

Original article

Polymorphic distribution of glutathione transferase activity with methyl chloride in human blood

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Received 13 April 1994 and accepted 7 July 1994

Interindividual variation in the *in vitro* conjugation of methyl chloride with glutathione by erythrocyte glutathione transferase was investigated in 208 healthy males and females from the southern and central parts of Sweden. It was found that 11.1% of the individuals lacked this activity, whereas 46.2% had intermediate activity and 42.8% had high activity. This distribution of three phenotypes is compatible with the presence of one functional allele with a gene frequency of 0.659 and one defect allele with a gene frequency of 0.341. The proportion of non-conjugators in this Swedish material was considerably smaller than that previously found in Germany (Peter *et al.*, *Arch Toxicol* 1989; 63, 351–355). The polymorphic distribution of another glutathione transferase, GST μ , was determined in the same individuals with a PCR method. No connection between the genotype for GST μ (*GSTM1*) and the glutathione conjugation with methyl chloride in erythrocytes was found.

Introduction

Methyl chloride is a methylating agent, mainly used in large-scale industrial processes (Bolt & Gansewendt, 1993). In humans methyl chloride can be metabolized by conjugation with glutathione (Redford-Ellis & Gowenlock, 1971). The metabolite *S*-methylcysteine has been identified in urine of workers exposed to methyl chloride (van Doorn *et al.*, 1980). Experiments performed on volunteers inhaling methyl chloride have revealed large interindividual differences in the rate of metabolism of this compound (Hake *et al.*, 1977; Putz-Anderson *et al.*, 1981; Nolan *et al.*, 1985).

Glutathione transferases (E.C. 2.5.1.18) are a group of isoenzymes that catalyse the conjugation of glutathione (GSH) with a wide variety of electrophilic, mainly hydrophobic, substances (Mannervik & Danielson, 1988). In contrast to all animal species investigated (mouse, rat, cattle, sheep, pig, rhesus monkey) (Peter *et al.*, 1989), human erythrocytes have been shown to contain a glutathione transferase isoenzyme that catalyses the conjugation of GSH with methyl chloride (Redford-Ellis & Gowenlock, 1971). Interestingly, a fraction of human blood samples appears to lack this activity; 25–40% of all individuals investigated were 'non-conjugators' (Peter *et al.*, 1989;

Hallier *et al.*, 1993). The isoenzyme has been partially purified and shown to presumably belong to class Theta (θ) (Schröder *et al.*, 1992). Methyl bromide, methyl iodide, methylene chloride and ethylene oxide are other substrates for this newly discovered glutathione transferase (Hallier *et al.*, 1990; Hallier *et al.*, 1993).

In order to further characterize the distribution of this isoenzyme the present investigation was performed. The glutathione transferase activity with methyl chloride was measured in blood samples from a Swedish control population. In addition, the distribution of another polymorphic glutathione transferase, GST μ (Warholm *et al.*, 1983; Seidegård & Pero 1985), was determined in the same population.

Materials and methods

Subjects

The study group consisted of 129 welders (all male) from the southern part of Sweden and of laboratory staff (48 males and 31 females) from the central part of the country. Median age and range of the populations are shown in Table 1. The study was approved by the Ethics Committees of the Karolinska Institute and the University of Lund.

Table 1. Distribution of GST θ phenotypes and GSTM1 genotypes among healthy controls

Populations	Sex	n	Median age years (range)	GST θ phenotypes				GSTM1 genotypes			
				+	(%)	-	(%)	+	(%)	-	(%)
Lab staff	Female	31	40 (24-71)	28	(90.3)	3	(9.7)	18	(58.1)	13	(41.9)
	Male	48	44 (24-79)	45	(93.8)	3	(6.2)	21	(43.8)	27	(56.2)
Welders	Male	129	48 (22-64)	112	(86.8)	17	(13.2)	61	(47.3)	68	(52.7)
Total	All	208	47 (22-79)	185	(88.9)	23	(11.1)	100	(48.1)	108	(51.9)

Blood sampling, preparation of erythrocyte cytosol and DNA isolation

Venous blood samples were collected in sodium citrate containing tubes and stored at -70°C . After thawing, the blood samples were centrifuged at $1200 \times g$ for 15 min. The supernatant, containing the cytosol of the lysed erythrocytes, was used for the measurement of glutathione transferase activity with methyl chloride.

The pellet, which consisted of white blood cells, was used for isolation of DNA. After lysis and proteinase digestion the samples were subjected to extraction with phenol-chloroform (Gustafson *et al.*, 1987) or to a modified salt out procedure (Miller *et al.*, 1988). DNA was then isolated after precipitation with ethanol.

Glutathione transferase activity with methyl chloride

The glutathione transferase activity with methyl chloride was measured essentially as described by Hallier and coworkers (Hallier *et al.*, 1990). Two ml supernatant was pipetted into 22 ml head space vials with addition of 10 mM GSH and 0.125 M sodium phosphate buffer, pH = 7.4, in a total volume of 5 ml. The vials were sealed and preincubated at 37°C for 30 min, while being rotated at 30 rpm. Thereafter 1.7 ml air from the gas phase of each vial was withdrawn and substituted by an equal volume of 1% methyl chloride in air, resulting in an initial concentration of 1000 ppm methyl chloride in the gas phase. The reaction was followed for 3 h. At 0, 30, 60, 120 and 180 min, 0.3 ml from the gas phase was withdrawn and the concentration of methyl chloride remaining was analysed by gas chromatography. A Varian gas chromatograph, model 3700, equipped with a Tenax TA 35/50 mesh column (2 m \times 3 mm) and a flame ionization detector, was used at 100°C with nitrogen (30 ml. min $^{-1}$) as carrier gas. The retention time for methyl chloride was 1 min. In control incubations the erythrocyte cytoplasm was substituted by water or 2% BSA. As a crude measure of the hemoglobin content of the blood samples the absorbance at 415 nm was determined.

PCR analysis of the GSTM1 polymorphism

The gene coding for GST μ is designated GSTM1 (Mannervik *et al.*, 1992). GSTM1 genotyping was performed essentially according to methods described by Brockmüller and collaborators (Brockmüller *et al.*, 1992). The detailed description is given elsewhere (Carstensen *et al.*, 1993). This PCR assay determines the presence or absence of intact GSTM1 since deficiency in GSTM1 activity has been ascribed to a homozygous deletion in the gene. Two separate PCR reactions with different primer pairs were performed and the products were analysed by agarose gel electrophoresis. An amplification product in both reactions indicate the GSTM1(+) genotype while absence of products in both reactions characterize the GSTM1(-) genotype.

Results

Figure 1 shows the decline in the concentration of methyl chloride in the gas phase with time in blood samples from three different individuals. The curves follow first-order kinetics and the glutathione transferase activity can thus be expressed as a function of the slope of the linearized curves. As can be seen, person 1 has no activity with methyl chloride, person 3 has a high activity, and the activity of person 2 is intermediate. In order to compare the activity between different samples the values were normalized by dividing the activity (expressed as the 'slope' per ml sample) with the absorbance determined at 415 nm (see Materials and methods).

The lack of activity in some samples was not an artefact caused by freezing and thawing the blood. The stability of the enzyme was checked by measuring the activity in fresh erythrocytes and then in frozen samples stored for various lengths of time at -20°C or -70°C . It was found that storage for 7 months at either temperature did not significantly affect the enzymatic activity. The glutathione transferase activity with methyl chloride in blood samples obtained from an individual at different occasions was also essentially the same.

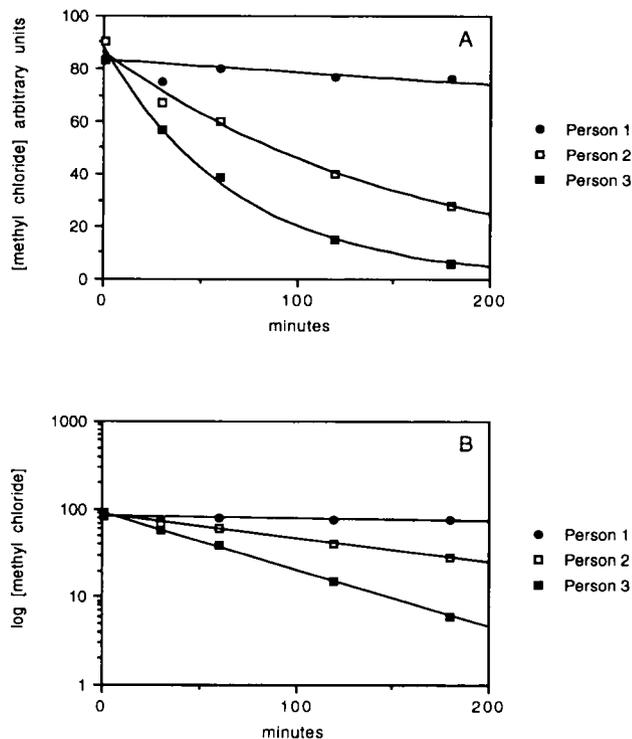


Fig. 1. Decline in the concentration of methyl chloride in the head space of incubation vials containing erythrocytes from three different individuals. Linear (A) or logarithmic (B) scale on the *y*-axis.

All individuals were classified as either conjugators (GST θ phenotype +) or non-conjugators (GST θ phenotype -). The results are shown in Table 1 along with the results of the *GSTM1* genotyping of the same individuals. In the total population, consisting of 208 individuals, 11.1% did not have any glutathione transferase activity with methyl chloride. This is a smaller value than that previously reported from Germany (Peter *et al.*, 1989; Hallier *et al.*, 1993). In contrast, the frequency of the two genotypes for *GSTM1* was similar to those previously reported from a Swedish study (Alexandrie *et al.*, 1994) and for various other European and US populations (Bell *et al.*, 1993; Brockmüller *et al.*, 1993; Fryer *et al.*, 1993). 51.9% of our total population had a deleted gene for *GSTM1*. We observed some variability between the

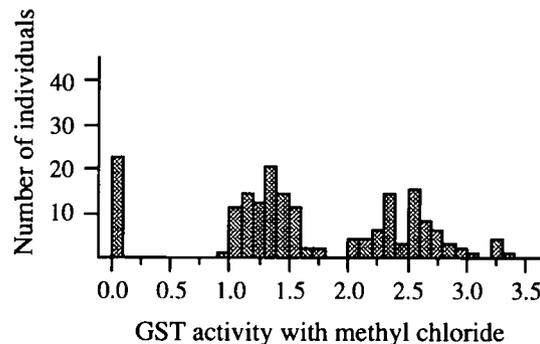


Fig. 2. Distribution of glutathione transferase activity with methyl chloride in human erythrocytes. The height of each bar represents the number of individuals within each activity range. The activity is expressed as 'slope' per ml per abs_{415} .

different subpopulations, defined by sex, age or occupation. However, none of these differences in GST θ or *GSTM1* distributions were statistically significant.

The erythrocyte glutathione transferase activities with methyl chloride, expressed as 'the slope' per ml sample per absorbance at 415 nm, of all individuals are plotted in Fig. 2. The pattern obtained indicates the presence of three different phenotypes; in addition to the group which lacks activity (phenotype --), there is one group of individuals with intermediate activity (phenotype +-) and one group with high activity (phenotype ++).

The value of 1.8 was chosen as the cut-off to differentiate between intermediate and high activity. The distribution of the three phenotypes is shown in Table 2. In the total population 42.8% had high activity (++), 46.2% had intermediate activity (+-) and 11.1% lacked glutathione transferase activity (--) with methyl chloride. It may be assumed that these three phenotypes are the result of the presence of two alleles, only one of which is functional. The corresponding genotypes for glutathione transferase θ can thus be designated as +/+, +/- and -/-. The allelic frequency for the functional allele (+) was calculated as 0.659 and the frequency for the defect allele was 0.341. The values obtained for genotype- and allele frequencies are compatible with

Table 2. Distribution of GST θ phenotypes and alleles among healthy controls

Populations	Sex	n	GST θ phenotypes			GST θ alleles	
			++ (%)	+ - (%)	-- (%)	+	-
Lab staff	Female	31	12 (38.7)	16 (51.6)	3 (9.7)	0.645	0.355
	Male	48	24 (50.0)	21 (43.8)	3 (6.2)	0.719	0.281
Welders	Male	129	53 (41.1)	59 (45.7)	17 (13.2)	0.640	0.360
Total	All	208	89 (42.8)	96 (46.2)	23 (11.1)	0.659	0.341

Hardy-Weinberg's law and indicate that our population is in genetic equilibrium ($\chi^2 = 0.15$, $p > 0.9$).

Discussion

The rate of metabolism of methyl chloride varies considerably between different individuals. There is a distinct pattern; one larger group of humans metabolizes methyl chloride faster than the other, smaller group (Hake *et al.*, 1977; Putz-Anderson *et al.*, 1981; Nolan *et al.*, 1985). It is probable that this difference is mediated by the polymorphic glutathione transferase 0 (Peter *et al.*, 1989), although other interindividual differences cannot be ruled out and may also contribute to the observed metabolic differences.

In the population studied in this work, consisting of 208 healthy individuals from the southern and central parts of Sweden, approximately 11% lacked glutathione transferase activity with methyl chloride in their red blood cells. The proportion of non-conjugators in our Swedish material is thus considerably smaller than that previously reported from Germany, where 40% of the individuals examined (20 males/25 females) were found not to conjugate methyl chloride (Peter *et al.*, 1989). In a more recent publication by the same research group it is stated that one quarter of the human population, the origin of which is not given, lacks this specific activity (Hallier *et al.*, 1993). We are not aware of any investigations of this particular polymorphism in any other ethnic population. However, since differences seem to exist even between related ethnic groups, it is important to initiate such studies.

In the German studies isolated erythrocytes were used for the measurements of glutathione transferase activity with methyl chloride, whereas frozen and centrifuged blood samples were used in the study described in this paper. It does not seem likely, however, that the presence of e.g. plasma proteins in our samples significantly affected the glutathione conjugation with methyl chloride. In a few cases isolated erythrocytes were also used in this work, with essentially similar results as with frozen blood (data not shown). The use of slightly different procedures cannot thus explain the observed difference in the percentage of non-conjugators.

Another human glutathione transferase, GSTM1 (GST μ), has previously been shown to be polymorphic (Warholm *et al.*, 1983; Seidegård & Pero, 1985). In about half of all individuals the gene for this particular GST isoenzyme is deleted (Seidegård *et al.*, 1988). There does not appear to be any connection between

GSTM1 polymorphism and the polymorphism for GST0. All possible combinations of these two polymorphisms were found in the population studied in this paper. Furthermore, there was no apparent correlation with gender, in accordance with previous observations (Peter *et al.*, 1989).

The GC method (Hallier *et al.*, 1990) used in this paper for the analysis of the conjugating capacity proved to be very reliable. There were no difficulties in discerning if an individual was a conjugator or a non-conjugator. Of particular importance in this context is the fact that there is almost no spontaneous conjugation between GSH and methyl chloride. We could also distinguish between individuals with high and intermediate activity with methyl chloride. A similar observation has previously been found in blood samples exposed to ethylene oxide, another substrate for GST0 (Hallier *et al.*, 1993).

For quantification of the conjugating capacity of an individual we used a crude measure, absorbance at 415 nm, instead of number of erythrocytes or hemoglobin content. The number of erythrocytes could not be counted as the erythrocytes were lysed in the freeze stored samples. The absorbance at 415 nm is highly correlated to the hemoglobin content, but easier to measure, and was considered a sufficiently accurate method. In some blood samples the number of erythrocytes was counted, but that did not change the classification of the sample (data not shown).

In addition to in erythrocytes, GST0 is probably also expressed in liver. We have measured the glutathione transferase activity with methyl chloride in a number of human liver samples and found a polymorphic distribution similar to what is found in blood (Warholm *et al.*, unpublished results). This may be due to the presence of some other GST isoenzyme(s) in liver, that conjugates methyl chloride. However, the finding that some liver samples completely lacked glutathione transferase activity with methyl chloride, but had normal activity with 1-chloro-2,4-dinitrobenzene, makes this suggestion less likely. It has not yet been possible to obtain erythrocytes and liver samples from the same individual for measurements of the activity with methyl chloride. That GST0 is expressed in liver is also suggested from a recent publication, where a polymorphic distribution in the *in vitro* conjugation of methylene chloride with GSH in human liver was found (Bogaards *et al.*, 1993). Methylene chloride is a substrate for the erythrocyte GST0 (Thier *et al.*, 1991).

Whether the polymorphism for glutathione transferase 0 is a relevant toxicological risk factor in humans is at present not known. However, individuals lacking GST0 might be more susceptible to industrial

chemicals, such as ethylene oxide and methylene chloride.

Acknowledgement

The work was supported by a grant from the Swedish Work Environment Fund.

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