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The accumulation of mercaptopurine metabolites in age fractionated red blood cells

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1 Red blood cells from four children with lymphoblastic leukaemia were age fractionated on Percoll density gradients into 'young', 'middle-aged' and 'old' cells.
2 The rates of accumulation of the mercaptopurine (MP) metabolites thioguanine nucleotides (TGNs) and methylmercaptopurine nucleotides (MeMPs) were measured in the cell fractions from the start of MP continuing chemotherapy.
3 TGNs and MeMP metabolites were present in all the red cell fractions after 3 days oral MP. There was no significant difference between the metabolite concentrations measured in either young, middle-aged or old cells (Mann-Whitney P = 1.0 to 0.12).
4 These observations suggest that MP metabolites do not enter red cells at the stem cell level at the start of therapy.
5 With respect to the monitoring of therapy, these results suggest that the concentration of TGNs after 7 to 10 days MP could be used to predict eventual steady-state concentrations using a simple model.

Keywords childhood leukaemia mercaptopurine thioguanine nucleotides red cell metabolism

Introduction

Mercaptopurine (MP) and methotrexate (MTX) have been the backbone of continuing chemotherapy in acute lymphoblastic leukaemia (ALL) for the last two decades and the most successful protocols for 'standard risk' disease include a prolonged period of daily MP coupled with weekly MTX. In children with ALL the concentration of MP intracellular metabolites has been shown to be clinically important. The red blood cell (RBC) concentration of MP derived thioguanine nucleotides (TGNs) appears to correlate with relapse free survival independently of other prognostic factors [1]. During MP treatment these metabolites accumulate slowly within RBCs on a time scale of days and weeks rather than hours [2]. That observation raises questions as to the source of the metabolites.

If TGNs are responsible for the therapeutic response to MP then an understanding of the influences involved in their generation and elimination becomes a part of any therapeutic drug monitoring programme. They could be formed entirely within RBCs during a course of therapy or come from MP metabolites originating elsewhere.

There are many interacting variables which can influence the formation of TGNs, but one of the most important is drug metabolism. MP undergoes extensive first-pass metabolism along three major pathways: oxidation, methylation and nucleotide formation [3]. The nucleotide pathway results in the eventual formation of TGNs, but the production of TGNs can be influenced by drug methylation [4]. The activity of the methylation enzyme (thiopurine methyltransferase; TPMT; EC 2.1.1.67) is controlled by a common genetic polymorphism [5]. There is an inverse relationship between the inherited level of TPMT activity and the concentration of TGNs produced at a standard dose of MP [6] and this helps to explain the large interindividual differences in RBC TGNs measured in children taking identical MP dosages. TPMT can produce a number of methylmercaptopurine metabolites (MeMPs). The nucleotide metabolites are easily measured in RBCs at the same time as the TGNs.

Evidence exists from both *in vitro* [7] and *in vivo* [8] studies that the delayed action of MP may indi-

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cate the incorporation of active metabolite(s) in DNA through the stem cells of bone marrow. To investigate the possibility of metabolite incorporation into red cell precursors in the bone marrow at the start of oral MP therapy we have studied TGN and MeMP metabolite concentrations in age fractionated RBCs. The fractionation of RBCs on Percoll density gradients reliably reflects red cell age with the youngest cells at the top and the oldest at the bottom [9]. The aim of this study was to gain a better understanding of the mechanisms involved in and the time course of the incorporation of active metabolites into RBCs.

Methods

Patients and study design

Four children (three boys and a girl) aged 2, 3, 3 and 6 years were recruited into the study. The patients had common ALL and were undergoing chemotherapy according to the Medical Research Council therapeutic protocol UKALL XI. The children were studied at the start of the continuing phase of chemotherapy (protocol week 8) from their first dose of MP. Two children (JB1 and MD2) were randomised to receive intrathecal MTX at weeks 9, 10, 11 and 12 whilst the remaining two children (AL3 and CW4) were randomised to intrathecal MTX plus high dose MTX with folinic acid rescue on weeks 9, 11 and 13. Basic maintenance chemotherapy consisted of daily MP, weekly MTX and monthly vincristine plus prednisolone.

Continuing chemotherapy per protocol included oral MP and weekly oral MTX given at standard doses of 75 mg m^{-2} and 20 mg m^{-2} respectively. Blood counts were done weekly and protocol directed parallel dose reduction of both MP and MTX were made if the neutrophil count fell below 1.0×10^9 l^{-1} and/or the platelet count fell below 100×10^9 l^{-1}. When the blood cell count recovered a protocol directed cycle of parallel dose increments followed. The children also received vincristine (1.5 mg m^{-2}, i.v.) and a 5 day pulse of oral prednisolone (40 mg m^{-2}), irrespective of blood count, at 28 day intervals.

The children were given their oral MP before breakfast and blood samples (5 ml, lithium heparin) were taken via their central venous catheters at least 5 h after the oral dose. The samples were taken every day for the first 3 to 4 days, when the child was an in-patient, and then twice weekly for the next 2–3 weeks. Red cells were not fractionated at the time of methotrexate therapy. Blood samples for cell fractionation were taken prior to and at least 3 days after the start of high dose i.v. MTX and/or i.t. MTX. All patient procedures were approved by the Sheffield Southern District Hospitals' Ethics Committee.

Blood fractionation

Leucocytes were removed from the blood samples by filtering through a 1:1 mixture of α-cellulose (Sigma C-8002) and microcrystalline cellulose (Sigmacell S-5504) [10]. The cellulose mixture was placed in 5 ml syringes to the 0.5 ml mark. The cellulose was wetted with 1 ml 0.9% NaCl prior to the addition of 1 ml blood. The blood was eluted with 2 ml 0.9% NaCl. The eluted red cells were washed and resuspended in Hanks balanced salt solution to a haematocrit of 40%.

The gradients were prepared by the method described by Salvo *et al.* [11] with modifications in the buffer system used. A sodium phosphate buffer was used with the concentration of the sodium and potassium chloride salts adjusted to keep the osmolarity of the system within the physiological range. Stock solutions of 3.5% bovine serum albumin (BSA, Sigma Cohn fraction V, pH 7.0) in Milli Q water (Millipore Ltd), 3.5% BSA in Percoll (Pharmacia) and a 0.4 M sodium phosphate buffer containing 2.3 M NaCl and 0.08 M KCl were prepared. The BSA-H_2O and BSA-Percoll stocks were stored at 4° C and brought to room temperature prior to gradient construction. Solution 1 was prepared by adding phosphate buffer to BSA-H_2O (1.0 ml to 19.0 ml respectively) and solution 2 by adding phosphate buffer to BSA-Percoll (0.82 ml to 19.18 ml respectively). Solution 1 ('0%' Percoll) and solution 2 ('100%' Percoll) were then mixed to produce gradients of '50', '57', '60', '63', '66' and '69'% (v/v) Percoll. The density of the gradients was measured using a 10 ml densitometry bottle and the osmotic pressure by a Micro-Osmometer (Model 3MD, Advanced Instruments Inc. USA).

The discontinuous Percoll gradients were assembled in 12 ml conical disposable test-tubes using 2 ml of each Percoll solution except for the 50% fraction, when 1 ml was used. The sharpest gradients were constructed by squirting the 69% Percoll directly into the bottom of the test-tubes and adding each subsequent gradient, drop by drop, to the resulting 'bubble' interface. Too smooth an interface at this stage resulted in mixing of the gradient layers. Leukocyte filtered blood (500 μl) was added to the top of each gradient and the tubes centrifuged at 1100 *g* for 10 min at 20° C.

After centrifugation the layer above the 50% gradient, which contained no red cells, was discarded. Each gradient fraction removed contained the cells in that fraction and lying on the interface layer beneath. All fractions of the same Percoll concentrations were pooled, washed twice and resuspended in Hanks balanced salt solution. The RBCs in each fraction were then counted. Control blood samples, taken from the same adult, were used to check the gradient fractionation procedure.

TGN and MeMP metabolite assays

The MP metabolites TGNs and MeMPs were assayed as previously described [12]. The lower limits of quantitation were 30 pmol and 120 pmol/8×10^8 RBCs for the TGNs and MeMPs respectively. To provide sufficient red cells for duplicate assay the fractions were pooled for analysis such that the two

top gradients (50 and 57% Percoll), the two middle gradients (60 and 63% Percoll) and the two bottom gradients (66 and 69% Percoll) were combined to produce top, middle and bottom fractions. The method of pooling was to take a volume of cells from each fraction such that the total volume taken contained 8×10^8 cells but the population of red cells from each fraction represented that isolated in each fraction. If, e.g. the 50% fraction contained a total of 1.12×10^9 cells and the 57% fraction a total of 3.21×10^9 cells (i.e. 25.9% and 74.1% of red cells respectively) the 8×10^8 red cells assayed would consist of 2.06×10^8 cells (25.9%) from the 50% fraction and 5.93×10^8 cells (74.1%) from the 57% fraction.

Results

Gradients

The measured density of Percoll was 1.134 g ml^{-1}, which was in good agreement with the density value stated by the producer (1.129 g ml^{-1}). The density of each 50, 57, 60, 63, 66 and 69% Percoll fraction was 1.058, 1.069, 1.074, 1.078 and 1.081 g ml^{-1} respectively and the osmotic pressure of the gradients ranged from 286 to 289 (median 288) mosmol kg^{-1} H_2O. The quoted range for paediatric plasma samples is 275 to 295 mosmol kg^{-1} H_2O. The pH values of gradients were consistently between 7.36–7.41.

Red cell population in fractions

The distribution of control RBCs fractionated on the gradients at the re-make of the Percoll stock solutions showed a small intra-person variation with 1 to 2% (median 1.5), 6 to 15% (median 8), 36 to 38% (median 38), 32 to 40% (median 36) and 15 to 16% (median 16) of cells harvested from the 50, 57, 60, 66 and 69% Percoll gradients respectively.

The children studied showed a large inter-individual variation in the percentage of RBCs in each gradient, but with the exception of 1 child (MD2), a low intra-individual variation. Table 1 shows the percentage of red cells in each gradient at weekly intervals throughout the study.

Metabolite concentration

The blood samples were obtained 6.25 to 7 h (median 6.5) post-dose. The red cell fractions obtained from these blood samples were assayed for MP metabolites in 10 separate 'runs' over a 3 month period. The coefficient of determination (R^2) for TGN analysis ranged from 0.96 to 1, median 0.99 and for the MeMPs 0.89 to 0.99, median 0.98. The interassay coefficient of variation for the assay quality control of 300 pmol TG and 3 nmol MeMP/8×10^8 RBCs over this period was 8.6% and 9.1% respectively. The concentration changes of TGNs and MeMPs in the top, middle and bottom gradients, representing young, middle-age and old red cell fractions respectively, are shown in Figures 1 and 2.

Table 1 The percentage of cells in each Percoll fraction at weekly intervals throughout the study for the individual children (JB1, MD2, AL3, CW4). The youngest cells remain in the top fractions, the middle-aged cells in the middle fractions whilst the older cells fall to the bottom fractions

	% red cells in each Percoll fraction																			
	JB1			*MD2*				*AL3*					*CW4*							
Percoll gradient	*Day 1*	*Day 7*	*Day 14*	*Day 1*	*Day 7*	*Day 15*	*Day 22*	*Day 1*	*Day 7*	*Day 15*	*Day 21*		*Day 1*	*Day 7*	*Day 14*	*Day 21*				
50%	3	3	3	6	35	6	11	18	17	14	10		7	8	5	5				
57%	6	6	14	5	23	17	18	31	38	36	30		25	19	20	16				
60%	29	33	35	10	18	37	24	27	24	24	26		29	39	32	41				
63%	43	43	30	14	12	17	25	13	9	14	18		14	15	20	21				
66%	11	10	10	22	3	11	11	5	6	6	9		7	9	10	8				
69%	8	6	8	44	1	11	11	6	6	7	8		18	11	12	9				

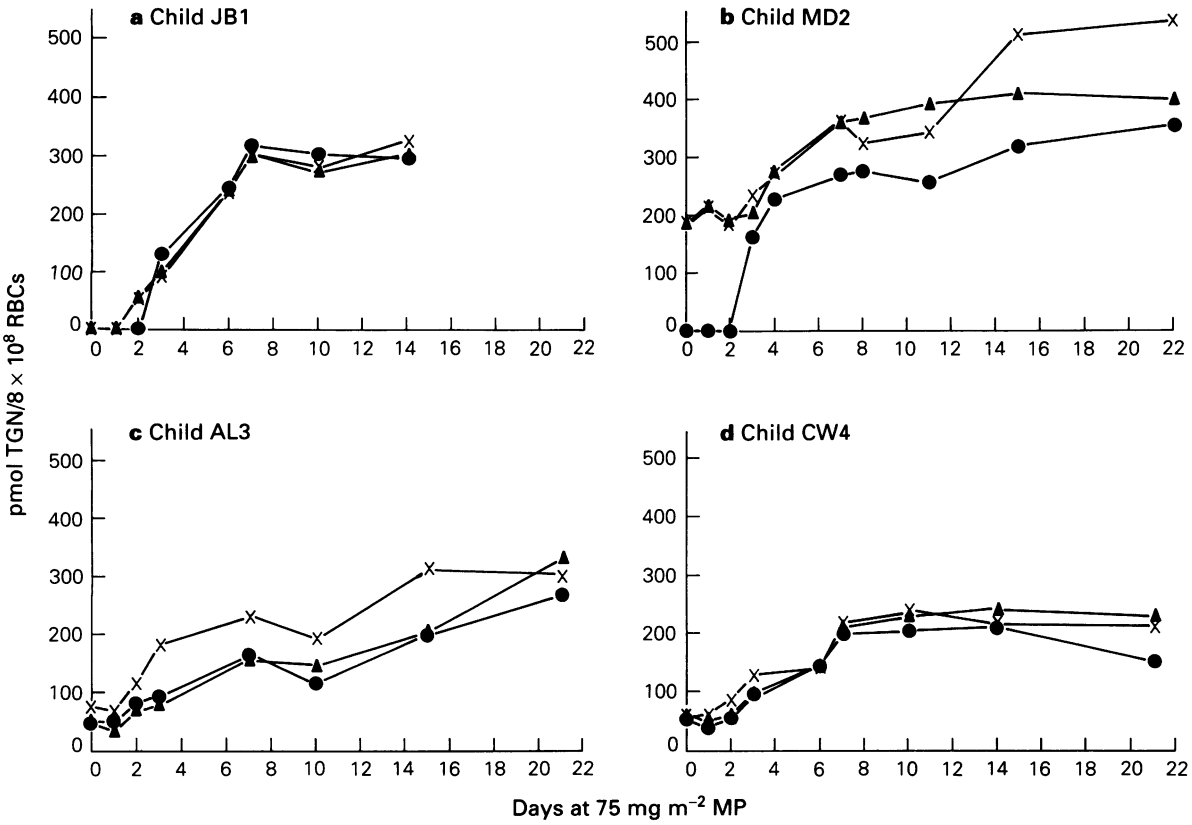

Figure 1 The concentration of TGN metabolites in young (50 and 57% Percoll fractions (●)), middle-aged (60 and 63% Percoll fractions (▲)) and old RBCs (66 and 69% Percoll fractions (×)).

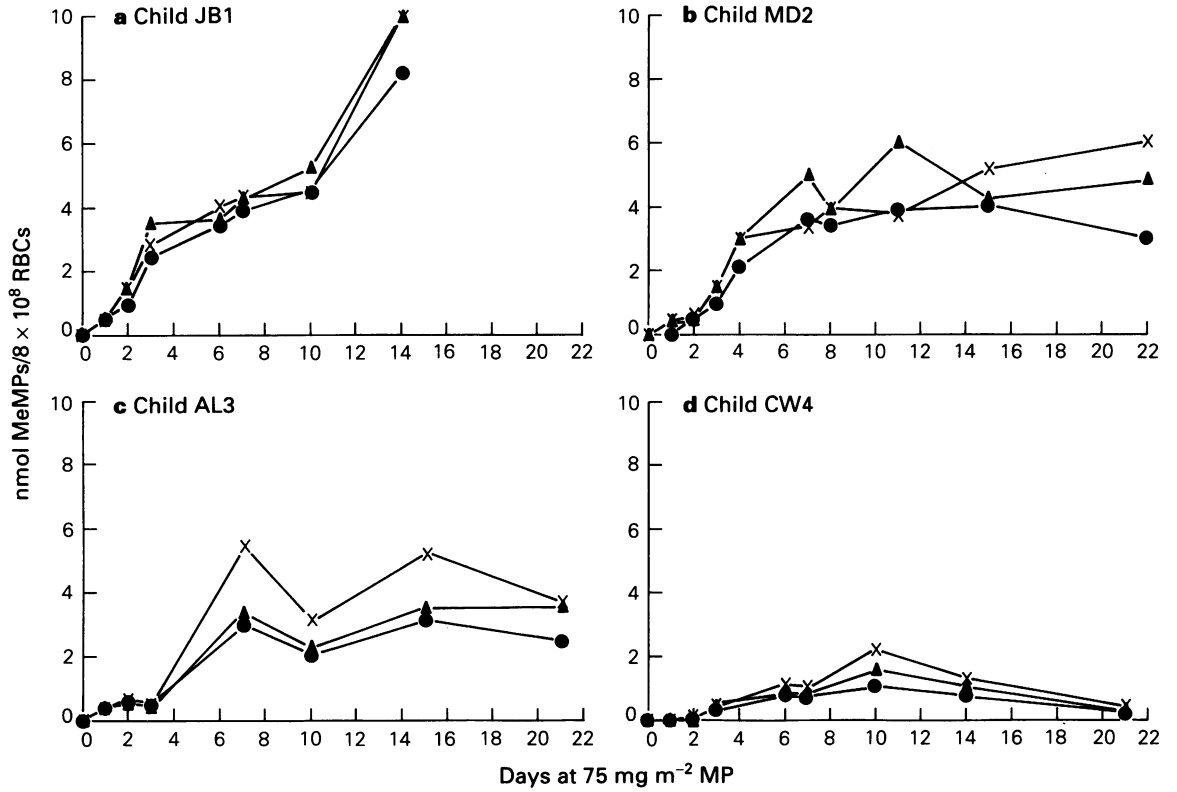

Figure 2 The concentration of MeMP metabolites in young (50 and 57% Percoll fractions (●)), middle-aged (60 and 63% Percoll fractions (▲)) and old RBCs (66 and 69% Percoll fractions (×)).

No TGN metabolites were detected in the pre-dose or day 1 blood samples taken from child JB1. The TGNs could be measured in the day 2 middle-aged and older fractions whilst the MeMPs could be measured in all the RBC fractions from day 1 onwards. Child MD2 had TGNs present in his pre-dose blood sample, taken 15 days after a thioguanine-containing intensification block. The TGN metabolites were present in the middle and older cell fractions in the pre-dose, day 1 and day 2 blood samples. All the cell fractions from the day 3 sample contained TGNs with the concentration gradient increasing from young to old cells. MeMPs were absent from the pre-dose sample and the young cell fraction of the day 1 blood sample. The pre-dose blood samples taken from children AL3 and CW4, 21 days post intensification, contained TGNs in all the RBC fractions, with the highest concentration in the oldest cells of child AL3. The day 1 sample contained the same concentrations of TGNs as the pre-dose sample, but by day 2 (AL3) or day 3 (CW4) the TGNs had started to increase in all cell fractions. The MeMPs were absent in the predose blood sample from AL3 and both the predose and day 1 samples from CW4.

There was no significant difference between the MP metabolite concentrations in the young *vs* the middle-aged or between the young *vs* the old RBC fractions (Mann-Whitney, $P = 1.0$ to 0.12).

Discussion

Circulating RBCs do not constitute a homogeneous population. They have a life span of 120 days and during that time undergo a progressive ageing process with a decrease in membrane components and water content with time. At the end of its life-span a RBC is smaller, has a higher concentration of solutes and is heavier than the younger red cell. Density gradient separation of RBCs is a well validated technique which yields cell fractions with a significant progressive shift in the average cell age with increasing gradient density [9]. The fractions isolated are heterogeneous but those fractions at the top are enriched with younger cells, middle-aged cells are, on average, of intermediate density whilst the older cells graduate to the bottom of the gradient.

At the start of MP chemotherapy the MeMP metabolites can be detected in all cell fractions within a few hours of the oral dose. The TGN metabolites accumulated in the cell fractions at a slower rate but after 3 days oral MP all the children had increasing TGNs in all three red cell fractions. The appearance of MP metabolites in all the cell age groups at an early stage in the study, before the time required for the development cycle of the stem cell, indicates that these metabolites did not originate in the bone marrow. The RBC metabolised the drug, or drug metabolites whilst in the circulation.

There is limited information available on the *in vitro* metabolism of thiopurine drugs by the RBC. Short term incubations of fresh whole blood have indicated that red cells alone, i.e. with no added cofactor supplements, readily metabolised MP to the MP nucleotide thioinosinic acid but further metabolism of this compound to TGNs did not occur [13]. Long term incubations indicate that the low activity of inosinate dehydrogenase in human RBCs could be a rate limiting factor governing TGN formation [14]. This may explain the slower rate of TGN accumulation within the RBC compared with the MeMP metabolites which are readily formed from thioinosinic acid.

The distribution of RBCs among the fractions showed an interpatient variation but, with the exception of one child, only a small intra-patient variation. The child with a large variation in RBC density distribution had 66% of his cells in the older cell fraction at the start of the study and he had very few young cells (reticulocytes). The number of circulating reticulocytes gives an important insight into the level of erythropoietic activity in the bone marrow [15] and the surge of reticulocyte production recorded in the day 7 fractionation is perhaps reflective of bone marrow recovery following the preceding intensification block.

In this study the concentration of MP metabolites did not differ between the RBC age fractions. This is not the case for methotrexate (MTX) [16]. RBCs separated at intervals after a 24 h MTX infusion showed that at 2 days post infusion there was no MTX in the youngest cells whilst 7 days after the infusion the highest MTX concentration was found in the youngest cells. By 10 to 14 days the MTX cells had moved down the gradient and by the time of the next MTX infusion (day 28) no MTX could be detected in the young RBCs [16]. These observations are in agreement with pharmacokinetic studies of MTX which suggest that i.v. MTX was incorporated into RBC precursors [17]. The accumulation of MTX within the RBC is due to the formation of polyglutamate derivatives, which are thought to serve as a slow release MTX store. Polyglutamation occurs only in immature RBCs [18]. After an i.v. dose MTX accumulates in the RBC, free MTX mirrors the plasma concentrations of the drug and falls rapidly. Over a period of days MTX polyglutamates accumulate in the RBC [18]. Child MD2, who showed marked erythropoietic activity (reticulocytosis) in the day 7 fractionation had lower concentrations of TGN metabolites in his younger cell fractions with no difference in the distribution of MeMPs between cell fractions. This latter observation suggests that the activity of TPMT did not differ between young and old RBCs.

TGNs accumulate slowly within RBCs and previous pharmacokinetic studies do not suggest that these metabolites could arise from red cell precursors [2]. Red cell TGNs continue to rise slowly during a course of oral MP whilst plasma drug concentrations are low and fall to the lower limit of detection by 5 h post-dose [19]. This suggests that a pool of MP metabolites could exist in tissues other than the bone marrow or plasma. By excluding the possibility of having RBC TGNs locked in at the stem cell stage

there would be no need to put a RBC production cycle in a predictive model for TGNs. The bone marrow can be considered as an effect compartment which is compatible with the delayed cytotoxic effects observed in the clinic [8]. This suggests that the concentration of TGNs at an early point in treatment could be used to predict eventual steady-state concentrations using a simple model.

MP is a highly metabolised drug and the many facets of its metabolism have been discussed elsewhere [4]. The role(s) of the MeMP metabolites are uncertain and the clinical relevance of these metabolites has not been substantiated. Because MeMPs exist at a much higher intracellular concentration than TGNs the possibility of the mediation of biological effects cannot be ruled out [20]. The ability of the TPMT reaction (i.e. MeMP formation) to deplete intracellular concentrations of the universal methyl donor S-adenosylmethionine may influence cellular mechanisms on many different levels [21].

The observation that RBC MP metabolites are not initially formed within the bone marrow does not mean that MP cannot have a direct influence on the stem cell population. The RBC can assimilate exogenously supplied purines and it has an elaborate system for their interconversion and catabolic disposal [22–25]. Such mechanisms could be used in the transfer of thiopurines to the bone marrow. Alternatively, RBC TGNs may simply reflect the overall body metabolism of MP to TGNs.

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