Homocysteine levels in patients with rheumatoid arthritis treated with low-dose methotrexate

Plasma homocysteine levels were determined in patients who participated in a randomized, double-blind placebo-controlled trial of folate supplementation (1 mg/day) during methotrexate therapy for rheumatoid arthritis. Plasma and red blood cell folate levels before methotrexate therapy were significantly negatively correlated with homocysteine levels. Homocysteine levels were not significantly correlated with the initial C_1 index (an assay that measures the folate status of blood mononuclear cells) or the C_1 index during methotrexate therapy. There was no significant difference in homocysteine levels between pretreatment and levels drawn at 3 or 6 months. Initial homocysteine levels were predictive of toxicities, such as gastrointestinal intolerance and elevations of liver enzymes in the placebo group. There was no significant correlation between occurrence of toxicity and initial homocysteine levels in the folic acid–supplemented group. Homocysteine levels were not predictive of the efficacy of methotrexate therapy. We conclude that plasma homocysteine levels are correlated with plasma and red blood cell folate levels before methotrexate therapy but is not correlated with folate status in blood mononuclear cells. (CLIN PHARMACOL THER 1991;50:547-56.)

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Homocysteine is a sulfur amino acid that is formed from *S*-adenosylmethionine as a product of transmethylation. Once formed, homocysteine may be remethylated to form methionine or condense with serine to form cystathionine. The former reaction is catalyzed in most tissues by the enzyme methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.13), which requires 5-methyltetrahydrofolate as a methyl donor and methyl cobalamin as a cofactor. Cystathionine is a product of the vitamin B_6-dependent enzyme cystathionine β-synthase.[1] Under conditions of imbalance between homocysteine production and metabolism, intracellular accumulation is limited by an increase in cellular homocysteine efflux. This is the mechanism for the increase in homocysteine concentration in plasma during vitamin B_6, vitamin B_{12}, and folate deficiency.[2]

Numerous studies have suggested that blood homocysteine levels may be indicative of folate status and may be markedly elevated during folate deficiency.[3-5] However, Stabler et al.[6] reported that homocysteine and serum folate levels are not significantly correlated in a sample from healthy blood donors.

There is a transient increase in plasma homocysteine concentration within a few days after the administration of the antifolate methotrexate. This has been demonstrated with a wide range of doses (25 mg to 33.3 gm/m^2) given to patients with psoriasis and patients with malignant diseases.[7-10] This shows that acute hyperhomocysteinemia is a consequence of methotrexate therapy and probably reflects the short-term effect of methotrexate on folate pools in cells exporting significant amounts of homocysteine into the extracellular space.

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Table I. Values at entry for folate, B_6, B_{12}, the C_1 index, homocysteine, and the homocysteine/cysteine ratio

Parameter	*Value*	*Range*	*n*
Median initial plasma folate (nmol/L)*	15.6	2.95-52.3	29
Median initial RBC folate (nmol/L)†	799.9	197-2420	29
Median initial vitamin B_{12} (pg/ml)‡	315	110-972	29
Median initial B_6 (A/C)§	1.50	1.14-3.36	27
Median initial plasma homocysteine concentration (μmol/L)	12.2	3.6-29.9	29
Median initial homocysteine/cysteine ratio	0.041	0.013-0.11	29
Median initial C_1 index (cpm/10^6 cells)	784	62-3885	28
Median C_1 index during methotrexate therapy (cpm/10^6 cells)	306	0-1971	27

RBC, Red blood cell; A/C, activity coefficient.
*Normal plasma folate levels, 4 to 22 nmol/L.
†Normal RBC folate levels, 550 to 2200 nmol/L.
‡Normal vitamin B_{12} levels, 150 to 750 pg/ml.
§Normal vitamin B_6 A/C, 1.15 to 1.89 A/C.

Low-dose methotrexate is used for the management of psoriasis, rheumatoid arthritis,[11-14] and other nonmalignant diseases.[15-17] The folate status of target and nontarget cells may be a determinant of toxicity and of the therapeutic effect of methotrexate.[18]

In this study we evaluated the hypothesis that homocysteine levels should be inversely related to folate status before methotrexate therapy and that homocysteine levels should increase with methotrexate therapy. We also hypothesize that homocysteine levels will be inversely correlated with the C_1 index, a measure of functional folate status in peripheral blood mononuclear cells.[19-21] This evaluation was done as a part of a 6-month randomized, placebo-controlled trial of folic acid supplementation (1 mg/day) during methotrexate therapy for rheumatoid arthritis. This trial demonstrated that folic acid supplementation significantly lowered the number of toxic manifestations, such as anorexia, stomatitis, and gastrointestional intolerance. In this study, 15 of 32 patients experienced some sort of toxicity, 33% were in the folic acid–supplemented group, and 67% were in the placebo group. Four patients in the placebo group had toxic events that were severe enough to require discontinuation of methotrexate. None of the patients in the folic acid–supplemented group stopped methotrexate therapy because of toxicity. The folic acid supplement did not alter the efficacy, which was measured by joint counts and joint indexes for pain, swelling, and tenderness and by physician and patient assessment of disease.

MATERIAL AND METHODS

Patients. Blood samples were analyzed from patients who participated in a trial of folic acid supplementation during methotrexate therapy for rheumatoid arthritis.[21] This trial was approved by the Institutional Review Board of the University of Alabama at Birmingham. All patients fulfilled the entry criteria for rheumatoid arthritis by the American Rheumatism Association.[22] At the time of enrollment, 80% of the patients were women and 20% were men. The median dose of methotrexate in both the placebo and the folic acid–supplemented groups was 7.5 mg/week. Table I lists the biochemical parameters of the patients at the time of enrollment.

Protocol. Fig. 1 shows the design of the trial. Thirty-two patients with rheumatoid arthritis were entered into a 24-week trial to evaluate the effect of folic acid supplementation (1 mg/day) versus placebo on the toxic manifestations of low-dose methotrexate therapy (median dose, 7.5 mg/week) such as gastrointestinal intolerance, stomatitis, and elevated liver function tests. Patients were seen before the start of methotrexate therapy and after approximately 3 and 6 months of therapy for follow-up. Patients were not accepted into the trial until gold salt, D-penicillamine, sulfasalazine, or hydroxychloroquine treatment had been discontinued for at least 10 days. Ninety-four percent of the patients were taking nonsteroidal antiinflammatory drugs or aspirin, which continued in stable doses throughout the trial. Nine patients in the folic acid group and eight patients in the placebo group were taking prednisone. If used, the total daily prednisone dose did not exceed 10 mg/day. Five patients in the folic acid–supplemented group and three patients in the placebo group were taking a folic acid–containing vitamin (mean dose, 400 μg/day) before the trial (difference not significant). Folate-containing vitamin preparations, if previously used, were stopped for the duration of the study. At the initial visit, a complete blood cell count including platelets, liver

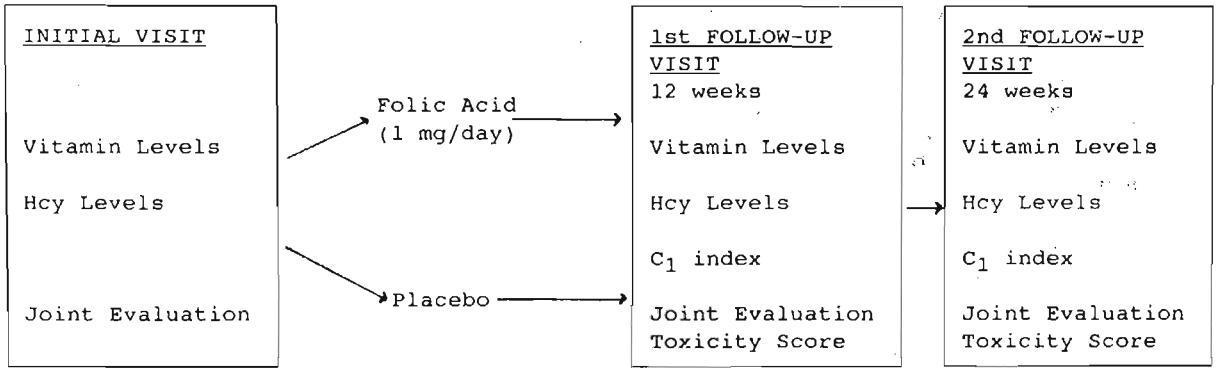

Fig. 1. Study design of the folic acid versus placebo supplementation trial in patients with rheumatoid arthritis treated with low-dose methotrexate. Hcy, Homocysteine.

and biliary enzyme levels (aspartate aminotransferase and alkaline phosphatase), rheumatoid factor, serum creatinine levels, plasma and red blood cell folate, vitamin B_{12}, pyridoxine levels, and the C_1 index in peripheral blood mononuclear cells were determined.

Patients also underwent clinical evaluations of disease activity, including joint examinations for pain, tenderness, and swelling. Changes in the joint examination were used as measures of drug efficacy. Oral methotrexate therapy was begun at a dosage of 2.5 to 7.5 mg per week and was increased in 2.5 mg increments at the discretion of the treating rheumatologist. The median dose in all patients was 7.5 mg/week. The methotrexate tablets were generally ingested in equal numbers on three consecutive occasions at 12-hour intervals, usually beginning on the same day of the week. At 3 and 6 months the patients were revaluated clinically, and blood samples for the C_1 index, vitamin B_{12}, and vitamin B_6 levels were redrawn. Microbiologic reassay for folate was not possible because a methotrexate-resistant *Lactobacillus casei* assay was not available to us at the time. Evaluation for toxic manifestations during follow-up was performed by calculation of a toxicity score that increased in proportion to the duration of toxic events, their intensity, and their clinical significance, and decreased with the time on the protocol at which the toxic manifestation first appeared.[21] Follow-up complete blood cell counts, creatinine, and liver function tests were performed at the discretion of the attending rheumatologist and were generally repeated at each follow-up visit.

Blood sample collection and processing. Nonfasting venous blood for homocysteine, cysteine, and vitamin levels was obtained by sterile technique in vacuum tubes containing ethylenediaminetetraacetic acid. Blood for peripheral blood mononuclear cells was collected in a heparinized tube. Red blood cell hemolysates for the analysis of red blood cell folate were prepared by freeze-thawing whole blood three times. Mononuclear cells were separated on a Ficoll-Hypaque gradient. Plasma samples were frozen at −70° C and sent to the clinical pharmacology unit in Bergen, Norway, for homocysteine and cysteine analysis.

Analytic methods. The C_1 index was performed in peripheral blood mononuclear cells by the method of Ellegaard et al.[19] and Morgan et al.[20,21] The C_1 index measures the activity of a folate-dependent enzyme system in peripheral blood mononuclear cells by assaying the formation of serine from glycine and radiolabeled formate.[19,20] Plasma and red blood cell folate levels were done by the microbiologic *L. casei* method.[23] Vitamin B_6 activity coefficients were determined by coenzyme activation of erythrocyte enzymes.[24]

Total homocysteine and total cysteine in plasma was determined by use of a modification of a fully automated assay described previously.[25] The procedure involves reduction of protein-bound sulfur amino acid with $NaBH_4$ followed by derivatization of reduced thiols with monobromobimane and finally quantization of the monobromobimane adducts with HPLC.[25] The modification introduced allowed the simultaneous quantization of homocysteine and cysteine in plasma, without sample clean-up or column switching. The monobromobimane adducts were separated on a 15 cm 3 μm octadecylsilane Hypersil column (Shandon Southern Products, Cheshire, England) eluted with an acetonitrile gradient (0% to 10% in 11.5 minutes) in 58 mmol/L ammonium nitrate/40 mmol/L ammonium formate buffer, pH 3.67. The flow rate was 2 ml/min. The effluent was monitored by fluorescence detection.

Table II. Correlations between homocysteine, the homocysteine/cysteine ratio, vitamin levels, and the C_1 index

Correlated parameter	*rho*	*p Value*	*n*
Initial plasma folate vs Hcy (all patients)	−0.58	<0.01	29
Initial plasma folate vs Hcy/Cys (all patients)	−0.67	<0.01	29
Initial RBC folate vs Hcy (all patients)	−0.38	<0.05	29
Initial RBC folate vs Hcy/Cys	−0.53	<0.01	29
Initial C_1 index vs Hcy	−0.05	>0.05	28
Initial C_1 index vs Hcy/Cys	−0.14	>0.05	28
Initial plasma B_{12} vs Hcy	−0.14	>0.05	30
Initial B_{12} vs Hcy/Cys	−0.20	>0.05	30
Initial B_6 A/C vs Cys	0.30	>0.05	27
Initial B_6 A/C vs Hcy/Cys	0.39	<0.05	27
C_1 Index during MTX therapy vs Hcy	−0.01	>0.05	27
C_1 Index during MTX therapy vs Hcy/Cys	−0.08	>0.05	27

Hcy, Homocysteine; Cys, cysteine; RBC, red blood cell; A/C, activity coefficient; MTX, methotrexate.

The homocysteine and cysteine adducts showed retention times of 11 minutes and 8.5 minutes in this system, respectively.

Data analysis. Correlations were determined by the Spearman rank correlation test.[26] The small numbers in many of the analysis groups did not justify the use of parametric statistics.[27] The difference between groups was determined by the Wilcoxon rank sum test.[26] *p* Values less than 0.05 were considered significant. Although 32 patients were entered into the trial, blood samples from each patient were not always available for all assays. The numbers of patients considered in each data analysis are noted.

RESULTS

Plasma homocysteine and other parameters before methotrexate exposure. Table II shows correlations between initial homocysteine levels and the homocysteine/cysteine ratio, plasma and red blood cell folate levels, and the C_1 index. Both initial plasma and red blood cell folate levels are significantly inversely correlated with homocysteine levels. Figs. 2 and 3 show the relationships between plasma and red blood cell folates and homocysteine levels and the homocysteine/cysteine ratio. The initial vitamin B_6 activity coefficient is also significantly correlated with homocysteine/cysteine ratios. In all cases, the relationship between the vitamin level is more strongly correlated with homocysteine/cysteine ratio than homocysteine level alone. There is no significant correlation between the C_1 index and plasma homocysteine levels or homocysteine/cysteine ratios before or during methotrexate therapy (Table II).

Long-term effect of methotrexate on homocysteine levels. Table III shows homocysteine levels and homocysteine/cysteine ratios before and during methotrexate therapy by treatment category. There is no significant difference in any treatment group between initial levels or levels drawn at the 3- or 6-month follow-up time points. However, there is a trend toward increased plasma homocysteine levels during methotrexate treatment, and this response is diminished by folic acid supplementation. Homocysteine levels are not significantly correlated with weekly or cumulative methotrexate dose.

Plasma homocysteine and toxicity from methotrexate. Table IV shows the relationship between final toxicity scores and initial homocysteine levels. Initial homocysteine levels are strongly predictive of toxicity in the placebo (folic acid–unsupplemented) group. Use of the homocysteine/cysteine ratio does not strengthen the correlation with toxicity. Patients with clinically important toxicities also tended to have elevated homocysteine levels or homocysteine/cysteine ratios during methotrexate therapy. For example, four patients had elevated liver function tests and two patients had cytopenias. The mean homocysteine levels for these two groups (14.7 and 18.0 μmol/L, respectively, normal value: 10.2 ± 2.9 μmol/L for age-matched control subjects) and the mean homocysteine/cysteine ratios (0.054 and 0.059, respectively) were elevated during therapy. Fig. 4 shows the relationship between homocysteine levels and the toxicity score in the placebo group. The fact that folic acid supplementation markedly decreased toxic manifestations of methotrexate renders a comparison of initial homocysteine levels and the toxicity score less useful in the folic acid–supplemented group.

Plasma homocysteine and therapeutic effect. Table V shows homocysteine levels in relationship to

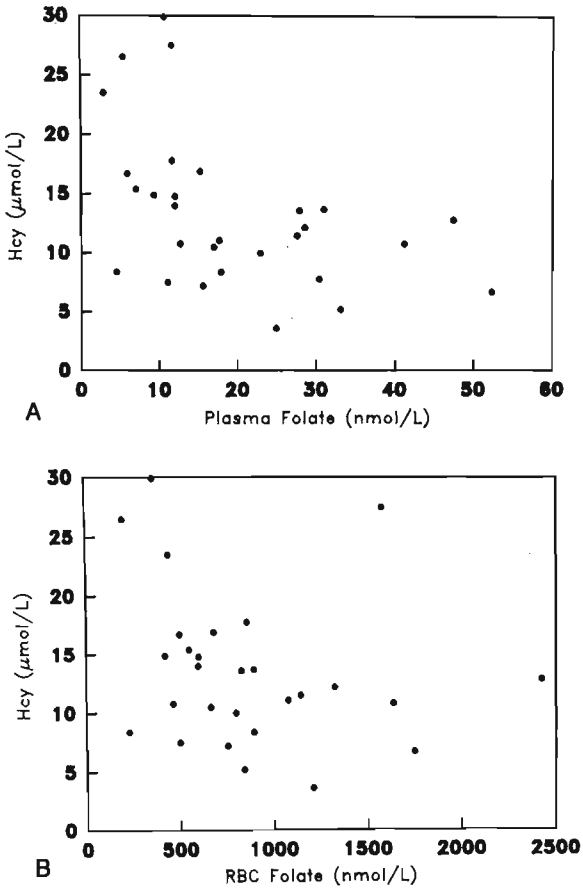

Fig. 2. Plasma and red blood cell (RBC) folate levels versus homocysteine (Hcy) level. **A,** Plasma folate levels versus Hcy level. **B,** RBC folate levels versus Hcy level.

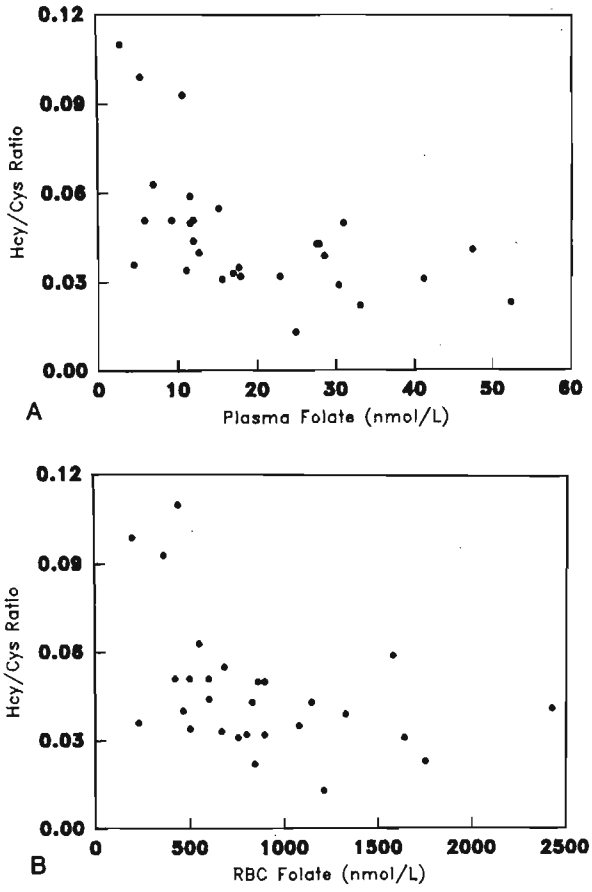

Fig. 3. Plasma and red blood cell (RBC) folate levels versus homocysteine/cysteine (Hcy/Cys) ratio. **A,** Plasma folate levels versus Hcy/Cys ratio. **B,** RBC folate levels versus Hcy/Cys ratio.

whether patients had an improvement of joint swelling of 30% or greater during the trial. Such a change is considered a therapeutic response (efficacy). No improvement is defined as no change in joint swelling or as deterioration in joint swelling. There was no significant difference in the initial minus final homocysteine levels or the homocysteine/cysteine ratio between the group that had improvement versus the group that had no improvement.

DISCUSSION

Plasma homocysteine levels may be affected by factors such as vitamin status and pharmacologic agents.[2] Mild to marked hyperhomocysteinemia develops in deficiencies of vitamin B_{12},[28] vitamin B_6,[29,30] or folate.[4,5] Plasma homocysteine is almost always elevated in clinical deficiency of folate[5] and the elevation probably reflects insufficient tissue folate for optimal homocysteine remethylation.[4] Kang et al.[4] demonstrated that total homocysteine was significantly negatively correlated with serum folate in patients with subnormal serum folate. Thus plasma homocysteine may be an indicator of whole body folate homeostasis, and elevation may occur only after depletion of cellular stores in organs playing a major role in the overall homocysteine remethylation, for example, the liver.

In this study, homocysteine levels were significantly negatively correlated with both plasma and red blood cell folate levels in patients with rheumatoid arthritis before beginning methotrexate therapy. The mean homocysteine concentration (13.1 ± 7.7 μmol/L) was not substantially above the mean value for hospitalized patients (10.2 ± 2.9 μmol/L), which is in agreement with findings obtained by Kang et al.[4] We also observed that the vitamin B_6 activity coeffi-

Table III. Homocysteine and homocysteine/cysteine ratios before and during methotrexate therapy

Patient group	*n*	*Before*	*Second and third visits*	*p Value*
		Homocysteine levels (μmol/L)		
All patients	17	13.1 ± 7.3	11.6 ± 4.9	0.3
Placebo group	3	13.0 ± 2.3	14.2 ± 1.7	0.3
Folic acid–supplemented group	14	13.2 ± 8.3	10.9 ± 5.3	0.2
		Homocysteine/cysteine ratio		
All patients	17	0.046 ± 0.023	0.041 ± 0.012	0.2
Placebo group	3	0.042 ± 0.009	0.047 ± 0.003	0.2
Folic acid–supplemented group	14	0.047 ± 0.026	0.039 ± 0.013	0.1

Data are mean values ± SD.

Table IV. Correlations between toxicity and homocysteine levels and homocysteine/cysteine ratios

Correlated parameters	*rho*	*p Value*	*n*
Final toxicity scores in folic acid–supplemented group vs Hcy (initial)	0.39	>0.05	14
Final toxicity score in folic acid–supplemented group vs Hcy/Cys (initial)	0.45	<0.01	14
Final toxicity score, placebo group vs Hcy (initial)	0.75	<0.01	10
Final toxicity score, placebo group vs Hcy/Cys (initial)	0.74	<0.01	10
Final toxicity score, all (2nd and 3rd visits)	0.51	<0.01	19
Final toxicity score: all patients vs Hcy/Cys (2nd and 3rd visits)	0.50	<0.01	19

cient was significantly correlated with the homocysteine/cysteine ratio. Before the start of methotrexate therapy, many of the patients with rheumatoid arthritis had been treated with D-penicillamine, which is a vitamin B_6 antagonist,[31] but this drug itself may also decrease plasma homocysteine through the formation of a mixed disulfide with homocysteine.[32,33]

The correlation between vitamin levels were generally stronger for the homocysteine/cysteine ratio than for the homocysteine level alone. It is possible that the ratio may correct for fluctuations in plasma homocysteine not related to folate, vitamin B_{12}, or vitamin B_6 levels, such as the dietary intake of sulfur amino acids. It is known that there is a complex relationship between plasma homocysteine and cysteine levels.[34] This possibility deserves further investigation.

The C_1 index measures the activity of a pathway that is dependent on the presence of reduced folates[19-21] and is an indicator of the folate status in the peripheral blood mononuclear cells. The C_1 index is not significantly correlated with either plasma or red blood cell folates[20] or plasma homocysteine levels (Table II), but it is significantly correlated with the folate content of the peripheral blood mononuclear cells.[20] The C_1 index was significantly lowered after methotrexate treatment of patients with rheumatoid arthritis,[20,21] whereas there was no statistical difference between homocysteine or the homocysteine/cysteine ratio before therapy or after 3 or 6 months of therapy. We found no correlation between the cumulative methotrexate dose and the C_1 index.[21] This suggests an ongoing partial restoration of the folate content of the blood mononuclear cells through redistribution of folates from pools not substantially depleted.[35]

The preservation of the whole body folate status during 6 months of low-dose methotrexate therapy is in fact indicated by essentially unaltered plasma homocysteine levels (Table III). In contrast, the folate status of blood mononuclear cells may be particularly affected by low-dose methotrexate. This may be explained by relatively low folate content of peripheral blood mononuclear cells, efficient retention of methotrexate in the polyglutamate form, and rapid turnover of the pool of reduced folates in these cells.[36] The sensitivity of these cells to methotrexate may be important in producing the anti-inflammatory effect of low-dose methotrexate therapy[37] and may also explain why reduction in the C_1 index during methotrexate treatment correlates with therapeutic efficacy. This hypothesis is supported by the fact that deterioration in

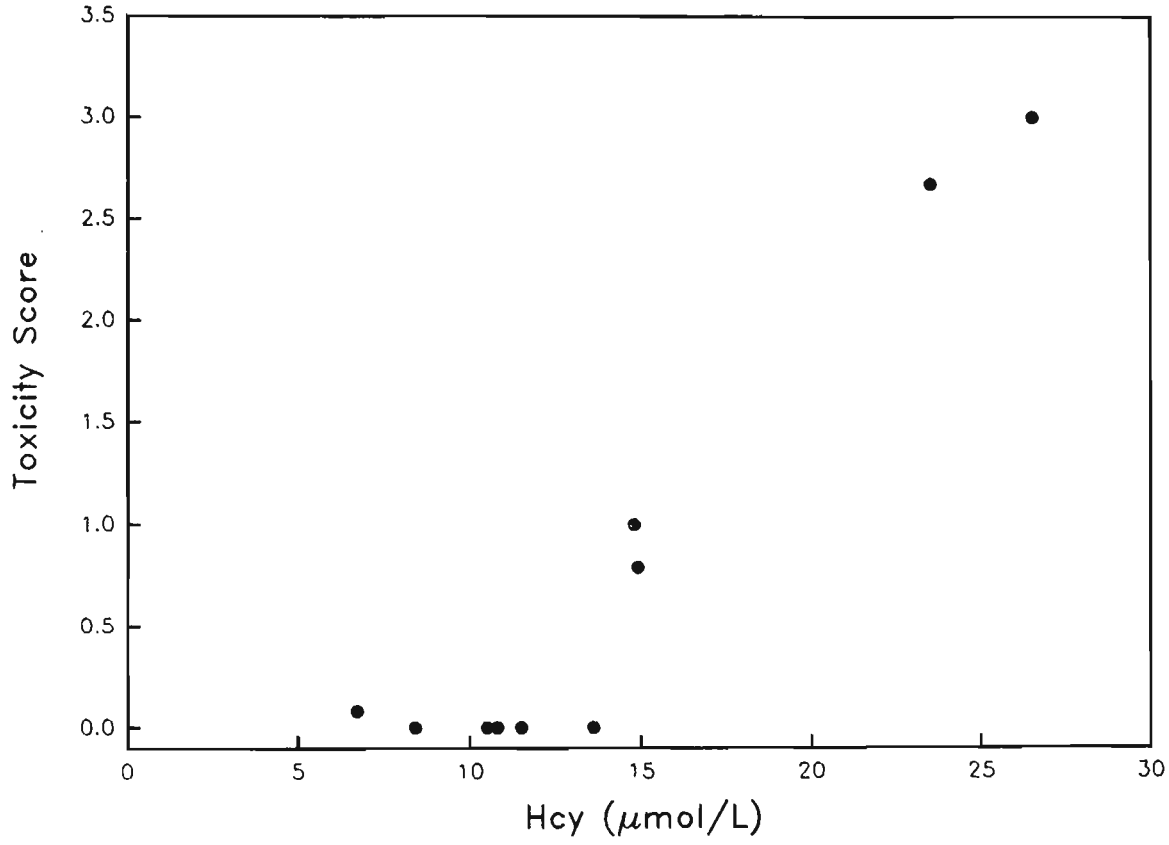

Fig. 4. The toxicity score (TS) in the placebo group versus the initial homocysteine (Hcy) level (in micromoles per liter). The toxicity score is calculated as follows: TS = Σ [(Duration of toxic event [weeks] × (intensity) × (clinical severity factor)/weeks on protocol]. In this equation, intensity is 1 (mild), 2 (moderate), or 3 (severe); and the clinical severity factor is 1 (alopecia, nausea, pruritis, anorexia or general gastrointestinal intolerance [such as pyrosis or cramps]), 2 (vomiting, diarrhea, stomatitis or rash), 3 (elevated liver enzyme levels or elevated serum creatinine level), or 4 (cytopenia, documented infections, or pulmonary toxicity).

peripheral blood mononuclear cells folate status, as measured by the C_1 index, was related to joint improvement, whereas changes in homocysteine levels were not related to the efficacy of methotrexate therapy.

Initial folate status as measured by serum or red blood cell folate, C_1 index,[21] homocysteine level in the plasma, and the homocysteine/cysteine ratio (Table IV) was predictive of ultimate toxicity indicated by the toxicity score. There seems to be a threshold of initial folate level, below which there is toxicity with the institution of methotrexate therapy. As expected, this correlation was stronger in the group of subjects who received placebo than in the group of subjects who received folic acid (Table IV). Although abnormal liver function tests occurred in only four of 32 patients, all four of those patients were in the placebo group. It has been suggested that inhibition of folate-dependent homocysteine remethylation can play a central role in methotrexate hepatotoxicity.[38-40] Thus the determination of plasma homocysteine levels may be a good indicator of impending hepatotoxicity. The reasons for initially low folate and vitamin B_{12} levels in this patient population were multifactorial, including impaired financial resources to buy food, poor food intake habits, and inability to prepared foods (particularly fruits and vegetables) because of crippling arthritis.

We have previously observed that low-dose methotrexate induced a transient hyperhomocysteinemia in

Table V. Relationship of homocysteine levels and homocysteine/cysteine ratios to efficacy*

Efficacy of methotrexate	*Change in parameter†*	*p Value*	*n*
	Homocysteine level (μmol/L)		
Improvement‡	−1.2 ± 3.9	>0.05	10
No improvement§	−1.4 ± 4.5	>0.05	10
	Homocysteine/cysteine ratio		
Improvement‡	−0.0033 ± 0.0129	>0.05	10
No improvement§	−0.0074 ± 0.0184	>0.05	10

Data are mean values ± SD.
*Efficacy refers to improvement in joint swelling.
†Initial value minus final value.
‡Improvement constitutes a >30% improvement in joint swelling.
§No improvement constitutes a <30% improvement in joint swelling, no improvement, or deterioration in joint swelling.

patients with psoriasis. The plasma homocysteine level normalized within 3 to 7 days, but levels between the methotrexate doses were essentially stable in most patients who were monitored for up to 4 weeks of treatment.[8] In this study, most of the samples were drawn 5 to 7 days after methotrexate administration, and no statistical difference between homocysteine or the homocysteine/cysteine ratio before low-dose methotrexate and after 3 or 6 months of therapy was noted, although a trend toward elevation of plasma homocysteine was observed (Table III). However, low-dose methotrexate treatment for this amount of time is known to affect folate status measured in white blood cells and in the serum.[21,41] This suggests that methotrexate may differentially affect the pools of reduced folates in different cells or tissues, which in turn determines parameters such as transient hyperhomocysteinemia, plasma levels between methotrexate doses, serum or red blood cell folate levels, and the C_1 index.

Folic acid supplementation did not significantly reduce plasma homocysteine, but a trend (from 13.2 to 10.9 μmol/L; Table III) was observed. Likewise, the folic acid–supplemented group showed a trend to lower plasma homocysteine levels than the placebo group (Table III). This contrasts to the finding of Brattström et al.[42,43] that 5 mg folic acid daily caused a marked reduction in plasma homocysteine levels, even in healthy subjects without overt folate deficiency. Similar observations have been made in patients with renal failure.[44] The fact that the plasma homocysteine was not markedly reduced in the patients with rheumatoid arthritis who received folic acid relative to control subjects may be explained by the coadministration of methotrexate, lower folic acid doses (1 mg versus 5 mg) than those used by Brattström et al.,[42,43] and intake of a substantial amounts of dietary folate in the control subjects.

In summary, we have found that plasma homocysteine concentration is significantly negatively correlated with serum folate and red blood cell folate in patients with rheumatoid arthritis. Initial homocysteine levels are also predictive of future toxicity during methotrexate therapy, but it does not correlate with the C_1 index or the efficacy of the drug. This may be explained if plasma homocysteine reflects whole body folate status, whereas the C_1 reflects perturbation of folate-dependent reactions only in the blood mononuclear cells, which may be the target cells for drug therapy in rheumatoid arthritis.

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