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Thiopurine Methyltransferase Biochemical Genetics: Human Lymphocyte Activity

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The level of human erythrocyte (RBC) thiopurine methyltransferase (TPMT) activity is inherited as a monogenic trait. Experiments were performed to determine whether the level of TPMT activity in the human lymphocyte is regulated in parallel with RBC TPMT. Supernatants of lymphocyte homogenates contained TPMT activity. Lymphocyte TPMT activity was maximal at a reaction pH *of 6.6. The apparent* K_m *value for 6-mercaptopurine, the thiopurine substrate for the reaction, was* 8.1×10^{-4} *M, and the apparent* K_m *value for* S-*adenosyl-*L*-methionine, the methyl donor for the reaction, was* 3.6×10^{-6} *M. The average TPMT activity in lymphocytes isolated from blood of 55 randomly selected subjects was 11.0 ± 0.4 units/*10^9 *cells (mean ± SE), with a range of from 4.8 to 17.7 units/*10^9 *cells. There was a significant correlation of relative RBC with relative lymphocyte TPMT activity in blood samples from these 55 subjects, with a correlation coefficient of 0.563 (*$P < 0.001$*). The correlation coefficient for RBC with platelet enyzme activities in these same subjects was also highly significant (*$r = 0.680$, $P < 0.001$*). Blood samples from four previously identified subjects who were homozygous for the allele* $TPMT^L$*, subjects who lacked detectable RBC enzyme activity, also lacked detectable lymphocyte and platelet TPMT activites. These results were compatible with the conclusion that the genetic polymorphism which regulates RBC TPMT activity also regulates the level of human lymphocyte and platelet TPMT activites.*

KEY WORDS: methyltransferase; thiopurine; lymphocyte enzyme; biochemical genetics; pharmacogenetics.

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INTRODUCTION

Thiopurine methyltransferase (TPMT) plays an important role in the catabolism of thiopurine drugs such as 6-mercaptopurine, 6-thioguanine, and azathioprine (Remy, 1963; Elion, 1967; Paterson and Tidd, 1975). This enzyme catalyzes the *S*-methylation of thiopurines with *S*-adenosyl-L-methionine as a methyl donor (Remy, 1963; Weinshilboum *et al.*, 1978). TPMT is found in a variety of tissues in experimental animals (Remy, 1963; Walker *et al.* 1981; Woodson *et al.*, 1981a), and the development of a sensitive radiochemical enzymatic assay has made it possible to measure TPMT activity in the human erythrocyte (RBC) (Weinshilboum *et al.*, 1978). The level of human RBC TPMT activity is inherited in a monogenic fashion (Weinshilboum and Sladek, 1980). A pair of alleles at a single locus, $TPMT^H$ for high activity and $TPMT^L$ for low activity, is responsible for most of the variation in human RBC TPMT activity. Individuals homozygous for the allele $TPMT^L$ have virtually undetectable enzyme activity, those homozygous for $TPMT^H$ have high activity, and heterozygous subjects have intermediate levels of activity. The gene frequencies of $TPMT^L$ and $TPMT^H$ are approximately 0.06 and 0.94, respectively (Weinshilboum and Sladek, 1980). It has been suggested that this "pharmacogenetic" polymorphism might represent one factor involved in individual variations in the catabolism of thiopurine drugs and in the therapeutic or toxic effects of these drugs (Weinshilboum, 1980; Weinshilboum and Sladek, 1980).

It would be important to determine whether the genetically regulated level of TPMT activity in the RBC reflects the level of the enzyme activity in nucleated human cells. It would be especially useful for future biochemical genetic and pharmacologic studies if these nucleated cells could be obtained easily from large numbers of subjects and maintained in culture *in vitro*. Lymphocytes are a nucleated cell type that can be obtained from large numbers of subjects. This study was designed to determine whether lymphocytes contain TPMT, to develop an accurate assay for lymphocyte TPMT activity, to characterize the properties of the lymphocyte enzyme, and to determine whether there was a significant correlation between lymphocyte TPMT activity and the genetically controlled level of enzyme activity in the RBC. Since it was necessary to measure platelet TPMT activity in the course of the experiments with lymphocytes, data were also obtained with respect to the platelet enzyme activity.

MATERIALS AND METHODS

Source of Blood Samples

Blood for the establishment of enzyme assay conditions and for characterization of the properties of lymphocyte TPMT was obtained from randomly

selected donors at the Mayo Clinic Blood Bank in Rochester, Minnesota. All blood donors were white adults. Blood samples for comparison of RBC with lymphocyte enzyme activity levels were obtained from 55 randomly selected adult laboratory personnel. Fifty-three of these subjects were white and two were black. Nine samples were obtained from members of families that included probands previously identified as homozygous for the allele $TPMT^{L}$. None of the subjects was taking any medication and none was either acutely or chronically ill. Written informed consent was obtained from all subjects and the studies were performed under guidelines approved by the Mayo Clinic Human Studies Committee.

Treatment of Blood Samples

Blood was obtained by venipuncture in 7-ml Vacutainer tubes that contained 10.5 mg disodium EDTA. Two 7-ml samples were obtained from each subject to yield an adequate quantity of lymphocytes. Blood samples were kept at room temperature prior to isolation of lymphocytes. After centrifugation in 13-ml plastic tubes at 200*g* for 10 min at room temperature, the platelet-rich plasma was removed and was used as a source of platelets for the platelet TPMT assay. The remaining blood was used for isolation of lymphocytes (see below). Lysates for use in the measurement of RBC TPMT activity were prepared exactly as previously described (Weinshilboum *et al.*, 1978).

Isolation of Lymphocytes

Paired 7-ml aliquots of blood from which platelet-rich plasma had been removed were each mixed with 9 ml of balanced saline solution. Lymphocytes were isolated from these preparations by density gradient centrifugation performed with Ficoll–Hypaque (Ficoll–Paque; Pharmacia Fine Chemicals Inc.) as described in detail elsewhere (Sladek-Chelgren and Weinshilboum, 1981). The "lymphocyte" layer obtained during Ficoll–Hypaque density gradient centrifugation was heavily contaminated with platelets (Sladek-Chelgren and Weinshilboum, 1981). Therefore, this preparation was subjected to sucrose density gradient centrifugation using a modification of the method of Perper *et al.* (1968) to remove platelets (Sladek-Chelgren and Weinshilboum, 1981). The resulting loosely packed lymphocyte pellet was then resuspended in the lower 5.5 ml of the 16% sucrose layer. Five-milliliter aliquots of each of the paired lymphocyte preparations from individual subjects were combined in one 15 × 75-mm plastic centrifuge tube. Of the cell suspension, 0.5 ml was diluted with 9.5 ml of Isoton II (Coulter Diagnostics Inc.) and was used to measure both lymphocyte and platelet numbers with a Coulter Model ZBI cell counter. The remaining 9.5 ml of resuspended

lymphocytes was centrifuged at 7000 *g* for 10 min at 4°C. The supernatant was discarded and the lymphocyte pellet was resuspended in 1 ml of 5 m*M* Tris-HCl buffer, *p*H 7.8, which contained 7 mg/ml of bovine serum albumin (BSA). The lymphocyte preparation was then homogenized for 30 sec with a Polytron tissue homogenizer, and the homogenate was centrifuged at 100,000*g* for 1 hr at 4°C. The final supernatant was used to measure lymphocyte TPMT enzyme activity. TPMT assay conditions were established and the biochemical properties of the enzyme were characterized with fresh pooled lymphocytes prepared daily with blood from 6 to 10 individual blood donors. All other data were obtained with preparations from individual subjects.

Lymphocyte TPMT Assay

The enzyme assay was a modification of the method of Weinshilboum *et al.* (1978). The procedure involved the conversion of 6-mercaptopurine (6-MP) to 6-methylmercaptopurine by TPMT in the presence of radioactively labeled *S*-adenosyl-L-methionine as a methyl donor. Specifically, 100-μl aliquots of the high-speed supernatant were placed in 15-ml conical glass centrifuge tubes. Either 5 μl of 6-MP (36 mg/ml) in dimethylsulfoxide or 5 μl of dimethylsulfoxide alone was added to the supernatant. Three different types of "blanks" were used. These blanks included samples with no 6-MP and with no enzyme preparation, samples containing enzyme that had been heated to 95°C for 5 min after which 6-MP was added, and unheated samples that contained enzyme but to which no 6-MP was added. The rationale for the use of each of the blanks is discussed below. Twenty-five microliters of 150 mM potassium phosphate buffer, *p*H 7, was then added to each tube. The enzyme reaction was initiated by the addition of 25 μl of a mixture of the following reagents (final concentration in 155 μl indicated): reduced glutathione, 1.5×10^{-2} M; allopurinol, 5×10^{-5} M; and ^{14}C-methyl-*S*-adenosyl-L-methionine, 2.3×10^{-5} M. The reaction mixture was incubated in a shaker water bath for 90 min at 37°C, and the reaction was terminated by the addition of 200 μl of 0.5 M borate buffer, *p*H 10. Two and five-tenths milliliters of 20% isoamyl alcohol in toluene was added to the reaction tubes, and they were mixed vigorously on a vortex mixer for 10 sec. After centrifugation at 700 *g* for 10 min, 1.5 ml of the organic phase was aspirated and was placed in a liquid scintillation vial that contained 0.5 ml of ethanol. Four milliliters of toluene fluor [5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene] was then added. Radioactivity was measured in a Beckman LS-7500 liquid scintillation counter. All results were corrected for the extraction of the radioactive product into the organic phase (50.4%) and

for counting efficiency. One unit of enzyme activity represented the formation of 1 nmol of 6-methylmercaptopurine/hr of incubation at 37°C. The results were expressed per 10^9 lymphocytes.

Platelet TPMT Assay

Platelet-rich plasma was obtained from each blood sample used for lymphocyte isolation. One milliliter of platelet-rich plasma was centrifuged at 16,000 *g* for 10 min at 4°C. The pellet was rinsed with 1 ml of balanced saline solution and was homogenized with a Polytron tissue homogenizer for 30 sec in 1 ml of 5 mM Tris–HCl, *p*H 7.8, that contained 7 mg/ml of BSA. The homogenate was centrifuged at 100,000 *g* for 60 min at 4°C, and the supernatant was used as a source of enzyme for the assay. The assay procedure was identical to that described above for lymphocytes. The results were expressed per 10^{11} platelets.

RBC TPMT Assay

RBC TPMT activity was measured by the method of Weinshilboum *et al.* (1978). One unit of enzyme activity represented the formation of 1 nmol of 6-methylmercaptopurine/hr of incubation. The results were expressed per milliliter of packed erythrocytes.

Thin-Layer Chromatography

The radioactive products of the enzyme reaction were identified by thin-layer chromatography performed as described previously (Weinshilboum *et al.*, 1978).

Partial Purification of Human Kidney TPMT

Human kidney TPMT was partially purified as described in detail elsewhere (Weinshilboum *et al.*, 1978). The specific activity of the partially purified preparation was 6.9 nmol 6-methylmercaptopurine formed/hr/mg protein. This represented a 15-fold purification over the specific activity of the enzyme in the supernatant of a human kidney homogenate after centrifugation at 100,000 *g* for 1 hr.

Kinetic Analysis

Michaelis–Menten (K_m) values were determined by the method of Wilkinson (1961) with a Fortran program written by Cleland (1963) and by the method

of Eisenthal and Cornish-Bowden (1974). A Cyber 170/720 computer was used for these calculations.

Materials

^{14}C-Methyl-*S*-adenosyl-L-methionine (sp act, 57.9 mCi/mmol) was obtained from New England Nuclear Corporation, Boston. Tris hydroxymethyl (aminomethane) base, 6-mercaptopurine, 6-methylmercaptopurine, reduced glutathione, *S*-adenosyl-*L*-methionine hydrochloride, dithiothreitol (Cleland's reagent), and sucrose were purchased from Sigma Chemical Company, St. Louis. Chelex-100 was purchased from Bio-Rad Laboratoriew, Richmond, California. Soluene-100 was obtained from Packard Instrument Company, Inc., Downers Grove, Illinois. Ficoll–Paque was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

RESULTS

Lymphocyte Isolation and Purity

It was important to determine the degree of "purity" of the lymphocyte preparations used in these experiments. The initial step in the lymphocyte isolation procedure was Ficoll–Hypaque density gradient centrifugation (Böyum, 1968). Unfortunately, lymphocytes isolated using this procedure are heavily contaminated with platelets (Sladek-Chelgren and Weinshilboum, 1981). For example, when blood samples from four individual subjects were studied, an average of $4.4 \pm 0.4 \times 10^8$ platelets (mean ± SE) was present in the lymphocyte preparations after the Ficoll–Hypaque step, and an average of $0.4 \pm 0.07 \times 10^8$ platelets remained after sucrose density gradient centrifugation. Therefore, the second step of the isolation procedure, the sucrose density gradient step, removed over 90% of the platelets contaminating the lymphocytes. Supernatants of platelet homogenates contained TPMT activity when assayed under conditions used to measure lymphocyte enzyme activity. This platelet enzyme activity was not due to contamination with erythrocytes. For example, when platelet-rich plasma from five individual subjects was studied, the samples contained an average of $5.3 \pm 0.3 \times 10^5$ platelets/μl (mean ± SE) but an average of only 17 ± 11 RBCs/μl. Erythrocytes could have been responsible for an average of only 0.03% of the TPMT activity measured in these preparations. In like fashion it was possible to calculate that the contribution of platelets to the enzyme activity measured in the final lymphocyte preparations obtained from 44 randomly selected individual subjects averaged 9.5 ± 0.7% (Fig. 1). A similar approach was used to determine the contribution of RBC contamination to the enzyme activity

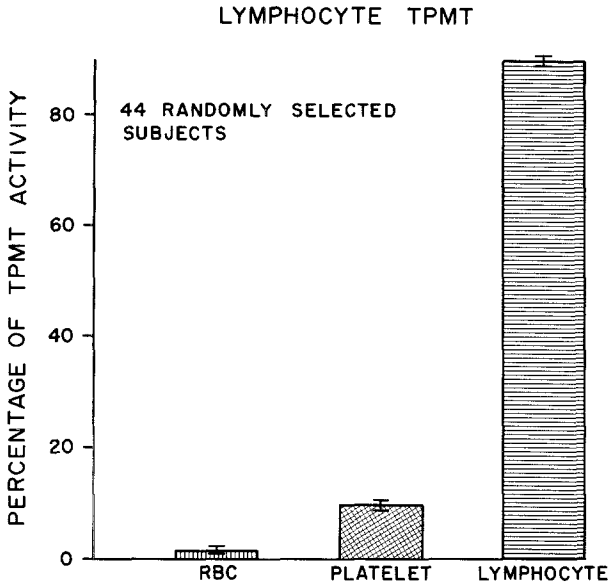

Fig. 1. The contribution of erythrocytes, platelets, and lymphocytes to TPMT activity measured in lymphocyte preparations. The results are average values ± SE for data from 44 randomly selected subjects.

measured in the lymphocyte preparations. When blood from the same 44 randomly selected subjects was studied, and when RBC contamination was determined by direct examination of Wright-stained slides of the final preparations, it was found that RBC TPMT could account for only an average of 1.5 ± 0.3% of the activity measured (Fig. 1). In all of the experiments described below, the contribution of platelet TPMT was determined directly by isolation of platelets and measurements of their enzyme activity. The number of platelets contaminating each lymphocyte preparation was measured, the platelet activity was subtracted from the total activity in the lymphocyte preparations, and the remaining enzyme activity was referred to as "lymphocyte TPMT." It must be remembered that, since the primary goal of the experiments was to measure lymphocyte TPMT, platelet activities were all determined under conditions that were optimal for the assay of TPMT in lymphocyte preparations.

Human Lymphocyte TPMT Assay

Choice of Blank. The choice of an appropriate blank is essential for the accurate determination of any methlytransferase activity (Weinshilboum *et*

al., 1979). Radioactivity removed during the organic solvent extraction step might represent nonpolar contaminants present in the radioactive methyl donor, ^{14}C-methyl-*S*-adenosyl-L-methionine (^{14}C-SAM); it might result from nonenzymatic interaction of the thiopurine substrate with the methyl donor to yield radioactively labeled material; or it might represent organic solvent extractable radioactive products resulting from the activities of other methyltransferase enzymes such as protein carboxyl methyltransferase (EC 2.1.1.24; O'Dea *et al.*, 1981). Each of these possibilities played a role in the assay of lymphocyte TPMT, and three separate blanks were used (Fig. 2). The first, blank A, was a measure of radioactivity extracted in the presence of ^{14}C-SAM alone. These samples contained no enzyme source and no thiopurine substrate. Blank A varied from 178 to 198 cpm. Blank B measured organic solvent-extractable cpm in the presence of all components of the reaction mixture, but the supernatant in these samples had been heated to 95°C for 5 min to inactivate enzymes. Radioactivity in blank B in samples from 55 randomly selected subjects varied from 211 to 369 cpm. Blank C was a measure of the activity of methyltransferase enzymes other than TPMT. These samples included supernatants and ^{14}C-SAM but did not include 6-mercaptopurine. The total cpm in blank C for the same 55 samples varied from 195 to 453. The average contribution of each of the different types of blanks for these 55 samples is shown graphically in Fig. 2. The total cpm in "active" samples

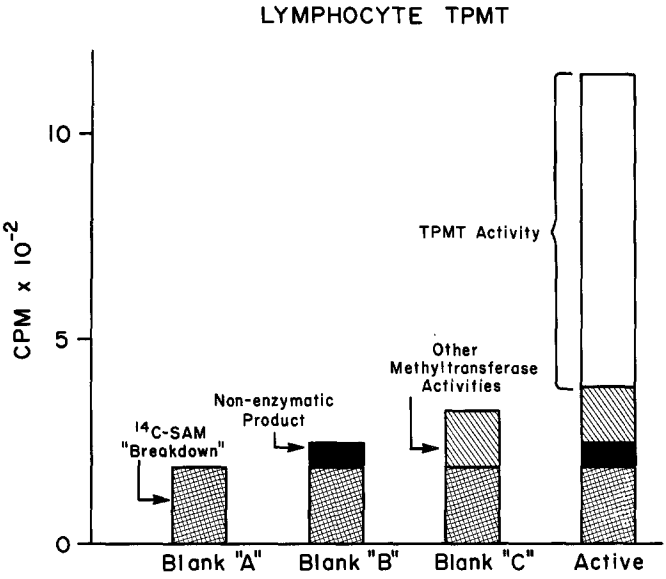

Fig. 2. Schematic representation of the types of blank samples used in the TPMT assay. See text for details.

varied from 546 to 2037, with an average value of 1145 ± 39. Calculation of the total cpm in blanks was performed as follows:

$$\text{total blank cpm} = \text{blank B} + \text{blank C} - \text{blank A}.$$

Although the use of three separate blanks might appear overly complex, it was necessary because the cpm in each blank varied from sample to sample. Detailed discussions of the need for multiple blanks in assays of methyltransferase enzyme activities in tissue homogenates have appeared elsewhere (Weinshilboum *et al.*, 1979; Sladek-Chelgren and Weinshilboum, 1981).

Linearity of Reaction with Enzyme Quantity and Incubation Time. TPMT activity in a pooled lymphocyte preparation increased with increasing concentrations of the high-speed lymphocyte supernatant in a linear fashion over a range equivalent to supernatant from 3.25 to 26 × 10^5 lymphocytes per assay tube (Fig. 3A). Individual lymphocyte preparations varied in the number of cells that they contained, but in all cases the number of lymphocytes assayed fell within this linear range. The time course of the enzyme reaction was linear for up to 150 min of incubation (Fig. 3B). All assays were performed with an incubation time of 90 min.

p*H Optimum.* The effect of *p*H on TPMT activity in lymphocyte supernatants was determined with 150 mM potassium phosphate buffer. All *p*H determinations were made at 20°C in the presence of lymphocyte supernatant and all components of the reaction mixture. Values of *p*H that varied from 6.1 to 7.7 were tested. Very little activity was present at either extreme of *p*H. The optimal *p*H for the reaction was 6.6. The use of a *p*H 7.0 potassium phosphate buffer resulted in a final reaction *p*H of 6.6.

Effect of Substrate Concentrations on TPMT Activity. Lymphocyte TPMT activity was measured in the presence of varying concentrations of 6-mercaptopurine (6-MP) and *S*-adenosyl-L-methionine (SAM), the two cosubstrates for the reaction (Fig. 4). Data obtained from these experiments were used to calculate apparent Michaelis–Menten (K_m) constants for both cosubstrates. The K_m values were 9.8 × 10^{-4} and 3.1 × 10^{-6} M for 6-MP and SAM, respectively, when calculated by the method of Eisenthal and Cornish-Bowden and were 8.1 × 10^{-4} and 3.6 × 10^{-6} M when calculated by the method of Wilkinson. The substrate concentrations chosen for use in the assay were approximately 10-fold greater than the apparent K_m values.

Effects of Glutathione and Bovine Serum Albumin on TPMT Activity. Sulfhydryl reducing agents such as dithiothreitol (DTT) and reduced glutathione have been shown to increase the activities of many methyltransferase enzymes including TPMT (Weinshilboum *et al.*, 1978; Walker *et al.*,

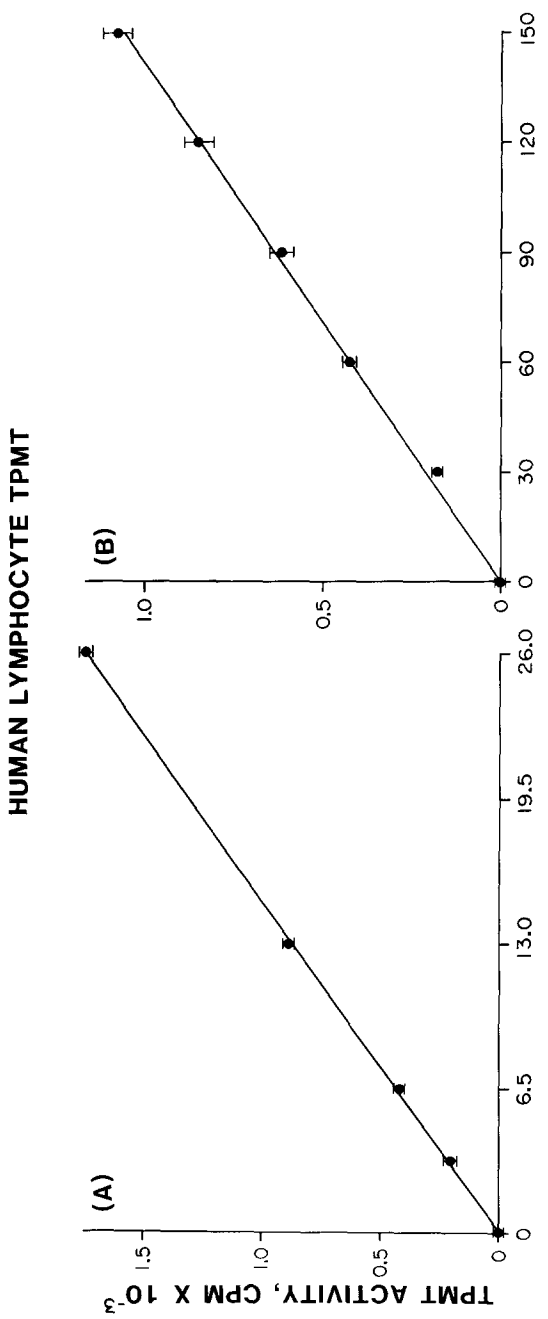

Fig 3. (A) Effect of lymphocyte cell number on TPMT activity. The incubation time was 90 min. (B) Effect of incubation time on lymphocyte TPMT activity. Each point represents the mean ± SE of three determinations.

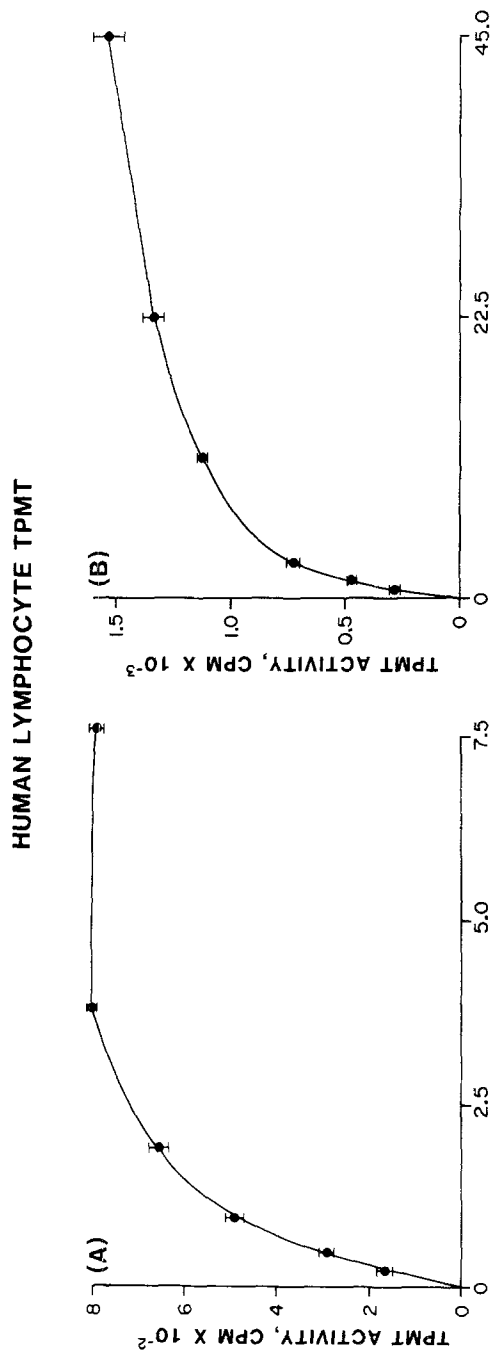

Fig. 4. (A) The effect of 6-mercaptopurine (6-MP) concentration on lymphocyte TPMT activity. (B) The effect of *S*-adenosyl-L-methionine (SAM concentration on lymphocyte TPMT activity. Each point represents the mean ± SE of three determinations.

1981), and little lymphocyte TPMT activity was observed in the absence of such agents. However, when increasing concentrations of DTT were tested with lymphocyte preparations, the sensitivity of the assay was low because the cpm in blank samples that contained no substrate (blank C) increased. These cpm presumably resulted from the methylation of DTT by the enzyme thiol methyltransferase (EC 2.1.1.9). Glutathione is not a substrate for thiol methyltransferase (Bremer and Greenberg, 1961), and its addition to the reaction mixture resulted in a striking increase in TPMT activity without an increase in cpm in blank samples. When a pooled lymphocyte preparation was assayed in the presence of various final concentrations of GSH, optimal TPMT activity was observed at a final concentration of 15 mM. The addition of concentrations of glutathione above 15 mM resulted in a decrease in TPMT activity. A final glutathione concentration of 15 mM was used in the assay.

Bovine serum albumin (BSA) has been shown to increase the activities of many dilute enzyme preparations. The addition of BSA also increased TPMT activity in lymphocyte preparations, but the point in the procedure at which BSA was added was critical. The addition of BSA after homogenization with the Polytron homogenizer did not result in an increase in TPMT activity. However, the addition of BSA before the homogenization step resulted in an approximate 40% increase in TPMT activity. BSA at a concentration of 7 mg/ml was added to all preparations prior to the homogenization step.

Thin-Layer Chromatography. Thin-layer chromatography of dried isoamyl alcohol–toluene extracts from assays of lymphocyte TPMT was performed with two different solvent systems (Fig. 5). In each system, over 98% of the total cpm migrated with authentic 6-methylmercaptopurine when the contributions of blank samples studied in parallel with "active" samples were subtracted.

Coefficient of Variation of the Assay. The coefficient of variation for the entire assay procedure including cell isolation was determined with a pool of blood from nine subjects. Five separate 14-ml aliquots of the pooled blood were made and were used to isolate lymphocytes. The coefficient of variation for the entire procedure including isolation of the cells and the enzyme assay was 10.0%. When a pooled sample was made after the ultracentrifugation step and this material was assayed 10 times to measure the coefficient of variation of the enzyme assay only, a value of 7.5% was found.

"Recovery" of Purified Enzyme. Because of the possibility that individual variations in lymphocyte TPMT activity might be due to varitions in the levels of endogenous enzyme activators, inhibitors, or competing enzyme systems, partially purified human kidney TPMT was added to lymphocyte homogenates obtained from 55 randomly selected subjects and the recovery of

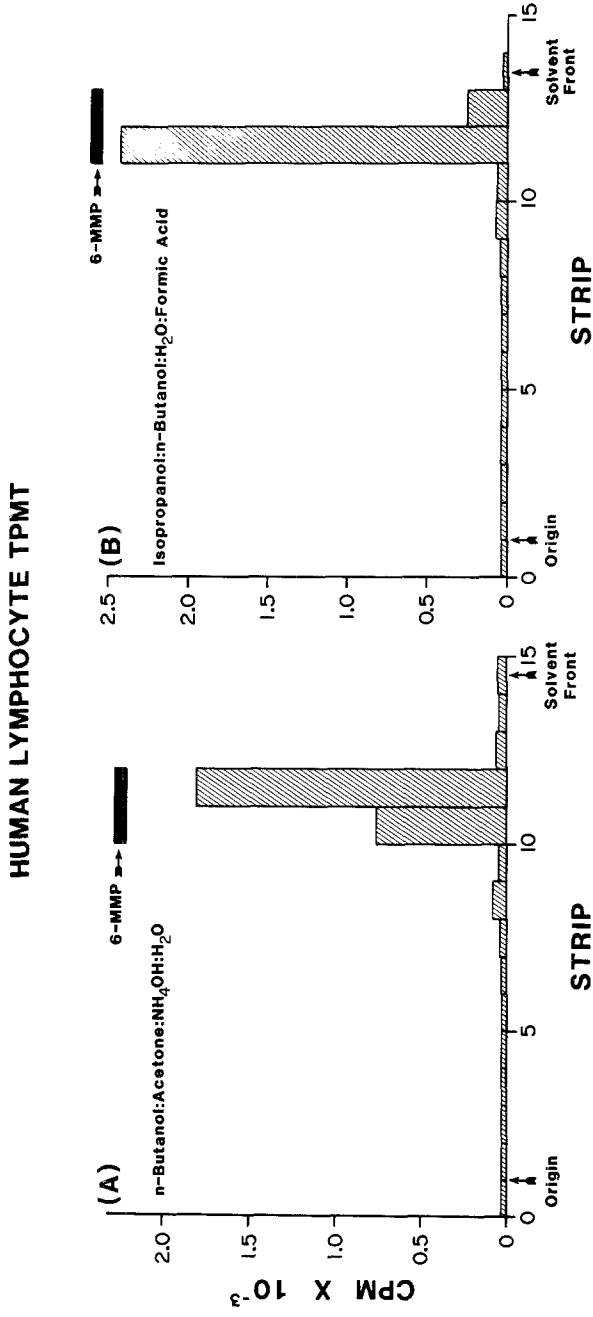

Fig. 5. Thin-layer chromatography of the product of the lymphocyte TPMT reaction. Filled bars represent R_f values for authentic 6-methylmercaptopurine (6-MMP).

enzyme activity was measured. The average recovery of the activity of partially purified enzyme added to these 55 samples was 101 ± 2% (mean ± SE). In addition, when data were compared from the upper and lower quartiles of the 55 samples based on the lymphocyte activities, recoveries of added activity were not significantly different. Recoveries of added enzyme activity were 98 ± 4 and 105 ± 4% from the lower and upper quartiles, respectively. The activity of the purified enzyme added to the samples was almost exactly the same as that present in the samples themselves, 1050 ± 31 vs 1144 ± 39 cpm, respectively. This was an important consideration since, if very high activities had been added, it might not have been possible to detect the effects of endogenous activators or inhibitors. These results made it unlikely that individual variations in lymphocyte TPMT activities were due to variations in the levels of endogenous enzyme activators, inhibitors, or competing enzyme systems.

Subcellular Localization of Lymphocyte TPMT

TPMT is a "soluble" cytoplasmic enzyme in the rodent liver and kidney (Remy, 1963; Walker *et al.*, 1981; Woodson *et al.*, 1981a). It was necessary to determine whether lymphocyte TPMT was also a soluble enzyme. Therefore, three separate pooled blood samples, each from four separate individuals, were used to isolate lymphocytes. It was necessary to use pooled samples to obtain an adequate number of cells to perform the experiment. Lymphocytes in each pooled sample were homogenized, and TPMT activity was measured in the homogenate. Enzyme activities were also measured in supernatants and resuspended pellets after centrifugation of the homogenates at 100,000 *g* for 1 hr. Of the activity present in the initial homogenates, 83.6 ± 2.3% (mean ± SE) was present in the supernatant and 18.7 ± 4.4% was present in the "unwashed" pellets. These results were compatible with the conclusion that TPMT was primarily a soluble cytoplasmic enzyme in the human lymphocyte.

Lymphocyte TPMT in Individual Subjects

RBC, lymphocyte, and platelet TPMT activities were measured in blood samples from 55 randomly selected subjects. RBC activity averaged 11.8 ± 0.3 (mean ± SE) units/ml RBCs, and values ranged from 5.8 to 15.6 units/ml RBCs. Lymphocyte activity averaged 11.0 ± 0.4 units/10^9 cells and ranged from 4.8 to 17.7 units/10^9 cells. Platelet activity averaged 21.7 ± 0.7 units/10^{11} platelets, with a range of from 10.1 to 38.6 units/10^{11} platelets. Average RBC, lymphocyte, and platelet activities for the 33 male subjects were 11.9 ± 0.4 units/ml RBCs, 10.9 ± 0.5 units/10^9 lymphocytes, and

21.9 ± 1.0 units/10^{11} platelets, respectively. Values for the 22 female subjects were not significantly different, averaging 11.6 ± 0.5, 11.1 ± 0.6, and 21.4 ± 0.9 for RBCs, lymphocytes, and platelets, respectively.

Individual subjects may be classified on the basis of their RBC TPMT activity levels as those with genetically "low" activity, presumed genotype $TPMT^L$ $TPMT^L$, those with "intermediate" activity, genotype $TPMT^H$ $TPMT^L$, and those with "high" activity, genotype $TPMT^H$ $TPMT^H$ (Weinshilboum and Sladek, 1980). In the initial description of the RBC TPMT polymorphism, it was found that 89% of the large population sample studied had high activity, activity greater than 9.5 units/ml RBCs, 11% had intermediate activity, 1 to 9.5 units/ml, and approximately 1 subject in each 300 studied had undetectable activity (Weinshilboum and Sladek, 1980). When a frequency distribution histogram was drawn for RBC TPMT activity in the 55 randomly selected subjects who were studied as part of the experiments described here (Fig. 6), 48 (87.3%) of the subjects had activity greater than 9.5 units/ml and 7 (12.7%), had activity less than 9.5 units/ml. These percentages were virtually identical to those found in the large population sample previously studied (Weinshilboum and Sladek, 1980). Correlations among RBC, lymphocyte, and platelet TPMT activities for these 55 subjects are shown in Fig. 7. The correlation coefficient for RBC and lymphocyte activities was 0.563 ($P < 0.0001$), that for RBC and platelet activities was 0.680 ($P < 0.0001$), and the correlation coefficient for lymphocyte and platelet activities was 0.285 ($P < 0.04$) (Figs. 7A, B, and C, respectively). Although these correlations were all significant, a correlation coefficient is not the most appropriate statistical test for the evaluation of these data. That is true because previous studies clearly demonstrated that subjects could be classified into three separate genetic groups based on their RBC enzyme activities (Weinshilboum and Sladek, 1980). There was no necessary reason why lymphocyte and platelet TPMT activities for these 55 subjects should have been related to their genetically determined RBC enzyme activities. However, the average RBC activities of individuals presumed to be of genotype $TPMT^H$ $TPMT^H$, those with activities of greater than 9.5 units/ml, were 12.5 ± 0.2 (mean ± SE) units/ml RBCs (Fig. 8). The lymphocyte and platelet activities for these same subjects averaged 11.6 ± 0.4 units/10^9 lymphocytes and 22.9 ± 0.6 units/10^{11} platelets. Similar values for the 7 subjects with RBC TPMT activity of less than 9.5 units/ml RBCs (presumed genotype $TPMT^L$ $TPMT^H$) were 6.8 ± 0.4 units/ml RBCs, 6.8 ± 0.7 units/10^9 lymphocytes, and 13.6 ± 1.4 units/10^{11} platelets (Fig. 8). Enzyme activity levels for lymphocytes and platelets were significantly lower ($P < 0.001$) in blood samples from subjects presumed to be heterozygous than in those from subjects with the genotype $TPMT^H$ $TPMT^H$ based on RBC enzyme activity levels (Fig. 8). It should be emphasized that the classification

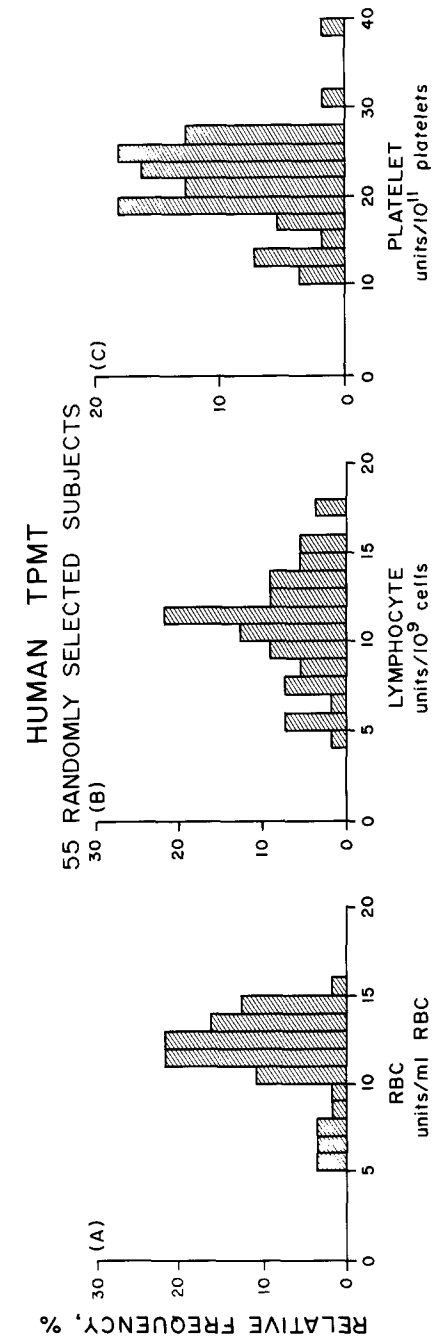

Fig. 6. Frequency distribution histograms for TPMT activity in (A) erythrocytes, (B) lymphocytes, and (C) platelets in blood samples from 55 randomly selected subjects.

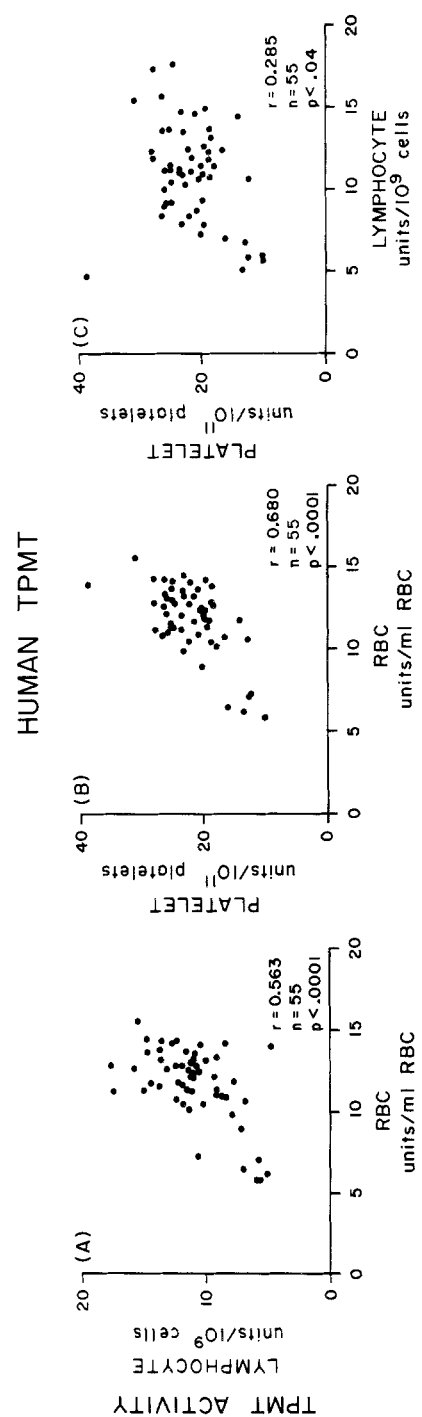

Fig. 7. Correlations of RBC, lymphocyte, and platelet TPMT activities in blood samples from 55 randomly selected subjects.

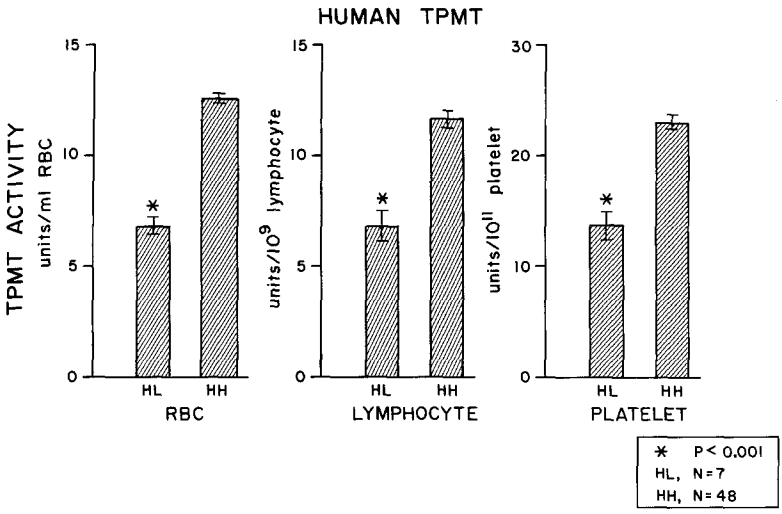

Fig. 8. RBC, lymphocyte, and platelet TPMT activities in subjects with intermediate (<9.5 units/ml RBC) and high (>9.5 units/ml RBC) RBC TPMT. Each value represents the mean ± SE for 7 subjects with intermediate and 48 subjects with high RBC TPMT activities.

of subjects was based entirely on the RBC activity level. All of these results were compatible with the conclusion that the genetic polymorphism regulating RBC TPMT also regulated the enzyme activity in lymphocytes and platelets. Another way to test this possibility was to measure lymphocyte enzyme activity in blood samples from subjects with known genotypes for RBC TPMT, subjects who were discovered in the course of the pedigree studies reported previously (Weinshilboum and Sladek, 1980).

Lymphocyte TPMT in Individuals with Known Genotypes

Our previous studies resulted in the identification of three subjects with undetectable RBC TPMT among 886 randomly selected individuals (Weinshilboum and Sladek, 1980). Identification of these three probands, subjects presumed to be homozygous for the allele for low enzyme activity, enabled us to study their relatives and to identify additional subjects with the genotype $TPMT^L$ $TPMT^L$. During the present study of lymphocyte enzyme activity, blood samples were obtained from four subjects with "low," four with "intermediate," and one with "high" activity among first-degree relatives of the three original probands of genotype $TPMT^L$ $TPMT^L$ (Table I). Pedigrees for the families in which more than one member was studied are shown in Fig. 9. As can be seen from the results shown in Table I, the four subjects with undetectable RBC TPMT also had undetectable lymphocyte and platelet

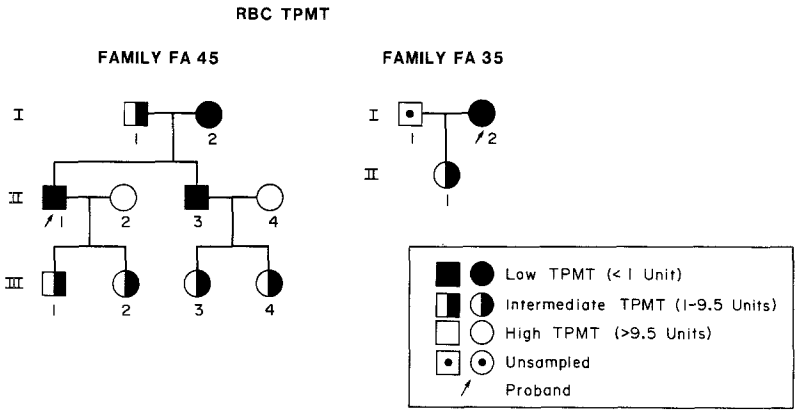

Fig. 9. Pedigrees of families with probands who had undetectable RBC TPMT. See text and Table I for details.

activities. Heterozygous subjects had intermediate activities in lymphocytes and platelets. However, the one subject with high RBC enzyme activity had an intermediate level of activity for both lymphocytes and platelets. With the exception of the results found in this one subject, these data were also compatible with the conclusion that the genetically regulated RBC enzyme activity level reflected the level of TPMT activity in lymphocytes and platelets.

Table I. Lymphocyte and Platelet TPMT Activities in Subjects from Pedigrees that Include a Proband with the Genotype $TPMT^{L}$ $TPMT^{La}$

Family	Subject	Genotype	TPMT activity		
			RBC	Lymphocyte	Platelet
FA 37	II-4[b]	LL	0	0	0
FA 35	I-2[b]	LL	0	0	0
	II-1	HL	5.6	1.2	1.8
FA 45	I-1	HL	8.1	4.8	4.0
	I-2	LL	0	0	0
	II-1[b]	LL	0	0	0
	II-2	HH	14.8	8.0	14.3
	III-1	HL	7.4	5.3	7.9
	III-2	HL	6.7	3.4	5.9

[a] Family and subject code numbers are those that were used previously to identify the subjects (Weinshilboum and Sladek, 1980). HH represents $TPMT^{H}$ $TPMT^{H}$, HL represents $TPMT^{H}$ $TPMT^{L}$, and LL represents $TPMT^{L}$ $TPMT^{L}$. Enzyme activities are expressed as units/ml RBCs, units/10^9 lymphocytes, and units/10^{11} platelets.
[b] Proband.

DISCUSSION

These studies were initiated to determine optimal conditions for the assay of human lymphocyte TPMT, to characterize the enzyme in lymphocyte homogenates, and to determine whether the level of lymphocyte TPMT activity was regulated in parallel with the genetically determined level of activity in the RBC. TPMT activity was present in highly purified preparations of human lymphocytes. Optimal conditions for the assay of the enzyme activity were determined, and both human lymphocyte and platelet TPMT activities were found to be regulated in parallel with RBC TPMT. The high degree of correlation among lymphocyte, platelet, and RBC TPMT activity is compatible with the conclusion that the genetic polymorphism that regulates TPMT activity in the human erythrocyte plays a major role in the regulation of the enzyme activity in other human cells and tissues. This hypothesis is further supported by the recent observation of a significant correlation between human RBC and kidney TPMT activities (Dunnette *et al.*, 1981).

Even though care was taken to be certain that the lymphocytes studied here were highly purified, these preparations still contained small numbers of monocytes (Sladek-Chelgren and Weinshilboum, 1981), and no attempt was made to separate T from B lymphocytes. Whether heterogeneity in TPMT activity or regulation occurs in these subtypes of mononuclear blood elements cannot be answered on the basis of these data.

The experiments described here have opened the way for the use of cultured human lymphocytes in biochemical genetic and "pharmacogenetic" studies of individual variations in the metabolism of 6-mercaptopurine, 6-thioguanine, and azathioprine. It should now be possible to use cultured lymphocytes to evaluate the potential functional significance of this polymorphism in a way that would be impossible in human subjects. The significance of such an evaluation is even greater since lymphocytes are the target cell for thiopurines when these drugs are used in the treatment of some forms of leukemia (Acute Leukemia Group B, 1963). The possibility of studying the functional importance of this polymorphism *in vitro* is especially important because of the toxicity of thiopurine drugs (Acute Leukemia Group B, 1963; Einhorn and Davidsohn, 1964; Paterson and Tidd, 1975).

Many questions with regard to the TPMT polymorphism remain unanswered. Whether this locus represents a "structural" or a "regulatory" gene is not clear, although the results of recent immunoprecipitation studies indicate that the relative level of RBC enzyme activity is directly related to the quantity of TPMT protein (Woodson *et al.*, 1981b). It is not known whether TPMT plays a role in cellular function other than in the metabolism of xenobiotic substances. The description of the cumulation of an endogenous TPMT substrate in the plasma of patients with chronic renal failure raises the possibility that the function of TPMT may extend beyond just the metabolism

of drugs (Pazmiño *et al.*, 1980). At the very least, increased understanding of the biochemical basis of the genetic regulation of this important enzyme may help to increase understanding of individual variations in the response of patients to thiopurine drugs and in the occurrence of reactions to these toxic therapeutic agents.

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