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Methionine Dependency of Cultured Human Lymphocytes (42330)

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Abstract. Human peripheral blood lymphocytes stimulated with phytohemagglutinin and a lymphocyte model consisting of the RPMI 6410 cell, a human virus-transformed B cell, required added methionine (Met) for growth of the cultures. This failure to meet all needs for Met via endogenous synthesis, which is characteristic of oncogenic transformation, occurred even in the presence of adequate homocysteine, methylfolate (5-CH_3-H_4PteGlu) and cobalamin (Cbl)-dependent methionine synthetase activity. Folinic acid (5-CHO-H_4PteGlu), which provides available folate independently of Cbl, improved growth only slightly in the absence of Met. Free Cbl at 222 n*M*, an amount great enough to alter other intracellular events, failed to increase growth in the absence of Met, but 0.22 n*M* Cbl bound to transcobalamin II did, however, enhance growth.

We reported in 1981 that cultured peripheral lymphocytes (PBL) from persons deficient in cobalamin (Cbl) synthesized and released less total protein and IgG than cells from healthy persons (1). Synthesis and release increased when Cbl was added to the cultures *in vitro*. We postulated that the link between Cbl and protein synthesis was in the need for Cbl in the synthesis of methionine (Met) which is in turn a precursor of *S*-adenosylmethionine (AdoMet), a product essential for many methylations including that of RNA (2). Further experiments demonstrated that PBL could not be cultured without Met in the medium. This form of Met dependency has been observed primarily in oncogenic transformation (3, 4), but also in cultures of virus-transformed cells (3, 5). The phenomenon has not been studied in normal human lymphocytes. Fujii *et al.* (6) did find that murine lymphoblasts required 40–60 μM exogenous Met, although the cells were able to synthesize endogenous Met via the Cbl-dependent synthetase reaction. These cells were, however, neoplastic. The present study, which has been reported partially in an abstract (7), was designed to evaluate the Met requirements of PBL of healthy persons and of a virus-transformed human B-cell line.

Methods. *Human peripheral lymphocytes.* PBL were isolated from healthy donor blood by the Ficoll–Hypaque sedimentation technique as described (1, 8). The preparation of crude phytohemagglutinin (PHA), M Form, Grand Island Biological Company, (GIBCO, Grand Island, N.Y.) was reconstituted to 5 ml and added to each culture at 0.1 ml/10 ml medium.

The RPMI 6410 cell line. The line, which was derived from a normal human B cell transformed by the Epstein–Barr (EB) virus, was purchased from Associated Biomedic Systems Inc. (Buffalo, N.Y.). It has been maintained by us for several years through serial cultures when in use and under liquid nitrogen when not in use.

Cultures. The experimental medium was RPMI 1640 (GIBCO) without Met, Hcy, Leu, Glu, folate, or Cbl. The following substances were added routinely: 50 μg/ml L-Leu (Sigma Chemical Co., St. Louis, Mo.); 0.3 mg/ml L-Glu (General Biochemicals, Chagrin Falls, Ohio); 100 units/ml penicillin; and 100 μg/ml streptomycin. The L-Met (GIBCO), DL-homocysteine (Hcy; Sigma), DL-methylfolate (5-CH_3-H_4PteGlu; Sigma), DL-5-formyltetrahydrofolate (5-CHO-H_4PteGlu, folinic acid; Sigma), and hydroxocobalamin (OH-Cbl; Sigma), and cyanocobalamin (CN-Cbl; Sigma) were manipulated according to the needs of each experiment. Stock cultures were maintained in T_{75} flasks in 30-ml volumes of RPMI 1640 medium containing 15 μg/ml (100 μM) Met but no Hcy, 1 μg/ml (1.5 μM) PteGlu, 5 ng/ml (3.7 n*M*) CN-Cbl, and 10% fetal calf serum (FCS; GIBCO). Doubling time was about 24 hr and every other day the stock cultures were diluted to 5×10^5 cells/ml. All experiments were conducted in T_{25} flasks (Cor-

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ning) in 10-ml volumes of RPMI 1640 medium with the additions and deletions as recorded for each experiment. Bovine serum albumin (BSA; Fraction V, Sigma) at 7 mg/ml was substituted for the FCS in order to eliminate a source of Cbl and of transcobalamin II (TC II). Cultures were started at 5×10^5 cells/ml and the 6410 cells were maintained for 3 days, although often with daily sampling. The PBL were cultured for up to 13 days depending on experimental requirements. The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. At the experimental time points the cells were counted manually, and viability was determined by trypan blue exclusion.

For some experiments Cbl bound to semipure TC II was added to the cultures. TC II was prepared from Cohn Fraction III by adsorption to and elution from CM-Sephadex C-50 (9). The preparation was equilibrated by dialysis against buffered saline and stored at −20°C. Prior to use, CN-Cbl or OH-Cbl was added to saturation. For some experiments the TC II, including that carrying Cbl, was removed by immunoadsorption with a pellet of Sepharose to which rabbit anti-human TC II had been coupled (9). This process reduces the TC II content by greater than 95% and, since the antibody was raised against pure TC II (9), removes only this protein as confirmed by electrophoresis.

Enzyme assay. The activity of the Cbl-dependent Hcy:5-CH_3-H_4PteGlu methyl-transferase (methionine synthetase; MS) was determined by the basic method of Taylor and Hanna (10) as applied by us (8). Holo activity was measured by the unmodified assay and total activity after the addition of CN-Cbl.

Statistical methods. The data reported in the tables were analyzed by the paired *t* test, in each instance comparing a change from the absence of an additive with the presence. The two-tailed section of the *t* table was used to determine the respective *P* value. Significance was considered to be at $P < 0.05$, but in many situations lower *P* values were obtained as noted.

Results. *Establishment of requirements for culture.* Through a series of preliminary experiments the basic requirements for Met and folate were established for both types of lymphocytes and were similar. Folate needs of cells replete in Cbl were met from Hcy and 5-CH_3-H_4PteGlu. Added Cbl was not required for growth of either PBL or the RPMI 6410 cell in the presence of Met; presumably adequate Cbl was carried over from the maintenance of stocks in medium containing Cbl.

Methionine auxotrophy. The basis for the present study is shown in Fig. 1; cultures of PBL could not be maintained without exogenous Met. This observation was repeated in 3-day cultures of RPMI 6410 cells and, although not shown, the pattern of Fig. 1 was the same as for the PBL. Deletion of Met decreased growth of 6410 cells profoundly, $P < 0.001$ (Table I). Providing the folate as equal amounts of 5-CHO-H_4PteGlu did not compensate for the deletion of Met (not illustrated). The effects of added Cbl were tested with Cbl either in the free form at 222 n*M* or at 0.22 n*M* bound to TC II. These amounts of the respective forms of Cbl were chosen because in work illustrated partially below and to be published in more detail elsewhere they induced equally the Cbl-dependent MS activity of lymphocytes. The much more efficient use of the TC II–Cbl originates in the function of TC II promoting Cbl entry into cells (6). The addition of 222 n*M* free OH-Cbl did not increase growth significantly in the absence of Met, $P > 0.1$ (Table I). In another set of six experiments, not shown, 2220 n*M* had no further effect. However, 0.22 n*M* TC II–Cbl allowed growth equivalent to that when exogenous Met was present (difference: $0.05 < P < 0.1$).

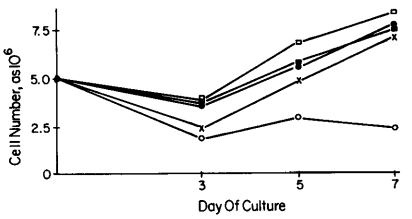

FIG. 1. Two experiments showing effects of varied amounts of methionine on the replication of PHA-stimulated PBL. The medium contained 7.0 mg/ml BSA, 300 μ*M* homocysteine, and 1.85 n*M* OH-Cbl. 5-CH_3-H_4PteGlu, 1.5 μ*M*, was added on Days 0 and 4 and the methionine varied: ○—none; ×—17 μ*M*; ●—33 μ*M*; □—50 μ*M*; ■—67 μ*M*.

TABLE I. REPLICATION OF RPMI 6410 CELLS (n = 10)

	67 μM Met/0 Cbl	0 Met/0 Cbl	0 Met/222 nM free OH-Cbl	0 Met/0.22 nM TC II OH-Cbl	67 μM Met/0.22 nM TC II OH-Cbl
Total cells, $\times 10^6$					
Mean	26.3	5.8	6.8	22.2	26.8
SD	5.9	1.0	1.2	4.8	4.3
% Viable	93	48	50	99	99

Note. All cultures contained 15 μM 5-CH_3-H_4 PteGlu and 300 μM DL-Hcy.

In seven sets of PBL from healthy donors (Table II and Fig. 2), the omission of Met, but the presence of free Cbl, impaired the growth during 7–10 days of culture, $P < 0.001$. In four of these experiments, 0.22 nM semipure TC II–Cbl invariably increased growth in the absence of Met, $P < 0.05$.

The present experiments were conducted with the forms and amounts of folate and Cbl close to those encountered in the human circulation. However, the classic studies of Met auxotrophy, which have been with cells other than lymphocytes, incorporated much larger amounts. In order to permit comparisons, a set of studies was conducted in RPMI 6410 cells under the conditions used in other studies. The Met and Hcy (L-thiolactone as used by others for these experiments) were at 100 μM and the free OH-Cbl was 1.5 μM. The folate was 100 μM folic acid (PteGlu; PGA). FCS (10%) replaced the BSA. The observations, not illustrated, did not differ from those of Tables I and II. Cell growth was not maintained in the absence of Met in spite of the increases in Cbl and folate. Growth was restored to an extent similar to that of Tables I and II by the replacement of the 1.5 μM free Cbl with 0.22 nM TC II–Cbl.

The active principle in the TC II–Cbl preparation. Although we have available pure TC II (9) it is unstable in prolonged culture at 37°C (Dr. Marijke Frater-Schroder, personal communication). Therefore, semipure TC II which we have found to be stable was chosen. Cohn fractionation and CM-Sephadex chromatography remove some contaminating proteins, including other binders of Cbl, from the original plasma. The subsequent dialysis would be expected to remove small molecules including Met. Nevertheless, there remained the possibility that nondialysable substances other than TC II were promoting growth. The question was approached in six experiments by comparing the effects on cell growth of two equal amounts of preparation. One preparation contained holo TC II and the other was treated by immunoadsorption to remove all TC II and Cbl (9). In the absence of Met the adsorbed preparation increased the 3-day growth of RPMI 6410 cells from 6.0 ± 0.5 $\times 10^6$ to 8.5 ± 0.6, but when the TC II–Cbl remained in the preparation there was a much greater ef-

TABLE II. REPLICATION OF STIMULATED PBL

	Met dependence (n = 5)		TC II–Cbl effect (n = 4)	
	33 μM Met/222 nM free Cbl	0 Met/222 nM free Cbl	0 Met/222 nM free Cbl	0 Met/0.22 nM TC II–Cbl
Total cells, $\times 10^6$				
Mean	13.2	3.8	3.5	17.1
SD	6.8	1.2	1.2	9.0
% Viable	90	79	81	96

Note. Cells stimulated with PHA and growth reported for Day 9 or 10 (day consistent with an experiment). DL-Hcy, 300 μM and 1.5–15 μM 5-CH_3-H_4 PteGlu. TC II–Cbl was tested in four of the five experiments.

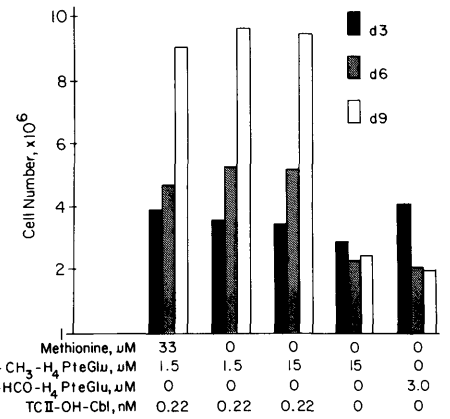

FIG. 2. An experiment showing the effect of TC II-Cbl on the methionine auxotrophy of stimulated PBL. Points increasing the 5-CH_3-H_4PteGlu to 15 μM were included and the effect of substitution of 5-CHO-H_4PteGlu for the 5-CH_3-H_4PteGlu was evaluated.

fect, the 3-day growth being $14.3 \pm 3.1 \times 10^6$ cells (intact vs adsorbed, $P < 0.01$). The adsorbed preparation was even less active in experiments with PBL. A preparation which contained apo TC II but no Cbl added to it was ineffective. In a third approach to the identification of the active principle in preparations of TC II, the Hcy content of the cultures were varied. Stock cells were cultured in medium containing 0.22 nM TC II–Cbl and 15 μM 5-CH_3-H_4PteGlu but no Met. It was reasoned that any specific effect of TC II in directing the Cbl *might* be dependent on the MS reaction and on the presence of Hcy, but any effect of the preparation unrelated to the Cbl would not. In a set of five experiments the mean number of cells on Day 3 was 15.3×10^6 when the medium contained 300 μM DL-Hcy and 8.8×10^6 when Hcy was omitted ($P < 0.01$).

The nature of the TC II effect. TC II binds Cbl to specific receptors or cells which initiates the internalization of the TC II–Cbl (11). Fujii *et al.* (6) found the Cbl of TC II–Cbl to promote growth of Cbl-depleted murine leukemic cells to the same degree as 2000-fold the amount of Cbl in the free form. Therefore, the effect of TC II in the present study could originate in part from more efficient entry of the Cbl bound to it. The question was approached by comparing the increase in MS activity of RPMI 6410 cells by free vs TC II-bound Cbl (Table III). The 0.22 nM TC II–OH Cbl increased total MS activity slightly more than the 222 nM free OH-Cbl while for the holo MS activity the pattern was reversed. Neither difference was significant. This increase in MS activity implied that equivalent amounts of free and TC II-bound Cbl were entering the cells (Table III), but the effects were not equivalent in compensating for the absence of Met (Table I).

Discussion. Generally the inability of cells to grow in the absence of Met but with Hcy as a potential precursor has been a characteristic of oncogenic transformation (3, 4). Auxotrophy for Met has not been described previously in nonmalignant lymphoid cells, but might be anticipated in virus-transformed lymphoblasts. Nonmalignant human fibroblasts transformed with Simian virus 40 are also auxotrophic for Met (3, 5, 12) and the 6410 line was transformed with the EB virus. Therefore, the phenomenon in the 6410 cells could be a function of virus transformation. The PBL stimulated with PHA, which were also auxotrophic, were neither virus-transformed nor malignant, and this most provocative observation is reported here for the first time.

The type of auxotrophy for Met described to date has been characterized by adequate synthesis of Met (5), reduced capacity to synthesize AdoMet (13), a reduced ability to use endogenous Met for the synthesis of AdoMet (3), a decline in the AdoMet/AdoHcy ratio (14), impaired methylation of macromolecules (15), and a reduced proportion of the synthesized Met in the free form (14). The need for

TABLE III. METHIONINE SYNTHETASE ACTIVITY OF DAY 3 RPMI 6410 CELLS

Met	OH-Cbl	Holo MS	Total MS
67 μM	0	64 ± 80[a]	327 ± 134
0	0	36 ± 22	94 ± 62
0	0.22 nM TC II-Cbl	139 ± 84	798 ± 324
0	222 nM Free Cbl	229 ± 210	637 ± 298

Note. These data were from the experiment shown in Table I. All cultures contained 15 μM 5-CH_3-H_4PteGlu and 300 μM DL-Hcy; $n = 10$.

[a] pg of Met per hour per 10^6 cells, means ± SD.

exogenous Met by the PBL and the transformed lymphoblasts may or may not be the same as this previously described form of auxotrophy. The characteristics listed above have not been tested. MS activity, but not whole cell Met synthesis, was, nevertheless, tested and the levels observed in the presence of free Cbl plus the ability of the cells to use 5-CH_3-H_4PteGlu suggest that the Met auxotrophy was related to a step in Met metabolism beyond Met synthesis.

Previously described cells auxotrophic for Met have been "immortal" through neoplasia or virus transformation. The auxotrophic PBL studied here were not. As collected they were in a resting phase and then transformed by PHA (8). The number usually declined, Days 0–3, and then increased (Figs. 1 and 2). Although the cultures were not maintained beyond Day 10 here, we have extended them to Day 14. Replication may persist until then or start to decline, but we have not found a way to make these cells immortal. By a week the cells have differentiated to the point where they make and release IgG (1). The unidentified factor that determines Met auxotrophy may be the same in PBL stimulated by PHA and in malignant cells, but the PBL do not otherwise behave like malignant cells. Stimulation with PHA is of course artificial, but PBL must naturally be stimulated with something in order to function.

The effect of TC II appeared to go beyond the one known function of TC II which is to bind Cbl to specific receptors on the cell and to initiate internalization. The TC II here may have directed the Cbl within the cell or set in motion an activity that Cbl alone does not possess. There may be an *in vivo* analogy. Deficiency of TC II has profound effects on lymphocyte function whereas simple Cbl deficiency does not (16).

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Note added in proof. It was not considered necessary to study the Met dependency of the stimulated PBL under the cultural conditions used by others with other types of cells (3–5). The RPMI 6410 cells were, however, studied under those conditions and expressed the same Met requirement and the same response to TC II–Cbl as under the cultural conditions considered by us to be more physiologic. Throughout the present studies, the requirements for folate, Cbl, and Met and the effects of TC II–Cbl in the absence of Met were identical for PBL and for RPMI 6410 cells. Neither were the experiments extended to cells other than lymphocytes. As stated in the opening sentence, the present study was a step toward determining the basis for the effect of Cbl on the synthesis of immunoglobulins, a process confined to lymphocytes.

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