0022-3565/91/2582-0559$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 1991 by The American Society for Pharmacology and Experimental Therapeutics
Vol. 258, No. 2
Printed in U.S.A.

Interaction between Methotrexate, "Rescue" Agents and Cell Proliferation as Modulators of Homocysteine Export from Cells in Culture[1]

HELGA REFSUM[2], BENEDICTE CHRISTENSEN, RUNE DJURHUUS and PER MAGNE UELAND
The Clinical Pharmacology Unit, Department of Pharmacology and Toxicology, University of Bergen, N-5021 Haukeland Hospital, Bergen, Norway
Accepted for publication May 6, 1991

ABSTRACT

Clinical studies on cancer and psoriasis patients have shown that plasma and urinary homocysteine (Hcy) responds to methotrexate (MTX) therapy, indicating that Hcy in extracellular fluids may be an indicator of the antifolate effect. However, the clinical data indicate that the burden of proliferating cells, cytotoxicity and the folate status are also determinants of extracellular Hcy. To evaluate this further, we investigated the modulation of cellular Hcy egress by MTX, rescue agents, cell proliferation and cytotoxicity. Nontransformed and chemically transformed fibroblasts and murine lymphoma cells, which are characterized by different growth behavior and MTX response, were used. The Hcy export rate was correlated positively with the proliferation rate in all cell types. 5-Formyltetrahydrofolate or 5-methyltetrahydrofolate added to fibroblasts not exposed to MTX reduced the Hcy export rate, whereas the export from the lymphoma cells was not affected. All cells types exposed to MTX were rescued by thymidine + hypoxanthine, and this allowed the assessment of Hcy export during MTX exposure without interference from cytotoxicity. In the fibroblasts, MTX with thymidine + hypoxanthine rescue induced a marked increase in Hcy export, and the dose-response paralleled the cytotoxicity curves obtained for MTX without rescue. Nontoxic concentrations of MTX without rescue enhanced the Hcy export. When MTX concentration was increased further, Hcy export was stimulated initially, and then declined rapidly as cell death ensued. MTX did not enhance the Hcy export from the lymphoma cells and, in the absence of rescue, the Hcy export from these cells declined in proportion to inhibition of cell growth. Our data show that Hcy export is modulated by growth rate, rescue agents and MTX, but the response differs between cell types. Furthermore, MTX may have a dual action on cellular Hcy egress due to the primary antifolate effect and subsequent inhibition of cell growth. These *in vitro* observations offer an explanation for the variations in plasma homocysteine observed in patients treated with regimens including MTX.

Hcy arises from *S*-adenosylhomocysteine, a product of *S*-adenosylmethionine-dependent transmethylation reactions. Intracellular Hcy is either converted to cysteine or is salvaged to methionine through remethylation. Remethylation is catalyzed by betaine-homocysteine transmethylase (EC 2.1.1.5.) in the liver and by methionine synthase (homocysteine-5-methyl-THF methyltransferase, EC 2.1.1.13.) which is widely distributed in mammalian cells. The latter enzyme requires 5-methyl-THF as a methyl donor and vitamin B_{12} as a cofactor (Finkelstein, 1990). An alternate fate of Hcy is export into the extracellular medium. This process occurs at a rate reflecting the balance between Hcy production and utilization (Svardal *et al.*, 1986a,b; Ueland *et al.*, 1986). Thus, extracellular Hcy may be an indicator of the function of enzymic processes or availability of cofactors or substrates involved in Hcy metabolism.

The concept of extracellular Hcy as a useful indicator of Hcy homeostasis has gained considerable support during the last few years (Ueland and Refsum, 1989). Clinical studies have shown that plasma Hcy is elevated in some inborn errors of metabolism (Mudd, 1989), in folate (Kang, 1987) and cobalamin (Lindenbaum *et al.*, 1988) deficiency states and in several diseases, including psoriasis (Refsum *et al.*, 1989a) and cancer (Kredich *et al.*, 1981).

Pharmacological agents may change the concentration of Hcy in plasma or alter the Hcy egress from cultured cells *in vitro*. Some of these compounds act by interfering with the folate-dependent remethylation of Hcy (Refsum and Ueland, 1990). We have demonstrated recently an increase in plasma Hcy in patients exposed to nitrous oxide for 90 min, showing that plasma Hcy is a particularly sensitive measure of nitrous

Received for publication January 8, 1991.
[1] This work was supported by grants from the Norwegian Cancer Society, from Astri og Birger Torsteds legat til bekjempelse av kreft and the Norwegian Research Council for Science and the Humanities.
[2] Was a Fellow of the Norwegian Research Council for Science and the Humanities.

ABBREVIATIONS: Hcy, homocysteine; THF, tetrahydrofolate; MTX, methotrexate; ALL, acute lymphoblastic leukemia; PBS, phosphate-buffered saline; BME, Basal Medium Eagle; Thd, thymidine; Hx, hypoxanthine; HPLC, high-performance liquid chromatography.

oxide-induced inactivation of methionine synthase (Ermens *et al.*, 1991). Effect of the antifolate drug MTX has been studied both *in vitro* and *in vivo*. It enhances the Hcy egress from confluent, quiescent fibroblasts in culture (Ueland *et al.*, 1986) and increases the plasma Hcy in patients treated with a wide range of doses (Refsum *et al.*, 1986, 1989a, 1991; Broxson *et al.*, 1989).

In patients with ALL or solid tumors exposed to high-dose MTX, the plasma Hcy is decreased upon administration of 5-formyl-THF rescue (Refsum *et al.*, 1986, 1991; Broxson *et al.*, 1989). A related observation has been made in healthy subjects and in folate-deficient patients with elevated plasma Hcy. In these subjects, high doses of folic acid significantly decreased the plasma Hcy concentration (Brattström *et al.*, 1988; Stabler *et al.*, 1988). These observations indicate that the plasma Hcy responds rapidly to changes in intracellular folate status. Furthermore, the effect on plasma Hcy from low doses of MTX in psoriasis patients suggests that Hcy is a particularly sensitive indicator of antifolate effect (Refsum *et al.*, 1989a).

The changes in plasma Hcy concentration during cancer chemotherapy with regimens including MTX (Broxson *et al.*, 1989; Refsum *et al.*, 1991) may represent the net result of several opposing or cooperative processes. Conceivable processes are proliferation of tumor cells, primary effect of MTX as an antifolate agent, the resulting cytotoxicity and finally supplementation of reduced folates, like 5-formyl-THF. To obtain a better understanding of the Hcy response in cancer patients before and after chemotherapy with MTX, we investigated interaction between these factors in a cell culture model system, by using computer assisted construction of Hcy export rate curves.

Materials and Methods

Chemicals. L-Methionine, L-homocystine, cyanocobalamin, folic acid, 5-formyl-THF, 5-methyl-THF, dithioerythritol and *o*-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium borohydride was obtained from Fluka Chemie AG (Buchs SG, Switzerland) and monobromobimane from Calbiochem, Behring Diagnostics (La Jolla, CA). MTX was a gift from Nycomed (Oslo, Norway).

Cell lines. Three different cell lines were used.

The murine T-lymphoma cell line, R 1.1 (referred to as lymphoma cells) was supplied by Dr. Dennis A. Carson at the Scripps Clinic (La Jolla, CA). This cell line arose spontaneously in a C58 mouse (Old *et al.*, 1965) and shares antigenic and metabolic properties with normal thymocytes (Ralph, 1973).

The nontransformed C3H/10T1/2 Cl 8 cells (Cl 8 cells) (Reznikoff *et al.*, 1973b) and the chemically transformed C3H/10T1/2 MCA Cl 16 cells (Cl 16 cells) (Reznikoff *et al.*, 1973a) were obtained from Dr. J. R. Lillehaug, Department of Biochemistry, University of Bergen. Both cell types are anchorage-dependent and were derived from mouse embryo fibroblasts (Reznikoff *et al.*, 1973a,b). The nontransformed Cl 8 cells are sensitive to postconfluence inhibition of cell division. Cell division of the transformed Cl 16 cells is not inhibited completely at confluence, and their growth rate is reduced moderately in methionine-deficient medium supplemented with Hcy (Djurhuus *et al.*, 1988).

Cell culture conditions and harvesting. Exponentially growing cells (Cl 8, Cl 16 and R 1.1) were washed with PBS, and seeded in tissue culture dishes (3.5 cm, Nunc, Roskilde, Denmark) at a density of 10,000 cells/dish in 3 ml of culture medium, when not otherwise indicated. The cells were grown in BME (Gibco, Paisley, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland) and containing 1 μg/ml of folic acid, 2 μg/ml of cyanocobalamin and 50 μM methionine. The cell lines were maintained at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 98%.

The cells were allowed to enter early logarithmic growth phase before the test substance(s) were added, and this was about 12 hr after seeding for the lymphoma cells and 36 to 48 hr for the fibroblasts.

In one experiment, Cl 8 cells which had just reached confluence, were used. The medium was changed 24 hr before the experiment, which was started by replacing the medium with fresh medium containing test substance(s).

The test substances, usually dissolved in PBS, were added (20–30 μl) directly to the dish, and control cells received an equal volume of PBS.

Samples of the medium were harvested from two parallel dishes in each group, either directly (fibroblasts) or after removing the cells by centrifugation (lymphoma cells). The samples were frozen at −20°C for the determination of Hcy, Thd and methionine. The fibroblasts were brought into suspension by trypsinization. Cell counts were determined using a Coulter Counter model ZM (Coulter Electronics Ltd., Luton, UK).

Exposure of cells to MTX, 5-formyl-THF, 5-methyl-THF and Thd + Hx. MTX (10 mM in PBS) was prepared immediately before use, and diluted in the culture medium to the concentrations indicated.

5-Formyl-THF and 5-methyl-THF (1 mM) were dissolved in ice-cold PBS immediately before use, and diluted in the culture medium (2 ml) to a final concentration of 10 μM.

Hx (21.8 mg) was dissolved in 10 ml of 0.1 M NaOH and Thd (9.7 mg) in 20 ml of PBS. Five milliliters of each solution were added to 10 ml of PBS. The final concentrations of Thd and Hx in the culture medium were 5 and 40 μM, respectively.

Determination of cytotoxicity. The cytotoxicity of 24-hr MTX exposure was evaluated by determination of total cell count (fibroblasts and lymphoma cells) or plating efficiency (fibroblasts) or by a soft agar-colony-forming assay (lymphoma cells). Cytotoxicity during continuous MTX exposure was evaluated by determination of cell count.

The fibroblasts were incubated in the presence of increasing concentrations of MTX for 24 hr, and the plating efficiency was determined after 8 days of culture in a drug-free medium. Only colonies comprising more than 30 cells were counted (Ueland *et al.*, 1986).

The cytotoxicity against the lymphoma cells was evaluated by a soft-agar-colony-forming assay (Dalen and Burke, 1971). After incubating the cells for 24 hr in the presence of MTX, the cells were plated at three different densities (100, 300 and 1000 cells/dish) in 2 ml of a drug-free medium containing 0.3% agar (Dulbecco's noble agar, Flow Laboratories, Irvine, Scotland) and 20% fetal calf serum. The dishes (3.5 cm, Nunc) were incubated, and colony formation (>30 cells/colony) counted after 10 days of growth, using an invert microscope. Platings were done in triplicate.

Determination of Hcy in the culture medium. Hcy in the culture medium was determined by a modification of an automated procedure developed for the determination of total Hcy in plasma (Refsum *et al.*, 1989b). The method measures both the major free fraction of Hcy and the small amounts of protein-bound Hcy in the culture medium.

The medium was treated with borohydride and then derivatized with monobromobimane. The reduction, derivatization and the injection into the HPLC column were carried out sequentially by a microprocessor controlled autosampler from Gilson, model 232-401 (Refsum *et al.*, 1989b). The Hcy-monobromobimane adduct was separated on a 15 cm 3 μm ODS Hypersil column eluted with an acetonitrile gradient (0–10% in 11.5 min) in 58 mM ammonium nitrate/40 mM ammonium formate buffer, pH 3.7. The flow rate was 2 ml/min. The effluent was monitored by fluorescence detection. The adduct showed a retention time of 11 min in this system.

Determination of methionine in the culture medium. Methionine was determined in the medium deproteinized with acid and neutralized. The amino acid was derivatized with *o*-phthaldialdehyde in the presence of mercaptoethanol in saturated borate buffer, pH 9.5 (Krishnamurti *et al.*, 1984). The derivatization procedure and the injection into an HPLC column were carried out sequentially by the

Gilson, model 232-401 autosampler. The methionine-o-phthaldialdehyde adduct was quantitated by reversed-phase liquid chromatography and fluorescence detection (Krishnamurti *et al.*, 1984).

Determination of Thd in the culture medium. Thd was determined in culture medium deproteinized with perchloric acid and then neutralized. Eighty microliters were injected into a 10 cm 3 μm ODS Hypersil column equilibrated with 15 mM acetate buffer, pH 4.5. The column was eluted at ambient temperature with a methanol gradient in acetate buffer. Methanol increased from 0 to 2.1% (6.5 min), and then to 4.5% (10 min). The flow rate was 2 ml/min. The absorbance of the effluent was measured at 260 nm using a variable absorbance detector, model Spectroflow 773 from Kratos Analytical Instruments (Ramsey NY). The retention time of Thd was 9.5 min.

Curve fitting and calculation of export rates and growth rates. We calculated the export rate per million cells and growth rate per 1000 cells. The calculations and presentation of data are as follows:

The cell number followed an exponential equation during the first and middle part of the growth period, but deviated upon reaching confluence (the fibroblasts) or when the medium was nutritionally depleted (lymphoma cells). Therefore, the growth curves were best fitted by a polynomial function. The data points for the growth and export were usually log-transformed to obtain a better curve fit. The cell number (N) was given by the equation:

$$\log N = a_0 + a_1t + a_2t^2 + a_3t^3 + \ldots + a_nt^n \quad (1)$$

where a_0, a_1, a_2, a_3 and a_n are constants and t is time after addition of test substance(s).

The concentration of Hcy in the medium (y) increased as a function of time, t. These curves were also usually best fitted to a polynomial:

$$\log y = b_0 + b_1t + b_2t^2 + b_3t^3 + \ldots + b_nt^n \quad (2)$$

In a few cases the export curves were best fitted to the equation:

$$1/y = c_0 + c_1t + c_2t^2 + c_3t^3 + \ldots + c_nt^n \quad (3)$$

The degree (n) of the polynomials, the constants ($a_0, a_1 \ldots a_n$; $b_0, b_1 \ldots b_n$, $c_0, c_1 \ldots c_n$) and the R values were obtained using a curve-fitting program, Multifit, version 1.51 for the Apple Macintosh, from Day Computing (Cambridge, UK).

Appropriate fitting of data included the criterion $R^2 > 0.98$. The differential, dN/dt, is the growth rate (g), which is given per unit of cell mass (N):

$$g = dN/dt/N \quad (4)$$

The differential, dy/dt, is the export rate (v), which is also given as per unit of cell mass (N):

$$v = dy/dt/N \quad (5)$$

Acceptable estimations of v and g can also be obtained by calculating the change in y or N (Δy or ΔN) during a short time interval (Δt). Δt of 1 hr was appropriate. The equations for v and g then become:

$$g = \Delta N/\Delta t/N \quad (6)$$

$$v = \Delta y/\Delta t/N \quad (7)$$

N is expressed as the mean cell number during

$$\Delta t{:}N = (N_t + N_{(t-\Delta t)})/2. \quad (8)$$

For growing cells, v and g were routinely plotted against the logarithm of cell density.

Results

Optimalization and evaluation of the culture conditions. All cells were cultured in BME. This medium contains only the small amounts of cobalamin contributed by the fetal calf serum. Preliminary experiments showed that cyanocobalamin reduced the Hcy export from the fibroblasts, but did not affect the export from the lymphoma cells. This indicates that serum does not provide sufficient cobalamin for maximal homocysteine remethylation in some cell types. The culture medium was therefore supplemented with 2 μg/ml of cyanocobalamin.

Thd + Hx was used to rescue cells exposed to MTX. We found that Thd at concentrations higher than 5 to 10 μM inhibited cell growth, as demonstrated previously by others (O'Dwyer *et al.*, 1987). We therefore used a rescue regimen containing 5 μM of Thd (and 40 μM Hx). Thd was used by the cells at a faster rate during MTX exposure (data not shown), probably due to inhibition of thymidylate synthase (Goldman and Matherly, 1985). Depletion of Thd terminated the protection of the cells against the cytotoxicity of MTX, and the quantitative relation was found that 5 μM Thd was sufficient to rescue about $0.5 \cdot 10^6$ cells/ml ($1.5 \cdot 10^6$ cells/dish).

Maximal cell density of the fibroblasts was about 0.6 to 1.5 $\cdot 10^6$ cells/dish (3 ml of medium), whereas the lymphoma cells reach 2 to 4 $\cdot 10^6$ cells/ml. When the lymphoma cells were seeded at the routine density ($\geq 0.5 \cdot 10^5$ cells/ml) and MTX was added after 12 hr, the rescue effect of 5 μM Thd lasted only about 2 doubling times. Therefore, to monitor export rate during cell division, the lymphoma cells were seeded at a low density of 3,300 cells/ml (10,000 cells/dish).

Clinical studies may suggest that MTX induces methionine depletion (Broxson *et al.*, 1989). Because MTX stimulates Hcy export from some cells, we investigated whether MTX increased the methionine consumption by cells in culture. Proliferating normal and transformed fibroblasts consumed about 15 to 30%, and the lymphoma cells up to 60 to 80% of the methionine present in the fresh medium (50 μM), but no effect of MTX on methionine concentration in the medium was detected (data not shown).

Growth and Hcy export. For the fibroblast cell lines, growth rate was highest at low cell density and then gradually decreased as a function of cell density, and proliferation ceased for the nontransformed cells as they reached confluence. The lymphoma cells showed maximal and stable growth rate during the midexponential growth phase and then proliferation declined (fig. 1A). The Hcy export rate plotted *vs.* cell density was also different for the fibroblasts *vs.* the lymphoma cells (fig. 1B). A plot of the export rate *vs.* growth rate shows clearly that the export rate was positively related to the rate of cell growth for all cell types (fig. 1C).

MTX cytotoxicity. The cytotoxicity of short-term (24 hr) MTX exposure of normal and transformed fibroblasts was evaluated by determination of cell count and plating efficiency, and LD_{50} values of 0.3 to 1 μM were obtained. The lymphoma cells were more sensitive toward MTX under these conditions, and LD_{50} was estimated to be 0.003 to 0.01 μM by cell count and clonogenic activity (data not shown).With continuous MTX exposure, LD_{50} values for the lymphoma cells (0.003 μM) and fibroblasts (Cl 8 and Cl 16) (0.01 μM) were less than 10-fold different (data not shown).

In the fibroblasts, the low MTX cytotoxicity of short-term in contrast to continuous exposure should be related to the observation that these cells (exposed to 1 μM MTX) underwent about two divisions before complete growth arrest occurred. The lymphoma cells ceased to grow almost immediately under these conditions (data not shown).

The effect of rescue agents on cell growth and Hcy export. Thd + Hx alone did not affect the growth rate of Cl 8

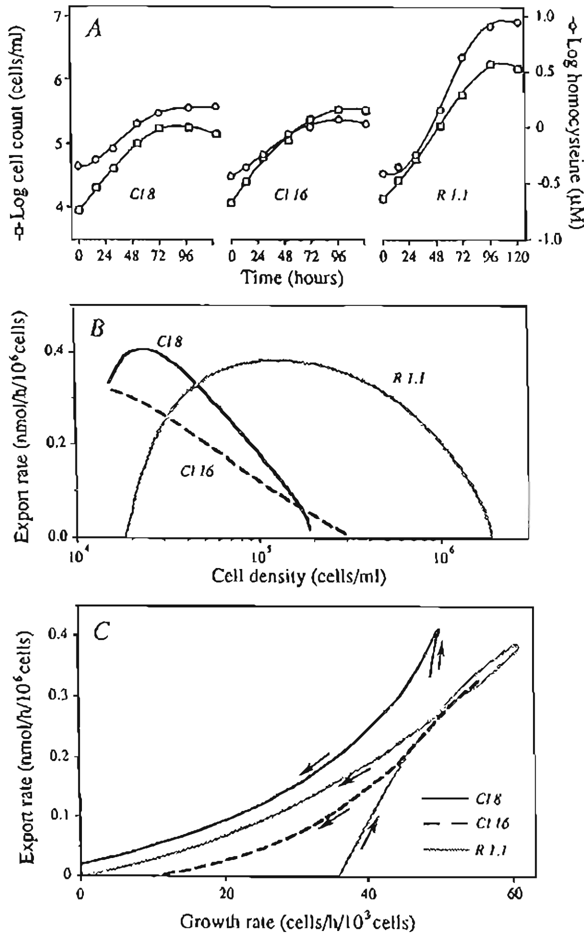

Fig. 1. Hcy export and its relation to cell growth in Cl 8, Cl 16 and lymphoma R 1.1 cells. A, cell count and amount of Hcy accumulating in the medium were determined for all three cell types. The curves for growth and Hcy export were fitted to polynomial functions after log transformation, as described under "Materials and Methods." B, the corresponding export rates (equations 5 and 7, "Materials and Methods") plotted *vs*. cell density. C, a plot of export rate *vs*. growth rate. Growth rates were determined from the data in "A" (using equations 4 and 6 under "Materials and Methods"). Arrows indicate the direction of increasing cell density.

and Cl 16 cells. The Hcy export rate was occasionally reduced (up to 30%) by this rescue regimen (data not shown). The effect of Thd + Hx on the lymphoma cells was somewhat different. The combination enhanced the growth rate about 2-fold and in most experiments moderately decreased the export rate from these cells (data not shown).

5-Formyl-THF and 5-methyl-THF did not affect the growth rate of Cl 8 and Cl 16 cells, but slightly (up to 20%) increased the growth rate of the lymphoma cells (fig. 2 and data not shown). 5-Methyl-THF reduced markedly the Hcy export from the Cl 8 (fig. 2) and Cl 16 cells (data not shown), whereas the lymphoma cells were not responsive (fig. 2). Essentially, identical results were obtained with 5-formyl-THF (data not shown).

Hcy export during MTX exposure and Thd + Hx protection. We used the Thd + Hx rescue regimen because it protected the cells against MTX cytotoxicity without providing the cells with reduced folates (Borsa and Whitmore, 1969; Howell *et al.*, 1981), and allowed the evaluation of the MTX effect on Hcy export without simultaneous cytotoxicity or

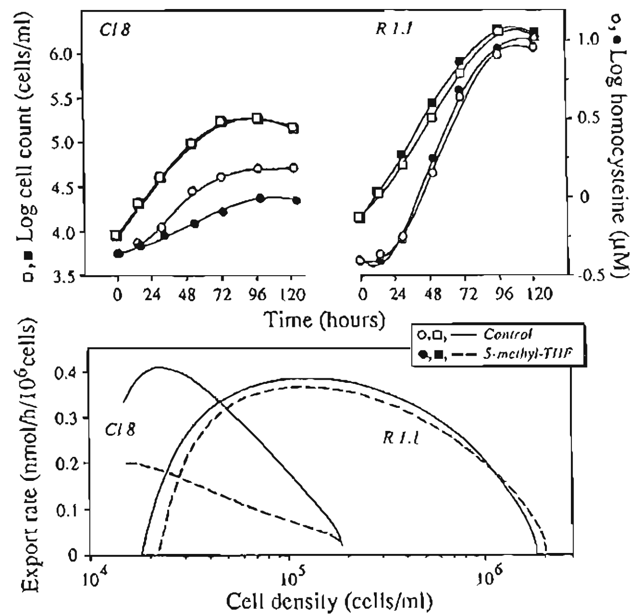

Fig. 2. Hcy export of Cl 8 and lymphoma R 1.1 cells exposed to 5-methyl-THF. Both cell types were incubated in a medium supplemented with 5-methyl-THF (10 μM) or without added folate (control). The growth and export curves were determined and fitted to polynomial functions after log transformation. From these equations the export rate was determined (from equations 5 and 7 under "Materials and Methods"), and plotted *vs*. cell density.

inhibition of cell growth. Under these conditions, MTX enhanced markedly the Hcy export rate from proliferating Cl 8 and Cl 16 cells (fig. 3). The Hcy export from proliferating lymphoma cells was not stimulated by MTX, but the Hcy export fell abruptly upon Thd depletion (fig. 3).

Hcy export and its relation to cytotoxicity at various MTX concentrations. We investigated the effect of increasing MTX concentrations on growth and Hcy export in all three cell lines during exponential growth, and on the Hcy export in confluent stationary Cl 8 cells. The experiment was performed in the absence and presence of Thd + Hx. Thd + Hx conferred complete protection against MTX cytotoxicity under the conditions of the experiment (fig. 4).

MTX induced a dose-dependent increase in Hcy export with half-maximal effect at about 0.01 μM in both Cl 8 and Cl 16 cells protected by Thd + Hx. In the absence of rescue, half-maximal inhibition of cell growth (LD_{50}) was observed at the same MTX concentration. Comparison of the growth and export curves shows that there was a slight stimulation of Hcy export at an MTX concentration (0.001 μM) exerting no effect on cell growth (fig. 4).

In confluent Cl 8 cells, MTX caused a dose-dependent increase in Hcy export. MTX had essentially no cytotoxic effect on confluent cells, and the Hcy export was the same in the absence and presence of Thd + Hx (fig. 4).

MTX induced an inhibition of growth of the lymphoma cells, and there was a parallel reduction in Hcy export. In the presence of Thd + Hx, the Hcy export became independent of MTX (fig. 4).

In the absence of Thd + Hx, the total Hcy export from Cl 8 and Cl 16 cells increased as a function of the MTX concentration up to 0.01 to 0.03 μM, and then declined at higher concentrations (fig. 4). To elaborate the kinetics of this biphasic response, we investigated the Hcy export rates of proliferating

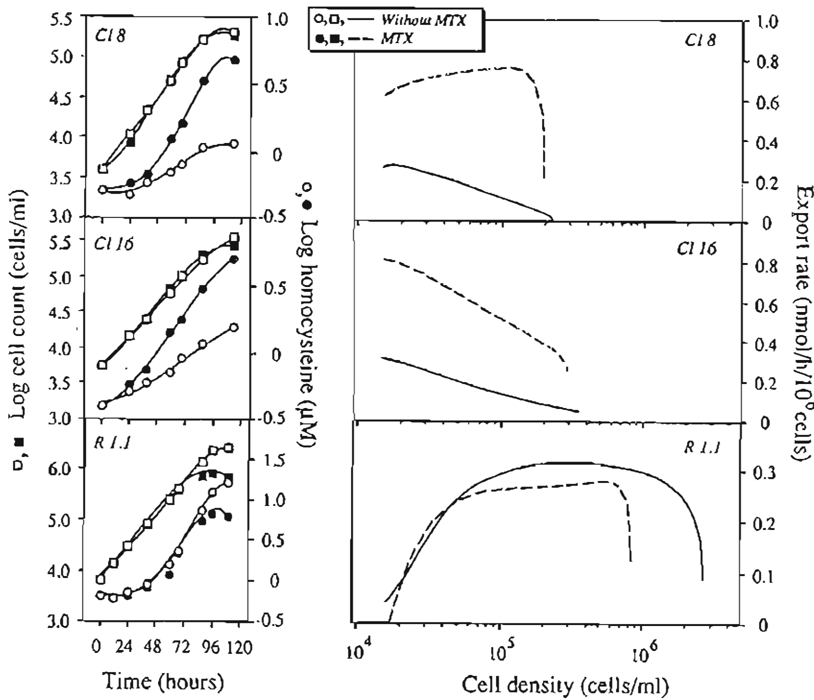

Fig. 3. Effect of MTX on Hcy export from Cl 8, Cl 16 and lymphoma R 1.1 cells protected with Thd + Hx. Left panels, growth and export curves for all cell lines cultured in the presence of Thd + Hx or MTX (1 μM) + Thd + Hx. The curves were fitted to polynomial functions after log transformation. Right panels, the export rates (determined from equations 5 and 7 under "Materials and Methods"), plotted *vs.* cell density.

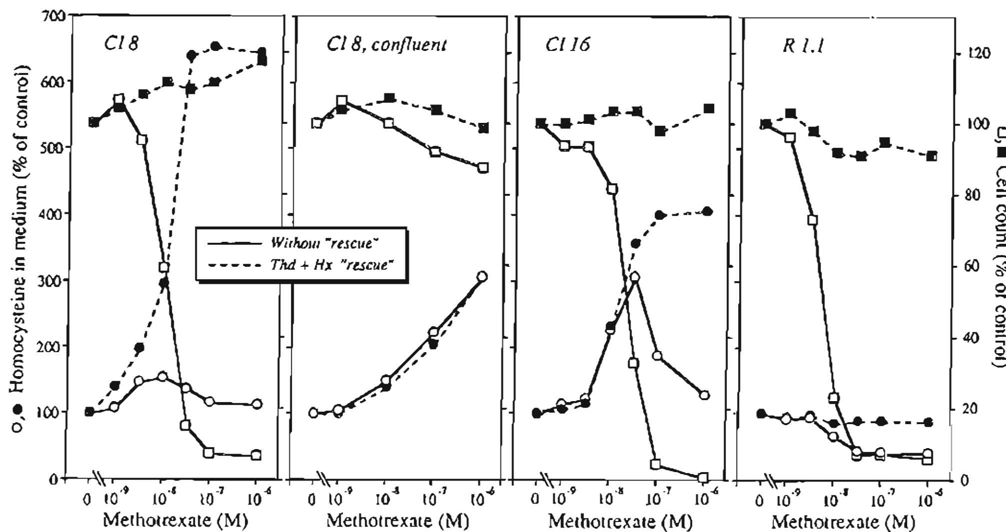

Fig. 4. Dose-response for the effect of MTX on Hcy export and cell growth in the absence and presence of Thd + Hx rescue. The cell count and Hcy accumulated in the cultured medium were determined for proliferating Cl 8, Cl 16, lymphoma R 1.1 cells and confluent Cl 8 cells incubated in the presence of various concentrations of MTX for 72 hr. Each point represents the mean of two determinations. The experiment was repeated 3 times, and a typical experiment is shown.

Cl 8 cells exposed to MTX at concentration inhibiting growth rate by about 50% (0.01 μM) and more (0.03 μM) (fig. 5).

MTX at a concentration of 0.01 μM stimulated the export rate in the time interval before inhibition of cell proliferation occurred (fig. 5, arrow). Thereafter the export declined rapidly. At a higher MTX concentration (0.03 μM), Hcy export rate was stimulated even more, but for a shorter time period (fig. 5).

Hcy export during exposure to other cytotoxic drugs. The effect of cytotoxic drugs other than MTX on Hcy export was investigated to evaluate whether increased export was related to cytotoxicity. Both vinblastine (LD_{50}, 0.001–0.01 μM) and cytarabin (LD_{50}, 0.03–0.3 μM) inhibited the growth of fibroblasts and lymphoma cells in a dose-dependent manner, and there was a parallel reduction in Hcy export (fig. 6).

Discussion

Only few reactions or tests are known to be influenced by MTX and can be used to monitor MTX pharmacodynamics *in vivo*. These include the deoxyuridine suppression test (Hwang *et al.*, 1989), the C_1 index (Morgan *et al.*, 1987) and homocysteine remethylation (Ueland and Refsum, 1989). Recent clinical studies suggest that such *in vivo* tests may provide useful guidelines during MTX therapy. In patients with rheumatoid arthritis receiving low-dose MTX therapy, the C_1 index of mononuclear cells have been shown to be an indicator of MTX efficacy (Morgan *et al.*, 1990) and plasma Hcy may forecast MTX toxicity (Morgan *et al.*, 1991).

The clinical studies on cancer (Refsum *et al.*, 1986, 1991; Broxson *et al.*, 1989) and psoriasis patients (Refsum *et al.*, 1989a) receiving MTX have demonstrated that the MTX-induced hyperhomocysteinemia is a sensitive, rapid and reproducible response. Moreover, plasma Hcy declines immediately upon supplementing the antidote, 5-formyl-THF (Refsum *et al.*, 1986, 1991; Broxson *et al.*, 1989), indicating that plasma Hcy mirrors the folate status. However, several factors complicate the interpretation of changes in the plasma Hcy level.

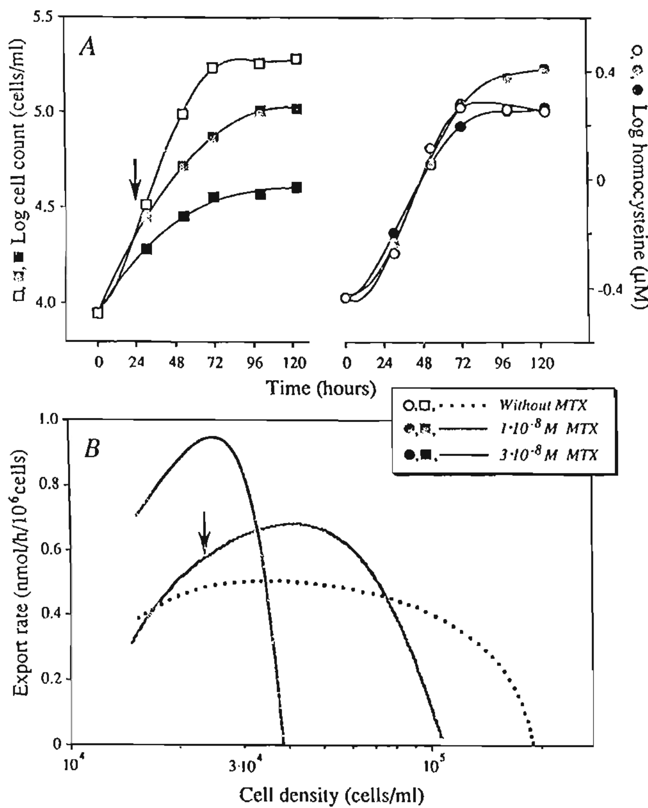

Fig. 5. The dual effect of cytotoxic concentrations of MTX on Hcy export from Cl 8 cells. Cl 8 cells were incubated without MTX and with 0.01 or 0.03 μM MTX. A, the raw data for the cell growth and Hcy export fitted to polynomial functions after log transformation. B, the export rates determined (from equations 5 and 7 under "Materials and Methods") and plotted *vs.* cell density. The arrow in A indicates the time point when cell growth in the presence of 0.01 μM MTX deviates from the control group. The arrow in B indicates the corresponding cell density.

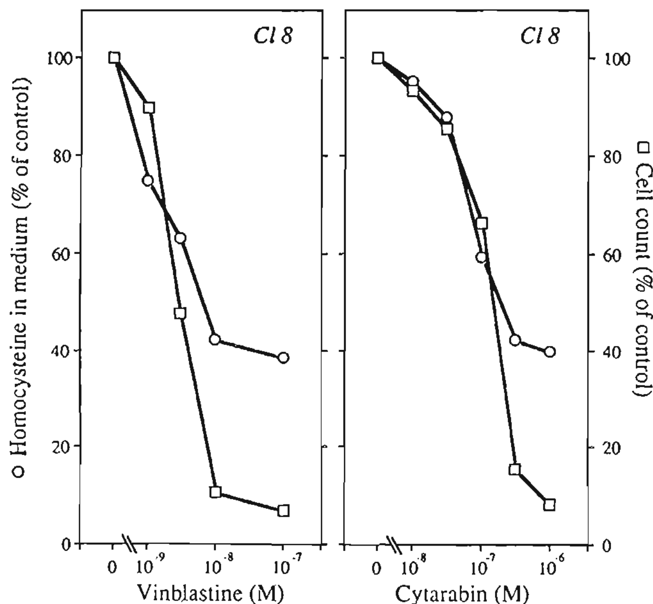

Fig. 6. Dose-response for the effect of vinblastine and cytarabin on Hcy export and cell growth. The cell count and Hcy accumulated in the culture medium were determined for proliferating Cl 8 cells incubated with various concentrations of vinblastine or cytarabin for 72 hr. Each point represents the mean of two determinations. The experiment was repeated 2 times.

Psoriasis and some malignant states like ALL, characterized by a large burden of proliferating cells, are associated with elevated plasma Hcy (Kredich *et al.*, 1981; Refsum *et al.*, 1989a, 1991), and cytoreductive therapy with regimens not containing MTX reduces plasma Hcy (Kredich *et al.*, 1981; Refsum *et al.*, 1991). Plasma Hcy has been measured following MTX doses varying 1000-fold, and no dose-response relationship has been observed (Refsum *et al.*, 1986, 1989a, 1991; Broxson *et al.*, 1989). In the present paper we have investigated whether these clinical findings have a corollary in a cell culture system.

Hcy export, cell growth and cancer. Most patients with solid tumors have normal plasma Hcy levels (Refsum *et al.*, 1986), whereas in children with ALL, the plasma Hcy is elevated and positively correlated with the white blood cell count, which reflects leukemic activity (Refsum *et al.*, 1991). A large burden of proliferating cells may release a substantial amount of Hcy into plasma and thereby contribute to the elevated plasma Hcy. This explanation is supported by the experimental data showing that cellular Hcy export was highest during early exponential growth (fig. 1 and Christensen *et al.*, 1991) and proportional to the specific growth rate (fig. 1 and Iizasa and Carson, 1985). We also found that cytotoxic drugs other than MTX inhibited Hcy export from all three cell lines in proportion to reduction in cell growth (fig. 6). This might be expected for a metabolite where the intracellular content is kept low by efficient efflux (Ueland and Refsum, 1989), and adds support to the possibility that the reduction in plasma Hcy in children with ALL during chemotherapy is due to eradication of leukemic cells (Refsum *et al.*, 1991).

The enhanced Hcy efflux from proliferating cells has been explained by an increased overall transmethylation rate (Iizasa and Carson, 1985) and redistribution of intracellular reduced folates (Christensen *et al.*, 1991). There is both experimental (Sirotnak, 1985) and clinical (Hoffbrand and Newscombe, 1967; Magnus, 1967; Saleh *et al.*, 1982) evidence of altered folate homeostasis in patients with leukemia or some forms of cancer, suggesting increased requirement of folate by malignant cells. In children with ALL, the plasma Hcy was correlated negatively with serum folate, which was subnormal in some patients (Refsum *et al.*, 1991).

Hcy export and rescue agents. We investigated the effect on the Hcy export from rescue agents for two reasons. First, 5-formyl-THF, 5-methyl-THF, Thd and purines have been used to modulate the MTX effect both in patients and in cell culture systems, and the 5-formyl-THF antidote is part of the high-dose MTX regimen (Ackland and Schilsky, 1987; O'Dwyer *et al.*, 1987). Secondly, the combination of Thd + Hx protects against cytotoxicity without supplying reduced folates, and is a useful tool to differentiate between altered Hcy export due to antifolate effect and inhibition of cell proliferation.

5-Methyl-THF reduced markedly Hcy export from proliferating fibroblasts (fig. 2). 5-Formyl-THF, which is probably converted to 5-methyl-THF (Matherly *et al.*, 1986), had a similar effect in these cells (data not shown). These experimental findings should be related to the observation that the increase in plasma Hcy after MTX infusion is reversed upon administration of 5-formyl-THF (Refsum *et al.*, 1986, 1991; Broxson *et al.*, 1989). An analogous observation is that p.o. folic acid, which is converted to 5-methyl-THF upon absorption (Straw *et al.*, 1984), reduces plasma Hcy even in subjects without overt folate deficiency (Brattström *et al.*, 1988).

In the lymphoma cells, supplementing reduced folates did

not reduce the Hcy export (fig. 2). Lack of effect from folates in these cells demonstrates clearly different response patterns among cell types, and addresses the important point that reduction in plasma Hcy after administration of folate may represent a response confined to some cells, not necessarily including the leukemic cells.

Hcy export from cells exposed to MTX. We observed that different cell lines exposed to MTX may have a different Hcy response. In the lymphoma cells protected with Thd + Hx, the Hcy export was not increased by MTX (fig. 3), whereas MTX enhanced markedly the Hcy export in a dose-dependent manner from stationary and proliferating fibroblasts rescued by Thd + Hx (figs. 3 and 4). Notably, in proliferating fibroblasts, half-maximal effect on export was observed at the same MTX concentration inhibiting the cell proliferation by 50% (fig. 4). These findings lead to the following conclusions. First, the increase in plasma Hcy during MTX treatment in patients (Refsum *et al.*, 1986, 1989a, 1991; Broxson *et al.*, 1989) is probably not due to a concert response of all cells. Secondly, the cellular Hcy efflux (from fibroblasts) induced by MTX is not dependent on MTX cytotoxicity, but both processes are mediated by the same or closely related metabolic derangements, most likely dihydiofolate reductase inhibition and the resulting folate redistribution.

MTX had a dual effect on Hcy efflux from responsive cells not protected by Thd + Hx. This is demonstrated by the experiments depicted in figure 4 showing that low concentrations of MTX (<0.01 μM) enhanced and higher concentrations decreased Hcy efflux from normal and malignant fibroblasts. In figure 5, the kinetics of this dual effect are characterized. The enhancement and inhibition of efflux predominated after short and prolonged exposure time, respectively. The balance between these opposing effects was dependent on the concentration of MTX, so that low MTX concentrations induced the highest response after prolonged exposure (fig. 5). The latter experiment (fig. 5) may serve as a model explaining the apparent paradox that low doses (25 mg) of MTX given to psoriasis patients (Refsum *et al.*, 1989a) induced a hyperhomocysteinemia of the same magnitude as that observed in cancer patients treated with high doses of 1 to 33 g/m^2 (Refsum *et al.*, 1986, 1991; Broxson *et al.*, 1989).

MTX at dose of 25 mg probably has an anti-inflammatory component distinct from its antiproliferative properties (Zanolli *et al.*, 1990), whereas high-dose MTX is a cytotoxic regimen.

In unprotected lymphoma cells exposed to MTX, the Hcy export was not stimulated but fell parallel to growth inhibition (fig. 4). Thus, these cells were neither responsive to folate supplementation (fig. 2) nor to MTX treatment. If there are cells *in vivo* which show a similar behavior, this may contribute to a reduction in plasma Hcy levels at high MTX doses.

Figures 4 and 5 also convey additional information on the relation between Hcy export and MTX cytotoxicity. The enhancement of Hcy export occurred at low MTX concentrations and even preceded the inhibition of cell growth. This is shown most clearly in figure 5 for Cl 8 cells. This is in line with the finding in a recent clinical study on psoriasis patients receiving low-dose MTX. It was concluded that extracellular (plasma) Hcy is a sensitive indicator of MTX pharmacodynamics (Refsum *et al.*, 1989a).

Possible mechanisms. MTX decreases the cellular content of reduced folates and, among these species, 5-methyl-THF, the methyl donor in the methionine synthase reaction, is depleted most efficiently (Allegra *et al.*, 1986; Baram *et al.*, 1987). This is the most likely mechanism behind the increased Hcy egress from the Cl 8 and Cl 16 cells after MTX exposure. The lack of Hcy response in the lymphoma cells is not clear but may be related to low Hcy remethylation rate in these cells. This is in line with the observation that inactivation of methionine synthase by nitrous oxide does not enhance Hcy export from the lymphoma cells, whereas the export from the fibroblasts is increased markedly (unpublished results).

Summary and conclusion. Hcy export from cultured cells was enhanced during proliferation, and the export from some cells was increased by MTX and decreased upon supplementing the medium with 5-formyl-THF. The export from other cells, like the MTX-sensitive lymphoma cells, was not increased during drug exposure. The MTX-dependent enhancement of Hcy export was not related to cytotoxicity or inhibition of cell growth, but probably reflects cellular depletion of 5-methyl-THF, inasmuch as it occurred in cells protected with Thd + Hx. Investigation of time course and concentration dependence suggests that increased Hcy export is an early and sensitive measure of the antifolate effect of MTX, but the increased export rate was antagonized and the export decreased when cytotoxicity and inhibition of cell growth ensued. This *in vitro* study may serve as a model to explain the variations in plasma Hcy and urinary excretion observed during various MTX regimens, including high-dose MTX infusion followed by 5-formyl-THF rescue.

Acknowledgments

The technical assistance of Gry Kvalheim, Elfrid Blomdal and Eli Gundersen is highly appreciated. We are grateful to Drs. Johan R. Lillehaug and Dennis A. Carson for providing cell lines used in this study.

References

ACKLAND, S. P. AND SCHILSKY, R. L.: High-dose methotrexate: A critical reappraisal. J. Clin. Oncol. **5**: 2017–2031, 1987.

ALLEGRA, C. J., FINE, R. L., DRAKE, J. C. AND CHABNER, B. A.: The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. Evidence for direct inhibition of purine synthesis. J. Biol. Chem. **261**: 6478–6485, 1986.

BARAM, J., ALLEGRA, C. J., FINE, R. L. AND CHABNER, B. A.: Effect of methotrexate on intracellular folate pools in purified myeloid precursor cells from normal human bone marrow. J. Clin. Invest. **79**: 692–697, 1987.

BORSA, J. AND WHITMORE, G. F.: Cell killing studies on the mode of action of methotrexate on L-cells *in vitro*. Cancer Res. **29**: 737–744, 1969.

BRATTSTRÖM, L. E., ISRAELSSON, B., JEPPSSON, J.-O. AND HULTBERG, B. L.: Folic acid—An innocuous means to reduce plasma homocysteine. Scand. J. Clin. Lab. Invest. **48**: 215–221, 1988.

BROXSON, E. H., STORK, L. C., ALLEN, R. H., STABLER, S. P. AND KOLHOUSE, J. F.: Changes in plasma methionine and total homocysteine levels in patients receiving methotrexate infusions. Cancer Res. **49**: 5858–5862, 1989.

CHRISTENSEN, B., REFSUM, H., VINTERMYR, O. AND UELAND, P. M.: Homocysteine export from cells cultured in the presence of physiological or superfluous levels of methionine: Methionine loading of non-transformed, transformed, proliferating and quiescent cells in culture. J. Cell. Physiol. **146**: 52–62, 1991.

DALEN, H. AND BURKE, H. J.: Some observations on the three dimensional growth of L5178Y cell colonies in soft agar culture. Exp. Cell Res. **65**: 433–439, 1971.

DJURHUUS, R., SVARDAL, A. M., UELAND, P. M., MALE, R. AND LILLEHAUG, J. R.: Growth support and toxicity of homocysteine and its effect on methionine metabolism in non-transformed and chemically transformed C^{3}H/10T1/2 cells. Carcinogenesis **9**: 9–16, 1988.

ERMENS, A. A. M., REFSUM, H., RUPREHT, J., SPIJKERS, L. J. M., GUTTORMSEN, A. B., LINDEMANS, J., UELAND, P. M. AND ABELS, J.: Monitoring cobalamin inactivation during nitrous oxide anesthesia by determination of homocysteine and folate in plasma and urine. Clin. Pharmacol. Ther. **49**: 385–393, 1991.

FINKELSTEIN, J. D.: Methionine metabolism in mammals. J. Nutr. Biochem. **1**: 228–237, 1990.

GOLDMAN, I. D. AND MATHERLY, L. H.: The cellular pharmacology of methotrexate. Pharmacol. Ther. **28**: 77–102, 1985.

HOFFBRAND, A. V. AND NEWSCOMBE, B. F. A.: Leucocyte folate in vitamin B12 and folate deficiency and in leukemia. Br. J. Haematol. **13**: 954–966, 1967.

HOWELL, S. B., MANSFIELD, S. J. AND TAETLE, R.: Thymidine and hypoxanthine requirements of normal and malignant human cells for protection against methotrexate cytotoxicity. Cancer Res. **41**: 945–950, 1981.

HWANG, S. M., MEADOWS, C. A. AND THENEN, S. W.: Methotrexate effects on folate status and the deoxyuridine suppression test in rats. Nutr. Res. **9:** 431–444, 1989.

IIZASA, T. AND CARSON, D. A.: Differential regulation of polyamine synthesis and transmethylation reactions in methylthioadenosine phosphorylase deficient mammalian cells. Biochim. Biophys. Acta **844:** 280–287, 1985.

KANG, S.-S., WONG, P. W. K. AND NORUSIS, M.: Homocysteinemia due to folate deficiency. Metabolism **36:** 458–462, 1987.

KREDICH, N. M., HERSHFIELD, M. S., FALLETTA, J. M., KINNEY, T. R., MITCHELL, B. AND KOLLER, C.: Effects of 2′-deoxycoformycin on homocysteine metabolism in acute lymphoblastic leukemia. Clin. Res. **29:** 541A, 1981.

KRISHNAMURTI, C. R., HEINDZE, A. M. AND GALZY, G.: Application of reversed-phase high-performance liquid chromatography using pre-column derivatization with o-phthaldialdehyde for the quantitative analysis of amino acids in adult and fetal sheep plasma, animal feeds and tissues. J. Chromatogr. **315:** 321–331, 1984.

LINDENBAUM, J., HEALTON, E. B., SAVAGE, D. G., BRUST, J. C. M., GARRETT, T. J., PODELL, E. R., MARCELL, P. D., STABLER, S. P. AND ALLEN, R. H.: Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. N. Engl. J. Med. **318:** 1720–1728, 1988.

MAGNUS, E. M.: Folate activity in serum and red cells of patients with cancer. Cancer Res. **27:** 490–497, 1967.

MATHERLY, L. H., BARLOWE, C. K. AND GOLDMAN, I. D.: Antifolate polyglutamylation and competitive drug displacement at dihydrofolate reductase as important elements in Leucovorin rescue. Cancer Res. **46:** 588–593, 1986.

MORGAN, S. L., BAGGOTT, J. E. AND ALTZ-SMITH, M.: Folate status of rheumatoid arthritis patients receiving long-term, low-dose methotrexate therapy. Arthritis Rheum. **30:** 1348–1356, 1987.

MORGAN, S. L., BAGGOTT, J. E., REFSUM, H. AND UELAND, P. M.: Homocysteine levels in rheumatoid arthritis patients treated with low-dose methotrexate. Clin. Pharmacol. Ther., submitted, 1991.

MORGAN, S., BAGGOTT, J. E., VAUGHN, W. H., YOUNG, P. K., AUSTIN, J. V., KRUMDIECK, C. L. AND ALARCON, G. S.: The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. **33:** 9–18, 1990.

MUDD, S. H., LEVY, H. L. AND SKOVBY, F.: Disorders of transsulfuration. *In* The metabolic basis for inherited diseases, ed. by C. R. Scriver, A. L. Beadet, W. S. Sly and D. Valle, pp. 693–734, McGraw-Hill, New York, 1989.

O'DWYER, P. J., KING, S. A., HOTH, D. F. AND LEYLAND-JONES, B.: Role of thymidine in biochemical modulation: A review. Cancer Res. **47:** 3911–3919, 1987.

OLD, L., BOYSE, E. AND STOCKERT, E.: The G (Gross) leukemia antigen. Cancer Res. **25:** 813–819, 1965.

RALPH, P.: Retention of lymphocyte characteristics by myelomas and q^+-lymphomas: Sensitivity to cortisol and phytohemagglutinin. J. Immunol. **110:** 1470–1475, 1973.

REFSUM, H., HELLAND, S. AND UELAND, P. M.: Fasting plasma homocysteine as a sensitive parameter to antifolate effect. A study on psoriasis patients receiving low-dose methotrexate treatment. Clin. Pharmacol. Ther. **46:** 510–520, 1989a.

REFSUM, H. AND UELAND, P. M.: Clinical significance of pharmacological modulation of homocysteine metabolism. Trends Pharmacol. Sci. **11:** 411–416, 1990.

REFSUM, H., UELAND, P. M. AND KVINNSLAND, S.: Acute and long-term effects of high-dose methotrexate treatment on homocysteine in plasma and urine. Cancer Res. **46:** 5385–5391, 1986.

REFSUM, H., UELAND, P. M. AND SVARDAL, A. M.: Fully automated fluorescence assay for determining total homocysteine in plasma. Clin. Chem. **35:** 1921–1927, 1989b.

REFSUM, H., WESENBERG, F. AND UELAND, P. M.: Plasma homocysteine in children with acute lymphoblastic leukemia. Changes during a chemotherapeutic regimen including methotrexate. Cancer Res. **51:** 828–835, 1991.

REZNIKOFF, C. A., BERTRAM, J. S., BRANKOW, D. W. AND HEIDELBERGER, C.: Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. **33:** 3239–3249, 1973a.

REZNIKOFF, C. A., BRANKOW, D. W. AND HEIDELBERGER, C.: Establishment and characterization of a clone of C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. **33:** 3231–3238, 1973b.

SALEH, A. M., PHEASANT, A., BLAIR, J. A., ALLAN, R. N., AND WALTERS, J.: Folate metabolism in man: Effect of malignant disease. Br. J. Cancer **46:** 346–353, 1982.

SIROTNAK, F. M.: Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: Biological significance and implications for improved therapy of human cancer. Cancer Res. **45:** 3992–4000, 1985.

STABLER, S. P., MARCELL, P. D., PODELL, E. R., ALLEN, R. H., SAVAGE, D. G. AND LINDENBAUM, J.: Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J. Clin. Invest. **81:** 466–474, 1988.

STRAW, J. A., SZAPARY, D. AND WYNN, W. T.: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res. **44:** 3114–3119, 1984.

SVARDAL, A. M., DJURHUUS, R., REFSUM, H. AND UELAND, P. M.: Disposition of homocysteine in rat hepatocytes and in nontransformed and malignant mouse embryo fibroblasts following exposure to inhibitors of S-adenosylhomocysteine catabolism. Cancer Res. **46:** 5095–5100, 1986a.

SVARDAL, A. M., DJURHUUS, R. AND UELAND, P. M.: Disposition of homocysteine and S-3-deazaadenosylhomocysteine in cells exposed to 3-deazaadenosine. Mol. Pharmacol. **30:** 154–158, 1986b.

UELAND, P. M. AND REFSUM, H.: Plasma homocysteine, a risk factor for vascular disease: Plasma levels in health, disease, and drug therapy. J. Lab. Clin. Med. **114:** 473–501, 1989.

UELAND, P. M., REFSUM, H., MALE, R. AND LILLEHAUG, J. R.: Disposition of endogenous homocysteine by mouse fibroblast C3H/10T1/2 Cl 8 and the chemically transformed C3H/10T1/2MCA Cl 16 cells following methotrexate exposure. J. Natl. Cancer. Inst. **77:** 283–289, 1986.

ZANOLLI, M. D., SHERRERTZ, E. F. AND HEDBERG, A. E.: Methotrexate—Anti-inflammatory or antiproliferative. J. Am. Acad. Dermatol. **22:** 523–524, 1990.

Send reprint requests to: Dr. Helga Refsum, Department of Pharmacology and Toxicology, University of Bergen, N-5021 Haukeland Hospital, Bergen, Norway