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6-Thioguanine nucleotide accumulation in red blood cells during maintenance chemotherapy for childhood acute lymphoblastic leukemia, and its relation to leukopenia

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Summary. In the present study of 12 boys and 19 girls 2–16 years of age (median, 7 years) on oral 6-mercaptopurine (6MP) and methotrexate (MTX) maintenance therapy (MT) for non-B-cell acute lymphoblastic leukemia (ALL), we found that (a) during MT, 6-thioguanine nucleotides (6TGN) (the major cytotoxic metabolite of 6MP) accumulate in the erythrocytes (E-6TGN); (b) for patients receiving an unchanged dose of 6MP, no significant correlation could be demonstrated between the mean E-6TGN (mE-6TGN) and the dose of 6MP ($r = -0.11$, $P = 0.28$) (31 patients); (c) among 21 patients receiving 50–75 mg/m^2 6MP, a variation of up to 3 orders of magnitude in mE-6TGN could be demonstrated, with the interindividual coefficient of variation (CV) in mE-6TGN for these patients being 0.31; (d) the median intraindividual CV in E-6TGN at an unchanged dose of 6MP was 0.11 (range, 0.04–0.18); and (e) the degree of myelodepression as measured by the mean white cell count was related to mE-6TGN ($r = -0.55$, $P = 0.0006$). These results indicate that E-6TGN could be a useful parameter for monitoring 6MP maintenance chemotherapy, although this needs to be explored in prospective studies.

Introduction

Daily oral 6-mercaptopurine (6MP) (50–90 mg/m^2) and weekly oral methotrexate (MTX) (20–30 mg/m^2) have been the backbone of maintenance chemotherapy (MT) for childhood acute lymphoblastic leukemia (ALL) for about two decades.

By itself, 6MP has little cytotoxic activity, but it is phosphorylated to thioinosine monophosphate by hypoxanthine-guanine phosphoribosyl transferase, using phosphoribosyl pyrophosphate as a cofactor, and is thereafter converted to its 6-thioguanine nucleotides (6TGN) [1], which mediate the cytotoxic effect of 6MP through their incorporation into DNA [20].

The cytotoxic effect of MTX is mainly due to intracellular polyglutamation of MTX (MTXpg), by which the affinity of binding to the folate-dependent target enzymes of MTX is enhanced [4]. In addition, polyglutamation leads to retainment of MTXpg in the cell, thus creating an intracellular pool of MTXpg [4].

The interindividual variations in the absorption, metabolism, and excretion of 6MP and MTX, as well as the intracellular build-up of 6TGN and MTXpg, are considerable [9, 11, 13, 18, 19, 21]. Although these variations in pharmacokinetics could be expected to influence the impact of therapy and, hence, the risk of relapse [7, 15], and some indications there of have been given [5, 16], little is actually known of their clinical significance.

Erythrocytes probably possess the enzymatic pathways necessary for the formation of 6TGN [10], which accumulates in erythrocytes (E-6TGN) during MT, although with large interindividual variation [13]. This interindividual variation could express genetically determined differences in the metabolism of 6MP and the ability to form 6TGN, as suggested by Lennard and Lilleyman [12] and Lennard et al. [14], as well as differences in the absorption and excretion of the drug [11, 21], i.e. systemic 6MP exposure. if this is the case, accumulation of 6TGN in red blood cells (RBC) may parallel the events in other tissues, including bone marrow stem cells; thus, the interindividual variation in E-6TGN could reflect differences in the intensity of 6MP MT.

In the present study we explored the inter- and intraindividual variations in E-6TGN, as well as their relation to

Abbreviations: ALL, acute lymphoblastic leukemia; E-MTX, erythrocyte concentration of methotrexate; E-6TGN, erythrocyte concentration of 6-thioguanine nucleotides; mE-6TGN, mean E-6TGN; mWBC, mean white cell count; 6MP, 6-mercaptopurine; MT, maintenance chemotherapy; MTX, methotrexate; MTXpg, methotrexate polyglutamates; WBC, white cell count

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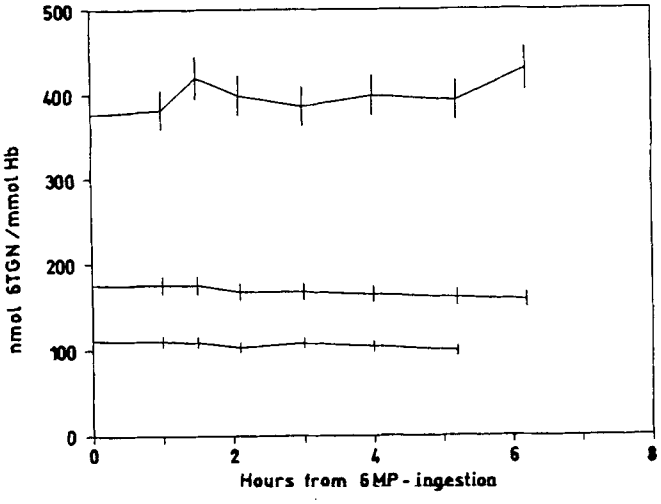

Fig. 1. Changes in E-6TGN during the hours following oral administration of 75 mg/m² 6MP to three patients with high, intermediate, and low steady-state E-6TGN. The *vertical lines* represent the standard deviation

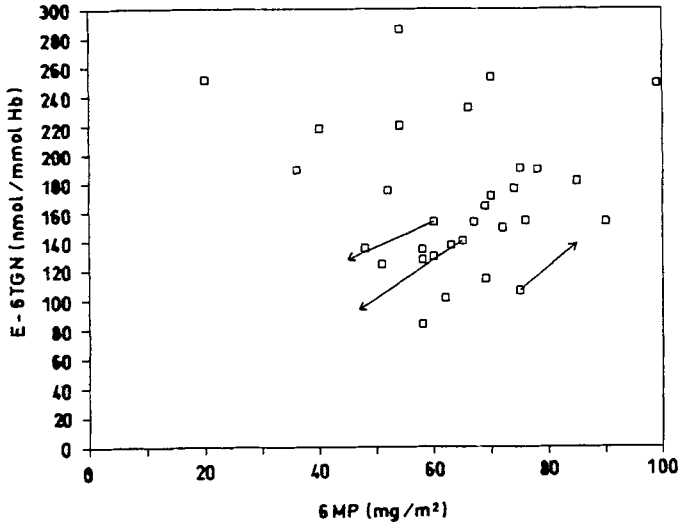

Fig. 2. Scattergram of steady-state levels of E-6TGN (mE-6TGN) in relation to the daily oral dose (per square meter) of 6MP. $r = 0.11$, $P = 0.28$; 31 patients. *Arrows* indicate changes in the dose of 6MP and corresponding steady-state E-6TGN for 3 patients

the 6MP dose and bone marrow depression as measured by the mean white cell count (mWBC) during MT. mWBCs have previously been identified as a prognostic factor for the risk of hematological relapse by standard-risk patients [16]. A Danish population-based study including all risk groups (122 patients diagnosed 1981–1985; median length of follow-up, 62 months) has confirmed a significant relationship between mWBC and the risk of hematological relapse ($P = 0.007$) as well as overall relapse risk ($P = 0.02$) (submitted).

Patients and methods

Patients. The patient cohort consisted of 12 boys and 19 girls with non-B-cell ALL who were >1 and ≤ 15 years of age at diagnosis, which includes all patients but one treated at the Pediatric Department, University Hospital, Copenhagen, during the study period (between March 1987 and October 1988). The last patient was excluded by a constantly changing dose of MTX or 6MP due to recurrent toxicity and febrile illness. At the time of the study, the median age of the patients was 6 years and 11 months (range, $2^2/_3$–$16^5/_6$ years). According to criteria given elsewhere [8], at diagnosis 6 patients were classified as being standard risk; 17, as being intermediate risk; and 8, as being high-risk ALL patients. All but one patient (with previous testicular relapse) were in first remission.

Therapy. Induction and consolidation therapy differed among the patients according to risk classification. In all cases, MT comprised daily oral 6MP (target dose, 50–75 mg/m²) and weekly oral MTX (target dose, 20 mg/m²), the doses of which were adjusted in an attempt to keep the WBC between 1.5 and $3.5–4.0 \times 10^9$/l. However, due to fluctuations in WBC, even at an unchanged drug dose [16], this was not achieved in all patients, and many subjects with intermittent WBC of $>4 \times 10^9$/l were given no more than the target dose of the drugs. The median prescribed dose of MTX and 6MP during the periods included in the analyses were 19 mg/m² (range, 10–22.5 mg/m²) and 65 mg/m² (range, 20–99 mg/m²), respectively. In addition to oral MTX and 6MP, six patients received vincristine (VCR)-prednisone reinductions at 3- to 4-month intervals as part of their MT. No other medication was given during the MT periods included in the analyses.

Hemoglobin values and platelet and WBCs, together with a physical examination, were done at least every 4 weeks during MT. At the time of inclusion in the study, all patients had been on MT for at least 3 months.

Analyses. For every patient, a mean WBC (mWBC) was calculated for a period of MT of at least 3 months (range, 3–13 months; median, 7 months), during which the dose of MTX and 6MP had been unchanged, and E-6TGN values were measured after a minimum of 5 weeks at an unaltered dose of 6MP. Three patients were studied for two MT periods at a different dose of 6MP.

MWBC was calculated as a weighted mean of all WBC measurements within a study period, using as weight the intervals between sampling. It was thus assumed in the analyses that any WBC was unchanged until the next was sampled. This gives a better index of the WBCs during the periods analysed than does a simple arithmetic mean of the WBC measurements available, which tend to favor periods with multiple WBC measurements due to leukopenia.

E-6TGN was measured using an HPLC method as previously described in detail elsewhere [3]. In short, 6 mg mercuric cellulose was added to 100 µl hemolysed RBCs to capture the 6TGN. Following washing of the cellulose, the 6TGN was released with β-mercaptoethanol, after which sulphuric acid was added, and set to heat at 98° C for 1 h to hydrolyse the 6-TGN to 6-thioguanine (6TG). Finally, 6TG was quantitatively detected at 342 nm on an HPLC system with a 5-µm Lichrosorb RP-18 (0.4 × 30 cm) column. E-6TGN was in all cases assayed in duplicate and expressed as nmol 6TGN/mmol hemoglobin (Hb). The within- and between-run CV of the assay in the analysis of samples with E-6TGN >100 nmol/mmol Hb was 5%–7%. No precautions were taken to avoid blood sampling during the hours following 6MP medication, since this factor influences neither the assay [3] nor E-6TGN values (Fig. 1). For each patient, E-6TGN was measured 2–8 times during the period of MT included in the analyses (median number of measurements, 4) and calculated as a mean of these measurements (mE-6TGN).

Statistical analyses. The correlation between variables was tested with linear regression analyses using the least-squares method (r = coefficient of correlation) as well as with Spearman's non-parametric rank-correlation analysis (r_s = Spearman's correlation coefficient).

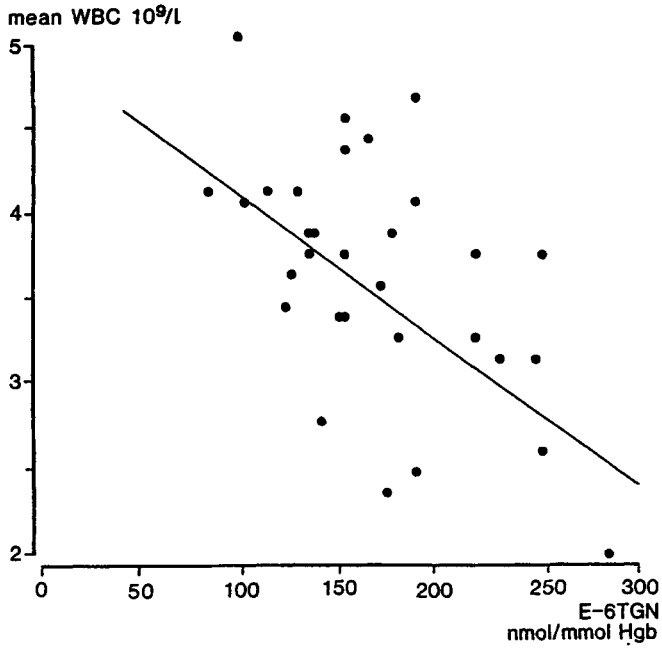

Fig. 3. Scattergram of mean WBC (mWBC) in relation to the steady-state levels of e-6TGN (mE-6TGN). $r = -0.55$, $P = 0.0006$; 31 patients (Y = 5.00-X*0.0079)

Results

E-6TGN

Among the patients, mE-6TGN varied from 85 to 286 nmol/mmol Hb (median, 154 nmol/mmol Hb), with an interindividual CV of 0.30. Even among patients receiving a comparable dose of 6MP (50–75 mg/m^2, 21 patients), a variation of up to 3 orders of magnitude in mE-6TGN was found (Fig. 2) (CV = 0.31). The median intraindividual CV for E-6TGN in patients in whom E-6TGN was measured at least 3 times during a study period (28 patients) was 0.11 (range, 0.04–0.18). The intraindividual CV for E-6TGN at an unchanged dose of 6MP was not significantly related to the E-6TGN concentration, the Hb value, or the dose of 6MP.

When all 31 patients were included, mE-6TGN was not significantly influenced by gender, age at the time of analysis, previous therapy (i.e., risk classification), the preceding total dose of 6MP given during MT, or the length of the preceding period of MT; neither was mE-6TGN related to the dose of 6MP ($r = -0.11$, $P = 0.28$; $r_s = 0.05$, $P = 0.40$) (Fig. 2). The lack of a correlation between mE-6TGN and the 6MP dose was demonstrated for both sexes. However, for three patients studied at two different periods of MT during which they received different doses of 6MP, a positive intraindividual correlation could be demonstrated between the dose of 6MP and E-6TGN (Fig. 2).

Patients with mE-6TGN values either below or above 154 nmol/mmol Hb (the median me-6TGN value of the study population) did not differ significantly in their prescribed dose of 6MP and MTX, red blood cell accumulation of MTXpg, gender, or age (Table 1).

mWBC

For each patient the WBC fluctuated considerably, with the median CV in WBC being 0.23 (0.10–0.41). Among the 31 patients, the mWBC varied from 2.0 to 5.1 × 10^9/l (mean, 3.6 × 10^9/l; CV, 0.20). The median SEM for mWBC was 0.3 × 10^9/l (0.1–0.6 × 10^9/l).

Neither sex, WBC at diagnosis, age at the time of analysis, previous medication (i.e., risk group), nor duration of MT significantly influenced mWBC. However, mWBC was found to be significantly related to mE-6TGN, as shown in Fig. 3 ($r = -0.55$, $P = 0.0006$; $r_s = -0.50$, $P = 0.0034$). Exclusion of the six patients who received VCR-prednisone reinductions only slightly altered this relationship between mWBC and mE-6TGN ($r = -0.51$, $P = 0.004$). The coefficient of correlation was almost identical for boys and girls ($r = -0.56$ and -0.58, respectively). The CV for the fluctuations in WBC was not related to E-6TGN ($r = 0.14$, $P > 0.20$; $r_s = 0.002$, $P > 0.40$).

A positive correlation existed between the dose of 6MP and mWBC ($r = 0.45$, $P = 0.004$; $r_s = 0.34$, $P = 0.003$); however, this only reflects that patients with the higher mWBC were given the larger dose of 6MP, as recommended in the protocols.

Discussion

In spite of impressive progress in the treatment of childhood ALL during the last 20 years, >30% of patients may still relapse [8]. This could at least partly be due to differences in the pharmacokinetics of the drugs used in the protocols, as has been suggested by a few studies [2, 5, 6, 16]. If interindividual variations in the pharmacokinetics of oral 6MP and MTX during MT have any impact on remission duration, there is a need for parameters that can be used in studying the prognostic significance of these

Table 1. Characteristics of patients with high and low E-6TGN

E-6TGN (nmol/mmol Hb)	Patients (*n*)	Mean age (years)	Sexratio (M/F)	Mean dose of 6MP (mg/m^2)	Mean dose of MTX (mg/m^2)	Mean E-MTXpg (nmol/mmol Hb)	mWBC (10^9/l)
All patients	31	$8^7/_{12}$	12/19	64	18	7.8	3.6
<154	16	$8^5/_6$	4/12	65	18	8.0	3.9
<154	15	$8^1/_3$	8/7	63	18	7.7	3.3

differences and that are applicable for dose adjustment. The low intraindividual CV for E-6TGN and the correlation of mE-6TGN to mWBC, as found in this study, suggests that E-6TGN could be of value in this respect. We have previously demonstrated that mWBC during MT is related to relapse risk [16], which emphasizes the significance of the relationship between this parameter and E-6TGN. The small intraindividual variation of E-6TGN (CV = 0.11) at an unaltered dose of 6MP seems to rule out the possibility that the relationship between mWBC and mE-6TGN merely reflects differences in patient compliance.

Lennard et al. [13] have previously demonstrated a relationship between E-6TGN and the absolute neutrophil count (ANC) measured 14 days later, which should reflect the latency of 6MP's determined bone-marrow cytotoxicity. However, since E-6TGN is stable at an unchanged dose of 6MP, a correlation with the ANC at other times of sampling would be expected. The failure of these investigators to demonstrate such a relationship could have been due to the bias of single ANC measurements, which, in contrast to the mean ANC (as well as mWBC), may be subject to considerable fluctuation [16].

In the present study we also could not confirm the relationship between mE-6TGN and the dose of 6MP that was reported by Lennard et al. [13]. However, since many of their patients were included in the analyses with several sets of "E-6TGN/6MP-dose" values, not every set of parameters was independent. In addition, these works included RBC samples from patients who had not received 6MP for $\geq$ 1 week; as expected these samples had only a small pool of 6TGN, if any. Although an intraindividual relationship exists between E-6TGN and the 6MP dose (Fig. 2), we doubt that a clinically significant interindividual correlation between these variables can be demonstrated.

It should be emphasized that we do not presently know whether red blood cells differ from malignant lymphoblasts in their ability to generate 6TGN and MTXpg; however, this could be the case. Thus, in a study of leukemic lymphoblasts at diagnosis and relapse, Zimm et al. [22] found multi-fold intraindividual changes in the activity of enzymes involved in 6MP metabolism, which to a certain extent contrast the low intraindividual variations in E-6TGN that we demonstrated. However, the question as to whether the development of these changes, which in some patients could have been responsible for therapy faialure, might be influenced by interindividual differences in systemic 6MP and MTX exposure remains unanswered, as does that concerning whether E-6TGN might be a clinically useful index for such changes. Lennard and Lilleyman [12] have also reported changes in the activity of RBC enzymes involved in 6MP metabolism during MT; however, the clinical significance of these and their relationship to changes in the malignant leukemic blasts remain to be explored.

We have previously reported an intraindividual stability in the accumulation of MTXpg in erythrocytes (E-MTX) at unchanged MTX dose and a negative correlation of mWBC with E-MTX [17]. To explore the independent and combined clinical significance of mWBC, E-6TGN and E-MTX as to relapse risk, as well as the relationship of E-6TGN and E-MTX to toxicity and the risk of severe infections during MT, a prospective clinical study has been initiated by the Nordic Society of Pediatric Hematology and Oncology (NOPHO).

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