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SHORT COMMUNICATION

Concordance between Enzyme Activity and Genotype of Glutathione *S*-Transferase Theta (GSTT1)

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ABSTRACT. Blood samples from 140 healthy German volunteers were used to further characterize the genetic polymorphism of the human theta class glutathione *S*-transferase 1 (GSTT1). For measurements of GSTT1 activity, hemolysates were incubated *in vitro* with different concentrations of dichloromethane. The resulting enzymatically mediated production of formaldehyde was determined colorimetrically by the Nash reaction. GSTT1 genotyping was performed by polymerase chain reaction (PCR) methods using genomic DNA from total white blood cells. The prevalence of homozygous deletion of the GSTT1 gene was 19.3% (95% confidence limits: 12.2–27.7%). There was a high agreement between genotyping and phenotyping data. The individuals with the null genotype had a rate of formaldehyde production below the limit of quantification. In addition, in the group of GSTT1-positive individuals, we could differentiate highly active people (35.7%) from individuals with an intermediate enzyme activity (45.0%). It can be concluded that the PCR method is suitable to quickly genotype large populations, whereas the phenotyping assay at present offers the advantage of differentiating heterozygously from homozygously active subjects. Our results confirm the ethnic differences in the prevalence of the homozygous deleted genotype which were previously observed and seem to exist even between closely related ethnic groups such as German and Swedish populations. BIOCHEM PHARMACOL **56**;9:1189–1193, 1998.

KEY WORDS. glutathione; glutathione *S*-transferase; GSTT1; polymorphism; genotyping; phenotype

The GST§ genes encode four classes of isoenzymes: alpha, mu, pi, and theta [1–7]. GSTs play an important role in detoxification of various electrophilic compounds. An activity of the theta class isoenzyme GSTT1 has been detected in human liver [8] and erythrocytes [7]. GST conjugation activities of GSTT1 have been measured toward several halogenated alkanes, such as methyl chloride, -bromide, -iodide, ethylene dibromide, and dichloromethane; toward epoxides such as ethylene oxide, 1,2-epoxy-3-(p-nitrophenoxy)-propane, monoepoxybutene, and diepoxybutane; toward 4-nitrobenzoylchloride and -iodide; and toward cumene hydroperoxide [1, 2, 7–16]. For dichloromethane, it has been reported that conjugation by GSTT1 leads to genotoxic metabolites [11]. GSTT1 conjugation of several compounds was shown to be genetically polymorphic [2, 5]. Genotyping [17–20] and phenotyping [1, 11] assays have been performed to determine the prevalence of deficiency of the GSTT1 allele. A comparison of these studies showed wide ethnic differences in the allele frequencies of the GSTT1 isoenzyme. For example, when a Swedish population of 208 individuals was phenotyped, 11.1% lacked the activity with methyl chloride as substrate [1]. In German populations, between 15 and 40% of GSTT1-negative individuals were found [2, 3, 4]. In comparative genotyping and phenotyping assays of only 16 German and British individuals, 38% (95% confidence limits: 11.6–66.5%) lacked the enzyme activity [5]. Only two small samples (N = 40 and N = 16) using comparative phenotyping and genotyping methods for GSTT1 have been performed so far [3, 5]. In one study, enzyme kinetic parameters were evaluated [11]. Because of the great variability of the deficiency of the GSTT1 even in closely related ethnic groups, more and larger studies are required.

In this study, we demonstrate genotyping and phenotyping assays with blood samples from 140 individuals. Genetic characterization was performed by PCR and phenotyping by determination of the individual activity with three concentrations of the specific substrate dichloromethane. For each individual, the enzyme kinetic parameters K_m and V_{max} were evaluated.

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§ Abbreviations: GSH, reduced glutathione; GST, glutathione *S*-transferase; GSTT1, glutathione *S*-transferase T1; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Reagents

Dichloromethane (99.9% purity) was obtained from Merck and GSH from Aldrich. Formaldehyde solution (37%) for the calibration curves was obtained from Ferak. The exact content of this solution was determined iodometrically. All other chemicals for the phenotyping assay were from Merck. The chemicals for the PCR buffer and the Taq-Polymerase were obtained from Perkin Elmer and the agarose from Biozym. Primers for the PCR reaction were synthesized by Molbiol.

Sample Collection

Blood samples were taken from 140 healthy German volunteers after written consent. Their mean age was 30.7 years, ranging between 22 and 49 years. For the genotyping assay, the lymphocytes were isolated as described earlier [21]. For the phenotyping assay, whole blood samples were drawn into 10 mL vials with ethylene diamine tetraacetic acid and stored at −80°. For the assays, the vials were thawed for 60 min at room temperature. The hemolysate was centrifuged (4°, 20,000 *g*, 30 min), the pellet was discarded, and the supernatant was used for the assay.

Phenotyping and Genotyping Assays

The phenotyping assay was performed essentially according to methods previously described [11] with the following differences. Two-mL Eppendorf vials contained 125 μL of individual hemolysate; 4 mM of GSH; dichloromethane as substrate in 31, 62, and 124 mM concentrations; and Tris-HCl buffer, 20 mM, pH 7.4, to a total volume of 1 mL. After a 5-min preincubation at 37°, the reaction was started by addition of the substrate with a 10 μL Hamilton syringe. After thorough mixing, the incubation was performed at 37° in a shaking Eppendorf thermomixer (700/min) for 90 min. Three measurements were performed for each concentration. Two substrate-free blood samples from each individual were analyzed under the same conditions. After incubation, each sample was treated with 333 μL of 20% trichloroacetic acid for denaturation of the enzyme. After centrifugation (15,000 *g*, 10 min, room temperature), 1 mL of the supernatant was added to 1 mL of Nash's reagent [22] into glass vials. After 30 min of incubation time at 60° in a water bath, the vials were cooled immediately to room temperature. The yield of 3,5-diacetyl-1,4-dihydrotoluidin generated by the reaction of formaldehyde with Nash's reagent was measured with a spectrophotometer at 415 nm against the blank of the individual calibration curve. Individual calibration curves were performed because of the different content of nonenzymatically formed formaldehyde in the individual hemolysate. The calibration samples contained 125 μL of hemolysate; 4 mM of glutathione; diluted formaldehyde solution (100–600 (M of formaldehyde); and Tris-HCl buffer 20 mM, pH 7.4, to a total volume of 1 mL. They were treated under the same conditions. The detection of a formaldehyde background in hemolysate without exposure to dichloromethane has already been reported [11]. Therefore, the yield of formaldehyde in the substrate-free hemolysate (mean) was subtracted from the yield of formaldehyde in the substrate-containing samples in each individual assay. For recording precision, hemolysate of a GSTT1-positive individual was stored in 125 μL portions at −80°. The intra-day precision was determined by measurement of a series of these samples with 62 mM of dichloromethane at one day. The coefficient of variation was 7.6% (N = 15). To evaluate inter-day precision, two samples were determined with a substrate concentration of 62 mM each day the assay was performed. The coefficient of variation was 6.7% (N = 15). For measurement of the time-course of the reaction, incubation samples contained 3 mL of individual hemolysate, 4 mM glutathione, 100 mM dichloromethane, and buffer to a total volume of 12 mL. Every 30 min, 1 mL of the sample was taken, treated with 333 μL of trichloroacetic acid, and analyzed as described. The genotyping assay was performed as described earlier [23].

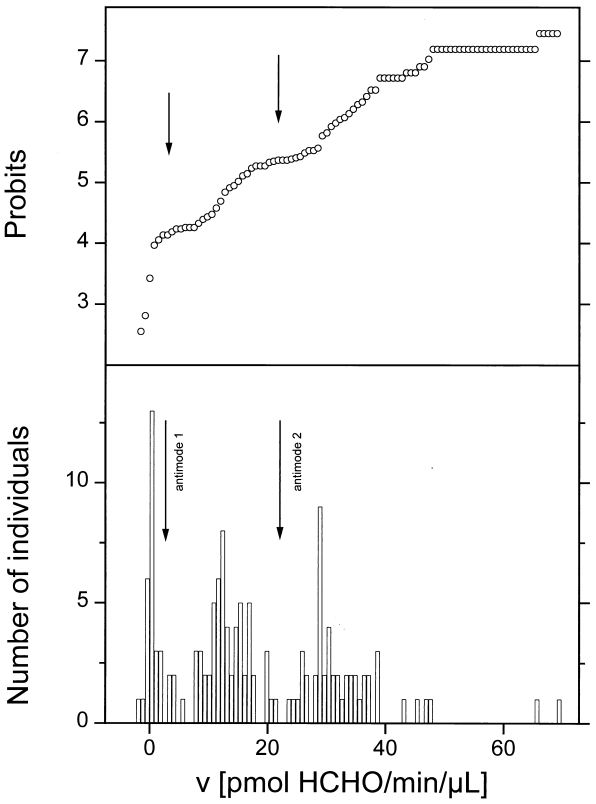

FIG. 1. Frequency distribution of formaldehyde production rate catalyzed by GSTT1 in 140 individuals incubated with 124 mM of dichloromethane.

RESULTS AND DISCUSSION

Conditions for the GSTT1 activity assay were studied, and the optimal conditions were used. The rate of formaldehyde production was constant over 180 min. For the subsequent

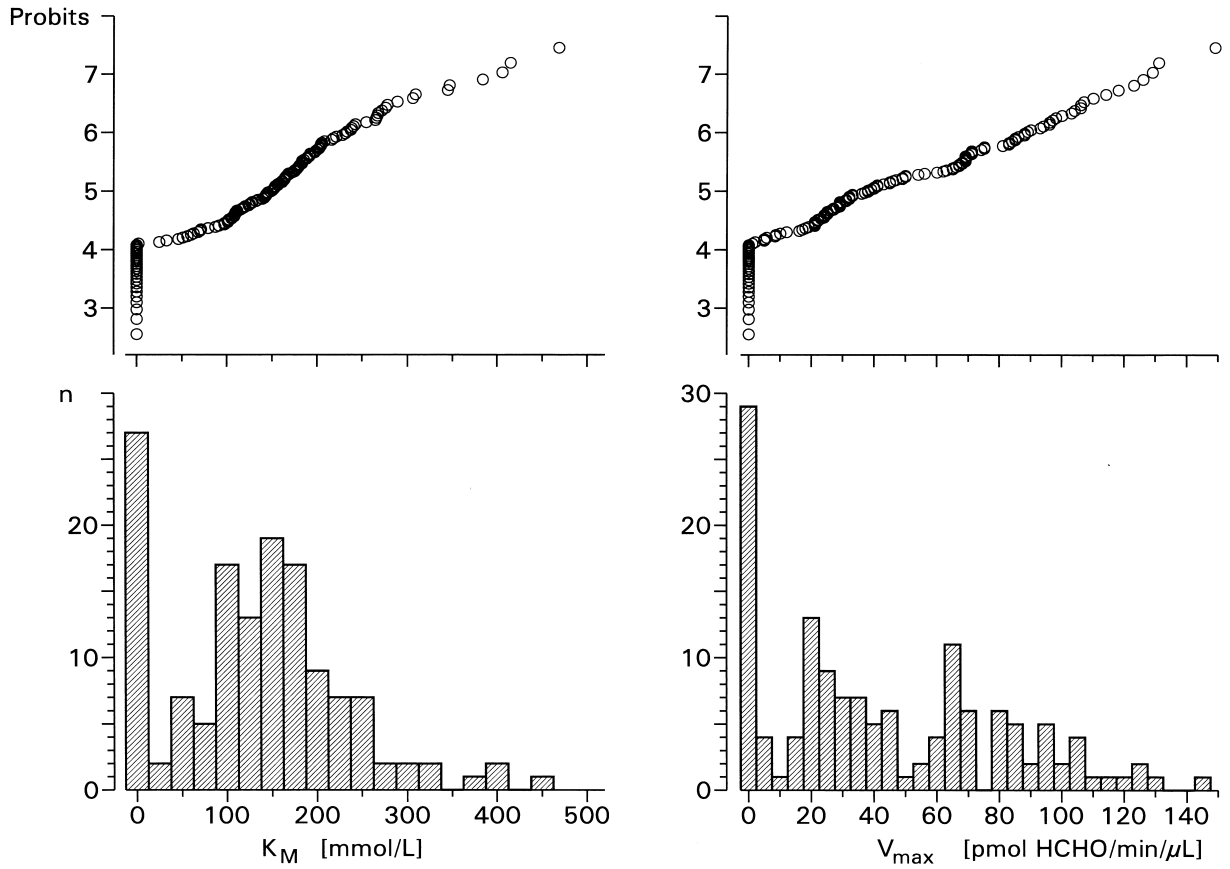

FIG. 2. Frequency distribution of K_m and V_{max} values of GSTT1 in 140 individuals measured with dichloromethane as substrate.

assays, 90-min incubation times were used. In our study, the activity of GSTT1 was measured in 140 subjects at three substrate concentrations. The distribution of activity with 124 mM dichloromethane is shown in Fig. 1 together with the probit plot. Three different phenotypes can be identified: one group with a deficient or very low activity, one with an intermediate activity, and one group with a high activity of the enzyme. The antimode between the deficient or very low and the other activities was at 2 pmol · min^{-1} per μL of hemolysate with 124 mM of dichloromethane. All individuals with an activity lower than 2 pmol · min^{-1} per μL hemolysate were considered functionally GSTT1-deficient. The prevalence of this GSTT1 deficiency was 19.3% (N = 27). For all individuals of this group, a homozygous deletion of the GSTT1 gene was found by PCR analysis, whereas all individuals with intermediate and high enzyme activity presented with the GSTT1 gene. Among the GSTT1-positive individuals, 45.0% (N = 63) had an intermediate and 35.7% (N = 50) a high activity with 124 mM of dichloromethane (Fig. 1). With the 62 mM substrate concentration (histogram not shown), the intermediate activity was between 2 and 15 pmol · min^{-1} per μL of hemolysate and the high activity between 15 and 55 pmol · min^{-1} per μL of hemolysate. All individuals showing high activity with 124 mM had a high activity with 62 mM, as well. At the 31 mM dichloromethane concentration (histogram not shown), the first antimode was at 2 pmol · min^{-1} per μL of hemolysate and the second at 8 pmol · min^{-1} per μL of hemolysate. In conclusion, the measurements of reaction velocity at the three substrate concentrations gave a concordant discrimination of intermediate and highly active people.

The K_m and V_{max} values were evaluated using Lineweaver–Burk plots. The variations for K_m and V_{max} among the individuals are shown in Fig. 2. The K_m of the GSTT1-positive individuals ranged between 26 and 468 mmol/L. The frequency distribution curve followed approximately a Gaussian curve. The V_{max} data showed a bimodal distribution in the GSTT1-positive individuals. The antimode between the intermediate and highly active persons is at 54 pmol · min^{-1} per μL of hemolysate.

A concordance was found between homozygous gene deletion and deficiency of GSTT1 activity. We compared the frequency of the 19.3% deficient individuals found in our study with the results of other studies. The deficiency of 11.1% (95% confidence limits: 6.6–16.8%) found in a population of 208 Swedish individuals [1] by phenotyping is significantly different from our results ($chi^2 = 4.604$, $P = 0.032$). No significant difference was found when our data were compared with the frequency of 15% of GSTT1-deficient people in a small German population numbering 40 subjects [3]. In conclusion, there may indeed exist differences in the prevalence of the GSTT1 gene deletion between different European ethnic subgroups.

TABLE 1. Frequency of GSTT1 genotypes and phenotypes in comparison with the expected values calculated according to Hardy-Weinberg's law

	Found by genotyping	Found by phenotyping	Expected from phenotyping
GSTT1*0/0	0.193	0.193 (n = 27)	0.193
GSTT1*1/0	0.807	0.450 (n = 63)	0.492
GSTT1*1/1		0.357 (n = 50)	0.315

It may be assumed that the individuals with the intermediate activity are heterozygous, and those with the high activity are homozygous for the GSTT1 alleles. Table 1 shows the allelic frequency found in our samples in comparison with the results calculated by Hardy–Weinberg's law. The difference between observed and expected results was not significant ($chi^2 = 0.41$), meaning that our population was in agreement with Hardy–Weinberg's law.

The unimodal distribution of K_m of GSTT1-positive individuals suggests that there is only one isoenzyme that catalyzes the conjugation of dichloromethane in these individuals and that functionally relevant amino acid variants of the enzyme are unlikely. Individuals with similar K_m had different enzyme activities with the same substrate concentration, resulting from differences in GSTT1 gene expression. In addition to a gene-dose effect, the influence of such factors as age, sex, smoking, drugs, or other environmental factors on the activity of the enzyme should be taken into consideration. It has recently been reported that the presence or lack of the GSTT1 gene influences the susceptibility to toxic side effects of certain compounds like tacrine [6]. These data have to be confirmed by additional genotyping studies including greater sample size and by additional studies on the involvement of GSTT1 in the metabolic pathway.

In conclusion, among the GSTT1 active people, there is still much variation in activity that is not explained by genotype, but deficient GSTT1 activity in the population can be reliably predicted using the genotyping assay. Investigations on the medical consequences of this GSTT1 polymorphism will remain a fascinating area of research.

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