Folate, Homocysteine, and Cobalamin Status in Patients with Rheumatoid Arthritis Treated with Methotrexate, and the Effect of Low Dose Folic Acid Supplement

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ABSTRACT. ***Objective.*** To investigate the effect of methotrexate (MTX) treatment of rheumatoid arthritis (RA) on folate metabolism, and to determine the effect of low dose folic acid on toxicity, efficacy, and folate status.

Methods. A 52-week prospective study of 81 patients with RA treated with MTX and self-administered low dose folic acid; 38 patients were included prior to MTX therapy, 33 patients continued established MTX therapy, and 10 patients were excluded. Drug efficacy and side effects were monitored with biochemical and clinical indicators.

Results. MTX treatment resulted in decreased concentrations of red blood cell (RBC) folate and a rise in plasma homocysteine. Intracellular concentrations of MTX were inversely correlated to RBC folate levels after treatment for a longer period (mean 41 months). Supplement with low dose folic acid prevented or diminished the influence of MTX on folate status and had a protective effect on MTX induced liver toxicity without changing the efficacy of MTX.

Conclusion. MTX interferes with folate and homocysteine metabolism, and the intracellular concentration of MTX may play a role. Our results indicate low dose folic acid supplementation has a beneficial effect on MTX toxicity. (J Rheumatol 2004;31:2374–81)

Key Indexing Terms:
RHEUMATOID ARTHRITIS METHOTREXATE ERYTHROCYTE FOLIC ACID

In treatment of rheumatoid arthritis (RA), low dose methotrexate (MTX) is frequently the drug of choice among disease modifying antirheumatic drugs, and the efficacy of MTX is well established[1-3]. MTX is also used for neoplastic diseases in high doses, and is known to inhibit the enzymes dihydrofolate reductase and thymidylate synthetase necessary for DNA synthesis. The mechanism of action in low doses for RA is a matter of controversy and various actions have been proposed[4-6]. It is clear, however, that MTX is an antifolate drug and that MTX accumulates intracellularly in erythrocytes during the first 6–8 weeks of treatment. The main obstacle for MTX therapy is the development of toxic side effects or lack of efficacy, which is experienced by almost one-third of the patients. These side effects have been correlated to folate deficiency, and folic acid supplementation for MTX toxicity has been widely investigated[7–13]. It is generally accepted that supplementation with folic acid decreases MTX toxicity without altering efficacy, but the appropriate dose of such folic acid supplementation is currently not well established[7,8].

The purpose of this open prospective study was to investigate short and longterm effects of MTX treatment in RA on folate status. MTX uptake, red blood cell (RBC) folate, plasma concentrations of homocysteine and cobalamins, and the effect of low dose folic acid supplement were investigated in patients starting MTX therapy and compared to folate status in RA patients treated for a longer period. Treatment efficacy and toxicity were evaluated clinically and biochemically in both groups.

MATERIALS AND METHODS

A total of 81 patients fulfilling the 1987 American College of Rheumatology (ACR) revised criteria for RA[14] were studied. Mean followup period was 52 weeks (range 27–80 weeks). Inclusion criterion was treatment with MTX; 38 patients were included prior to initiation of MTX therapy (Group 1) after at least 4 weeks of washout if the patient had received other disease modifying antirheumatic drugs. All patients were seen by a rheumatologist who was responsible for their treatment independent of this study. The initial weekly dose was 5 mg in 4 patients, 7.5 mg in 33 patients, and 10 mg in one patient. Thirty-three patients had been treated with MTX for 41 ± 25 months (mean ± SD) at the time of inclusion (Group 2). Folic acid supplement was taken by 13 patients in Group 1 (mean daily intake: 7 patients 200 µg, 4 patients 400 µg, 2 patients > 400 µg) and by 14 patients in Group 2 (mean daily intake: 8 patients 200 µg, 5

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patients 400 µg, one patient > 400 µg). In both groups, folic acid 200 µg and 400 µg was self-administered as a vitamin pill also containing other vitamin supplements including cobalamin, whereas supplement with higher doses of folic acid was prescribed by a rheumatologist independently of this study.

Patient questionnaires were administered at Weeks 0, 1, 2, 4, 6, 8, 12, 16, 20, 24, 28, and 52 regarding morning stiffness (< 30 min, 30–60 min, 60–120 min, > 120 min), number of swollen joints (maximum 40 joints), number of tender joints (maximum 42 joints), patient's global assessment of disease activity using a numeric rating scale (NRS) from 1 (no disease activity) to 10 (highest possible disease activity), patient's score of pain using NRS from 1 (no pain) to 10 (worst possible pain), and modified Health Assessment Questionnaire (HAQ score)[15].

Clinical evaluation was performed by the same physician at Weeks 0, 12–16, 28, and 52 and consisted of number of swollen joints (maximum 38 joints), number of tender joints (maximum 40 joints), global assessment of patient's general condition from 0 (very good) to 4 (very poor), and duration of morning stiffness (minutes). MTX response was evaluated according to preliminary ACR core criteria[16] and the 3 response categories were none, 20%, or 50%.

Toxicity was assessed by patient questionnaires regarding the presence of gastrointestinal, mucocutaneous, cerebral, pulmonary, and urogenital symptoms. Laboratory assessment was performed as described below, and physician's evaluation was performed at Weeks 0, 12–16, 28, and 52.

Laboratory assessment at Weeks 0, 1, 2, 4, 6, 8, 12, 16, 20, 24, 28, and 52 included measurement of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), complete blood cell count, fraction of neutrophils, platelet count, and serum analyses of alanine aminotransferase (ALT, reference limits 10–40 U/l), alkaline phosphatase, and creatinine. Analyses of these variables were by routine methods. Folate status was evaluated by measurement of the following indicators at Weeks 0, 12, 28, and 52: RBC folate (reference limit > 350 nmol/l) and plasma cobalamins (reference limits 200–600 pmol/l) determined on the ACS:Centaur™ Automated Chemiluminescence System (Bayer, Tarrytown, NY, USA) by a competitive protein binding assay; and homocysteine levels determined by an immunologic method using an IMx instrument (Abbott, Wiesbaden, Germany) (upper reference limits 7.9 µmol/l for women and 11.2 µmol/l for men < age 60 yrs, and 11.9 µmol/l for subjects aged > 60 yrs).

Erythrocyte MTX analysis was performed by a radiochemical ligand-binding assay[17]. Briefly, erythrocyte concentrate was lysed with phosphate buffer and the hemoglobin (Hb; mmol/l) determined photometrically (wavelength 540 nm). The samples were boiled for 10 min, centrifuged at 3000 rpm for 10 min, and the clear supernatants were stored at –80°C until tested for MTX content. This was performed with bovine dihydrofolate reductase as binder, [^{3}H]MTX (Moravek Biochemicals, Brea, CA, USA) as tracer, and NADPH tetrasodium salt as cofactor. Unlabeled MTX (Bie & Berntsen, Roedovre, Denmark) diluted with phosphate buffer was prepared in concentrations from 2 to 12 nmol/l and used as standards. The reaction was performed in reaction buffer (phosphate buffer and NADPH tetrasodium salt) and terminated with ice-cold (4°C) dextran coated charcoal (Sigma). This was followed by centrifugation at 2600 rpm for 15 min to pellet the charcoal, and radioactivity in the supernatants was counted in a liquid scintillation beta counter. The sensitivity of the assay was < 1 nmol/l and coefficient of variation of the assay was < 20%. Steady-state erythrocyte MTX was calculated as the mean level from Week 6 unless erythrocyte MTX increased even further, in which case steady-state erythrocyte MTX was calculated from Week 8.

Statistical analysis was by paired (or unpaired when appropriate) Student t-test, and Wilcoxon matched-pairs signed-ranks test if data were not normally distributed. Correlation of data was by linear regression analysis. Comparison of frequencies was by chi-square analysis. Differences were considered significant if p values were < 0.05 and all statistics tests were performed using GraphPad Prism software.

RESULTS

Eighty-one patients were included in the study and 10 were excluded because they proved not to meet the inclusion criteria (additional disease, death, or other) or withdrew for personal reasons. There were no differences in the demographic data between Group 1 and Group 2, as shown in Table 1.

Disease activity and the effect of MTX. Disease characteristics of the 2 groups at the start of the study are shown in Table 1. As expected, a number of the variables are significantly different in the 2 groups, showing higher disease activity in Group 1.

Seventeen patients (68%) in Group 1 without folic acid supplement responded to MTX therapy, compared to 8 patients (62%) in the group of patients supplemented with folic acid. There was no significant difference between the groups in any variables measured for disease activity ($p > 0.05$; Table 2).

Toxicity. The presence of side effects in both groups is shown in Table 3. Gastrointestinal or mucocutaneous side effects (nausea, mouth ulcers, stomatitis, diarrhea) were reported most frequently. Other side effects reported were rashes, cough, flu-like symptoms, and itch. There was no significant difference in the frequency of clinical side effects in patients with or without folic acid supplementation (Table 3).

Three patients in Group 1 without folic acid supplements discontinued MTX therapy because of clinical or laboratory signs of MTX toxicity (leukopenia, consistent ALT increase, alopecia) and the MTX dose was reduced because of ALT increase (2-fold higher than the upper reference limit) in 3 patients. Only one patient had a dose reduced because of liver toxicity in the group supplemented with folic acid; this difference did not reach the level of statistical significance. However, a small but significant increase in ALT was observed after MTX therapy for 15–18 weeks in the subgroup of patients not supplemented with folic acid, which was less pronounced and not significant until Week 52 in the group of patients receiving folic acid (Table 4).

Intracellular concentration of MTX and folate. The concentration of MTX measured in hemolysed erythrocytes (erythrocyte MTX) increased during the first 6 to 8 weeks of therapy and reached a steady-state level in most patients at this time. Intracellular concentrations of MTX measured in 33 patients starting MTX therapy with 7.5 mg weekly are illustrated in Figure 1. Data are not shown if the dose was either increased or diminished, in which case the curve does not continue until Week 28. It is clear that the intracellular levels of MTX vary considerably among patients treated with the same dose.

The levels of RBC folate were significantly higher in Group 1 at study start compared to Group 2 (mean ± SD, 740 ± 386 vs 552 ± 281 nmol/l; Figure 2). After MTX treatment

Table 1. Demographic data and baseline characteristics of the 2 study groups. Values are mean (SD) unless otherwise indicated.

	Group 1 n = 38	Group 2 n = 33	
Demographic data			
MTX therapy, yrs	0	3.5 (2.1)	
Age, yrs	53.6 (13.4)	57.7 (10.7)	NS
Disease duration, yrs	11.0 (10.1)	12.8 (8.3)	NS
Sex, % female	68	75	NS
Concomitant treatment, % of subjects			
Corticosteroids	39	33	NS
Mild analgesics (NSAID, acetaminophen)	78	71	NS
Opioid analgesics	24	30	NS
Physician assessment			
No. swollen joints (max. 38)	7.2 (6.7)	1.7 (2.4)	$p < 0.001$
No. tender joints (max. 40)	10.9 (9.9)	4.5 (4.6)	$p < 0.001$
Duration of morning stiffness, min	63 (55)	74 (240)	NS
Global score (NRS 0–4)	1.97 (0.99)	1.28 (0.76)	$p < 0.001$
Disease activity (NRS 1–10)	5.4 (1.9)	3.5 (1.9)	$p < 0.001$
Patient assessment			
Pain (NRS 1–10)	4.2 (2.5)	3.5 (2.1)	NS
Disease activity (NRS 1–10)	4.6 (2.4)	3.3 (2.3)	$p < 0.05$
HAQ	0.87 (0.61)	0.73 (0.51)	NS
Laboratory assessment			
CRP, nmol/l	266 (216)	175 (137.9)	$p < 0.05$
ESR, mm/h	32 (23)	27.1 (18.5)	NS

Table 2. Disease activity variables before and after starting MTX therapy in patients supplemented or not supplemented with folic acid. Values are mean (SD) unless otherwise indicated.

	MTX – Folic Acid, n = 25 Weeks Therapy				MTX + Folic Acid, n = 13 Weeks Therapy			
	0	16	28	58	0	16	28	58
Physician assessment								
Global assessment (0–4)	2.12 (1.03)	1.26 (0.92)	1.74 (0.55)	1.07 (0.85)	2.00 (0.97)	1.28 (0.88)	1.70 (0.69)	1.10 (0.91)
Disease activity (NRS 1–10)	5.2 (2.2)	3.2 (2.6)	3.9 (2.1)	3.3 (2.4)	5.3 (2.0)	3.2 (2.4)	3.8 (2.1)	3.4 (1.9)
No. of tender joints (max. 40)	10.2 (9.5)	7.3 (9.2)	10.2 (10.5)	7.7 (9.5)	10.5 (8.7)	7.2 (7.9)	9.2 (9.3)	6.4 (7.1)
No. of swollen joints (max. 38)	6.2 (6.5)	4.3 (4.4)	5.5 (5.2)	4.9 (6.7)	6.8 (6.2)	4.1 (4.2)	4.5 (4.4)	3.5 (5.1)
Patient assessment								
Morning stiffness, min	77 (60)	51 (53)	64 (48)	44 (55)	65 (56)	47 (47)	52 (46)	37 (46)
Pain (NRS 1–10)	3.8 (2.3)	3.3 (2.3)	3.1 (2.1)	2.7 (2.1)	4.1 (2.4)	3.3 (2.0)	3.2 (2.0)	3.2 (2.0)
Disease activity (NRS 1–10)	4.5 (2.1)	3.4 (2.4)	3.6 (2.3)	2.8 (1.7)	4.6 (2.3)	3.3 (2.1)	3.6 (2.1)	3.2 (1.6)
HAQ	0.80 (0.45)	0.68 (0.48)	0.63 (0.46)	0.50 (0.43)	0.87 (0.58)	0.75 (0.56)	0.72 (0.54)	0.61 (0.48)
Laboratory assessment								
ESR, mm/h	28 (21)	27 (24)	23 (23)	21 (19)	31 (21)	27 (21)	25 (23)	25 (22)
CRP, nmol/l	258 (220)	246 (307)	196 (227)	157 (172)	272 (213)	251 (300)	202 (242)	151 (148)

for 12–19 weeks the RBC folate levels declined to levels similar to those of Group 2 unless the patients also received folic acid supplements. In this case the RBC folate levels remained stable during the study period (Figures 3A, 3B).

Correlation between steady-state erythrocyte MTX and RBC folate in patients treated with MTX for up to 28 weeks is illustrated in Figure 4A. All patients who reached a steady-state erythrocyte MTX level taking any MTX dose within 28 weeks are included in these data. There was no correlation between erythrocyte MTX and RBC folate levels in Group 1, whereas a significant inverse correlation was observed in patients in Group 2 ($r^2 = 0.36$, $p < 0.05$; Figure 4B). There was no significant difference in mean erythrocyte MTX levels in patients supplemented and those not supplemented with low dose folic acid (mean ± SD, 29.0 ± 12.8 vs 32.6 ± 17.7 nmol/l) or in the weekly MTX dose (12.4 ± 3.6 mg vs 10.5 ± 3.6 mg).

Plasma homocysteine and cobalamins. Plasma homocys-

Table 3. Number of patients reporting symptoms of MTX toxicity. There was no significant difference between the 2 groups or between patients supplemented or not supplemented with folic acid.

	Presence of Clinical Side Effects			
	Group 1		Group 2	
	+ Folic Acid	– Folic Acid	+ Folic Acid	– Folic Acid
Occurrence (No. of visits)				
0	2	6	6	5
1 or 2	2	6	1	2
≥ 3	9	13	7	12
Side effects				
Gastrointestinal/mucocutaneous symptoms	7	16	7	10
Other symptoms	4	3	1	4

Table 4. Plasma alanine aminotransferase during study period in Group 1 with or without supplementation of folic acid. SD is one standard deviation. P values are results of paired t-test analysis comparing ALT values at Week 0.

	Week			
	0	15–18	28	52
+ Folic acid		NS	NS	$p < 0.05$
Mean (SD)	23 (14.5)	32 (24.2)	23 (7.2)	27 (12.9)
Median (min-max)	18 (13–67)	22 (17–105)	23 (14–36)	22 (14–49)
– Folic acid		$p < 0.05$	$p < 0.05$	$p < 0.005$
Mean (SD)	20 (7.4)	28 (18.9)	32 (33.2)	35 (16.8)
Median (min–max)	19 (9–40)	23 (9–74)	22 (10–177)	33 (13–70)

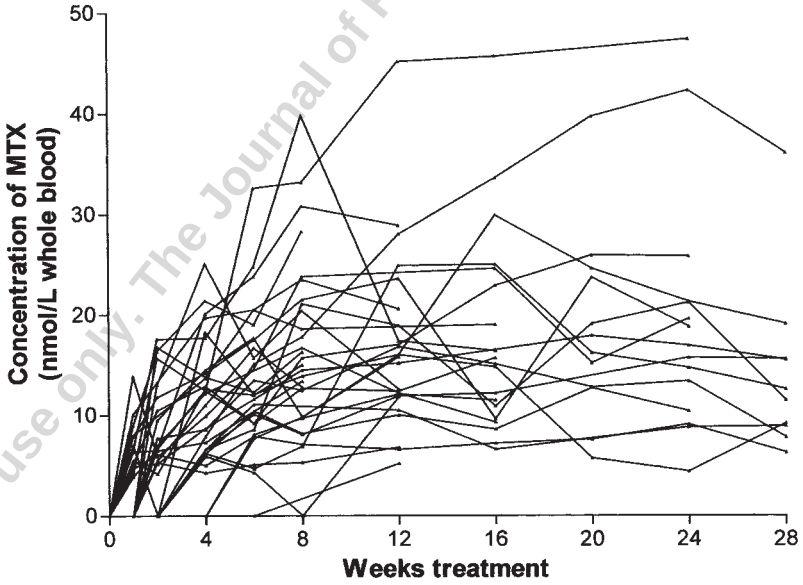

Figure 1. MTX concentrations in erythrocytes measured in patients starting MTX therapy. Data are illustrated from patients treated with 7.5 mg MTX/week and are discontinued if the weekly dose was changed either way. Results are expressed as MTX in mmol/l whole blood.

teine levels were above or in the upper range of the reference interval in the majority of the patients and were inversely correlated to RBC folate levels in patients with no daily intake of folic acid ($r^2 = 0.26$, $p < 0.001$). Patients starting MTX therapy had significantly lower plasma homocysteine levels than patients well established on MTX (Table 5). After starting MTX therapy the plasma homocysteine levels increased further (Figure 5). The increase was significant after 28 weeks in the subgroup of patients not supplemented with low dose folic acid, and after 52 weeks

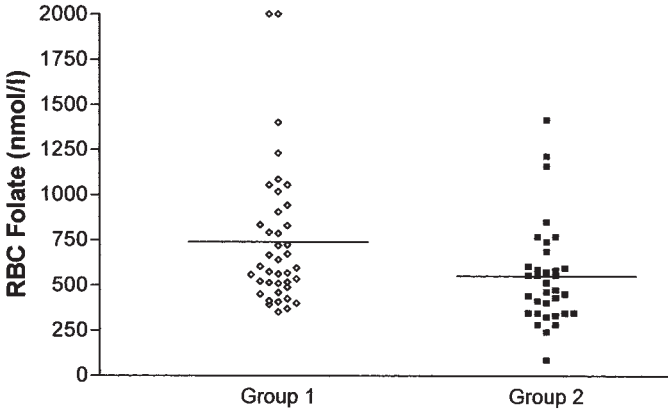

Figure 2. RBC folate levels in Group 1 before MTX treatment and in Group 2 treated with MTX for 41 ± 25 months. Line indicates mean value.

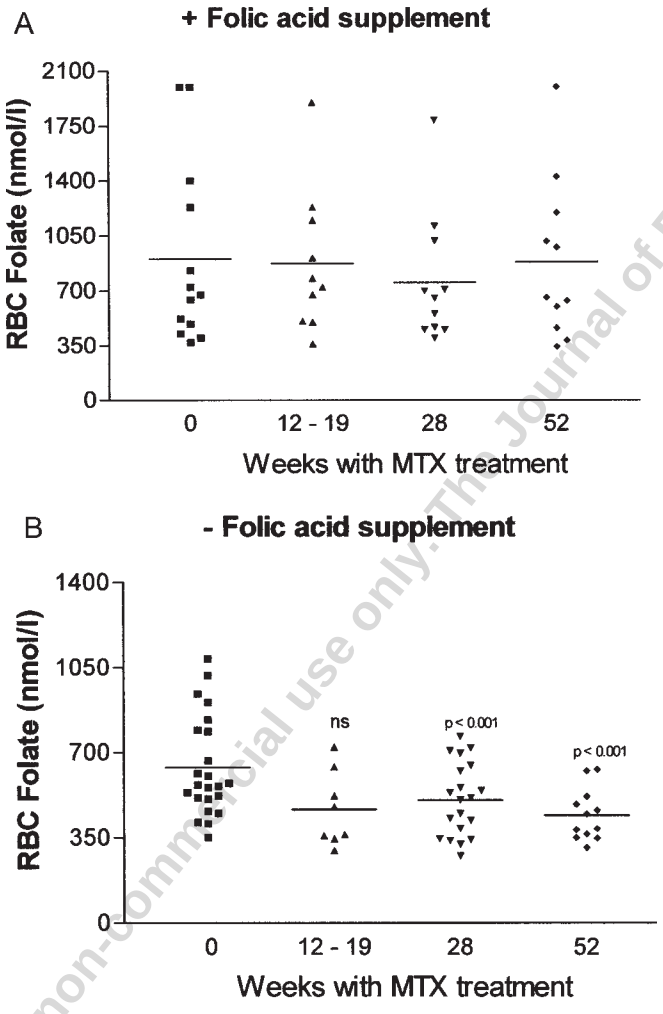

Figure 3. Development of RBC folate levels in patients starting MTX therapy with (A) or without (B) folic acid supplement. Line indicates mean value.

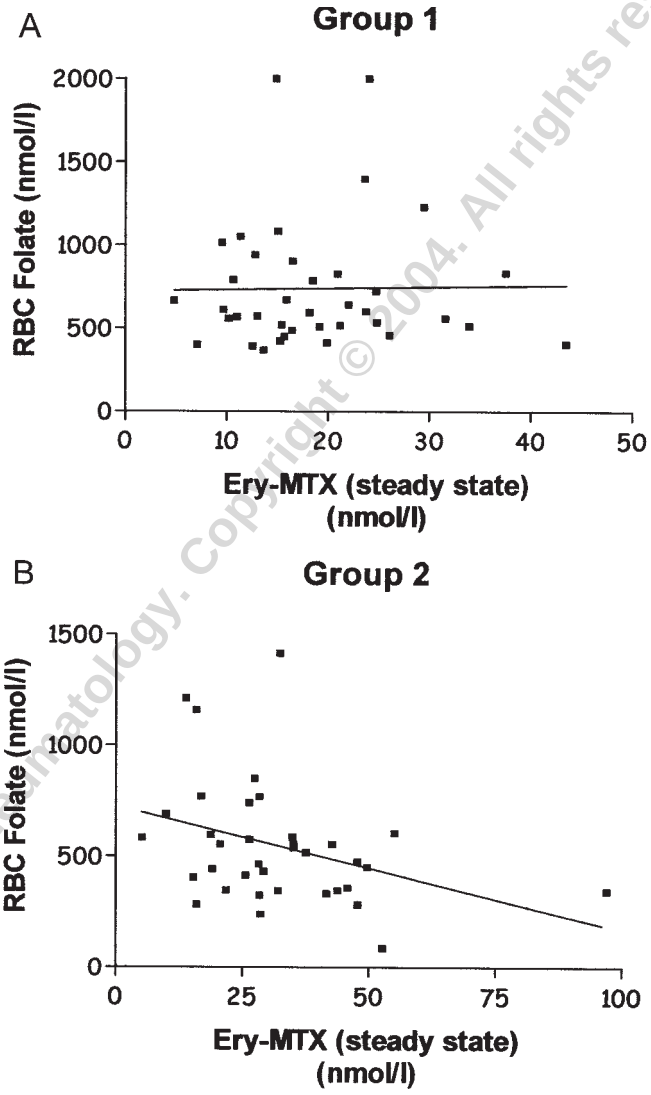

Figure 4. Correlation of RBC folate and steady-state erythrocyte MTX (Ery-MTX) in Group 1 (A) and in Group 2 (B). Linear regression analysis was performed.

Table 5. Plasma homocysteine levels in Group 1 and 2 and homocysteine levels in patients supplemented or not with folic acid in the 2 groups. Results are expressed as mean (SD) in µmol/l.

Group 1			Group 2		
11.1 (3.8)			13.8 (5.6)		p < 0.05
+ Folic acid	– Folic acid		+ Folic acid	– Folic acid	
9.7 (3.3)	11.5 (3.8)	NS	11.3 (3.3)	15.6 (6.2)	p < 0.05

of treatment there was also a small but significant increase in the group of patients receiving daily folic acid supplement. Finally, in Group 2 there was no change in the plasma homocysteine levels over time, but there was a significant difference between patients receiving folic acid in low doses and patients not supplemented with folic acid measured at initiation of study (Table 5). Creatinine levels were meas-

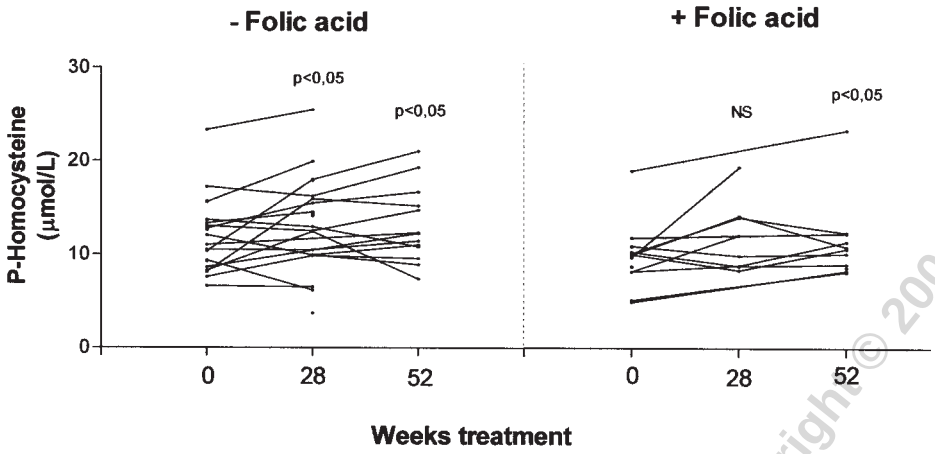

Figure 5. Plasma homocysteine levels in patients starting MTX therapy with or without folic acid supplement. Absolute values from each patient in Group 1 are plotted; p values are results of paired t-test analysis.

ured at study start in order to exclude kidney insufficiency and were found to be within normal range in all patients.

The levels of plasma cobalamins were similar in the 2 groups and were unaffected by MTX therapy in both groups. There was, however, a small but significant increase in plasma cobalamin in the subgroup of patients supplemented with folic acid, but in 12 of these patients the folic acid was taken as a vitamin pill also containing vitamin B12 (≥ 100% of the recommended daily allowance). There was no correlation between plasma concentrations of cobalamins and homocysteine ($p > 0.05$).

DISCUSSION

We studied the effects of MTX therapy and low dose folic acid supplement on folate status variables in patients with RA. Our data show considerable variation in erythrocyte MTX concentrations between patients receiving identical weekly dose of MTX, which is in accord with the known interindividual variation in MTX absorption[18]. It was also observed that intracellular concentrations of MTX were inversely correlated to RBC folate levels. Thus it is tempting to speculate that RBC folate concentrations decline concurrently with the accumulation of intracellular MTX, and that there may be a causal relationship between the 2 variables. To further investigate the antifolate effect of MTX therapy we also measured plasma homocysteine and plasma cobalamins. Plasma homocysteine levels were in the upper range or above the upper reference limit, in accord with other studies of plasma homocysteine in patients with RA[19–21], and increased even further during MTX therapy. This is in agreement with a study showing an increase in plasma homocysteine to a maximum after 24 weeks of MTX treatment[22]. Morgan, *et al* did not find a significant increase in plasma homocysteine during MTX therapy, possibly due to the relatively small number of patients in their study[23]. Concomitant ingestion of folic acid limited this increase, and in patients well established on MTX this resulted in significantly lower levels of plasma homocysteine compared to patients not supplemented with folic acid. Other studies have also shown that supplement with folic acid counteracts the observed enhancement in plasma homocysteine levels compared to placebo, but in these studies folate doses were higher (5 mg/week to 5 mg/day)[19,24-26]. In some of these studies there are no data regarding the habitual intake of vitamins[19,24], or there is no distinction between patients supplemented and those not supplemented with folic acid either as vitamins[26] or in higher doses[22].

Patients with RA have increased mortality that is mainly caused by an excess of cardiovascular and cerebrovascular diseases[27]. Since homocysteine may promote the development of cardiovascular diseases and RA patients commonly have increased concentrations of homocysteine it has been proposed that hyperhomocysteinemia may partly explain the high incidence of vascular diseases[28,29]. The MTX-induced enhancement of homocysteine and the protective effect of small doses of folic acid is of immense clinical importance, since even minor changes of plasma homocysteine are associated with considerable reduction (or elevation) of the risk of cardiovascular disease[30,31].

Plasma cobalamin levels were not affected by MTX treatment and there was no correlation between plasma concentrations of cobalamins and homocysteine, although the cobalamins are important and necessary components of homocysteine-methionine metabolism. This is in agreement with a study by Leeb, *et al*[31], who found that serum levels were similar in patients treated with MTX and in healthy controls. We found a clear inverse correlation between plasma homocysteine and RBC folate, indicating that RBC folate is a better indicator than vitamin B12 for plasma homocysteine levels. This is in accord with a metaanalysis showing that reduction of plasma homocysteine by folic acid supplement was influenced by the pretreatment levels

of plasma homocysteine and folate, but not by the concentration of vitamin B12[33].

The mechanism of MTX-induced liver toxicity is uncertain, but is considered to be caused by the accumulation of MTX in hepatocytes[13,34,35]. A recent study found no correlation between liver concentrations of MTX and liver function tests[36]. Similarly, in our study there was no direct correlation between erythrocyte MTX and level of ALT, but we show a beneficial effect of supplement with low dose folic acid on the development of liver toxicity as measured by ALT. This finding is in agreement with other studies, although the dose of folate supplement was higher or was given as leucovorin[13,37]. The hypothesis that intracellular concentrations of MTX affect the hepatocytes via an antifolate mechanism resulting in elevated ALT levels is supported by the observations in our study.

Although the exact mechanism of action of MTX as an antirheumatic agent is not completely understood, it is generally accepted that the adverse effects are mediated mainly by the antagonistic action of MTX on folate metabolism, and that supplement with folic acid has a protective effect on the mucosal and gastrointestinal toxic effects of MTX[10,38]. The lack of correlation between clinical toxicity and folic acid supplement in this study may be due to the low dose of folic acid in the majority of patients or to the relatively low number of individuals in this group. To elucidate such relationships it may be necessary to register adverse effects more unambiguously and to observe a larger number of patients supplemented with the same dose of folic acid. Presently the dose of such folate supplement is not well established and a definitive folate supplement regime does not exist. The question whether all patients or only patients experiencing side effects should receive folic acid supplement remains unanswered. The major objection against administration of folic acid (or folinic acid) is a possible reduction of the efficacy, which, however, seems to be dose related and not statistically significant in doses ≤ 5 mg/week[9,12,39-41]. Our results indicate that small doses of folic acid interfere with MTX-related changes in folate metabolism and should therefore be considered in future studies.

We conclude that low dose MTX therapy affects folate status and that an inverse relationship exists between the concentration of folate and MTX in erythrocytes. We also conclude that a minimal daily dose of folic acid neutralizes the decrease in RBC folate observed with MTX therapy. Further, low dose folic acid supplement diminishes the development of side effects observed with MTX therapy with no influence on MTX efficacy, and may therefore be recommended until more substantial studies conclude otherwise.

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