day 2	Lysed cell MS activity pmole Met/hr/10^6 cells		Intact cells Met synthesis pmole/hr/10^6
Met vs Hcy	Cbl		Holo	Total	
a Met+; Hcy−	Free CN-Cbl, 220 nM	1.97 ± 0.02	83.6 ± 7.89	418.0 ± 128.0	4.6 ± 0.4
b Met−; Hcy+	No Cbl	0.66 ± 0.04	93.4 ± 11.0	198.0 ± 26.2	1.1 ± 0.1
c Met−; Hcy+	Free CN-Cbl, 220 nM	0.79 ± 0.04	107.6 ± 16.8	392.0 ± 98.2	4.5 ± 0.5
d Met−; Hcy+	TC II-CN Cbl, 0.22 nM	1.39 ± 0.04	75.6 ± 10.2	381.0 ± 96.6	4.6 ± 0.7
e Met−; Hcy+	Adsorbed Preparation	0.62 ± 0.03	98.0 ± 12.0	219.0 ± 26.3	1.5 ± 0.3
		c–b; $P < 0.05$	c–b; $P > 0.1$	All $P > 0.1$	c–b; $P < 0.01$
		d–c; $P < 0.001$	c–d; $P < 0.1$		d–c; $P > 0.1$

Note. Cultures were started at 0.5 × 10^6 cells/ml in medium containing 10% FCS and either Met 33 μM (met+; Hcy−) or Hcy thiolactone 160 μM (Met−; Hcy+) and 5-CH_3-H_4PteGlu 1.5 μM. In all tables, n = the number of separate experiments, each including all of the experimental points, performed in successive weeks. Lysed cell MS assays were performed without added Cbl (holo) and with (total). The incubations for 18 hr intact Met synthesis were performed in duplicate. P values were derived by the paired t test, with the differences between pairs determined within each experiment.

TABLE III. THE EFFECTS OF Cbl ON PBL FROM HEALTHY PERSONS STIMULATED WITH PHA (Mean ± SEM, $n = 5$)

Cultural conditions		Cell number × 10^6/ml		Lysed cell MS activity Day 9 cells pmole Met/hr/10^6 Cells		Intact cell Met synthesis Day 6 cells pmole/hr/10^6 cells
Met vs Hcy	Cbl	Day 6	Day 9	Holo	Total	
a Met+; Hcy−	Free CN-Cbl, 220 n*M*	2.06 ± 0.12	2.51 ± 0.18	65.1 ± 14.0	371 ± 49.2	1.5 ± .03
b Met−; Hcy+	No Cbl	0.62 ± .03	0.68 ± 0.03	77.7 ± 11.4	99.2 ± 13.3	0.3 ± .003
c Met−; Hcy+	Free CN-Cbl, 222 n*M*	0.83 ± 0.04	0.92 ± 0.02	95.8 ± 7.72	284 ± 61.9	0.7 ± .01
d Met−; Hcy+	TC II-CN Cbl, 0.22 n*M*	1.19 ± .07	1.40 ± 0.04	93.2 ± 17.3	204 ± 31.6	1.0 ± .01
e Met−; Hcy+	Adsorbed preparation	0.66 ± .03	0.62 ± 0.02	103.0 ± 11.7	130 ± 7.18	0.2 ± .003
		c–b; d–b; d–c; d–e; All $P < 0.01$	c–b; $P < 0.001$ d–b; $P < 0.02$ d–c; $P < 0.001$ d–e; $P < 0.001$	c–b; d–b; All $P > 0.1$	c–b; $P < 0.05$ d–b; $P < 0.1$ c–d; $P > 0.1$	c–b; $P < 0.02$ d–b; $P < 0.01$ d–c; $P < 0.01$ d–e; $P < 0.01$

Note. Cells were started at 0.5 × 10^6 cells/ml in medium containing 0.7% BSA, PHA, either Met at 33 μM (Met+; Hcy−) or Hcy at 160 μM (Met−; Hcy+) and 5-CH_3-H_4PteGlu at 1.5 μM which was renewed every third day.

on cell division. There was a much greater effect when 1/1000 as much Cbl was provided in the form of TC II-Cbl. Removing the TC II and the Cbl from the preparation of TC II rendered it inert.

Met dependency of stimulated PBL. The results, Table III, were essentially the same as for the cultured lymphoblasts. The cells replicated poorly when Hcy replaced the Met even in the presence of free Cbl. The amount of 1/1000 Cbl bound to TC II partially restored cell division and simultaneously increasing the synthesis of Met by intact cells but without any additive effect on the MS activity of the lysed cells. In the interpretation of these experiments it must be recognized that MS activity of cell extracts is an *in vitro* assay where all required substances other than the enzyme are provided in excess. The capacity of the intact cell to transfer the label to Met, and then to other products, depends on the cell for not only the enzyme but for the reduction of the Cbl, the provision of *S*-adenosylmethionine (AdoMet), and the synthesis of MeCbl. In this set of experiments 0.7% bovine serum albumin (BSA) replaced the FCS of the medium. In earlier experiments (1, 2, 10) BSA was used to avoid even bovine TC II in the basic medium. As experiments progressed it was learned that Met auxotrophy was as easily demonstrated in the presence of FCS. Nevertheless, for the crucial experiments with PBL there was a reversion to the original medium.

The TC-II effect in Cbl G-mutation cells. When Hcy replaced Met in the absence of Cbl the 2-day cell increase was reduced from 28 to 14%. The inclusion of free Cbl or TC II-Cbl, in the absence of Met, improved cell increase to 75%, Table IV, but in contrast to the observations in other lymhoid cells, TC II-Cbl was no more effective than free Cbl. Stepwise increases in the free Cbl and TC II-Cbl to 2.0 μM and 2.0 nM, respectively, did not restore cell division (not illustrated). The remainder of Table IV points out the nature of the defect in these cells. The MS activity of cell extracts was not diminished but was, in fact, greater than that of RPMI 6410 cells after culture with TC II-Cbl. Nevertheless, the intact cells produced half as much Met from Hcy plus 5-CH_3-H_4PteGlu as RPMI 6410 cells even in the presence of TC II-Cbl. In the absence of Met there was even less Met synthesis, Table IV, and synthesis was further reduced by the omission of any Cbl in either the presence or absence of Met. The Cbl G defect inhibited the TC II effect observed in other cells.

Discussion. As before, both the EBV-transformed normal human B cells and human PBL stimulated by a mitogen replicated poorly when the Met of the medium was replaced with Hcy. TC II-Cbl (0.22 nM) purified of "nonspecific" growth factors partially restored cell division to the levels achieved with Met. Free Cbl (220 nM) had an effect on growth in the presence of Hcy,

TABLE IV. THE EFFECTS OF Cbl ON Cbl G-MUTATION LYMPHOBLASTS AT 48 hr OF CULTURE (Mean ± SEM, $n = 5$)

Cultural conditions		Cell number $\times 10^6$/ml, Day 2	Lysed cell MS activity pmole Met/hr/10^6 cells		Intact cell Met synthesis pmole/hr/10^6
Met vs Hcy	Cbl		Holo	Total	
a. Met+; Hcy−	Free CN-Cbl, 220 n*M*	2.09 ± 0.10	89.6 ± 15.8	529.0 ± 54.0	2.6 ± 0.5
b. Met+; Hcy−	None	1.90 ± 0.10	63.6 ± 12.3	406.4 ± 70.7	0.6 ± 0.07
c. Met−; Hcy+	None	0.57 ± 0.04	124.0 ± 35.7	359.8 ± 26.8	0.8 ± 0.10
d. Met−; Hcy+	Free Cn-Cbl, 220 n*M*	0.87 ± 0.07	166.0 ± 47.1	575.0 ± 25.9	1.6 ± 0.2
e. Met−; Hcy+	TC II-CN-Cbl, 0.22 n*M*	0.88 ± 0.04	188.0 ± 43.3	782.0 ± 61.6	2.3 ± 0.4
		a–d; $P < 0.001$	e–b; $P < 0.05$	d–a; $P > 0.1$	a–d; $P < 0.05$
		a–e; $P < 0.001$	e–c; $P < 0.05$	e–a; $P < 0.05$	a–e; $P > 0.1$
		d–c; $P \ll 0.001$		e–d; $P < 0.05$	e–d; $P < 0.10$
		e–c; $P \ll 0.001$	others NS	d–c; $P < 0.05$	d–c; $P < 0.02$
		e–d; $P > 0.1$		e–c; $P < 0.01$	e–c; $P < 0.02$

Note. All conditions were as in the experiment of Table II.

but it was much less than with the TC II-Cbl. The TC II effect was not produced by the one known property of TC II which is to promote entry of Cbl into cells. The expected 1000-fold promotional effect of the TC II was, in fact, observed, but through adjustment of concentrations of Cbl, equal amounts of Cbl were put into the cells. Cellular Cbl was not greater when the Cbl in the medium was bound to TC II. The consistent observation of equivalent induction of lysed cell MS activity, an enzyme of the cytosol, and known to be induced by Cbl (2), by either 220 n*M* free Cbl or by 0.22 n*M* TC II-Cbl was further evidence suggesting equivalent cell entry of Cbl.

In the classic studies (11, 12) of the auxotrophy for Met of cultured neoplastic cells the concentrations of folate, as folic acid (PteGlu), and Cbl were much greater than those in the medium in the present study. It has been suggested that the failure of some cultured cells to utilize Hcy as a replacement for Met may be a consequence of too little Cbl and folate in the medium (13). In planning our conditions of culture we selected the concentrations of the constituents of the medium on an experimental basis as described under *Methods.* The concentrations used were closer to those encountered by cells *in vivo* than the amounts generally used in similar models (11–14). TC II-Cbl at 0.22 n*M* is threefold greater than the mean circulating amounts in man, ~0.07 n*M*, but less than the capacity of circulating TC II to bind Cbl. Free Cbl at 220 n*M* is artifactual but this concentration is required to internalize equivalent amounts in the absence of TC II. Methylfolate as used here, and not PteGlu, is the dominant form in the human circulation. The amounts in the medium initially were much higher than *in vivo* but we observed rapid loss of preparatory experiments. The basal concentrations were, therefore, less than the original. Earlier (1) we observed RPMI 6410 cells to be auxotrophic for Met even with PteGlu at 100 μM and free OH-Cbl at 1.5 μM.

Auxotrophy for Met by some types of cells in culture has probably been given more emphasis than justified. Human cells are not "cultured" in the absence of Met *in vivo* and certainly auxotrophy for Met is not confined to malignant cells since none of the cultures of the present study originated from neoplastic cells. The EBV transformed lines were, nevertheless, immortalized and the PBL transformed by a mitogen if not immortalized. There are probably gradations of Met dependency among human cells. Rather than being an endpoint of the experiments, the auxotrophy for Met should be considered to be a probe of quantitative, or perhaps even qualitative, differences in the Met-Hcy-Cbl metabolism of human cells.

The principal contribution of the present study was the demonstration for the first time that TC II has an activity in Cbl metabolism within the cell and beyond the well recognized property of promoting the entry of Cbl. This function of TC II could, among other possibilities, be in directing the produc-

tion of certain active forms of Cbl within the cell or in directing placement. The location of the active principle as in the TC II was achieved by an indirect approach, the removal of activity by removal of TC II from the preparation. Pure TC II was not stable enough in prolonged cultures (1) and a semipure preparation was therefore used. Immunoabsorption of TC II removed the growth promoting factor, but there is the remote possibility of removal of some substance other than TC II as well. The antibody raised against pure human TC II (15) appeared to be specific (16), but the possibility remains. However, the preparation of TC II-Cbl was no more effective than free Cbl in restoring growth of Cbl G cells in the absence of Met. Had the preparation contained growth factors independent of Met synthesis, it would have been active for these cells as well as for RPMI 6410 cells and PBL.

The most important, and still unanswered question is "What is the basis of the TC II effect?" Clearly, any enhancement of the MS activity of cell extracts was not responsible for enhancement of growth in the absence of Met. Activity in both the assay without added Cbl and that with, referred to for convenience as holo and total activity, respectively, was increased as much or more by free Cbl in the culture as by TC II-Cbl. Yet the free Cbl did not enhance growth in the absence of Met to the same degree. TC II could act by promotion of synthesis of Met by intact cells, an *in vivo* activity in contrast to the MS assay of cell extracts which is much influenced by *in vitro* conditions. In only the PBl was there significantly greater Met synthesis when the Cbl was bound to TC II. However, in the Cbl G cells where Met synthesis is impaired by defective formation of MeCbl, TC II-Cbl was no more effective than free Cbl in permitting cells to divide in the absence of Met. Therefore, the TC II effect appeared to require a fully functioning Cbl-dependent Met synthesis.

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