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Red blood cell methotrexate and folate levels in children with acute lymphoblastic leukemia undergoing therapy: a Pediatric Oncology Group pilot study

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Summary. We enrolled children with acute lymphoblastic leukemia (ALL) in a Pediatric Oncology Group (POG) pilot study to monitor erythrocyte (RBC) methotrexate (MTX) and folate (F) levels before and during treatment. The mean value for RBCF at diagnosis was 0.86 ± 0.46 nmol/ml RBC in the 214 patients who achieved remission and 1.21 ± 0.74 nmol/ml RBC in the 10 patients who did not ($P = 0.020$). Folate levels tended to increase during remission induction, but they dropped following an intensive consolidation with methotrexate to levels that were sustained throughout chemotherapy treatment. Methotrexate levels reached mean values of approximately 0.15 nmol/ml RBC at the end of an intensive methotrexate consolidation, then fell to levels that were sustained throughout maintenance therapy. There was a weak correlation between improved event-free survival and higher RBCMTX levels after consolidation, but no correlation was found between improved survival and the level of RBCMTX or RBCF during maintenance therapy. A larger study with more complete data is needed to determine whether RBCMTX or RBCF might be useful in predicting event-free survival in patients with ALL.

Introduction

The treatment of acute lymphoblastic leukemia (ALL) of childhood includes methotrexate (MTX) [11]. Although many regimens have shown marked success in treating the disease, with disease-free survival rates as high as 70%–80% being reported [6, 9, 33], the optimal use of MTX is not known. Doses of MTX used for the treatment of ALL have ranged from 15–20 mg/m^2 given weekly for maintenance therapy [1, 4, 19] to 500–2,000 mg/m^2 given every 2–4 weeks for consolidation therapy [12, 15, 30] to 33.6 g/m^2 given as part of consolidation [32] or CNS leukemia therapy [2].

MTX accumulates in normal tissues. Its pharmacology in erythrocytes (RBC) has been well characterized. After its uptake by erythroblasts [8], the drug is polyglutamylated [3, 39] and most of it is retained throughout the RBC life span [26]. Since RBCMTX appears to rise with increasing exposure of the RBC to the drug [34], it has attracted interest as a potential measure of overall drug exposure [36, 37]. MTX exposure might correlate with compliance, bioavailability, toxicity, and, possibly, treatment outcome [24, 25, 34, 35].

MTX is an analog of folic acid, and at least part of its antileukemic effect derives from inhibition of the enzyme dihydrofolate reductase, which reduces folates (F) needed for thymidylate synthesis. Kamen et al. [24, 25] have shown that RBCF levels tend to be low in patients treated with MTX, presumably because MTX competes with F for entry into cells [16]. Diet [29], malabsorption [28], and therapy with other antifols and antibiotics [7, 28] may also affect MTX and F levels.

We performed serial studies of RBCF and RBCMTX levels in an effort to catalog changes in those values during the treatment of childhood ALL. In addition, we attempted to correlate the outcome of therapy with the levels of RBCF and RBCMTX determined at preselected intervals.

Patients and methods

Study design. Patients were eligible if they were younger than 21 years of age and had non-B-, non T-cell ALL for which they had received no prior treatment. Pediatric Oncology Group (POG) study 8036 opened in 1980 for the treatment of ALL in children. This was a randomized treatment study stratified for high- or low-risk features as defined in Table 1. After undergoing a common induction and intensification therapy (detailed in Fig. 1), lower-risk patients received either intrathecal therapy (IT) every 8 weeks during maintenance (treatment S) or intravenous MTX

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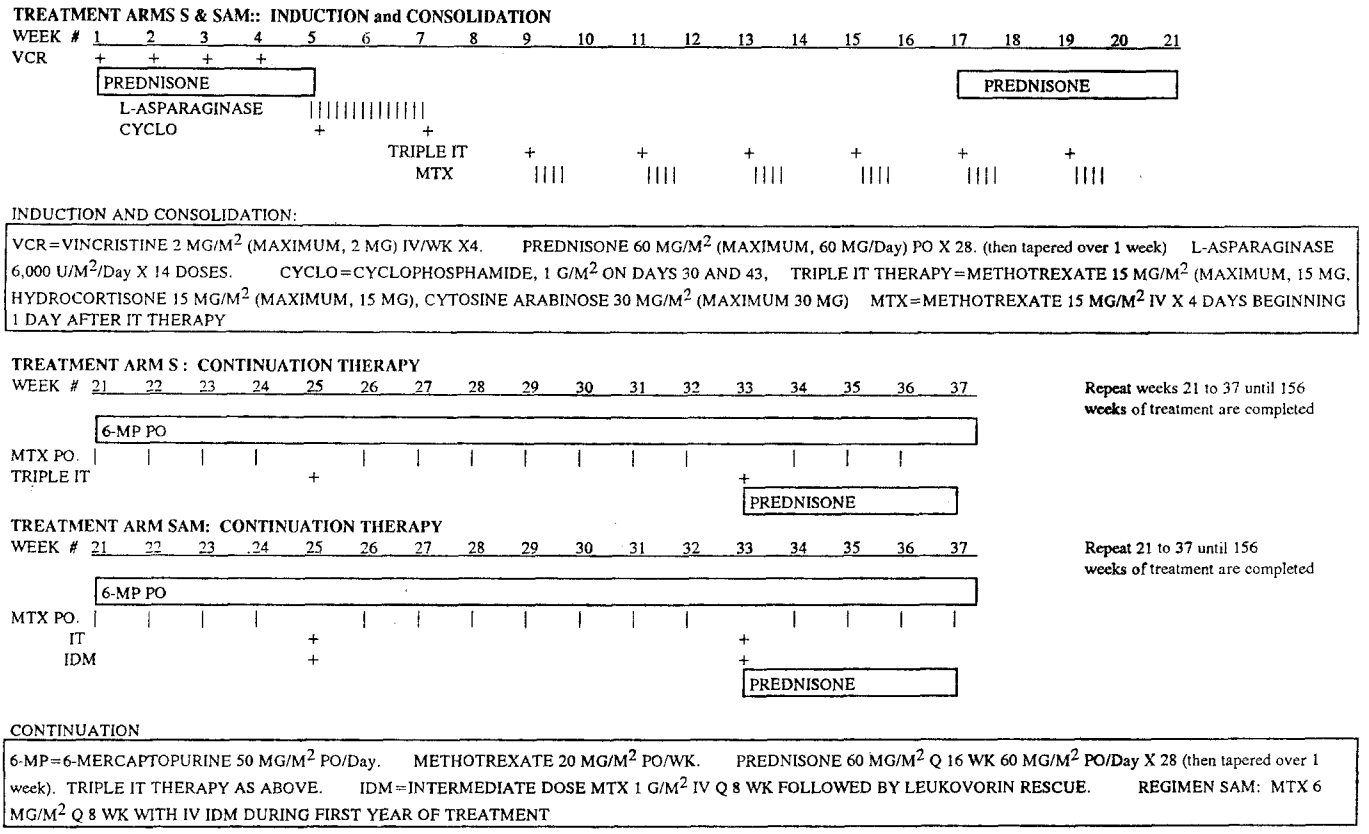

Fig. 1. Chemotherapy treatment plan

Table 1. Criteria for risk classification

Feature	Age (years)				
	<1	1–3	3–6	6–10	>10
WBC level ($\times 10^{-9}$/l):					
<10	B	A	A	A	B
10–99	B	B	A	B	B
≥100	B	B	B	B	B
Liver or spleen below umbilicus or extramedullary leukemia	B	B	B	B	B

A, Good risk; B, poor risk

(1,000 mg/m^2) with leucovorin rescue [and MTX (6 mg/m^2) IT during the first four treatments] every 8 weeks (treatment SAM). High-risk patients were randomized between these two treatments and a third regimen (SARA) with several rotating chemotherapy pulses patterned after the LSA_2L_2 regimen [41]. Patients on the SARA arm were excluded from all analyses described below except that for RBCF at diagnosis.

Beginning in July 1983, investigators were requested to send blood samples to a central laboratory at The Johns Hopkins Hospital for RBCF and RBCMTX analysis. Samples were requested at diagnosis, at the time of achievement of remission, at the end of the L-asparaginase/cyclophosphamide and IT/i. v. MTX consolidation phases, at 4 weeks after the start of continuation therapy, and every 8 weeks thereafter until relapse or discontinuation of therapy (Table 2). During continuation therapy, samples were obtained prior to IT or high-dose pulses of MTX. The cutoff point for analysis was May 1990.

Samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), which were sent by overland mail to the laboratory. Previous work from our laboratory (unpublished data) had demonstrated that RBCF and RBCMTX measurements did not change when blood samples were sent by carrier and kept in the laboratory at ambient temperatures for up to 10 days in evacuated tubes. RBCMTX and RBCF were measured using previously described radioligand binding [20, 23].

For the folate assay, 100 µl blood was added to 900 µl of 50 mM potassium phosphate buffer (pH 7.5) supplemented at 1 mg/ml each with bovine serum albumin and ascorbic acid. The processed samples were then frozen at –20° C until assay. For the methotrexate assay, 300 µl blood was added to a mixture of 1,200 µl 50 mM TRIS and 150 mM β-mercaptoethanol in 5 mM EDTA buffer adjusted to pH 8.0. The processed samples were boiled for 10 min and then spun in a microcentrifuge for 5 min. The resulting supernatant was frozen at –20° C until assay. The specificities of the MTX assay are such that a 100-fold excess of F does not alter MTX levels [20]; similarly, a 10-fold excess of MTX over folate (a quantity far above that seen in out study) does not alter folate levels [21].

Statistical considerations. Since patients were randomized only after they had achieved a complete remission and since treatment arm 3 (SARA) was given only to higher-risk patients, the major analyses were restricted to treatment arms 1 (S) and 2 (SAM), the lone exception being the correlation of pretreatment RBCF with induction response. The major analyses of outcome were conducted on the basis of the duration of complete remission, the time from the achievement of a complete remission or the time at which the remission sample was obtained (whichever was later) to the earliest of the parameters relapse, death, or last clinical contact. For assessment of the prognostic significance of RBCF or RBCMTX levels, the log-rank test [31] was used following stratification for treatment (S versus SAM). Student's *t*-test [14] was used to compare responders with nonresponders on the basis of pretreatment RBCF, whereas correlations among laboratory and clinical measures were evaluated using Pearson's correlation coefficients [14].

Table 2. RBCF and RBCMTX levels measured during the course of the present study

Week of treatment	At risk	Treatment S								At risk	Treatment SAM							
		Folate				Methotrexate					Folate				Methotrexate			
		N	Mean	SD	Med	N	Mean	SD	Med		N	Mean	SD	Med	N	Mean	SD	Med
0	121	101	0.83	0.41	0.71					103	89	0.85	0.39	0.78				
4	121	62	0.98	0.41	0.90					103	55	0.90	0.41	0.83				
9	116	52	1.12	0.55	1.01					99	47	1.02	0.41	0.99				
21	115	49	0.75	0.29	0.68	44	0.16	0.08	0.18	95	39	0.65	0.18	0.66	40	0.15	0.25	0.14
25	115	53	0.74	0.35	0.68	47	0.15	0.09	0.12	94	50	0.67	0.29	0.64	49	0.13	0.08	0.11
33	115	63	0.71	0.27	0.69	54	0.11	0.06	0.11	92	56	0.78	0.26	0.81	56	0.12	0.06	0.10
41	110	58	0.71	0.25	0.66	56	0.13	0.06	0.12	92	50	0.72	0.25	0.68	51	0.11	0.06	0.10
49	106	54	0.73	0.24	0.68	54	0.12	0.07	0.11	90	47	0.79	0.26	0.81	46	0.11	0.06	0.11
57	106	49	0.73	0.24	0.67	51	0.11	0.07	0.09	89	49	0.82	0.26	0.79	48	0.12	0.05	0.10
65	103	42	0.73	0.28	0.66	41	0.10	0.05	0.11	89	52	0.91	0.33	0.91	49	0.12	0.05	0.11
73	100	42	0.74	0.21	0.68	42	0.11	0.08	0.10	88	45	0.86	0.29	0.81	44	0.11	0.06	0.10
81	97	43	0.80	0.26	0.73	41	0.11	0.06	0.11	88	45	0.97	0.38	0.84	44	0.10	0.04	0.10
89	96	37	0.84	0.25	0.86	37	0.10	0.05	0.09	84	39	0.91	0.29	0.88	38	0.10	0.05	0.10
97	95	35	0.82	0.35	0.82	34	0.10	0.05	0.10	83	39	0.87	0.29	0.85	38	0.10	0.06	0.10
105	91	33	0.82	0.27	0.76	32	0.12	0.08	0.11	83	40	0.87	0.33	0.89	39	0.10	0.04	0.09
113	90	32	0.74	0.25	0.71	31	0.12	0.07	0.10	83	35	0.93	0.42	0.85	34	0.11	0.05	0.11
121	88	29	0.83	0.30	0.82	28	0.10	0.06	0.11	80	32	0.91	0.37	0.85	30	0.12	0.05	0.13
129	85	35	0.84	0.37	0.8	33	0.13	0.06	0.13	79	27	0.99	0.33	0.89	25	0.11	0.05	0.11
137	83	37	0.88	0.33	0.86	36	0.11	0.06	0.11	79	24	0.86	0.32	0.80	24	0.11	0.04	0.11
145	79	28	0.78	0.34	0.74	29	0.12	0.07	0.12	79	20	0.90	0.32	0.79	20	0.10	0.04	0.10
153	79	23	0.78	0.41	0.68	23	0.09	0.05	0.10	77	16	0.80	0.27	0.75	15	0.09	0.04	0.09

Folate and methotrexate levels are expressed in nanomoles per milliter of RBC. At risk, Number of patients on study from whom a sample was requested; N, samples received and processed; SD, standard deviation; Med, median value

Results

Of the 793 patients who were enrolled in the parent treatment study (POG 8036) after July 20, 1983, 272 were entered in the pharmacology pilot study. The number of samples received at the time points indicated in Table 2 diminished throughout the therapy, far in excess of the number of patients who relapsed or stopped therapy prematurely. As the significant dropoff in the quantity of samples may have resulted in a selection bias, all inferences must be made with caution.

RBCF levels at diagnosis

The mean RBCF level at diagnosis was 0.87 ± 0.47 nmol/ml RBC. The median was 0.71 nmol/ml RBC. The published range for healthy volunteers is 0.45–1.75 nmol/ml RBC [25]. Of the 224 values obtained at diagnosis, 185 (83%) were above 0.50 nmol/ml. To assess the relationship of folate levels at diagnosis with clinical features, Pearson correlations between baseline folates and WBC, platelet count, hemoglobin level, and age were calculated for patients with pretreatment folate values (including 34 who were later randomized to the SARA arm). Although none of the correlations was clinically significant ($r = 0.17$, $P = 0.01$ for WBC; $r = -0.06$, $P = 0.40$ for platelets; $r = -0.09$, $P = 0.17$, for hemoglobin; $r = -0.17$, $P = 0.1$ for age), WBC count and age correlations were statistically significant, with a slight tendency for folates to increase with WBC and to decrease with age. Our assay measures whole-blood folate, and there may have been contributions from leukemia cells and other leukocytes that have high folate contents [18, 38]. However, neither age nor WBC accounted for more than 3% of the variation in folate.

The mean levels of RBCF at diagnosis were 0.86 ± 0.46 nmol/ml RBC for the 214 patients who achieved remission and 1.21 ± 0.74 nmol/ml RBC for the 10 patients who failed to do so ($P = 0.020$). As noted above, only this analysis included patients who were treated on the third arm (SARA) of the treatment protocol.

For analysis of the correlation of the pretreatment folate level with the duration of remission, levels of <0.5, 0.5–0.75, 0.76–1.0, and >1.0 nmol/ml RBC were contrasted. Although pretreatment folate (Table 3) correlated significantly with age and WBC at diagnosis (two powerful predictors of outcome in POG ALL studies), no clear correlation of outcome with pretreatment folate was found, as noted in Table 3. The division of levels shown in Table 3 indicates a very high recurrence rate for patients with folate levels of between 0.50 and 0.75 who received treatment S; such a poor outcome was not noted for patients receiving treatment SAM, and no significant trend was noted for levels higher and/or lower than the 0.50–0.75 range. Because of the unusual pattern observed for the patients with folate levels of between 0.5 and 0.75, there was an overall tendency for patients with lower pretreatment folate levels to have poorer outcomes on treatment SAM. The reason for this is unclear.

Table 3. Pretreatment folate levels and event-free survival

Pretreatment folate	Treatment S			Treatment SAM		
	N	Fail	Expected	*N*	Fail	Expected
<0.5	20	4	7.8	12	3	2.7
0.5–0.75	36	20	10.6	31	5	7.0
0.76–1.0	22	6	8.2	27	6	5.5
>1.0	23	6	9.4	19	5	3.9
Totals	101	36	36	89	19	19

Folate levels are expressed in nmol/ml RBC. *n*, Number of patients
P values: Within treatment S (4-way), $P = 0.008$; within treatment SAM (4-way), $P = 0.80$

Table 4. Methotrexate levels measured at week 21

RBCMTX	Treatment S			Treatment SAM		
	n	Fail	Expected	*n*	Fail	Expected
0–0.15	20	7	5.4	24	7	5.2
>0.15	24	6	7.6	16	2	3.8

Methotrexate levels are expressed in nmol/ml RBC. *n*, Number of patients
P value (adjusted for treatment) for <0.15 vs >0.15 nmol MTX/ml RBC: $P = 0.15$

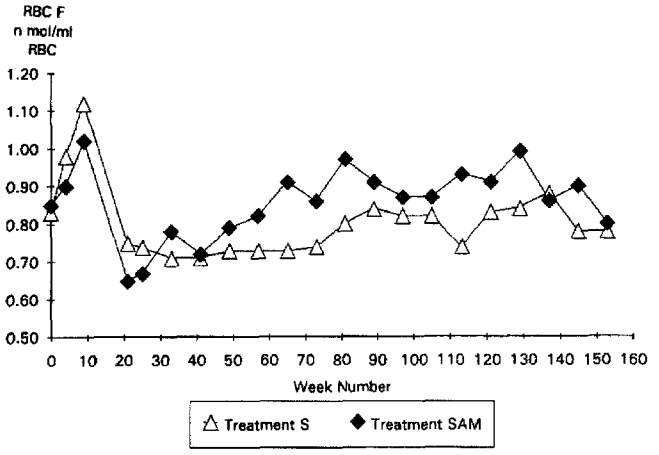

Fig. 2. Changes in Mean RBCF for Patients on Treatment Arms S and SAM During Chemotherapy for ALL

RBCF levels during therapy

Folate levels are plotted in Fig. 2. They tended to rise during remission induction and asparaginase consolidation, which may have reflected recovery from the effects of leukemia, improved dietary intake, or blood transfusions. At the conclusion of MTX consolidation (week 21), RBCF levels declined substantially (Table 2). Mean values of 0.75 ± 0.29 and 0.65 ± 0.18 nmol/ml RBC were obtained in treatment groups S and SAM, respectively. These were well below the mean values of 1.12 ± 0.55 and 1.02 ± 0.41 nmol/ml RBC obtained for the S and SAM groups, respectively, just before the start of the MTX consolidation. The fall in RBCF was significant (two-sided *P* value, <0.001 for both treatments according to the matched Z-test). This confirms the data of Kamen et al. [24], whose MTX-treated patients had RBCF levels much lower than could be accounted for by replacement with MTX alone.

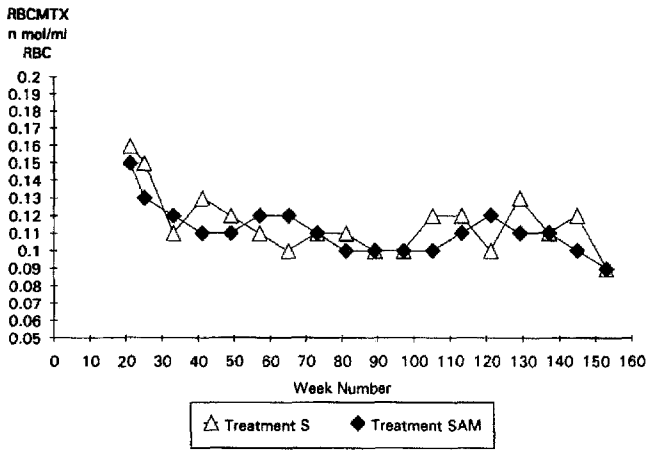

Fig. 3. Changes in Mean RBCMTX for Patients on Treatment Arms S and SAM During Chemotherapy for ALL

Folate levels changed relatively little throughout maintenance therapy. RBCF levels at weeks 9 (prior to MTX consolidation), 21 (at the end of MTX consolidation), 33 (during early maintenance), and 81 (during later maintenance) were also studied with respect to remission duration. No significant correlation was noted between the RBCF levels measured at those times and event-free survival (data not shown).

RBCMTX levels

RBCMTX levels are plotted in Fig. 2. MTX was first given at week 9 in both regimens. At week 21, the end of this consolidation, a median RBCMTX level of 0.18 nmol/ml RBC (mean, 0.16 ± 0.08 nmol/ml RBC) was noted in patients on treatment S and a median value of 0.14 nmol/ml RBC (mean, 0.15 ± 0.06 nmol/ml RBC) was observed in patients on treatment SAM. These findings were not significantly different. Treatments did not diverge until after this point. Week 21 RBCMTX levels were analyzed with respect to outcome and the results are recorded in Table 4. There was a weak trend toward improved remission duration in patients with higher RBCMTX levels ($P = 0.15$ for patients with ≤ 0.15 vs >0.15 nmol/ml RBC; Table 4).

Analysis of RBCMTX at week 33 (after 12 weeks of S or SAM therapy) showed no significant correlation with remission duration (data not shown). Despite the much greater MTX dose given to patients on the SAM regimen as compared with those on the S regimen (1 g/m^2 given i.v. $\pm$ 6 mg/m^2 IT every 8 weeks vs 15 mg/m^2 IT every 8 weeks, with all patients receiving weekly oral MTX for the other 7 weeks of every 8-week cycle) RBCMTX levels were similar in the two treatment groups.

Discussion

Previous work has suggested that MTX pharmacokinetics may have an impact on the outcome of antileukemia treatment. Craft et al. [10] demonstrated that patients with ALL undergoing treatment on an intensive MTX schedule who absorbed the drug more slowly had a higher relapse rate. Evans et al. [13] have recorded higher early-relapse rates for patients showing faster systemic clearance of MTX following infusions of 1 g/m^2. Borsi and Moe [5] noted the same prognostic significance of rapid clearance in patients who received consolidation treatment of up to 8 g/m^2.

Previous work conducted in our laboratory has suggested a significant correlation between postconsolidation RBCMTX levels and mean steady-state MTX-infusion levels for patients receiving a series of 12 infusions at 1 g/m^2 MTX every 2 weeks [17]. RBCMTX has the advantage of paralleling what may be incorporated into and retained by target cells (e.g., lymphoblasts) rather than reflecting only the serum levels to which the cells are exposed. For example, the usefulness of high-dose MTX has recently been questioned [22] on the basis of observations in other tumor systems that 100- to 1,000-fold increase in MTX concentration may enhance some intratumor levels by only 2 or 3 times [40]. This, in turn, would suggest that a measure of net drug uptake, such as RBCMTX, might be useful as a measure of antileukemia efficacy, although our data failed to confirm this.

It is noteworthy that RBCMTX levels were similar in our study in patients who were treated with or without high-dose MTX pulses. This may reflect the observation that MTX is taken up by RBC only during the erythroblast stage and that the percentage of circulating RBC that are exposed during the erythroblast stage to the 2- or 3-day period of circulating high MTX levels may be too small to alter RBCMTX levels significantly. Alternatively, RBCMTX may have a turnover more rapid than the 56 days between pulses. In our study, RBCMTX levels obtained at week 25 (4 weeks after the end of the MTX consolidation) remained high, reflecting the intense exposure during consolidation. Levels obtained at week 33 (12 weeks after the end of the MTX consolidation) lay within the range measured during later continuation therapy, suggesting that RBCMTX levels reflect a period of exposure of between 4 and 12 weeks.

Schroder [35] saw no significant difference in RBCMTX during continuation therapy in 4 patients who later relapsed as compared with 46 who remained relapse-free. Our study also showed no significant correlation between RBCMTX during maintenance therapy and the outcome of therapy. The relatively narrow range of maintenance MTX doses (usually 15–30 mg/m^2) may not produce differences in exposure large enough to result in differences in outcome. Another possibility is that very small doses of MTX are well above necessary cytotoxic thresholds. Alternatively, recent work [27] has suggested that patients with higher 6-mercaptopurine (6MP) metabolite levels attain much higher rates of disease-free survival. The marked excess of delivered 6MP as compared with MTX (350 vs 20 mg/m^2 weekly) may make the 6MP-metabolite levels of greater importance in determining the outcome of therapy.

In summary, we found a small trend toward improved remission duration in patients showing higher RBCMTX levels after undergoing MTX consolidation. We saw no significant correlation between remission duration and the MTX or folate levels measured during maintenance therapy. The relatively low number of patients investigated in our study (as compared with the chemotherapy group as a whole) and the variability in the completeness of sample collection require us to exercise caution in interpreting our results. A larger pharmacology study including much greater patient accrual and more complete sample collection is under way in a subsequent POG trial and will attempt to establish definitive correlations of RBCMTX and RBCF levels with leukemia-free survival.

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