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Human Erythrocyte Protein L-Isoaspartyl Methyltransferase: Heritability of Basal Activity and Genetic Polymorphism for Thermal Stability[1]

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Protein L-isoaspartyl methyltransferase (PIMT) is believed to play an important role in the disposition of age-damaged proteins by catalyzing the repair of abnormal isoaspartyl linkages resulting from the spontaneous deamidation of asparaginyl residues or isomerization of aspartyl residues. As a step toward testing the hypothesis that human disease- or age-related pathology might be associated with a deficiency in PIMT, we investigated basal activity and thermal stability of PIMT in erythrocyte lysates from 299 U.S. family members. Thermal stability was measured because it is a sensitive measure of variation in amino acid sequence. Basal activity was normally distributed with a mean ± SD of 558 ± 43 units/ml erythrocytes. Statistical analysis of the data revealed that basal PIMT activity exhibited a high degree of heritability. Enzyme thermal stability showed a skewed bimodal frequency distribution, and segregation analysis of family member pedigrees was consistent with Mendelian inheritance of two major alleles. No DNA was available from the family samples, so we tested two additional population samples for a known Ile/Val polymorphism at codon 119 and for PIMT activity and thermal stability, using blood donated by 25 Norwegians and by 20 Koreans. Single-stranded conformational polymorphism analysis using polymerase chain reaction revealed a 100% correlation between thermal stability grouping and this polymorphism. The high thermal stability samples were all homozygous Ile, the low thermal stability samples were all homozygous Val, and the intermediate thermal stability samples were all heterozygous. Furthermore, this polymorphism was responsible, in part, for the variation observed in basal erythrocyte PIMT activity. These results will help provide a foundation for future studies aimed at correlating levels of PIMT activity, or other properties of this enzyme, with human disease.

Key Words: **isoaspartate; protein repair; polymorphism.**

Protein L-isoaspartyl methyltransferase (EC 2.1.1.77, PIMT)[3] is an ubiquitous enzyme found in bacteria, plants, and animals (1–5). Its ability to recognize and methylate the unusual α-carboxyl group on the side chain of isoaspartate in proteins and peptides *in vitro* is well documented (6–9) and supports the hypothesis that PIMT functions to repair damaged proteins. The presence of isoaspartate within a polypeptide results in the insertion of a methylene group into the peptide backbone, which may lead to loss of enzymatic activity or other functional consequences. Isoaspartyl residues are generated within susceptible amino acid sequences by deamidation of aspara-

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[3] Abbreviations used: PIMT, protein L-isoaspartyl methyltransferase; RBC, erythrocytes; SSCP, single-stranded conformational polymorphism; PCR, polymerase chain reaction.

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gine or isomerization of aspartate (10, 11). Spontaneous breakdown of isoaspartyl methyl esters, formed by the action of PIMT, leads to the production of isoaspartate and aspartate in, approximately, a 3:1 ratio (12). Repeated cycles of methylation/demethylation at physiological pH have been shown to effectively convert abnormal isoaspartyl linkages to normal aspartyl linkages in model peptides (12–14) and in a spontaneously deamidated form of the *Escherichia coli* HPr phosphocarrier protein (15). PIMT-catalyzed methylation has been shown to restore a calcium-dependent enzyme activating function in calmodulin which had accumulated isoaspartate during *in vitro* aging (16). Evidence for a repair function for PIMT *in vivo* is supported by studies showing that the level of isoaspartate-bearing proteins increases dramatically in rat PC12 cells grown in the presence of a methyltransferase inhibitor (17) and in mice in which the gene encoding PIMT has been selectively disrupted (18). The PIMT-deficient mice grow more slowly than their normal counterparts and undergo fatal seizures between 26 and 60 days after birth.

PIMT is apparently crucial for optimal cell function, and low levels of PIMT might contribute to the pathophysiology of human disease. Since isoaspartate formation is bound to occur with a wide range of rates in most proteins as they age, the possible role of PIMT deficiency in selected age-related diseases has been investigated. No difference was found in PIMT-specific activity in postmortem brain samples from Alzheimer's disease patients compared with controls (19), and no significant deficit in PIMT levels compared with controls was found in cultured fibroblasts obtained from donors with Werner's syndrome or progeria (B. A. Johnson and D. W. Aswad, unpublished work). The gene encoding PIMT has been structurally characterized (20) and localized to chromosomal region 6q22.3–6q24 in humans (21). Genetic polymorphisms of the enzyme have been described at the nucleotide level (22) but these polymorphisms have not yet been associated with any phenotypes.

As one step in exploration of a possible role of PIMT in human disease, we have undertaken a study of PIMT levels in erythrocyte (RBC) lysates from 299 healthy donors representing 52 families. Red blood cells (RBCs) offer a convenient source of PIMT (2, 23–25) and have previously proven useful as an indicator of general tissue levels of several small-molecule methyltransferases, including catechol *O*-methyltransferase (26, 27) and thiopurine methyltransferase (28). Population and family studies on the levels of these methyltransferases as well as RBC histamine *N*-methyltransferase (29) have provided important insights into their patterns of inheritance and have elucidated the molecular basis for clinically important individual variation in neurotransmitter metabolism and sensitivity to xenobiotics, including chemotherapeutic agents (30, 31). In an attempt to take a similar approach to determine the role of inheritance in the regulation of erythrocyte PIMT activity or other properties, we measured basal RBC PIMT activity and thermal stability of the enzyme in blood samples obtained from family members. Thermal stability was measured since it has been shown to be a sensitive indicator of amino acid changes as a result of genetic polymorphisms (30).

The current study was carried out to establish the normal range of PIMT levels in healthy subjects and to determine the extent to which heredity might influence individual variation in the level of PIMT activity or its thermal stability. We show here that PIMT levels in erythrocytes of healthy individuals fell within a narrow range, and the level of activity appeared to be governed substantially by inheritance. Thermal stability data from a population sample of 299 donors suggested the presence of two major alleles which were subsequently shown to be associated with the presence of an Ile/Val polymorphism recently described by Tsai and Clarke (22) at PIMT amino acid residue 119. These alleles were inherited in a simple autosomal Mendelian fashion. These findings should provide a foundation for future attempts to determine the role of inheritance in the regulation of PIMT in humans and the possible role of PIMT in human disease.

MATERIALS AND METHODS

Materials. Radiolabeled *S*-adenosyl-[*methyl*-^{3}H]-L-methionine was purchased from NEN Dupont and diluted to the desired specific activity with unlabeled *S*-adenosyl-L-methionine (Sigma Chemical Co.) which had been purified by carboxylmethyl cellulose chromatography (32). Bovine γ-globulins were purchased from Sigma Chemical Co. Rat recombinant PIMT was purified as described (33).

Blood samples. All blood samples were obtained after informed, written consent of the donors or, in the case of minor children, their parents.

Minnesota, U.S.A. Blood was obtained from 299 healthy human donors (29) and erythrocyte lysates were prepared as described previously (34). The donors consisted of members of 52 families. Some kindreds included grandparents as well as parents and children in nuclear families. Additionally, a pool made with randomly selected human erythrocyte lysates from healthy blood donors (designated the Minnesota reference lysate) was used to correct for both intraday and interday assay variations as described subsequently. Lysates were stored at −80°C until used.

Norway and Korea. Blood was donated by 25 healthy, genetically unrelated Norwegian adults. Erythrocyte lysates were prepared and assayed for PIMT activity, and genomic DNA was isolated from whole blood or buffy coats using the QIAamp Blood Kit (QIAGEN, Inc., Chatsworth, CA). Blood was also donated by 20 healthy, genetically unrelated Korean children and processed as described above for the Norwegian samples. The samples from the Norwegian and Korean donors were originally obtained for another, independent investigation of thiopurine methyltransferase activity (35). We found no significant correlation between PIMT and thiopurine methyltransferase activities or thermal stabilities (data not shown). Thus, these sam-

ples can be considered to have been randomly selected with respect to PIMT activity and thermal stability.

California, U.S.A. Blood was drawn from seven genetically unrelated healthy human adults, equal volumes were pooled, and an erythrocyte lysate was prepared (34). PIMT activity was measured on the same day the lysate was prepared.

PIMT assay. Frozen erythrocyte lysates were thawed on ice. On each day, a new vial of Minnesota reference lysate was used. From each sample, lysate was removed and split into two equal portions. One portion was kept on ice and the other was heated in a shaking water bath at 52°C for 15 min. These time and temperature parameters had been determined previously to achieve 50% inactivation of PIMT in the Minnesota reference lysate. Heated samples were placed back on ice until assayed. Samples were assayed in duplicate using a modification of the methanol diffusion method (19, 36) as described below. Assay tubes contained 10 μl of a buffer composed of 310 mM sodium phosphate, 100 mM sodium citrate, 10 mM sodium EDTA, pH 6.0, 10 μl of 25 mg/ml bovine γ-globulins in 10 mM HCl, 10 μl H_2O, 10 μl erythrocyte lysate, and 10 μl of 100 μM *S*-adenosyl-[*methyl*-^{3}H]-L-methionine (1.54 nCi/pmol). Blanks contained H_2O instead of erythrocyte lysate. Tubes were incubated at 37°C for 2 min prior to the addition of the *S*-adenosyl-[*methyl*-^{3}H]-L-methionine to start the reactions. After 5 min at 37°C, the reactions were stopped by the addition of 700 μl of 7% trichloroacetic acid, vortexed, and kept on ice for 5 min. The pellets were collected by centrifugation at 11,900*g* for 30 s at room temperature followed by supernatant aspiration. Pellets were washed once with 700 μl H_2O and then dissolved in 100 μl of 0.2 *M* NaOH/2% MeOH. Methyl esters (formed by the action of PIMT and *S*-adenosyl-[*methyl*-^{3}H]-L-methionine on the isoaspartyl groups in the γ-globulins substrate) were hydrolyzed, producing radioactive methanol. A portion (80 μl) of this solution was spotted onto accordion-pleated filter paper lodged in the cap of a shell vial containing 2.5 ml Liquiscint (National Diagnostics, Atlanta, GA) scintillation cocktail. The vials were heated at 40°C for 1 h to facilitate the diffusion of methanol into the Liquiscint, the filter papers were removed, and the vials were counted in a Beckman Model LS 7500 liquid scintillation counter. One enzyme unit (U) was defined as 1 pmol methyl groups transferred/min at 37°C.

Thermal stability of PIMT in lysates. Thermal stability was calculated by dividing the PIMT activity measured in the heated lysates by the PIMT activity measured in the unheated or control (basal) lysates. This calculation yielded the reported H/C ratios.

Assay variation corrections. To correct for intraday and interday variation in assay conditions, each daily set of samples included duplicate Minnesota reference lysate samples, both at the beginning of the assay set (early reference lysate samples), and at the end of the assay set (late reference lysate samples). The Minnesota reference lysate was stored frozen in aliquots and a new tube was thawed for each day's set of assays. The PIMT activity of all late reference lysate samples was 1.41 $\pm$ 0.05 U/mg protein (mean $\pm$ SD), while the PIMT activity of all the early reference lysate samples was 1.54 $\pm$ 0.06 U/mg protein (mean $\pm$ SD). Thus, an average downward drift of 8.4% occurred during the period between initiating the early assays and the late assays on any given day. To account for this drift, all experimental samples were corrected upward to a no-drift equivalent by using a correction factor that depended on the relative position of the sample in the assay set. For example, the PIMT activity obtained for sample 30 (of 40 total daily experimental samples) was corrected upward by a factor of 1.06 (30/40ths of 8.4%). In addition to this daily sample-position drift correction, assays were also corrected for interday variation of the Minnesota reference lysate from the mean of all Minnesota reference lysate sample assays measured on all assay days. Thus, if the PIMT activity of the Minnesota reference lysate samples on day 7 were 5% higher than the mean value of all day's Minnesota reference lysate samples, then all of the drift-corrected samples assayed on day 7 were corrected downward by 5%. Corrections of the PIMT assay data for heated and unheated lysate samples were both adjusted by the procedure described above, using heated and unheated Minnesota reference lysate samples, as appropriate.

Protein determination. Protein was measured by the method of Lowry (37) following precipitation with a final concentration of 5% trichloroacetic acid. Bovine serum albumin was used as a standard.

Single-stranded conformational polymorphism (SSCP) DNA analysis (38). Genomic DNA was amplified by PCR using primers specific for exon 5 containing the major polymorphism at amino acid position 119 of the PCMT1 gene. The DNA containing this polymorphism was amplified with primer 3 (5′-GTTGGATGTACTGGAAAAGTCATAGG-3′) and primer 4 (5′-CAAGCTGTACTCTCCCTGAAGACAG-3′) to give a 118-bp product (22). The PCR reaction mixture contained 1× reaction buffer (Promega, Madison, WI), 20 μM dNTPs, 0.165 μM [α-^{32}P]dCTP (3000 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA), 2.5 pmol of each primer, about 200 ng DNA, and 1 U *Taq* DNA polymerase (Promega). The amplification procedure began with a "hot start" using HotWax Mg^{+2} beads (Invitrogen, Carlsbad, CA) in a 10-μl reaction volume to give a final Mg^{+2} concentration of 7.5 mM. The cycling parameters consisted of an initial denaturation and melting step of 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 55°C for 15 s, and 72°C for 30 s. The PCR was concluded with a final extension step of 72°C for 5 min. For the analysis of SSCPs, the PCR products were denatured at 95°C for 5 min and loaded onto a 0.5× Hydrolink MDE polyacrylamide gel (J. T. Baker, Phillipsburg, NJ) (40 cm long × 0.4 mm thick) in 0.6× TBE/10% glycerol and subjected to electrophoresis in 0.6× TBE running buffer at 3 W for 18–20 h at room temperature. The gel was vacuum dried and autoradiography was performed. Assignments were made by comparing the banding patterns with those of individuals with known genotypes.

Enzyme data analysis. Statistical analyses were performed on an IBM PC using either NWA STATPAK, Multi-function Statistics Library version 3.1 (Northwest Analytical, Inc., Portland, OR) or the StatPac Statistical Analysis Package (Walonick Associates Inc., Minneapolis, MN). Correlation coefficients were calculated using a Pearson product–moment formula to compare data which had a gaussian distribution or the nonparametric Spearman rank correlation coefficient for data which had a nongaussian distribution.

RESULTS

Stability of PIMT in frozen lysates. Initial experiments were carried out with erythrocyte lysates obtained in 1986 from 299 healthy donors in Rochester, Minnesota. Since these erythrocyte lysates had been stored at −80°C for several years, it was important to compare the activity of PIMT in the stored Minnesota reference lysate (defined under Materials and Methods) with lysate made from freshly drawn and pooled blood (California lysate; composition described under Materials and Methods). The stored Minnesota reference lysate had a mean $\pm$ SD PIMT specific activity of 1.40 $\pm$ 0.09 U/mg protein and the freshly prepared California lysate had a mean $\pm$ SD PIMT specific activity of 1.38 $\pm$ 0.12 U/mg protein, indicating that the enzyme in frozen samples was not affected by storage. We also noted that three cycles of freezing and thawing did not significantly alter the PIMT activity in the Minnesota reference lysate.

PIMT activity in family members. PIMT basal activity in the erythrocyte lysates from Minnesota had a normal frequency distribution with a mean of approxi-

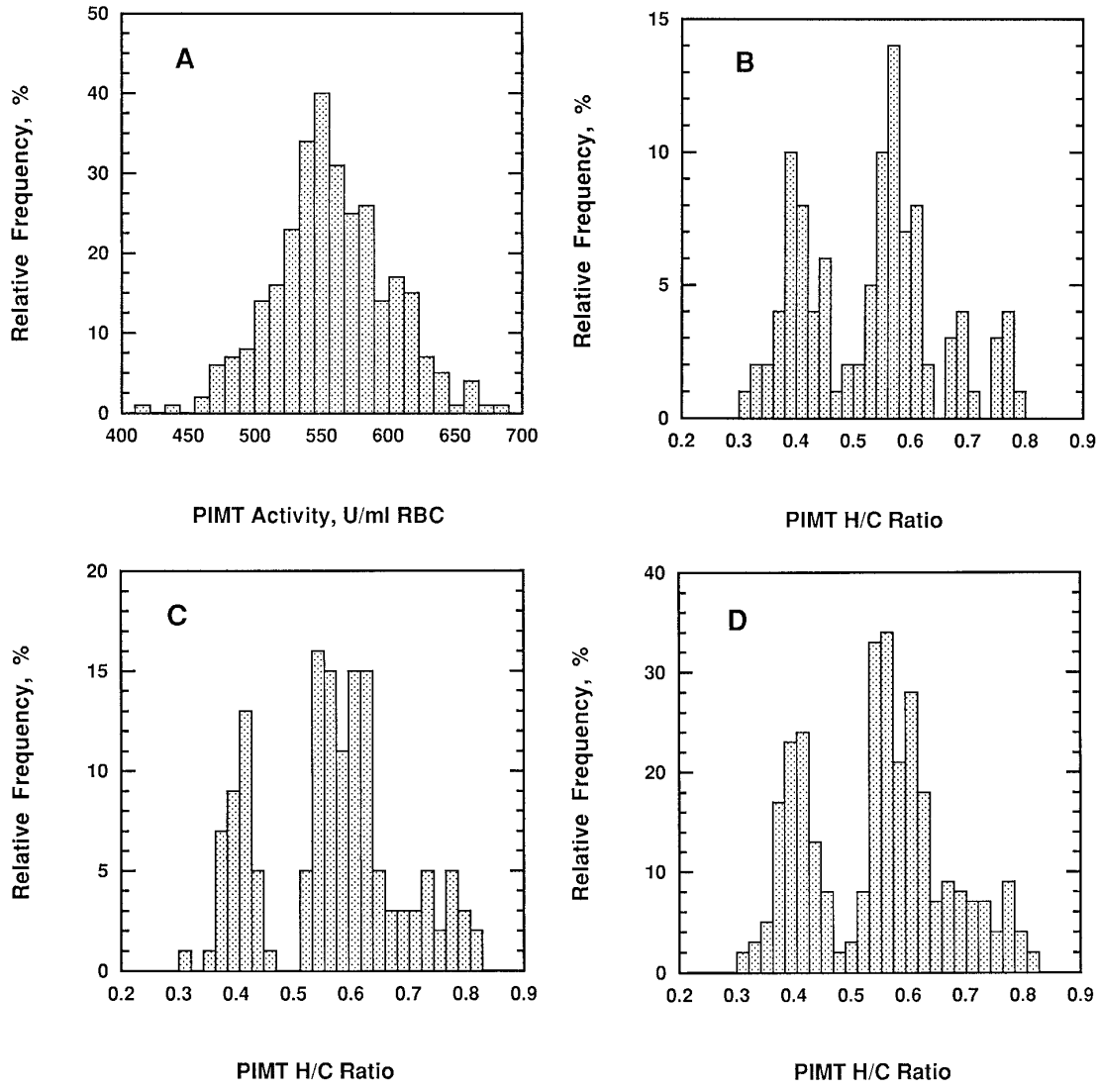

FIG. 1. Frequency distribution histograms of erythrocyte PIMT activity and thermal stability in Minnesota population sample. Assays were performed as described under Materials and Methods. (A) Basal (unheated) PIMT activity, all family members (N = 299). (B–D) Thermal stability as measured by H/C ratio (PIMT activity after heating at 52°C for 15 min (H) divided by unheated (control, C) activity); B, unrelated adults (N = 104); C, children (N = 146); D, all family members (N = 299).

mately 560 U/ml RBC (Fig. 1A) and a range from 420 to 687 U/ml RBC. There were no significant age- or gender-related differences in levels of enzyme activity; males averaged 556 ± 43 U/ml RBC and females averaged 559 ± 42 U/ml RBC (means ± SD). Since family members share genes, data from the 299 family members included in the study were used to determine correlations among family members as a first step toward a determination of the possible role of inheritance in the regulation of erythrocyte PIMT level of activity (Table I). For this analysis, only data for oldest sons or oldest daughters were used to avoid bias on the basis of family size. If the heritability of a quantitative trait is 1.0, i.e., if the variance is due entirely to the effects of additive inheritance, then sibling–sibling or parent–offspring pairs would have a correlation coefficient of 0.5 (39). In that same situation, the midparent value (the average of values for mother and father in each family) would be 0.71 (39). It can be seen that all of the correlations for basal activity listed in Table I, with the exception of those for genetically unrelated pairs, i.e. the parents, were highly significant, an indication of very high heritability for the trait of level of erythrocyte PIMT activity. These data are compatible with the conclusion that the major factor regulating RBC PIMT activity level in this population sample was inheri-

TABLE I

Familial Correlations of RBC PIMT Basal Activity and Thermal Stability[a]

Comparison	Basal activity			Thermal stability	
	N	r_P	*P*	r_S	*P*
Father vs mother	46	0.244	0.103	−0.002	0.989
Oldest son vs oldest daughter	38	0.638	<0.0001	0.415	0.009
Father vs oldest son	35	0.418	0.012	0.375	0.026
Father vs oldest daughter	39	0.435	0.005	0.433	0.006
Mother vs oldest son	41	0.631	<0.0001	0.262	0.099
Mother vs oldest daughter	45	0.540	<0.0001	0.321	0.031
Midparent vs oldest son	35	0.679	<0.0001	0.514	0.001
Midparent vs oldest daughter	38	0.637	<0.0001	0.584	<0.0001

[a] Human erythrocyte lysates from Minnesota donors. Basal activity represents enzyme activity in unheated samples. Thermal stability was determined by H/C ratio as defined under Results. *N*, number of samples; r_P, Pearson product–moment coefficient; r_S, Spearman rank correlation coefficient; *P*, probability. Midparent equals the average of parental values for the indicated parameter. Activities and thermal stabilities were compared using the statistical test indicated. Statistical significance was judged to be $P < 0.05$.

tance. The next issue to be addressed is the mechanism responsible for that effect. One possibility would be a genetic polymorphism that alters the encoded amino acid, a mechanism already shown to be responsible for significant variation in the levels of other cytosolic methyltransferase enzymes in humans (40–42). A sensitive test for differences in amino acid sequence that has been used frequently in biochemical genetic studies is thermal stability (30). Therefore, the next step in the analysis involved the measurement of PIMT thermal stability in these same samples.

Thermal stability of PIMT in family members. In contrast to the results for basal erythrocyte PIMT activity noted above, the frequency distribution of erythrocyte PIMT thermal stability as measured by H/C ratio (see Materials and Methods for a definition) was bimodal and was skewed at the upper end of the higher mode (Figs. 1B–1D). This is a situation very similar to that reported for RBC catechol *O*-methyltransferase which has a genetic polymorphism that changes a single amino acid, resulting in a sharp difference in enzyme thermal stability (40). There were no significant age- or gender-related differences in PIMT thermal stabilities. However, a skewed bimodal frequency distribution of H/C ratios was observed when the data for the parents (Fig. 1B) or for their children (Fig. 1C) were plotted, suggesting that inheritance may play a role in the observed differences in thermal stability of erythrocyte PIMT. That suggestion was further supported by the correlation analysis (Table I), which demonstrated a striking familial aggregation of erythrocyte PIMT thermal stability.

When H/C ratios were plotted against basal PIMT activity (Fig. 2A), the data fell into two distinct low and high H/C ratio clusters, with the high H/C cluster skewed and possibly composed of two subgroups. The presence of these distinct groups, when coupled with the correlation data from the family studies which demonstrated familial aggregation of PIMT thermal stability, suggested that the trait of PIMT H/C ratio might be inherited in a simple Mendelian fashion, as discussed in the following section.

Segregation analysis of PIMT thermal stability. If PIMT thermal stability is inherited as a monogenic trait, this trait should segregate within families as predicted by the rules of Mendelian inheritance. In order to perform segregation analysis of the data, however, it would be necessary to classify each family member as having low, intermediate, or high thermal stability, presumably reflecting genotypes low/low, low/high, and high/high H/C ratios. Unfortunately, although it is relatively straightforward to separate the low subgroup by use of the nadir of the H/C ratio frequency distribution shown in Fig. 1D ($\leqslant 0.48$), the hypothetical intermediate and high H/C ratio subgroups overlap. To make it possible to test the hypothesis of Mendelian inheritance, we used the Hardy–Weinberg theorem to estimate frequencies for proposed alleles for high and low thermal stability using only data for the 104 unrelated adult subjects studied. This was done to avoid a biased sample as a result of differences in family size. As demonstrated subsequently, these hypothetical alleles regulating the trait of RBC PIMT thermal stability proved to be directly correlated with a genetic polymorphism at the DNA level that alters the amino acid encoded by PIMT codon 119. The results of the Hardy–Weinberg analysis indicated that 36% of these 104 subjects were apparent genotype low/low for the trait of H/C ratio, while 48% would be expected to be low/high

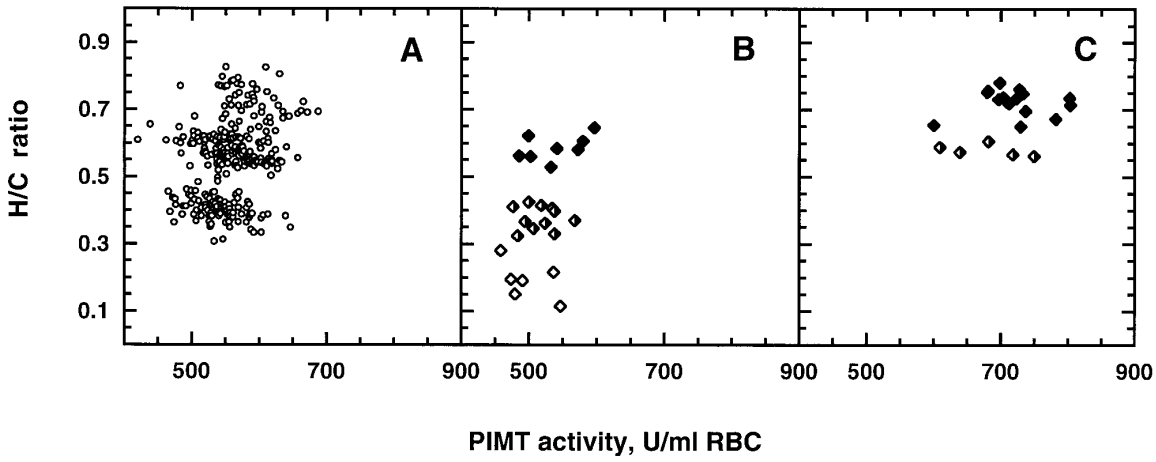

FIG. 2. Thermal stability and activity of PIMT in erythrocyte lysates and genotype at codon 119. PIMT activity after heating (H) divided by unheated (C, control) activity (H/C ratio) plotted against basal (unheated) PIMT activity. (A) Minnesota population sample (N = 299): open circles, genotypes not determined. (B) Norwegian population sample (N = 25): open triangles, homozygous Val at PIMT residue 119; half-filled triangles, heterozygous Ile/Val at PIMT residue 119; filled triangles, homozygous Ile at PIMT residue 119. (C) Korean population sample (N = 20): half-filled triangles, heterozygous Ile/Val at PIMT residue 119; filled triangles, homozygous Ile at PIMT residue 119.

and 16% high/high, with a cutoff between those with intermediate and high H/C ratios at a value of ⩾0.67. If those cutoff values were used to classify all subjects in the families studied, it was possible to predict both the relative frequency of matings of individuals with different genotypes for this trait and the outcomes of those matings, assuming Mendelian inheritance. The results of the segregation analysis shown in Table II were consistent with Mendelian inheritance of the trait of erythrocyte PIMT thermal stability. In only one case, a high/high × low/low mating that yielded a high/high offspring instead of the expected high/low (intermediate) phenotype, the results did not fit the model's prediction. This is not a major inconsistency, however, since both the high/high parent and the child in this family had H/C ratios that were very near the cutoff value of 0.67.

TABLE II

Segregation Analysis of the Trait of Erythrocyte PIMT Thermal Stability in a Minnesota Population Sample

Parents	N (families)	Children			Predicted H/C ratio phenotype[b]
		H[a]	I	L	
L × L	4	0	0	10	L
H × H	1	3	0	0	H
H × L	7	1	23	0	I
H × I	4	5	7	0	H, I
L × I	12	0	24	13	I, L
I × I	14	12	16	6	H, I, L

[a] H, high H/C ratio ⩾0.67; I, intermediate H/C ratio 0.48 < H/C < 0.67; L, low H/C ratio ⩽ 0.48.
[b] Based on a Mendelian (monogenic) model of inheritance.

Genotypic variation in PIMT. Partial sequencing of DNA from 30 individuals has previously suggested that there are three sites of polymorphism in the human PIMT gene (22). The site with the highest variation corresponds to amino acid 119, which has a calculated allelic frequency of 0.77 for Ile and of 0.23 for Val within a racially mixed population sample (N = 30). To determine whether the observed phenotypic differences in PIMT activity and thermal stability might be related to this known allelic variation, we used SSCP analysis to examine the genomic DNA sequence corresponding to PIMT amino acid residue 119. DNA was not available from the Minnesota donors, so we used DNA and erythrocyte lysates obtained more recently from a group of 25 unrelated healthy Norwegian adults and 20 unrelated healthy Korean children. As shown in Fig. 2B, all individuals from Norway who had low PIMT H/C ratios were homozygous for Val, all individuals who had intermediate H/C ratios were heterozygous Ile/Val, and, finally, all individuals who had high H/C ratios were homozygous for Ile. Similarly, in the Korean population sample studied, all individuals who had high H/C ratios were Ile homozygous and all individuals who had lower PIMT thermal stability were heterozygous Ile/Val (Fig. 2C). Note that none of the individuals in this Korean population sample were homozygous for Val. This latter observation might be explained by the small sample size (N = 20), but it is also consistent with a previous observation that the frequency of the allele that encodes Val 119 is low in Asian

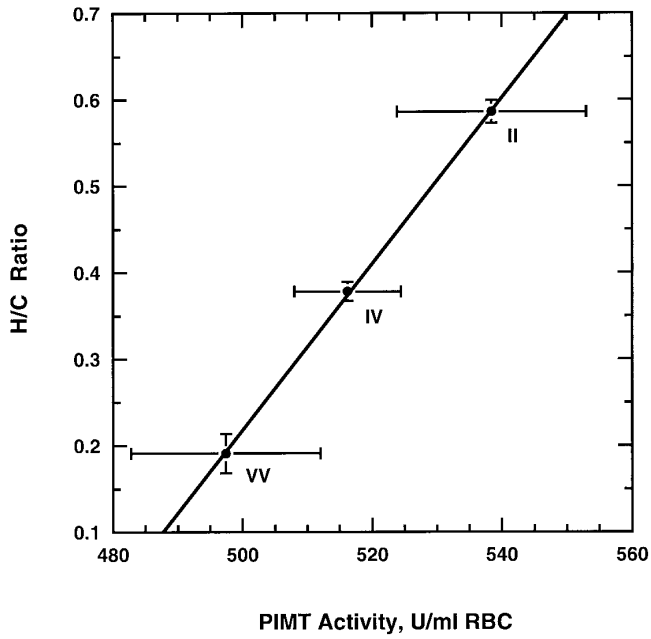

FIG. 3. Correlation of PIMT basal activity and thermal stability with respect to genotype in the Norwegian population sample (N = 25). Values for PIMT basal activities and H/C ratios were averaged based on the genotype of the samples at PIMT residue 119. II, homozygous Ile (N = 8); IV, heterozygous Ile/Val (N = 11); V, homozygous Val (N = 6). Error bars represent ±SE. Linear regression correlation coefficient was 0.9997.

populations (C. G. DeVry and S. Clarke, unpublished work).

Basal erythrocyte PIMT activity also seems to be influenced by the identity of amino acid residue 119. In the two population samples for which we have genotypic data, Norway and Korea, slightly higher erythrocyte PIMT activity was seen for the Ile homozygous form compared to Ile/Val and to the Val homozygous form (Figs. 2B and 2C). Furthermore, there was a linear relationship between H/C ratios and basal PIMT activity for the Norwegian population sample studied when these parameters were stratified on the basis of genotype at amino acid 119 (Fig. 3). Statistical analysis (t test) between group means revealed that the H/C ratios for each genotype were significantly different ($P < 0.0001$) and that the difference in mean PIMT activities for the two homozygous forms was also significant ($P < 0.044$).

Population comparisons. Although the erythrocyte lysates from the Minnesota, Norway, and Korea population samples were not assayed concurrently, we believe we can usefully compare values for the different populations since assays of the Minnesota reference lysate showed very little variation (Table III). Mean basal erythrocyte PIMT activity for the Minnesota and Norwegian population samples were very similar (558 ± 43 and 519 ± 36 U/ml RBC, respectively, (means ± SD)), while the basal erythrocyte PIMT activity for the Korean population sample was significantly higher (710 ± 54 U/ml (mean ± SD)) (Fig. 2). These observed variations between populations in erythrocyte PIMT basal activities may be due to differences in gene regulation. Erythrocyte PIMT thermal stabilities for the Minnesota and Korean population samples were identical, but the Norwegian population samples had a lower mean H/C ratio for each stability group (Fig. 2 and Table IV). In fact, comparing the Minnesota and Norwegian populations, the thermal stability groups for the Norwegians were shifted downward by approximately one stability group; e.g., the high thermal stability group in the Norwegian population sample was equivalent to the intermediate thermal stability group in the Minnesota population sample (Figs. 2A and 2B). We presume this variation represents a systematic difference in collection or in handling of the Norwegian samples prior to assay.

The tight correlation between genotype for the polymorphism at codon 119 and H/C ratio made it possible to extend the analysis of the relationship of that polymorphism to the level of basal erythrocyte PIMT activity and, thus, to include the Minnesota population sample in a similar analysis. As already described under "Segregation Analysis of PIMT Activity," that population sample could be separated into groups with low, intermediate, and high PIMT thermal stability, with the clear understanding that some samples would be misclassified because of overlap between the intermediate and high H/C subgroups. Since these three subgroups correlate with Val/Val, Val/Ile, and Ile/Ile genotypes at codon 119, respectively, the analysis depicted graphically in Fig. 3 was repeated for the parents, children, and for all family members (Fig. 4). That analysis used H/C ratio as a marker for PIMT codon 119 genotype and con-

TABLE III

Consistency of PIMT Activity in a Minnesota Reference Lysate Monitored in Parallel with PIMT Assays of Samples from Minnesota, Norway, and Korea

	Reference lysate	
Parallel assay set[a]	PIMT activity (U/mg protein)	Thermal stability (H/C ratio)
Minnesota	1.48 ± 0.09	0.514 ± 0.038
Norway	1.44 ± 0.09	0.479 ± 0.054
Korea	1.47 ± 0.09	0.526 ± 0.047

[a] Composition of the Minnesota reference lysate was identical for each assay set (see Materials and Methods for a description). Values reported are means ± SD.

TABLE IV

Comparison of H/C Ratios Determined for PIMT in RBC Lysates[a]

Thermal stability group	Population sample		
	Minnesota	Norway	Korea
High H/C ratio[b]	0.734 ± 0.043	0.586 ± 0.037	0.723 ± 0.039
Intermediate H/C ratio	0.579 ± 0.038	0.378 ± 0.035	0.580 ± 0.018
Low H/C ratio	0.401 ± 0.033	0.191 ± 0.056	None

[a] Values reported are means ± SD.
[b] High, intermediate, and low H/C ratios are defined in the footnote to Table II for the Minnesota population sample. For the population samples from Norway and Korea, the groupings were made on the basis of the observed genotype at codon 119.

firmed the observation shown in Fig. 3. Specifically, it confirmed that this polymorphism was responsible for part, but not all, of the effect of inheritance on basal level of erythrocyte PIMT activity.

Low PIMT thermal stability was not due to enzyme inactivators in lysates. We verified that there were no endogenous substances in the low thermal stability lysates that may have contributed to the inactivation of PIMT by performing the following experiment. Using lysates from the Minnesota population sample, we made two lysate pools, one from six individuals with high H/C ratios and one from six individuals with low H/C ratios. None of the individuals was genetically related. We then added an excess of purified rat recombinant PIMT to the lysate pools, before and after heat treatment (52°C, 15 min), and assayed the samples for PIMT activity as outlined under the Materials and Methods section. We found no difference between the two lysate pools in the methyltransferase activity contributed by the rat recombinant enzyme (data not shown).

DISCUSSION

Erythrocyte PIMT activity seems to be tightly controlled within a given population. For the largest population sample studied, Minnesota, PIMT activity was 558 ± 43 U/ml RBC (mean ± SD) (Fig. 1A). This finding was in agreement with the concept that PIMT is a constitutively expressed enzyme necessary for cell survival (43, 44). Thus, very low levels of the enzyme may be deleterious or fatal. Not all erythrocyte methyltransferases show such a narrow activity range. Histamine *N*-methyltransferase activity, assayed in a subset of the Minnesota population sample reported here, has a much wider degree of variation, 137 ± 33 U/ml RBC (mean ± SD) (29). Thiopurine methyltransferase also displays a wide range of activity (45). Within a population sample of 298 unrelated healthy white adults from Minnesota, the activity of this methyltransferase had a mean ± SD of 12.76 ± 2.76 U/ml RBC. Frequency distributions of enzyme activity fell into three well-defined groups representing high, intermediate, and low (or nondetectable) activity. The high group (N = 264) had a mean ± SD of 13.50 ± 1.86 U/ml RBC, the intermediate group (N = 33) had a mean ± SD of 7.20 ± 1.08, and the low group (N = 1) had no detectable thiopurine methyltransferase activity.

Our results from statistical analysis of the basal RBC PIMT activity and thermal stability data from family member samples showed that both parameters had a high familial aggregation that was probably due to the effects of inheritance (Table I). Genetic factors also govern the activities of several other methyltransferase enzymes in humans (26–29).

One possible mechanism responsible for inherited differences in enzyme activity is amino acid changes due to genetic polymorphisms. Thermal stability is a sensitive measure of differences among proteins in amino acid sequences (30). Our results from RBC PIMT thermal stability studies, when coupled with SSCP DNA analysis, indicated that thermal stability of erythrocyte PIMT was directly related to an Ile/Val polymorphism at residue 119 (Fig. 2). Since only one gene encodes PIMT in humans, this polymorphism represents allelic variation (21). In addition to influencing PIMT thermal stability, the Ile/Val polymorphism also affected the level of basal enzyme activity. Higher RBC PIMT activity was associated with the Ile homozygous form compared to the Val homozygous form in the Norwegian population sample studied (Fig. 3). This relationship was also seen in the larger Minnesota population sample when the individuals were assigned a genotype at codon 119 based on their measured PIMT H/C ratios (Fig. 4).

To date, the three-dimensional structure of PIMT has not been determined; therefore, the location and significance of residue 119 in the enzyme reaction is unknown. Ingrosso *et al.* have identified three conserved amino acid sequence motifs in *S*-adenosyl-L-methionine-utilizing enzymes including PIMT (46). These

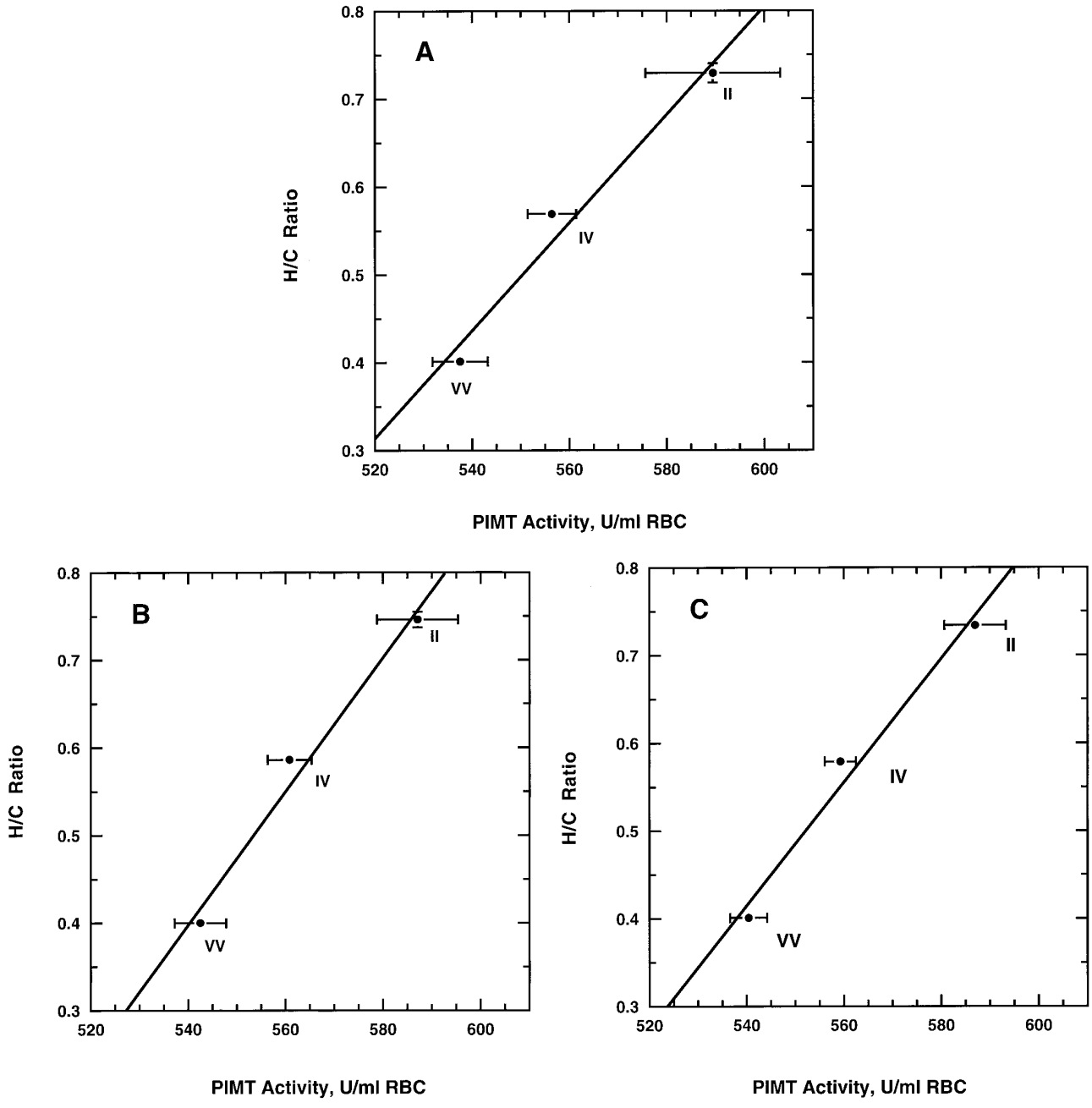

FIG. 4. Correlation of PIMT basal activity and thermal stability with respect to genotype in the Minnesota population sample. Values for PIMT basal activities and H/C ratios were averaged based on the presumed genotypes of the samples at PIMT residue 119. II, homozygous Ile; IV, heterozygous Ile/Val; VV, homozygous Val. Error bars represent ±SE; in cases where H/C ratio error bars are not evident, they are contained within the symbol. (A) Unrelated adults (N = 104): II, N = 15; IV, N = 51; VV, N = 38; linear regression correlation coefficient was 0.9713. (B) Children (N = 146): II, N = 25; IV, N = 82; VV, N = 39; linear regression correlation coefficient was 0.9787. (C) All family members (N = 299): II, N = 47; IV, N = 157; VV, N = 95; linear regression correlation coefficient was 0.9781.

motifs have been proposed to be involved with *S*-adenosyl-L-methionine and/or *S*-adenosyl-L-homocysteine binding (47). Residue 119 does not occur in any of these motifs; instead, it resides midway between motif I and II. If residue 119 is involved in the isoaspartyl peptide binding site, the subtle difference between Ile and Val side chains may allow for discrimination among protein substrates. If there are differences in protein substrate binding between the isozymes, it may be beneficial for an organism to be heterozygous for this trait, thus increasing the range of damaged proteins that can be effectively repaired.

This study indicates that erythrocyte lysates provide a useful and reliable indicator of heritable PIMT properties. The fact that PIMT activity in erythrocyte lysates from healthy individuals fell over a narrow range suggests that very low levels of PIMT may not be well tolerated. Thus, screening for PIMT activity in erythrocyte lysates of patients with medical syndromes of unknown etiology might provide a link between PIMT deficiency and one or more human diseases.

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