EXTENDED REPORT

Purine enzymes in patients with rheumatoid arthritis treated with methotrexate

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Objectives: To study (*a*) purine metabolism during treatment with methotrexate (MTX) in patients with rheumatoid arthritis (RA) and (*b*) the relation of purine metabolism with efficacy and toxicity of MTX treatment.

Methods: One hundred and three patients with active RA who started treatment with MTX were included. The initial MTX dosage was 7.5 mg/week and raised to a maximum of 25 mg weekly if necessary. The purine enzymes 5′-nucleotidase (5′NT), purine-nucleoside-phosphorylase (PNP), hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), and adenosine-deaminase (ADA) were measured before the start, after six weeks, and after 48 weeks or at study withdrawal. The laboratory results were related to measures of efficacy and toxicity of MTX treatment.

Results: Baseline values of 5′NT and PNP (16.9 and 206.8 nmol/10^6 mononuclear cells/h, respectively) were similar to those in former studies. Activities of HGPRT and ADA were relatively low at the start (8.7 and 80.3 nmol/10^6 mononuclear cells/h, respectively). After six weeks purine enzyme activities showed no important changes from baseline. After 48 weeks of MTX treatment a decrease of the enzyme activities of ADA (–21.6 nmol/10^6 mononuclear cells/h; 95% CI –28.6 to –14.7), PNP (–78.9 nmol/10^6 mononuclear cells/h; 95% CI –109.0 to –48.7), and HGPRT (–2.0 nmol/10^6 mononuclear cells/h; 95% CI –3.1 to –0.9) was found. No association was shown between the enzyme activities of ADA, PNP, and HGPRT, and the efficacy or toxicity of MTX treatment. In contrast, enzyme activity of 5′NT showed a decrease in the subgroup of patients discontinuing MTX treatment because of hepatotoxicity.

Conclusion: MTX treatment in patients with RA leads to a significant decrease of the purine enzyme activities of ADA, PNP, and HGPRT that is not related to the anti-inflammatory efficacy or toxicity of MTX. Hepatotoxicity was related to a decrease in the enzyme activity of 5′NT. These changes may be explained by direct or indirect (via purine de novo and salvage metabolism and the homocysteine pathway) effects of MTX.

Despite extensive research, the precise mechanism of action of MTX in vivo is still unknown.[1 2] MTX, a folic acid antagonist, influences several metabolic pathways, including purine metabolism. Purines are necessary for the synthesis of nucleic acids (adenosine monophosphate and guanosine monophosphate) and eventually desoxyribonucleic and ribonucleic acid. The purine metabolism is composed of a de novo synthesis and a salvage route. The latter involves the enzymes 5′-nucleotidase (5′NT), purine-nucleoside-phosphorylase (PNP), hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), and adenosine deaminase (ADA) (fig 1).

Purine metabolism may have an interesting role in patients with rheumatoid arthritis (RA). Firstly, several disturbances of purine metabolism have been associated with immune disorders.[3–11] In RA, in which a dysregulation of the immune system also may play a part, changes in purine enzymes have been reported.[11–13] Secondly, the outcome of treatment with azathioprine (efficacy as well as toxicity) in patients with RA has been associated with low activities of purine enzymes.[14–16] Thirdly, the anti-inflammatory effect of MTX may be dependent on adenosine release.[2] Also, some of the MTX related toxicity may be caused by its inhibition of purine metabolism.[1]

As far as we know this is the first study investigating the effects of MTX treatment on purine enzymes in patients with RA. The relation of purine enzyme levels with efficacy and toxicity of MTX treatment was also studied.

PATIENTS AND METHODS

One hundred and three patients with RA according to the American College of Rheumatology (ACR) criteria were included in the study.[17] They comprised a sample from 411 participants in a 48 week, multicentre, double blind, placebo controlled, randomised clinical trial investigating the effect of folic acid and folinic acid supplementation on the efficacy and toxicity of MTX treatment.[18] Because measurement of purine enzyme activities requires special blood drawing and laboratory conditions, only patients from 9/22 participating centres could enrol in this study. Assignment of patients to treatment was stratified for each centre. Patients were randomly distributed between three treatment modalities: MTX plus placebo, or MTX plus folic acid (1.0 mg/day), or MTX plus folinic acid (2.5 mg/week). The initial MTX dosage was 7.5 mg/week and could be increased to a maximum of 25 mg/week if necessary. The dosages of folic and folinic acid were doubled when the MTX dosage reached ⩾15 mg/week.

For inclusion in the study the arthritis had to be active, defined as a disease activity score (DAS) ⩾3.0. Preceding treatment with MTX was not allowed. A washout period of two weeks for other disease modifying antirheumatic drugs was obligatory. Corticosteroids and non-steroidal anti-inflammatory drugs were permitted in stable doses from at

Abbreviations: ADA, adenosine deaminase; AICAR, amino-imidazolcarboxamide ribosyl-5-phosphate; ALT, alanine aminotransferase; DAS, disease activity score; ESR, erythrocyte sedimentation rate; HGPRT, hypoxanthine-guanine-phosphoribosyltransferase; MTX, methotrexate; 5′NT, 5′-nucleotidase; PNP, purine-nucleoside-phosphorylase; RA, rheumatoid arthritis; THF, 5,10-methylene-tetrahydrofolate

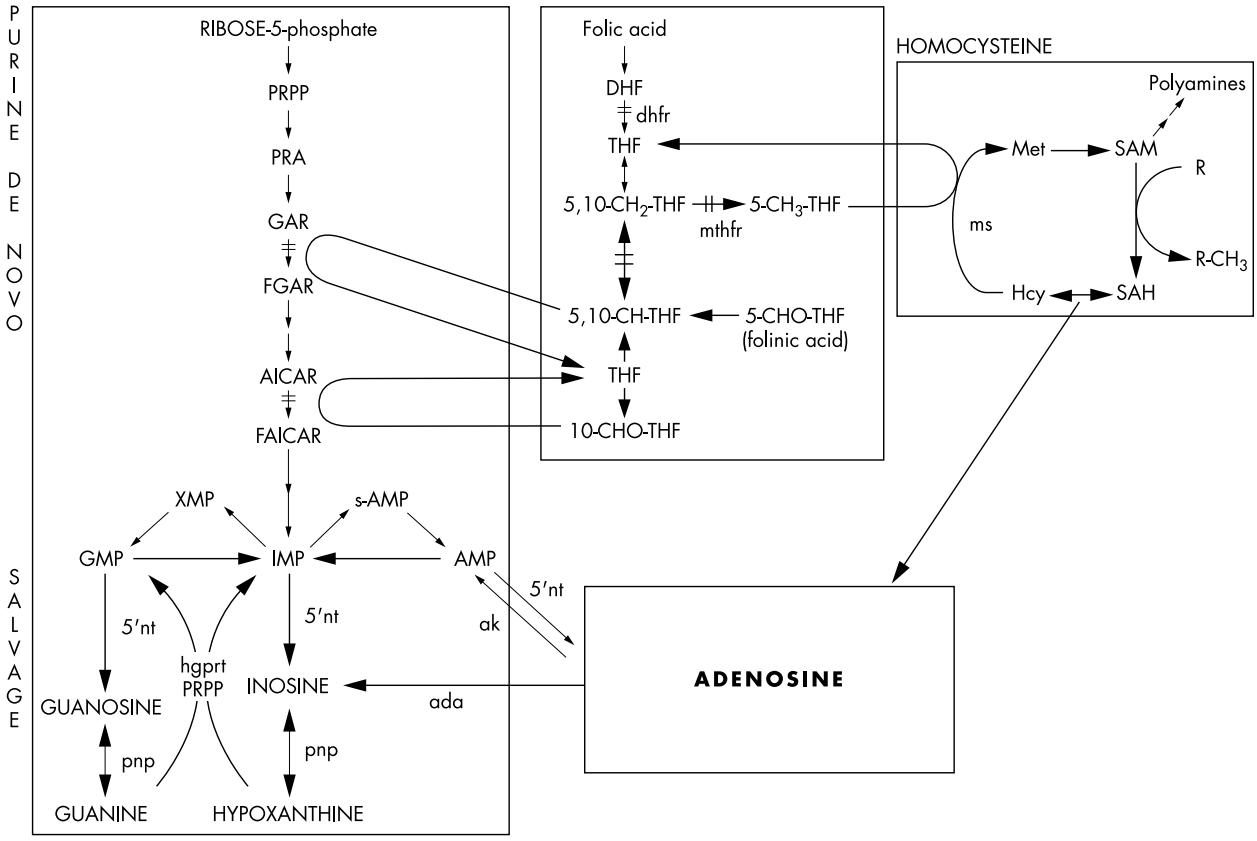

Figure 1 Simplified metabolic scheme illustrating folate metabolism and its relation to purine and homocysteine metabolism. Known inhibition of enzymes by methotrexate is indicated by two short lines across an arrow. DHF, dihydrofolate; dhfr, dihydrofolate-reductase; THF, tetrahydrofolate; 5,10-CH_2-THF, 5,10-methylene tetrahydrofolate; 5,10-CH-THF, 5,10-methenyl tetrahydrofolate; 10-CHO-THF, 10-formyl tetrahydrofolate; mthfr, methylene-tetrahydrofolate reductase; 5-CH_3-THF, 5-methyl tetrahydrofolate; 5-CHO-THF, 5-formyl tetrahydrofolate (folinic acid); Met, methionine; SAM, S-adenosyl-L-methionine; ms, methionine synthetase; Hcy, homocysteine; SAH, S-adenosyl-L-homocysteine; R, methyl acceptor; PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, phosphoribosylamine; GAR, glycinamide ribosyl-5-phosphate; FGAR, form-glycinamide ribosyl-5-phosphate; AICAR, amino-imidazolcarboxamide ribosyl-5-phosphate; FAICAR, form-amino-imidazolcarboxamide ribosyl-5-phosphate; IMP, inosine monophosphate; s-AMP, succinyl-adenosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; XMP, xanthine monophosphate; 5′nt, purine-5′nucleotidase; pnp, purine nucleoside phosphorylase; hgprt, hypoxanthine-guanine phosphoribosyl transferase; ada, adenosine deaminase; ak, adenosine kinase.

least one month before enrolment until the end of the study. Alcohol was permitted if <20 alcoholic drinks a week.

Efficacy

Efficacy of MTX treatment was measured by the DAS every six weeks.[19 20] Spearman's rank correlation was used to express the correlation between changes in plasma purine enzyme activities and the erythrocyte sedimentation rate (ESR; mm/1st h) and DAS at baseline and after 48 weeks of MTX treatment. Finally, response categories (good, moderate, or no response) were defined according to the European League Against Rheumatism (EULAR) response criteria.[21]

Toxicity

Toxicity was assessed every three weeks using a standard toxicity questionnaire including 36 items designed by Fries *et al*, and special forms for other symptoms and complaints.[22] Routine laboratory measurements (three weekly) included ESR (mm/1st h), haemoglobin (mmol/l), white blood cell count ($\times 10^9$/l), platelet count ($\times 10^9$/l), serum creatinine level (µmol/l), and alanine aminotransferase (ALT; units/l).

Gastrointestinal toxicity included all symptoms and complaints of the mouth and the upper or lower abdominal tract, except laboratory abnormalities.

An increase in liver enzyme activities was defined as raised ALT values <3 times the upper limit of normal levels occurring on at least at two of four consecutive (three-weekly) evaluations (mild) or as raised ALT values ≥3 times the upper limit of normal range (moderate). When adverse events occurred the protocol allowed for reduction in the dosage.[18]

Purine enzymes

For this study blood samples were taken before the start of MTX treatment, after six and 48 weeks (end of study), 16 hours after the intake of MTX. If MTX was discontinued before the end of the study, blood was drawn at study withdrawal. The purine enzyme activities of 5′NT, PNP, HGPRT, and ADA were measured by high performance liquid chromatography.[23] Blood was collected in 10 ml Vacutainer tubes containing polystyrene granules (Becton and Dickinson, Rutherford, NJ). Mononuclear cells were obtained by Ficoll-Isopaque (density 1.077 g/ml, Nycomed) gradient centrifugation of defibrinated blood, and the enzyme activities were measured in these cells. Enzyme activities are expressed in nmol/10^6 mononuclear cells/h of incubation. All enzyme assays were carried out in triplicate, results represent the mean value. Reference values were obtained by former studies carried out in our laboratory and partly published by Stolk *et al*.[24]

Statistical analysis

Baseline characteristics and changes in laboratory variables were compared using Student's *t* test. Linear regression analyses were performed to find associations between the change in

Table 1 Demographics and disease characteristics in the 103 patients with rheumatoid arthritis at baseline*. Results are shown as mean (SD), except where indicated otherwise

Age (years)	55.6 (12.7)
Sex (% female)	69
Disease duration (months)	75.9
Rheumatoid factor (% positive)	87.5
Disease activity score	4.6 (1.0)
Ritchie articular index	17.4 (8.0)
No of painful joints (maximum 53)	21.7 (11.4)
No of swollen joints (maximum 44)	17.3 (7.2)
ESR (mm/1st h)	42.0 (21.9)
VAS pain (0–100 mm scale)	51.3 (19.3)
VAS general health (0–100 mm scale)	49.3 (18.8)

*The range of the visual analogue scale (VAS) was from 0 (best status) to 100 (worst imaginable status).
ESR, erythrocyte sedimentation rate.

purine enzyme activities and several independent variables: age, sex, and folate supplementation. Folate supplementation was defined as the use of either folic or folinic acid as co-medication during treatment with MTX. Spearman rank correlation coefficients were used to express the correlation between changes in plasma purine enzyme activities and clinical variables of efficacy. In subsequent analyses values of patients who ended the study prematurely were taken into account until drop out. Finally, the mean change in purine enzyme activities was compared between the group of patients who continued all 48 weeks of MTX treatment and patients who discontinued MTX treatment because of (hepato)toxicity.

RESULTS

A total of 103 patients enrolled in the study: 34 were treated with MTX plus placebo, 33 with MTX plus folic acid, and 36 with MTX plus folinic acid supplementation. Table 1 gives details of patient characteristics and baseline variables. There were no significant differences between the three treatment groups (data not shown). Sixty three patients consumed alcoholic drinks: 25 patients <1 alcoholic drink/week, 30 patients 1–10 alcoholic drinks/week, and eight patients 11–19 alcoholic drinks/week.

Purine enzymes

Changes in purine enzyme activities were not associated with age or sex, and no differences were found in subgroups with or without folic or folinic acid supplementation (data not shown). Therefore in subsequent analyses all patients, whether given treatment with folic or folinic acid supplementation or placebo, were considered as one group.

Table 2 shows purine enzyme activities at the start and the changes after six and 48 weeks of MTX treatment. After six weeks no patients had discontinued MTX treatment and all patients were treated with the same dosage of 7.5 mg MTX/week. Purine enzyme activities showed no significant changes from baseline. However, after 48 weeks when 76 patients were still being treated with MTX (15 treated with MTX plus placebo, 29 treated with MTX plus folic acid, and 32 treated with MTX plus folinic acid), PNP, HGPRT and ADA all showed a significant decrease ($p=0.0001$, $p=0.002$, and $p<0.00001$, respectively). In contrast, 5'NT did not change. The mean MTX dosage after 48 weeks was 17.0 mg/week (95% CI 15.8 to 18.3 mg/week). Additional analysis showed no correlation between the MTX dose and changes in purine enzyme activities.

Efficacy

Efficacy was measured in the group of 76 patients still receiving MTX treatment after 48 weeks. Changes in enzyme activities of ADA, PNP, and HGPRT showed no correlation with the ESR and DAS at baseline nor with the change in ESR and DAS after 48 weeks. In contrast, 5'NT correlated with DAS at baseline ($p=0.01$) and the change in ESR after 48 weeks ($p=0.03$)

Table 2 Purine enzyme activities at baseline and changes from baseline after six and 48 weeks*. Values are shown as the mean (95% confidence interval).

	Baseline (n=103)	Week 6 – baseline (n=103)	Week 48 – baseline (n=76)
5'NT	16.9 (15.0 to 18.8)	2.1 (–0.6 to 4.8)	0.4 (–1.2 to 2.1)
ADA	80.3 (74.9 to 85.7)	–1.5 (–10.2 to 7.3)	–21.6 (–28.6 to –14.7)
PNP	206.8 (182.6 to 230.9)	–6.5 (–40.7 to 27.7)	–78.9 (–109.0 to –48.7)
HGPRT	8.7 (8.0 to 9.4)	–0.1 (–1.1 to 0.8)	–2.0 (–3.1 to –0.9)

*Enzyme activities are expressed in nmol/10^6 mononuclear cells/h of incubation. Reference values (from our laboratory obtained in patients with rheumatoid arthritis and healthy controls in former studies and partly published by Stolk *et al*[24]): 5'NT = 13.7 (SD 3.6) for men, 20.4 (SD 4.7) for women; ADA = 134 (SD 46); PNP = 212 (SD 54); HGPRT = 12.3 (SD 4.2).

Table 3 Spearman correlation coefficients between measures of disease activity (ESR and DAS) and changes in purine enzyme activities during 48 weeks of methotrexate treatment in patients with rheumatoid arthritis

	ESR t=0 (n=103)	Change in ESR after 48 weeks (n=76)	DAS t=0 (n=103)	Change in DAS after 48 weeks (n=76)
5'NT	0.16 p=0.11	0.25 p=0.03	0.23 p=0.01	0.11 p=0.34
ADA	–0.06 p=0.55	–0.10 p=0.39	0.04 p=0.68	0.10 p=0.38
PNP	–0.09 p=0.37	–0.16 p=0.19	–0.07 p=0.47	–0.09 p=0.47
HGPRT	–0.06 p=0.53	–0.04 p=0.71	0.13 p=0.18	0.07 p=0.54

*ESR, erythrocyte sedimentation rate; DAS, disease activity score.

Table 4 Relation between changes in purine enzyme activities from baseline and toxicity-related discontinuation of methotrexate treatment. Values are the mean (95% confidence interval)

		Toxicity related discontinuation (n=27)	
	No discontinuation (n=76)	All toxicity (n=27)	Hepatotoxicity (n=14)
5′NT	0.4 (–1.2 to 2.1)	–1.9 (–7.9 to 4.1)	–6.1 (–12.9 to 0.7)*
ADA	–21.6 (–28.6 to –14.7)	–19.1 (–31.4 to –6.8)	–14.9 (–25.5 to –4.3)
PNP	–78.9 (–109.0 to –48.7)	–38.7 (–86.6 to 9.2)	–67.6 (–133.6 to –1.5)
HGPRT	–2.0 (–3.1 to –0.9)	–0.9 (–3.2 to 1.4)	–3.4 (–6.5 to –0.4)

*No significant between-group differences were found with the exception of the decrease of 5′NT from baseline in the group of patients discontinuing methotrexate because of hepatotoxicity compared with no discontinuation (p=0.007).

(table 3). According to the EULAR response criteria, 35 patients (46%) showed a good response, 38 patients (50%) a moderate response, and three patients (4%) no response.[21] No relation was seen between changes in purine enzyme activities and response category (data not shown).

Toxicity

Discontinuation of MTX treatment because of adverse events occurred in 27/103 (26%) patients; in 14 patients withdrawal was due to raised ALT values and in 13 patients for a variety of reasons.

As a consequence of the premature discontinuation of MTX treatment, the mean MTX dosage was lower in the group of patients withdrawing from MTX treatment because of any adverse event (10.3 mg/week, SD 4.0) and in the subgroup of patients withdrawing from MTX because of hepatotoxicity (9.3 mg/week, SD 3.5) than in the group of patients continuing MTX treatment for 48 weeks (17.0 mg/week, SD 5.1).

Table 4 shows the results of changes in purine enzyme activities in patients who continued 48 weeks of MTX treatment compared with patients who discontinued MTX because of (hepato)toxicity. No significant between-group differences were found with the exception of the decrease of 5′NT from baseline in the group of patients discontinuing MTX because of hepatotoxicity (p=0.007).

DISCUSSION

In this study four enzymes from the purine salvage pathway were measured before and during MTX treatment in patients with RA. The main result was the statistically significant decrease of ADA, PNP, and HGPRT during MTX treatment, which had not been reported before in either rheumatology or oncology publications. These changes in purine enzyme activities were not associated with the efficacy of MTX treatment. This is confirmed by the finding that the greatest decline in the DAS occurred during the first six weeks of MTX treatment, while the purine enzyme activities at that time showed no significant changes. Also, no relation was shown between changes in these purine enzyme activities and the toxicity of MTX treatment.

In contrast, the enzyme activity of 5′NT decreased in the subgroup of patients discontinuing MTX because of hepatotoxicity. Analogously to the 5′NT deficiency that has been associated with azathioprine related bone marrow toxicity we expected, but did not find, low baseline 5′NT activity in this patient group.[25] Also, after six weeks' MTX treatment 5′NT activity had not yet changed from baseline. Apparently, 5′NT decreases at some point between six weeks and the last measurement at study withdrawal. It is uncertain whether the decrease in 5′NT is causally related to the occurrence of hepatotoxicity. The decrease in 5′NT activity during MTX treatment in a subgroup of patients might make them more prone to the hepatotoxic effects of MTX. The association of 5′NT with the DAS at baseline and the change in ESR after 48 weeks is probably due to chance, because we found no significant changes in 5′NT activity during the study.

While considering the meaning of the changes in purine enzyme activities during MTX treatment, we should first discuss the metabolism of adenosine, which has anti-inflammatory properties.[1 2] Two separate pathways lead to the formation of adenosine; the purine de novo synthesis and the homocysteine re-methylation route (fig 1). Degradation of adenosine is catalysed by ADA and indirectly by PNP via inosine.

In vitro studies show that MTX leads to adenosine release in fibroblasts and endothelial cells. Cronstein *et al* were the first to prove that MTX exerts an adenosine-induced anti-inflammatory effect in vivo in a mouse model.[1 2] In purine de novo synthesis, MTX inhibits glycinamide ribosyl-5-phosphate formyltransferase and amino-imidazol-carboxamide ribosyl-5-phosphate (AICAR) formyltransferase, leading to increment of AICAR and its precursor AICAribonucleoside. The effect on the subsequent formation of adenosine is not clear. Cronstein proposes two possible mechanisms by which MTX leads to increment of adenosine: (*a*) accumulation of AICAR causes inhibition of adenosine monophosphate deaminase with subsequent increase of adenosine monophosphate and finally adenosine, and (*b*) accumulation of AICAR inhibits ADA and subsequent increase of adenosine.[2] The latter fits well with the decrease of ADA activity we have noted during MTX treatment.

Homocysteine metabolism is affected by MTX, because inhibition of 5,10-methylene-tetrahydrofolate (THF) reductase leads to diminished availability of 5-methyl-THF and consequently reduced homocysteine remethylation; whether this has an effect on the formation of adenosine is not known. Inhibition of several metabolic steps by MTX is dose dependent. The dosages of MTX used in RA lead to partial inhibition and the resulting hyperhomocysteinaemia may lead to subsequent increase of S-adenosyl-L-homocysteine and adenosine. On the other hand, reduction of the formation of methionine, S-adenosyl-L-methionine and S-adenosyl-L-homocysteine may lead to a decrease of adenosine.

The effects of MTX on the enzyme activities of ADA and PNP are unknown. The decrease of ADA and PNP we observed during MTX treatment may be explained in several ways. Firstly, MTX might have a direct inhibiting effect on ADA and PNP. Secondly, MTX might inhibit ADA and PNP indirectly through, for instance, AICAR and its metabolites. Inhibition of the degradation route would subsequently lead to an increase of adenosine. Thirdly, ADA and PNP may be inhibited indirectly to compensate for the decrease of adenosine during MTX treatment, caused by inhibition of purine de novo synthesis and homocysteine remethylation. Theoretically, decrease of ADA and PNP would lead to increment of adenosine and subsequent anti-inflammatory effects. Unfortunately, we could not find an association between the decrease of ADA and PNP and the efficacy of MTX treatment in this study.

The effect of MTX on the enzyme activity of HGPRT is also not clear. MTX exerts an inhibiting effect on purine de novo synthesis, leading to an increase of 5-phosphoribosyl-1-pyrophosphate (PRPP). Because HGPRT is a co-substrate of PRPP one would expect an increase of HGPRT during MTX treatment. The decrease of HGPRT we found in our study may be explained by the declining demand of intracellular nucleotides, because MTX inhibits the DNA and especially RNA synthesis.

Finally, some points need to be discussed. The reason for blood sampling 16 hours after MTX intake was a concomitant study investigating the influence of MTX treatment on homocysteine metabolism. No data are available about the influence of time between intake of MTX and blood sampling for determining purine enzyme activities. Another point is whether the results are influenced by a change in the cells tested. In this study no measurements were done in lymphocyte subsets. However, a former study from our laboratory using the fluorescence activated cell sorter in patients with RA and healthy controls showed no differences between purine enzyme activities measured in B or T lymphocytes or CD4 and CD8 subsets (data not published). Although MTX itself may influence the division of various subsets of lymphocytes, it seems unlikely that the results from our present study are a simple reflection of a change in the cells tested.

In conclusion, this is the first study that shows changes in purine enzyme activities during MTX treatment of patients with RA. The decrease in enzyme activities of ADA, PNP, and HGPRT showed no association with the efficacy or toxicity of MTX treatment. In contrast, hepatoxicity was related to a decrease of the enzyme activity of 5′NT. Further studies are needed to determine the precise role of purine metabolism in RA and in treatment with MTX. Although adenosine measurements are technically difficult because it has a very short half life, it seems necessary to involve adenosine in future studies.

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