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Acute and Long-Term Effects of High-Dose Methotrexate Treatment on Homocysteine in Plasma and Urine[1]

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ABSTRACT

The amino acid, homocysteine, is not supplied by food but is a product formed by cleavage of *S*-adenosylhomocysteine; a product of transmethylation. Homocysteine is further salvaged to methionine. Since this reaction is in most tissues dependent on 5-methyltetrahydrofolate, we investigated the effect of the antifolate drug, methotrexate (MTX), on homocysteine in patients treated with this drug against cancer. Free and protein-bound homocysteine in plasma and urinary excretion of this amino acid were monitored in seven patients before, during, and after infusion with MTX (1–13.6 g). Each patient was investigated during one to five consecutive MTX treatments, which were separated by intervals of 1 to 4 weeks. Three components of the homocysteine response could be distinguished. (*a*) An acute effect appeared after a lag period of about 6 h, lasted for about 24 h, and was characterized by a transient increase in free and protein-bound homocysteine and a concomitant increase in urinary excretion of homocysteine. Some patients showed a marked plasma response, whereas in others, enhancement of urinary excretion predominated. (*b*) A long-term effect developed within 48–72 h after each infusion and was characterized by a progressive decrease in both plasma homocysteine and urinary excretion of homocysteine to amounts below those observed prior to the infusion. This effect lasted for at least 4 weeks. In this way the amount of homocysteine in plasma and urine decreased as a function of the number of MTX infusions. (*c*) This long-term effect was associated with a decrease in acute homocysteine response in plasma and/or urine. Notably, MTX induced no acute or long-term effect on plasma methionine, suggesting that the homocysteine response is not caused by an imbalance in methionine metabolism due to malignant disease or chemotherapy. The cause and possible consequences of altered homocysteine metabolism during MTX therapy are discussed.

INTRODUCTION

The antifolate drug, MTX,[3] has been widely used as an antineoplastic agent in the treatment of malignant diseases like acute lymphoblastic leukemia and several solid tumors (1). A therapeutic regimen which has been adopted during the last 10 years involves a rapid infusion of high doses of MTX, followed by leucovorin rescue (1, 2). It has been proposed that the biological effects of MTX therapy are related to the product of the drug concentration times duration of exposure (3, 4). Thus, high-dose MTX treatment may offer a clinical condition in which metabolic effects of MTX in humans can be assessed within a relatively short time period.

MTX is a tight-binding inhibitor of dihydrofolate reductase, the enzyme responsible for the regeneration of tetrahydrofolate from dihydrofolate (5). Inhibition of this enzyme induces cellular depletion of THF cofactors, including 5-methyl-THF, and thereby blocks several metabolic processes dependent on reduced folates. These include synthesis of purines, thymidylate necessary for DNA synthesis, as well as salvage of homocysteine to methionine (5).

Homocysteine is an amino acid which is not supplied by food but is a product formed from the endogeneous transmethylase inhibitor, AdoHcy. The reaction is catalyzed by the enzyme, AdoHcy hydrolase (6). Homocysteine is further metabolized either to cystathionine via the so-called transsulfuration pathway or is converted to methionine. The latter pathway is in most tissues catalyzed by an enzyme which requires 5-methyl-THF as methyl donor (7). These metabolic reactions are outlined in Fig. 1.

Cultured cells release large amounts of homocysteine into the extracellular medium and only small amounts are retained within the cells. The homocysteine egress is markedly increased following exposure to MTX, and the MTX-dependent homocysteine egress is inhibited by leucovorin (8). Based on knowledge on the metabolic relations depicted in Fig. 1, we suggested that the effect of MTX on homocysteine egress was related to inhibition of 5-methyl-THF-dependent conversion of homocysteine to methionine.

The aim of the present work was to investigate whether the effect of MTX on homocysteine observed with cultured cells had its parallel *in vivo* in humans. We therefore decided to monitor homocysteine levels in extracellular media like plasma and urine from patients undergoing high-dose MTX therapy.

MATERIALS AND METHODS

Chemicals. MTX and leucovorin were obtained from Nyco, Oslo, Norway. Sources of reagents ([^{14}C]adenosine, DL-homocysteine, dithioerythritol, AdoHcy hydrolase, 2′-deoxycoformycin) used for the homocysteine assay have been given previously (9). L-Methionine was purchased from Sigma Chemical Co., St. Louis, MO, and triethylamine and phenylisothiocyanate were from Pierce, Rockville, IL. Columns (0.46 x 10 cm) for reversed-phase liquid chromatography were packed with 3-μm octadecylsilane Hypersil (from Shandon Southern Products, Ltd., Cheshire, United Kingdom).

Patients. Seven patients were included in this study, and they all received high-dose MTX infusion as treatment against malignant disease. Their clinical characteristics are listed in Table 1. All patients gave their informed consent to participate in the study.

Protocol, Blood, and Urine Sampling. Five patients received MTX at a dose of 1 g, patient H. B. between 2 and 3.8 g, and patient K. A. L. 13.6 g. Each dose was given as a 2–4-h i.v. infusion. Twenty-four h after start of the infusion, the patients received "rescue" therapy with leucovorin (5-formyl-THF, 15 mg in 11 doses).

The MTX doses were separated by intervals of 1–4 weeks. During these intervals, some patients received chemotherapy with drugs other than MTX.

Details on the chemotherapy given to these patients are outlined in Table 2 and in Refs. 10 and 11.

Blood samples were collected by venipuncture prior to the MTX infusion and 12, 24, 48, and 72 h after start of the MTX infusion. In some cases, more frequent blood samples were drawn.

Urine was routinely collected for 24 h before start of MTX infusion, either as a pooled sample or as fractionated samples obtained by collecting each portion of voided urine. After the start of the MTX infusion the urine was collected for the following 72–96 h as fractionated samples.

Processing of Blood Samples. Blood samples were collected in cooled vacutainers containing EDTA; whole blood was cooled on ice for 3 min

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[3] The abbreviations used are: MTX, methotrexate; 5-methyl-THF, 5-methyltetrahydrofolate; AdoHcy, *S*-adenosylhomocysteine.

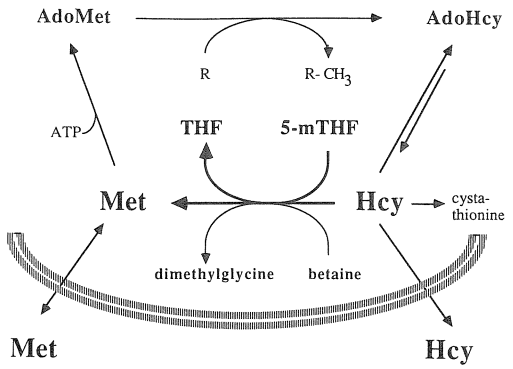

Fig. 1. Metabolism of homocysteine (*Hcy*), methionine (*Met*), and related compounds. *AdoMet*, *S*-adenosylmethionine; *THF*, tetrahydrofolate.

Table 1 *Patient characteristics and diagnosis*

Patients	Age (yr)	Sex	Wt (kg)	Diagnosis
K. A. L.	18	M	62	Ewing's sarcoma
I. S.	62	F	69	Non-Hodgkin's lymphoma
B. T.	23	F	50	Angioimmunoblastic lymphadenopathy
I. H.	15	M	37	Non-Hodgkin's lymphoma
A. L.	46	M	122	Non-Hodgkin's lymphoma
H. B.	20	M	69	Non-Hodgkin's lymphoma
I. S. V.	47	M	74	Non-Hodgkin's lymphoma

Table 2 *Methotrexate dose schedule, plasma drug concentration, and treatment with other drugs than methotrexate*

Patients	MTX treatments	Intervals between MTX dosing (wk)	Dose (mg)	MTX in plasma[a] (mM)	Chemotherapy
K. A. L.	1	1	13,600	1.84	M[b]
	2		13,600	2.90	
	3		13,600	2.80	
I. S.	1		1,000	0.75	CHOP/M[c]
B. T.	1		1,000	0.26	CHOP/M[c]
I. H.	1	1	1,000	0.42	M
	2		1,000	0.45	
	3		1,000	0.17	
A. L.	1	3	1,000	0.12	MACOP-B[d]
	2		1,000	ND	
	3		1,000	0.38	
	4		1,000	0.35	
	5		1,000	0.22	
H. B.	1	3	3,840	0.27	CHOP/M[e]
	2		2,000	0.21	
	3		2,000	0.42	
	4		2,000	0.29	
I. S. V.	2	4	1,000	0.17	MACOP-B[f]
	3		1,000	0.06	

[a] 24 h after start of MTX infusion.
[b] The designations of the regimens are based on the abbreviations: A, Adriamycin; B, bleomycin; C, cyclophosphamide; H, hydroxydaunomycin (Adriamycin); M, methotrexate with leucovorin rescue; O, Oncovin; P, prednisone. Further details on these regimens are given in Ref. 10 and 11. ND, not determined.
[c] MTX given 14 days after CHOP.
[d] MTX given 10 days after ACOP-B.
[e] MTX given 10 days after CHOP.
[f] MTX given 14 days after ACOP-B.

and then centrifuged at 1000 × *g* for 5 min at 2°C. A portion (600 μl) of the plasma was then immediately deproteinized by mixing with perchloric acid containing EDTA (final concentrations, 0.6 N and 10 mM, respectively). The precipitated protein was removed by centrifugation, and the supernatant was neutralized to pH 7 by adding 1.44 N KOH-1.2 N $KHCO_3$. Plasma and the deproteinized fraction were stored at −80°C until analysis.

Determination of Homocysteine in Plasma and Urine. Free homocysteine was determined in plasma deproteinized with perchloric acid, and total homocysteine was assayed directly in plasma. Homocysteine in urine was assayed in urine diluted with phosphate buffer. The radioenzymic method for the determination of homocysteine in plasma and urine has been described in detail previously (9).

Determination of Methionine in Plasma. Methionine was assayed in deproteinized plasma with a slight modification of the method of Bidlingmeyer *et al.* (12). Methionine and other amino acids were converted to their phenylthiocarbamyl derivatives in the presence of phenylisothiocyanate. These derivatives were separated by high-pressure liquid chromatography on an octadecylsilane Hypersil column (10 x 0.46 cm). The mobile phase was composed to optimize the separation of phenylthiocarbamylmethionine from interfering compounds. A gradient system was obtained by mixing two solutions (A and B). Solution A was 0.14 N sodium acetate, pH 6.35, containing 0.5 ml triethylamine/liter. Solution B was 30% acetonitrile in Solution A. The column was equilibrated with 20% of Solution B and 80% of Solution A and eluted with this mobile phase for 0.5 min after injection of sample. At this time point the composition was changed to 40% Solution B and 60% Solution A, and then there was a linear gradient which reached 50% Solution B at 3 min. Then the column was eluted isocratically with 50% Solution B. The flow rate was 2 ml/min. The absorbance was recorded at 260 nm, using a Spectroflow model 773 variable wavelength detector from Kratos. The retention time of phenylthiocarbamylmethionine was 7.72 min.

The recovery of methionine by this assay was evaluated by determination of methionine in plasma samples before and after addition (50 nmol/ml) of methionine. The recovery of added methionine was 97 ± 6% (SE; $n = 4$).

Determination of MTX in Plasma. MTX was assayed using an enzyme multiplied immunoassay technique (EMIT; Syva Co., Palo Alto, CA).

Definitions. The designations, phase I, II, and III, used in Table 3, refer to before, during, and after MTX infusion, respectively.

Values for homocysteine in plasma and urine obtained in the period before start of the MTX infusion are referred to as pretreatment values or phase I values.

Phase II refers to the time during or immediately after MTX infusion. Thus, homocysteine in plasma during phase II is defined as the plasma concentration determined 24 h after start of the infusion. Urinary excretion of homocysteine during treatment, *i.e.*, during phase II, is defined as the maximum excretion during a 24-h period within 48 h after start of infusion. These conventions were made on the basis of the observed lag periods before maximum homocysteine response in plasma and urine occurs.

Plasma and urinary homocysteine after treatment, *i.e.*, phase III, is defined as values determined at least 48 h after start of the infusion.

Statistical Methods. The validity of using normal distribution function and parametric tests for homocysteine in plasma from healthy volunteers has been documented previously (9). However, the number of observations during phase I ($n = 6$) and values for homocysteine excretion in urine in two cancer patients (K. A. L., I. S.) far beyond the 95% confidence interval obtained for healthy persons (9) did not justify use of parametric tests. Therefore, the statistical evaluation of the present data was based on nonparametric tests.

Friedman test (two-way nonparametric analysis of variance) was used for comparison between three groups (values for phase I, II, and III). If significant differences between groups were obtained, paired comparison were made using an ordinary Wilcoxon (matched paired sign rank) test.

Possible effect of the number of MTX infusions on homocysteine in plasma or urine (during phase I, II, or III) could be tested for when the value for the first infusion was consistently higher than all corresponding values observed during the following infusions in the same patient. The probability that this should occur by chance in one particular individual is given by the expression $1/n$ (where n = the number of infusions). According to the multiplication law, the probability that this should occur by chance in all patients could be expressed as the product of the separate probabilities.

Correlations between homocysteine in plasma and urine were tested

for in phase I, II, and III in patients receiving their first MTX infusion, using the Spearman rank correlation coefficient.

All P values were given as two-tailed, except the P values obtained by the Wilcoxon test after performing the Friedman test, which were given as one-tailed. For these paired comparisons, the final P values were corrected using the Bonferroni correction.

RESULTS

Plasma and Urinary Homocysteine before Start of MTX Therapy. The normal values for free homocysteine in plasma were 2.27 ± 0.11 nmol/ml for men and 1.95 ± 0.13 nmol/ml for women, whereas the normal values for protein-bound homocysteine in plasma were 6.51 ± 0.32 nmol/ml for men and 7.29 ± 0.65 nmol/ml for women (9). The amount of homocysteine in plasma (total, free, and protein-bound) from the cancer patients included in this study (Table 3) was not significantly different from these plasma homocysteine values obtained in healthy volunteers (9). Notably, three patients (K. A. L., I. S., and A. L.) excreted large amounts of homocysteine into the urine. In these patients, the homocysteine excretion (Table 3) was beyond the 95% confidence interval of the urinary excretion (about 6 μmol/24 h) observed in healthy subjects (9).

Acute Effects of the First MTX Dose on Homocysteine in Plasma and Urine. Homocysteine in plasma was monitored before, during, and after the MTX infusion, according to the schedule described in "Materials and Methods." After a lag period of 4–6 h, plasma homocysteine increased progressively (20–100%) and reached a maximum at 24 h, as shown for patients K. A. L. (Fig. 2) and H. B. (Fig. 3). This plasma value is therefore referred to as plasma homocysteine during treatment (phase II). The patients received "rescue" therapy with leucovorin at this time point, and homocysteine in plasma gradually declined for the following 24–72 h and often reached concentrations below the pretreatment values. Data for all patients are summarized in Table 3.

Homocysteine was determined in the fractionated urine samples from the patients before, during, and after treatment. As shown for patients K. A. L. and H. B. in Figs. 2 and 3, urinary excretion of homocysteine showed diurnal variations, and larger amounts were excreted during the day than during the night. All patients showed a enhanced excretion between 6 and 36 h after MTX infusion. Data for all patients are summarized in Table 3.

Long-Term Effects. The effect of MTX on homocysteine in plasma and urine was investigated in most patients during 3–5 consecutive MTX infusions, as described in the preceding paragraph. The treatments were separated by intervals of 7 days to 4 weeks (Table 2). In most patients, both the amount of homocysteine in plasma and urinary excretion of homocysteine prior to (phase I) and during MTX infusion (phase II) decreased as a function of the number of MTX infusions. After 2–5 doses, essentially no homocysteine response was observed in some patients (K. A. L., A. L., H. B.). Thus, a state gradually developed which was characterized by low plasma homocysteine, reduced excretion of homocysteine into urine, and small acute effect of MTX on these parameters. This was observed irrespectively whether the homocysteine response involved an increase in plasma homocysteine or homocysteine excretion or both (Figs. 2 and 3; Table 3). Notably, this state was developed within 1–2 treatments corresponding to about 2 weeks in a patient (K. A. L.) receiving high MTX doses, whereas MTX treatment must be repeated at least 4 times in patients (A. L., H. B.) receiving lower doses (Table 2 and 3).

Relation between Free and Protein-bound Homocysteine in Plasma and Homocysteine in Urine. Free, total, and protein-bound (*i.e.*, total minus free) homocysteine were routinely determined in plasma from patients under study. Alterations in total homocysteine during MTX exposure reflect almost parallel changes in both free and protein-bound homocysteine. The ratio between free and bound was about 0.4 (Figs. 2 and 3; Table 3), as previously demonstrated for plasma from healthy

Table 3 *Homocysteine and methionine in plasma and urinary excretion of homocysteine in patients treated with high-dose methotrexate*

Patients	MTX treatment	Free Hcy[a] in plasma[b]			Bound Hcy in plasma[b]			Total Hcy in plasma[b]			Met in plasma[b]			Urinary excretion of Hcy[c]		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
K. A. L.	1	1.91	2.84	1.15	4.57	10.0	4.79	6.48	12.9	5.95	26.7	29.2	29.2	23.5	52.5	16.5
	2	1.30	1.51	1.21	4.10	4.30	3.53	5.40	5.81	4.74	37.8	38.9	30.4	14.1	22.9	16.5
	3	1.23	1.21	1.29	2.98	3.49	3.68	4.21	4.70	4.97	16.1	30.4	30.3	ND	15.4	11.0
I. S.	1	4.42	4.84	2.25	11.3	13.9	9.44	14.8	18.7	11.7	28.5	22.3	22.8	23.9	46.3	9.7
B. T.	1	1.84	2.32	1.06	3.28	6.39	3.47	5.12	8.71	5.53	32.9	25.9	40.7	5.1	9.3	7.1
I. H.	1	2.18	4.23	1.77	5.38	11.2	4.72	7.56	15.4	6.49	56.8	49.0	56.0	4.8	5.7	2.3
	2	ND	1.99	1.28	ND	7.74	4.20	ND	9.73	5.48	ND	28.8	48.9	2.9	5.1	4.0
	3	1.47	1.75	ND	3.51	6.25	ND	4.98	8.00	ND	34.8	43.5	ND	ND	5.1	4.0
A. L.	1	3.05	3.08	1.99	4.99	7.42	4.55	8.04	10.5	6.54	31.6	18.0	31.8	16.3	25.8	14.8
	2	2.24	3.26	2.04	6.09	8.20	6.29	8.33	11.46	8.33	16.5	25.6	13.7	15.9	25.5	ND
	3	1.52	2.13	1.72	4.81	8.76	5.30	6.33	10.9	7.02	17.9	29.8	31.1	14.1	19.9	ND
	4	1.43	1.55	1.36	5.60	7.00	5.20	7.03	8.55	6.56	20.3	28.7	21.2	8.8	12.1	ND
	5	1.60	2.24	1.43	5.25	7.10	4.75	6.85	9.34	6.18	37.0	32.7	33.5	8.2	10.6	7.5
H. B.	1	2.26	4.15	1.71	8.27	15.7	4.83	10.5	19.8	6.54	22.0	23.9	33.8	8.0	18.4	11.4
	2	1.01	1.80	1.08	2.35	6.68	3.14	3.36	8.48	4.22	26.2	64.5	20.9	5.8	11.3	ND
	3	1.47	1.80	1.31	4.94	6.19	3.22	6.41	7.99	4.53	37.6	30.4	47.5	ND	7.35	ND
	4	1.32	1.70	1.11	3.29	4.67	3.52	4.61	6.37	4.63	29.4	53.5	28.9	ND	6.05	ND
I. S. V.	2	1.51	2.59	ND	4.25	6.31	ND	5.76	8.90	ND	25.7	49.0	ND	4.4	8.7	ND
	3	1.60	1.70	1.23	3.96	5.75	3.26	5.56	7.45	4.49	54.5	22.9	ND	6.9	8.1	ND

[a] Hcy, homocysteine; Met., methionine; ND, not determined.
[b] The amounts of free, protein-bound, total Hcy and Met in plasma are given as nmol/ml plasma.
[c] The urinary excretion of Hcy is determined as the average excretion during a 24-h period and is given as μmol Hcy excreted per 24 h. I, phase I, prior to the MTX infusion; II, phase II, during and immediately after the MTX infusion; III, phase III, after the MTX infusion. Details are given under "Definitions" in "Materials and Methods."

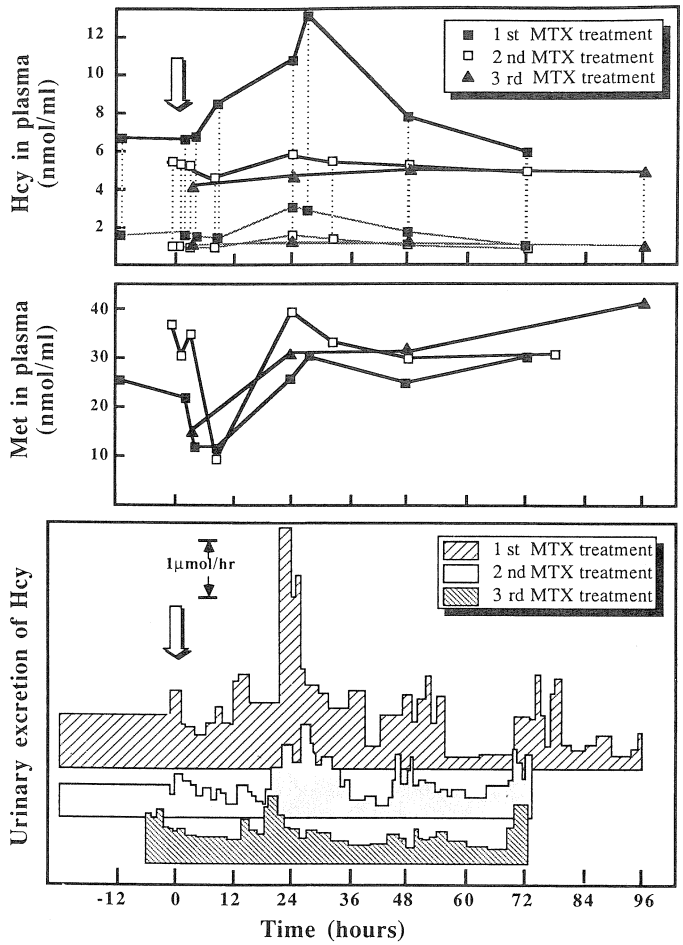

Fig. 2. Homocysteine and methionine in plasma and urinary excretion of homocysteine in a patient (K. A. L.) during three consecutive MTX infusions. *Arrows*, time of start of MTX infusion. *Top*, total amount (——) and free (· · · · ·) of homocysteine (*Hcy*) in plasma. The height of the *vertical dotted lines* connecting each pair of observations indicates the amount of protein-bound homocysteine. *Middle*, amount of methionine (*Met*) in plasma. *Bottom*, urinary excretion of homocysteine. Patient characteristics and drug schedule are listed in Tables 1 and 2.

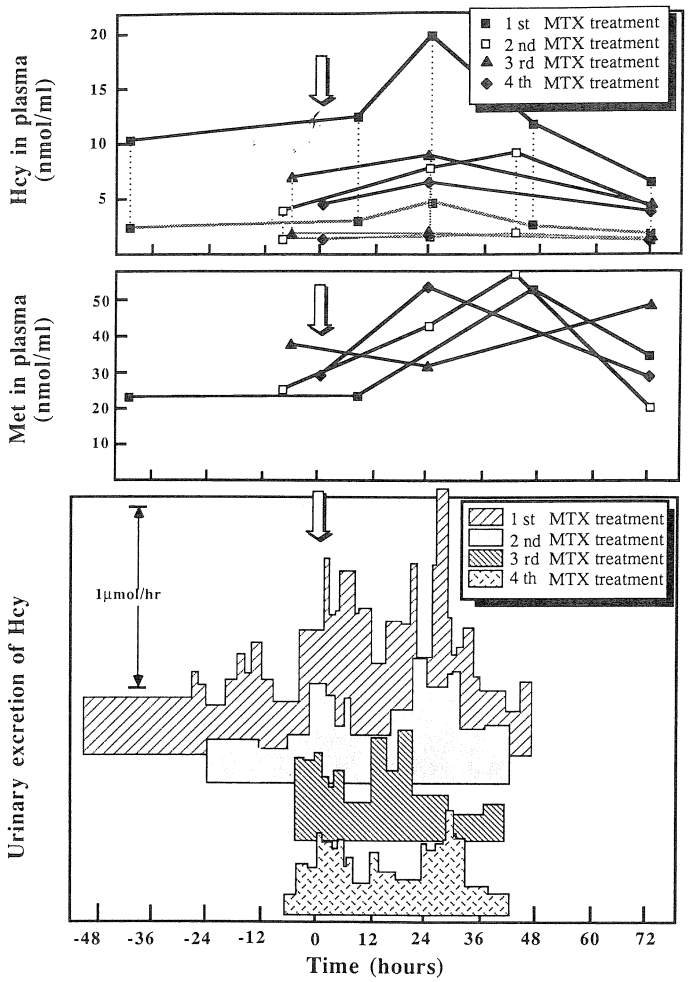

Fig. 3. Homocysteine (*Hcy*) and methionine (*Met*) in plasma and urinary excretion of homocysteine in a patient (H. B.) during four consecutive MTX infusions. *Arrows*, time of start of MTX infusion. For further details, see legend to Fig. 2.

volunteers (9). Notably, when analyzing data from all patients, there was only a weak, but not significant, positive correlation between homocysteine in plasma and the amount excreted into the urine (r 0.31–0.54).

Methionine in Plasma. Methionine concentration in plasma from patients treated with MTX was within the range (10.1–56.8 nmol/ml) reported for plasma from healthy volunteers (13). Notably, methionine in plasma did not show any acute increase following MTX infusion, and there was no progressive decrease in plasma methionine following each MTX dosing (Figs. 2 to 3; Table 3).

For 7 MTX treatments in 5 different patients (K. A. L., I. S., B. T., A. L., H. B.), blood samples were obtained within 12 h after start of infusion. In patient K. A. L. there was a transient drop in plasma methionine during this time period (Fig. 2). In other patients, plasma methionine showed only a slight decrease (from 32.9 to 21.7 at 3.5 h in patient B. T.), no alteration (patients H. B., I. S.) or an increase (patients H. B., A. L.) in this time period.

Statistical Evaluation of Data. Values for free homocysteine in plasma during phases I, II, and III were significantly different both when comparing these values during the first infusion ($n = 6$, $P < 0.005$) and when values from all treatments where analyzed together ($n = 16$, $P < 0.001$). Free homocysteine during phase II was significantly higher than during phase I ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$), the values for phase II were higher than the values for phase III ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$) and the values for phase I were higher than for phase III ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.005$).

Protein-bound homocysteine during the three phases was significantly different ($n = 6$, $P < 0.01$; $n = 16$, $P < 0.001$). The values were higher during phase II compared with phase I ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$) or phase III ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$), but protein-bound homocysteine was not statistically different between phases I and III ($P > 0.10$).

Total homocysteine in plasma during the three phases was also significantly different ($n = 6$, $P < 0.005$; $n = 16$, $P < 0.001$). The values were higher during phase II compared with phase I ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$) or phase III ($n = 6$, $P < 0.05$; $n = 16$, $P < 0.001$), but total homocysteine was not statistically different between phases I and III ($P > 0.10$).

Urinary excretion of homocysteine was also significantly different between the three phases (I, II, and III), both when only the first MTX infusions were analyzed ($n = 6$, $P < 0.01$), and when three additional treatments (in which excretion were determined in all three phases) were included ($n = 9$, $P < 0.005$). Urinary excretion was higher during phase II than during phase I ($n = 6$, $P < 0.05$; $n = 9$, $P < 0.01$) and phase III ($n = 6$, $P < 0.05$; $n = 9$, $P < 0.01$), but no difference was found between phases I and III ($P > 0.10$).

Urinary excretion of homocysteine was significantly higher during the first MTX infusion compared with the following treatments during both phase I ($P = 0.05$) and phase II ($P < 0.02$).

A similar trend exists for the amount of homocysteine in plasma, *i.e.*, a decrease in plasma homocysteine in phase I or II after the first MTX infusion, but this could not be verified by statistical analysis, probably because there was no alteration of homocysteine in plasma in the patient (A. L.) receiving MTX doses of only 1 g.

DISCUSSION

Site of Production and Disposition of Homocysteine. Homocysteine is a product of transmethylation and if formed from AdoHcy through the action of AdoHcy hydrolase, which is widely distributed in mammalian tissues (6). Once formed, homocysteine may be either further metabolized (Fig. 1) or exported into the extracellular medium. Pronounced homocysteine egress has been demonstrated with isolated hepatocytes (14) and various cells in culture (8, 15, 16). The presence of significant amounts of homocysteine in extracellular media like plasma and urine (9) and low homocysteine concentrations in whole tissues (14, 17) suggests that homocysteine efflux may operate *in vivo*. The amount of homocysteine excreted into the urine of normal individuals (9) and MTX-treated patients (Table 3) is low relative to the total flux through homocysteine in humans (18). This does not imply that homocysteine egress is not quantitatively important in some cells since the utilization of methyl groups and thereby the total homocysteine formation is mainly accounted for by the creatinine synthesis in liver (7). Besides, extracellular homocysteine can be taken up and metabolized by cells (19). Thus, the homocysteine egress from some cells into the extracellular compartment may be important for the maintenance of intracellular homocysteine within a certain range and may therefore be a measure of the balance between homocysteine formation and utilization.

Effect of MTX on Homocysteine in Cultured Cells and in Patients. From the metabolic relations depicted in Fig. 1, one would expect that MTX inhibits the salvage of homocysteine to methionine. This possibility has been investigated in cultured fibroblasts which export large amounts of homocysteine in the presence of MTX (8). The homocysteine egress seems to prevent accumulation of intracellular homocysteine under these conditions.

The present paper is the first report showing that MTX affects the disposition of homocysteine in patients. The clinical findings show some similarities with the observations made with cultured cells. Both the homocysteine egress from cultured cells (8) and the increase in plasma homocysteine (Figs. 2 and 3) take place over a period of days, outlast the MTX exposure, and are reversed following administration of leucovorin.

Some features of the homocysteine response in patients have not been demonstrated in cultured cells. These are the long-term effects characterized by a progressive decrease in homocysteine in plasma and/or urine prior to (phase I) and during (phase II) MTX infusion as a function of the number of MTX treatments.

Possible Mechanisms. The transient increase in homocysteine in plasma and urine following the first MTX infusions (Figs. 2 and 3; Table 3) is probably related to cellular depletion of 5-methyl-THF required for the conversion of homocysteine to methionine. Under these conditions, homocysteine may be formed in amounts exceeding the capacity of the homocysteine-consuming pathways (Fig. 1). Thus, homocysteine egress may reflect and be a measure of lack of intracellular reduced folates, including 5-methyl-THF, relative to the metabolic demand. This explanation is supported by the finding that the homocysteine response could be reversed in cells by addition of 5-formyl-THF (8) and the homocysteine content in plasma and urine declines following administration of 5-formyl-THF to patients (Figs. 2 and 3; Table 3). Notably, in cultured cells the MTX-dependent homocysteine egress was demonstrated under conditions where MTX exerted essentially no cytotoxic effect, *i.e.*, in confluent cells and in the presence of "rescue" agents like thymidine and hypoxanthine (8). This indicates that stimulation of homocysteine egress is not intimately associated with MTX cytotoxicity.

Data on the effect of MTX on 5-methyl-THF are scanty. A moderate decrease in 5-methyl-THF in the liver has been demonstrated in rats during chronic MTX exposure (20). More interestingly, Kesavan *et al.* (21) have recently found that low doses of MTX induced a rapid and pronounced depletion of 5-methyl-THF in tumor cells grown either i.p. or in culture. Reduction in pools of other reduced folates was not observed under these conditions and required higher doses of MTX (21, 22). The lability of the 5-methyl-THF pool during MTX exposure may explain the rapid homocysteine response in cells (8) and in patients treated with this drug and points to homocysteine in extracellular media as marker for antifolate effect.

The long-term effect of MTX on homocysteine metabolism in patients may represent an adaptive mechanism. The ability of MTX to reduce the amount of 5-methyl-THF required for homocysteine metabolism may be gradually lost. Alternatively, prolonged inhibition of the 5-methyl-THF-dependent salvage of homocysteine to methionine may induce the capacity of alternate metabolic routes for homocysteine, as for example the betaine-dependent conversion to methionine in liver and kidney or the transsulfuration pathway (7). This, in turn, may have a sparing effect on the utilization of reduced folates. Induction of the former pathway has been demonstrated in rat fed a protein-deficient diet (23).

It was conceivable that the decrease in homocysteine in plasma and urine results from alteration in methionine metabolism due to the chemotherapy or the malignant disease. This possibility was supported by observation with isolated liver cells. The homocysteine egress from these cells depends on the metabolic flux through the AdoHcy hydrolase pathway, which in turn depends of the availability of exogenous methionine (14). However, no consistent alteration in plasma methionine in patients treated with MTX (Table 3) suggests that the decrease in plasma and urinary homocysteine is not related to negative methionine balance.

Implications and Perspectives. In patients with genetic defects causing interference of 5-methyl-THF-dependent methylation of homocysteine, the urinary homocysteine excretion and the homocystinemia (24–28) are generally higher than in our patients treated with MTX (Table 2). This difference in urinary and plasma homocysteine in human genetic disorders *versus* patients treated with MTX does not imply that these effects of MTX reflect only slight alterations without biological significance. The reasons for this are as follows. (*a*) In genetic diseases, the enzymatic defects are present in all cell types and tissues whereas pronounced perturbation of homocysteine metabolism following MTX treatment is probably confined to MTX-sensitive cells. This possibility is supported by data with cultured cells, showing that induction of homocysteine egress predominates in MTX-sensitive cells (8). Besides, in the intact organism,

reuptake and metabolism of homocysteine by cells not sensitive to the acute effect of MTX, like the hepatocytes, may occur. (*b*) High-dose MTX treatment causes cellular depletion of reduced folates in sensitive cells, lasting for days or weeks. This contrasts to the presence of a permanent metabolic defect in genetic disorders.

It is conceivable that the transient increase in homocysteine in extracellular medium like plasma and in urine may be a measure of the ability of MTX to induce depletion of reduced folates in sensitive cells and tissues. The relation between this acute response and the burden of MTX-sensitive tumor cells should be further investigated. Such an investigation should be stimulated by the finding of high urinary excretion of homocysteine before and during MTX treatment in three cancer patients [K. A. L., I. S., and A. L. (Table 3)] included in this study and some children with acute leukemia.[4]

The observation that MTX affects homocysteine metabolism in humans points to the possibility that this may be responsible for some effects or side effects of MTX. Based on data from experiments with rats, it has been suggested that the hepatotoxic effect of MTX (29, 30) may be related to the inhibition of 5-methyl-THF-dependent conversion of homocysteine to methionine (31). The present paper supports this possibility by demonstrating the effect of clinical use of this drug on homocysteine metabolism. Prolonged enhancement of betaine-dependent synthesis of methionine in liver, a hypothetical cause of reduced amount of homocysteine in plasma and urine, may induce hepatic depletion of the lipotropic agent, choline, which in turn may be responsible for fatty metamorphosis in the liver (30, 31). Possible alterations in homocysteine metabolism in patients receiving other MTX regimens, like the chronic low dose used in the management of psoriasis, should be investigated, because these patients are at high risk of liver damage (32, 33).

The present report adds a cautionary note to the use of MTX in combination with other drugs interfering with homocysteine metabolism. The possible hazard of the drug combination MTX plus nucleoside analogues inhibiting AdoHcy hydrolase, like the antiviral drug 9-β-D-arabinofuranosyladenine, was first mentioned by Cantoni *et al.* (34). They suggested, on theoretical grounds, that "rescue" therapy with 5-formyl-THF would fail if homocysteine synthesis is blocked, because of lack of the methyl acceptor in the 5-methyl-THF homocysteine methyltransferase reaction. The anesthetic agent, nitrous oxide, irreversibly oxidizes vitamin B_{12} and thereby inhibits the cobalamin-dependent enzyme, 5-methyl-THF homocysteine methyltransferase. Therefore, interaction between MTX and nitrous oxide should also be considered (35).

Several questions arise from the finding that MTX affects homocysteine metabolism in humans. For example, to what extent can untoward MTX effects, like hepatotoxicity, be prevented or enhanced by compounds affecting homocysteine metabolism, as for example methionine or adenosine (36)? Furthermore, will altered flux through the homocysteine-metabolizing pathways (37) affect cell kill induced by MTX?

Conclusion. High-dose MTX treatment induced acute and long-term effects on the disposition of homocysteine in cancer patients. The acute increase in plasma and urine homocysteine is probably related to cellular depletion of reduced folates and may be a measure of the ability of MTX therapy to affect folate metabolism. The chronic effect, characterized by a low concentration of homocysteine in plasma and urine, clearly shows that this drug has a profound effect on homocysteine metabolism, which greatly outlasts the short infusion period. Altered homocysteine metabolism observed following clinical use of MTX should stimulate investigation of the possible exploitation of homocysteine in cancer chemotherapy.

[4] H. Refsum *et al.*, unpublished results.

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