Human glutathione S-transferase Theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism

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In humans, glutathione-dependent conjugation of halomethanes is polymorphic, with 60% of the population classed as conjugators and 40% as non-conjugators. We report the characterization of the genetic polymorphism causing the phenotypic difference. We have isolated a cDNA that encodes a human class Theta GST (GSTT1) and which shares 82% sequence identity with rat class Theta GST5-5. From PCR and Southern blot analyses, it is shown that the GSTT1 gene is absent from 38% of the population. The presence or absence of the GSTT1 gene is coincident with the conjugator (GSST1+) and non-conjugator (GSTT1−) phenotypes respectively. The GSTT1+ phenotype can catalyse the glutathione conjugation of dichloromethane, a metabolic pathway which has been shown to be mutagenic in *Salmonella typhimurium* mutagenicity tester strains and is believed to be responsible for carcinogenicity of dichloromethane in the mouse. In humans, the enzyme is found in the erythrocyte and this may act as a detoxification sink. Characterization of the GSTT1 polymorphism will thus enable a more accurate assessment of human health risk from synthetic halomethanes and other industrial chemicals.

INTRODUCTION

Most cancers are caused by exposure to environmental carcinogens and not by the inheritance of specific susceptibility genes such as those of retinoblastoma or the Li–Fraumeni syndrome (Marx, 1991). However, genes that encode enzymes involved in the metabolism of carcinogens or environmental toxins can be polymorphic and such polymorphisms may be related to an increased risk of cancer in some individuals (Nebert, 1991). These xenobiotic metabolizing enzymes are classically divided into two categories: the 'phase I' enzymes, which are almost exclusively cytochromes *P*-450, and the 'phase II' enzymes such as the glucuronyl-, glutathione- (GSH) and sulpho-transferases.

This paper is concerned with the soluble glutathione transferases (GSTs; EC 2.5.1.18), which comprise a supergene family of dimeric proteins catalysing the conjugation of GSH with a wide variety of electrophiles (Coles and Ketterer, 1990). The subunits of the soluble GST family are placed into four multigene classes, Alpha, Mu, Pi and Theta according to their amino acid sequence identities (Mannervik et al., 1985; Meyer et al., 1991).

One gene locus of the Mu family (GSTM1) has attracted interest because it is present in only 55% of the population (Board, 1981) and yet its product (GSTM1-1) can detoxify potential carcinogens arising from man-made risks such as cigarette smoking. Thus the absence of the gene may result in an increased risk of lung cancer caused by cigarette smoking, a possibility that has been the subject of several epidemiological studies (Seidegard et al., 1990; Zhong et al., 1991; Nazar-Stewart et al., 1993; and references therein). These epidemiological studies are justifiable because the risk is man-made and does not have a natural selection pressure with equivalent exposure.

An unrelated GST null phenotype has been described for the GSH-dependent detoxification of naturally occurring monohalomethanes. In human erythrocytes, the monohalomethanes are detoxified by conjugation with GSH (Redford-Ellis and Gowenlock, 1971; Peter et al., 1989; Hallier et al., 1990). Approximately 60–70% of the human population are able to carry out this metabolic reaction ('conjugators') whereas the remainder are unable to do so ('non-conjugators') (Peter et al., 1989). Further characterization of this phenotype showed that GSH conjugation of the industrially useful chemicals dichloromethane (DCM) and ethylene oxide (EO), which is also a metabolic product of ethylene in animals and humans (Filser and Bolt, 1983), could only be catalysed by blood samples from the conjugator population (Thier et al., 1991; Fost et al., 1991). However, positive conjugator status is not necessarily beneficial because conjugation of monohalomethanes and EO is detoxifying whereas conjugation of DCM yields a mutagenic metabolite. For example, the GST Theta class enzyme of rat, GST5-5, is responsible for dehalogenase activity towards DCM in this species (Meyer et al., 1991). DCM metabolism proceeds via *S*-chloromethyl GSH, yields formaldehyde as a final product, and does not require any phase I enzyme. Using heterologous expression of GST5-5 from cDNA, this pathway has been shown to be mutagenic in bacterial test systems (Thier et al., 1993). Given that the monohalomethanes, EO, DCM and other man-made alkyl halides have wide industrial uses as methylating agents, soil fumigants, pesticides and solvents (IARC, 1986, 1987), any polymorphic locus that may be involved in their metabolism would have epidemiological interest.

In this paper, we report the isolation of the first human GST Theta cDNA, GSTT1, and show that this is the product of the gene locus responsible for the observed polymorphism. We also show that the null phenotype results from the absence of the GSTT1 gene.

MATERIALS AND METHODS

Materials

Restriction and modifying enzymes used for cloning procedures were obtained from Gibco-BRL, Pharmacia and New England

Abbreviations used: DCM, dichloromethane; EO, ethylene oxide; GST, glutathione transferase.

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Biolabs. Nylon membrane (Hybond N) used in Southern blotting was obtained from Amersham International. NA45 paper was supplied by Schleicher and Schuell. Qiagen columns used in the preparation of plasmid DNA were supplied by Hybaid (Teddington, Middx, U.K.). PCR reactions were carried out with Amplitaq kits from Cetus Corporation using oligonucleotides obtained from Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K. The sources of methyl bromide and DCM are stated in Hallier et al. (1993). All other chemicals were from Sigma Chemical Co. or BDH Ltd.

Preparation of cDNA fragments using PCR

HepG2 first-strand cDNA was prepared from total RNA using the method described by Pemble et al. (1986) and was used without any further purification in subsequent PCR reactions. A 1 μl portion of first-strand cDNA was used per 50 μl of reaction mixture. PCR reactions were carried out as described in Pemble and Taylor (1992) using 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

cDNA cloning of GSTT1

A cDNA clone encoding human GSTT1-1 was produced using PCR. Primer 297R was a sense 21mer (ATGGGTCTGCAGCTCTACCTG) designed to be complementary to the N-terminus of GST Theta (MGLELY). In order to use a non-degenerate oligonucleotide, the glycine residue was assumed to be encoded by GGT (as it is for rat GST subunit 12) and the remainder of the N-terminal coding sequence was assumed to be identical to that of rat GST subunit 5. The antisense primer 298R (TCACTGGATCATGGTCAGCA) is a 20mer complementary to the 3′ coding sequence of rat GST subunit 5 and was chosen to include the TGA stop codon. These primers were used to amplify the coding sequence of GSTT1 using HepG2 first-strand cDNA as template and an annealing temperature of 60 °C. From this sequence, another sense primer, 593Y (ATTCTGAAGGCCAAGCAGTTC), was designed to be complementary to a sequence 80 bp from the stop codon. 593Y was used in combination with a non-specific oligo(dT) anti-sense primer 89/535 (GCGGCCGCTTTTTTTTTTTTTTT) in order to clone the overlapping 3′ end of the cDNA. PCR reaction conditions, cycling conditions, preparation of cDNA, subcloning and sequencing of plasmids were all as described previously (Pemble and Taylor, 1992).

Measurement of the conjugation of methyl bromide and DCM in human erythrocyte lysates

Whole blood samples from volunteers were drawn into heparinized vials. After centrifugation at 400 *g* for 10 min, plasma and white blood cells were removed by aspiration. The erythrocytes were washed twice in saline and subsequently lysed with an equal amount of distilled water at 4 °C for 1 h. The haemolysate was dialysed overnight against 20 mM sodium phosphate buffer, pH 7.4, and 2 mM EDTA and then centrifuged for 10 min at 2000 *g* to remove membranes.

The determination of conjugator status using methyl bromide as a substrate was measured by g.c. according to Hallier et al. (1993). The metabolism of DCM to formaldehyde was determined as follows: 9 ml headspace vials were filled with 1.8 ml of haemolysate, 4 mM GSH and 20 mM Tris/HCl buffer, pH 7.4, to a total volume of 7.8 ml. Samples were incubated at 37 °C for 1 h. No haemolysate was added to the control reactions. Samples (1 ml) were removed every 20 min and protein was precipitated by addition of 333 μl of 20% trichloroacetic acid. The supernatant (1 ml) was analysed for formaldehyde as described by Nash (1953).

PCR analysis and Southern blotting of DNA isolated from blood

Genomic DNA was isolated from blood samples as described by Maniatis et al. (1982). PCR reactions were carried out in 50 μl of 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, 1 mM 2-mercaptoethanol, 1% (w/v) gelatin, 1 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of Amplitaq and 1 μg of genomic DNA. PCR conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 1 min. Primers used were TTCCTTACTGGTCCTCACATCTC and TCACCGGATCATGGCCAGCA. PCR products were analysed on a 1.5% (w/v) agarose gel. For Southern blotting, 5 μg of genomic DNA was digested with *Hin*dIII, separated on a 0.6% (w/v) agarose gel, blotted onto Hybond N membrane and hybridized to insert from pGSTT1. The final wash was performed using 0.2 × SSPE (i.e. 30 mM NaCl, 0.2 mM EDTA, 2 mM NaH_2PO_4, pH 7.4) at 65 °C. DNA fragments for probes and radiolabelling were prepared as described in Maniatis et al. (1982).

RESULTS

The cDNA sequence of human GSTT1

The sequence of the human GSTT1 cDNA clone is shown in Figure 1. The cDNA is 1002 bp long, and comprises the coding

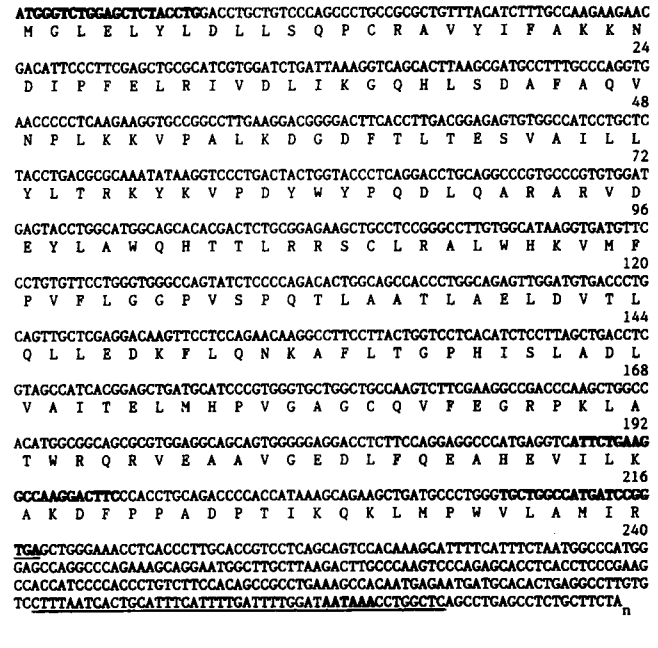

Figure 1 The nucleotide and deduced amino acid sequence of GSTT1

Numbers refer to the deduced amino acid sequence. The three oligonucleotide sequences shown in bold, together with the oligo(dT) anti-sense primer 593Y, were used to obtain the two clones by PCR (pgst297 using primers 297R/298R and pgst593 using primers 593Y/535). These clones had an overlap of 84 bp which was perfect excluding a 2 bp mismatch arising from the 298 primer itself (a rat subunit 5 deduced sequence; see the Materials and methods section). The AATAAA polyadenylation signal is also highlighted in bold. The underlined sequences are the TGA stop codon and the 48 bp 3′ sequence which shares 75% sequence identity with a similar region in rat GST subunit 5. Meyer et al. (1991) reported the N-terminal sequence of human GST Theta as GLELYLDLLSQPXRAVYIFAKKNDIPFELRIVDLIKGQHLXSDFAQ.

Table 1 Determination of conjugator status using the conjugation of methyl bromide

Quantitative values were determined for the German samples (1–10) and qualitative (+ + + or n.d.) for the British samples (11–16). n.d., not detectable. Also shown is the conjugator status for DCM (measured by formaldehyde yield) although two German subjects were unavailable (**) and samples from British subjects were not tested (*).

Subject no.	Conjugation of methyl bromide (nmol/min per mg of haemoglobin)	Formation of formaldehyde (pmol/min per mg of haemoglobin)	Conjugator phenotype
1	n.d.	n.d.	−
2	n.d.	n.d.	−
3	n.d.	n.d.	−
4	n.d.	n.d.	−
5	1.75	17.7	+
6	3.32	15.4	+
7	0.56	17.0	+
8	0.40	**	+
9	1.01	**	+
10	2.22	17.8	+
11	+ + +	*	+
12	+ + +	*	+
13	n.d.	*	−
14	+ + +	*	+
15	+ + +	*	+
16	n.d.	*	−

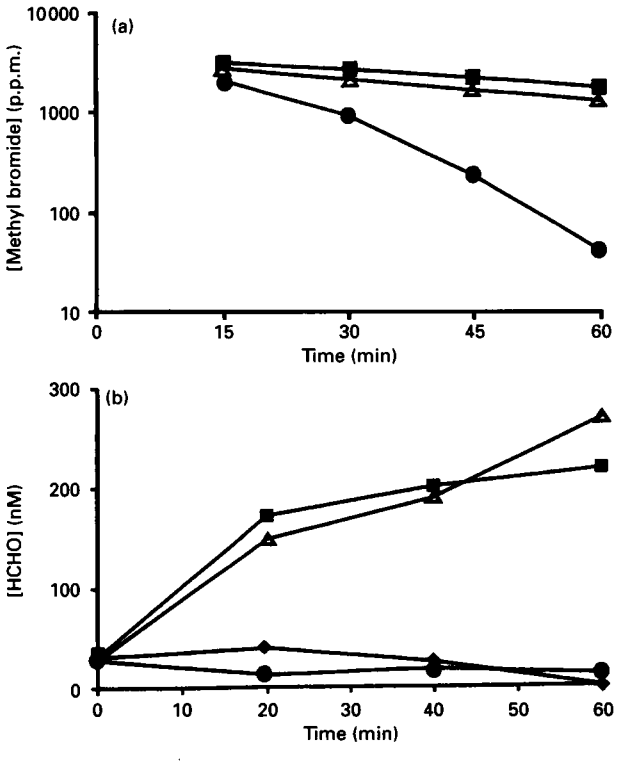

Figure 2 Differences in methyl bromide conjugation and of formaldehyde formation arising from conjugator status

(**a**) Disappearance of methyl bromide in the headspace of incubation vials. The initial concentration was 5000 p.p.m. △, Controls incubated with heated haemolysate; ●, incubation with haemolysate of a conjugator; ■, incubation with haemolysate of a non-conjugator. (**b**) Yield of formaldehyde in incubations of haemolysate with DCM. ■, △, Incubation with haemolysate of two conjugators; ◇, ●, incubation with haemolysate of two non-conjugators.

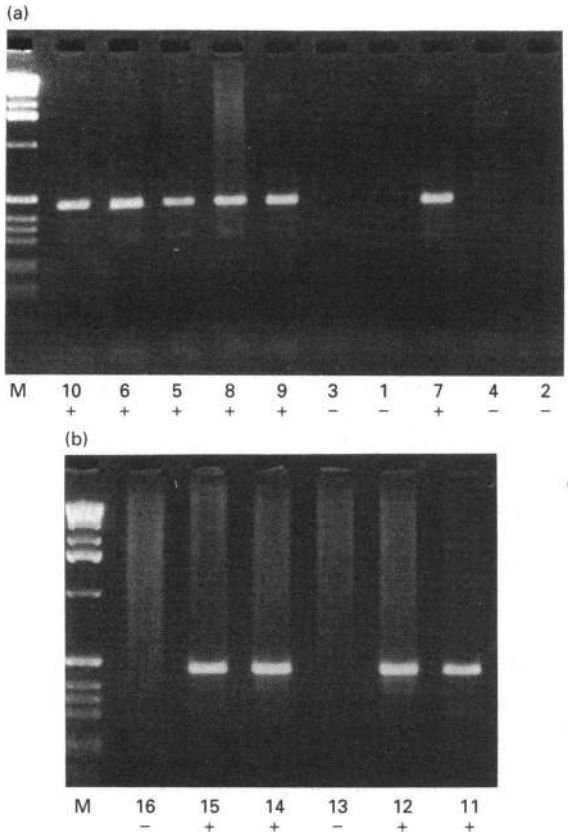

Figure 3 PCR analysis of DNA isolated from conjugators and non-conjugators

PCR products were analysed on a 1.5% (w/v) agarose gel. An aliquot (25 μl) of each PCR reaction was loaded in each track. Gel (**a**) shows the analysis of ten individuals from Germany and gel (**b**) an analysis of the six British subjects. The numbers below each track refer to the individual numbering system used in Table 1. These are not consecutive because the analysis was done blind; however, for convenience, the conjugator status (+ or −; Table 1) is marked under each track. Markers (M) are the 1 kb ladder from Gibco-BRL. Primers were TTCCTTACTGGTCCTCACATCTC and TCACCGGATCATGGCCAGCA. Control reactions using primers for the GST class Mu processed psuedogene (S. Pemble, unpublished work) gave a positive yield for all 16 DNA preparations (results not shown). PCR analysis of the same DNAs using a different pair of primers gave the same result (results not shown).

sequence of 720 bp (including the initiating methionine residue) and a 282 bp non-coding 3′ end. It is unlikely in such a short clone that a PCR-generated nucleotide substitution is present; however, the possibility of such an artifact should be noted. The deduced amino acid sequence would give rise to a mature protein of 239 amino acids, which is the same length as rat GST subunit 5 and is again a longer protein than those found in other GST families (Pemble and Taylor, 1992). The deduced N-terminal sequence is consistent with that of human GST Theta (Meyer et al., 1991; see legend to Figure 1). Comparison of the sequence with rat GST subunit 5 yields 82% identity at the nucleotide level and 80% at the amino acid level. In addition, a 48 bp sequence within the 3′ non-coding areas of pGSTT1 and within rat GST subunit 5 share 75% identity (see Figure 1). A similar area of sequence identity has previously been noted between rat GST subunit 5 and three human class Mu cDNAs; two from liver-derived, allelic cDNAs of GSTM1 and one from a muscle-derived cDNA of GSTM2 (Pemble and Taylor, 1992). Such

conservation between 3′ non-coding sequences of two GST families, both within and between species, suggests that the sequence is functional.

Preliminary PCR analyses of individual DNA samples suggested that the GSTT1 locus is polymorphic (results not shown) and could be related to an observed phenotypic polymorphism. Schroeder et al. (1992) proposed that the methyl bromide-conjugating activity of human erythrocytes was attributable to a GST of the Theta family. This suggestion has circumstantial support. First, rat GST 5-5, GST Theta and the human erythrocyte enzyme all fail to bind to a GSH-affinity column or to use the model GST substrate chlorodinitrobenzene (Meyer et al., 1991; Schroeder et al., 1992). Second, significant sequence identity exists between the N-terminal sequences of rat GST subunit 5, GST Theta and that of DCM dehalogenase of *Methylobacterium* (La Roche and Leisinger, 1990; Meyer et al., 1991; Pemble and Taylor, 1992). The latter enzyme enables the bacterium to use DCM as a sole carbon and energy source. The possibility that GSTT1 is the gene locus responsible for individual differences in the conjugator phenotype was tested by genotype association using the PCR and Southern blotting.

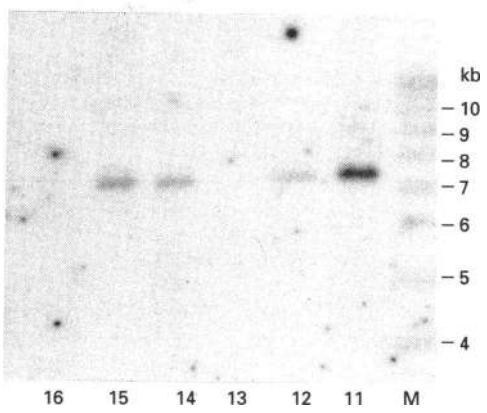

Figure 4 Southern blot of DNA from the laboratory populations

5 μg of DNA was digested with *Hin*dIII and loaded in each track. Lanes correspond to subject 11–16 (see Table 1). Markers (M) are the 1 kb ladder from Gibco-BRL. There is no *Hin*dIII restriction site in the GSTT1 cDNA.

Assignment of conjugator/non-conjugator status

The conjugator status of 16 individuals from our laboratories was determined using methyl bromide as substrate (Table 1). Absolute quantitative values were determined for ten people from Germany (nos. 1–10) and qualitative (+ or −) values were determined for six British subjects (11–16). An example of the difference between a conjugator and a non-conjugator as determined by this method is shown in Figure 2(a). For haemolysate from non-conjugators, the disappearance of methyl bromide parallels that of the control sample, showing that no enzymic conjugation occurs, whereas samples from conjugators catalyse a rapid decrease in methyl bromide. Examples of the difference seen in the formation of formaldehyde from DCM arising from conjugator status are shown in Figure 2(b). Formaldehyde is only produced by samples taken from conjugators and not from those of non-conjugators. On this basis, the conjugator status of the 16 people is that shown in Table 1.

Table 2 Comparison of conjugator status with GST genotypes

The GSTM1 genotypes of the six British subjects were determined in addition to their conjugator status and GSTT1 genotype. Subjects 11 and 15 are null for GSTM1 but have a positive conjugator phenotype, whereas the GSTT1 genotype correlates with conjugator status in all cases.

Subject no.	Conjugator phenotype	GSTT1 genotype	GSTM1 genotype
11	+	+	− −
12	+	+	+ −
13	−	−	− −
14	+	+	+ −
15	+	+	− −
16	−	−	− −

Identification of the polymorphism in the GSTT1 locus

A pair of primers complementary to the 3′ coding section of the GSTT1 sequence gave a PCR product of 480 bp. Analysis of this 480 bp PCR band using sub-cloning and sequencing showed that it contained an intron of 130 bp; however, the sequence of the intron has not been confirmed by fully sequencing both strands. These primers were used in a blind analysis of the DNA of the 16 subjects. The results of this PCR analysis are shown in Figure 3. Of the 16 samples, 10 gave a PCR product of the expected size and 6 gave no PCR product at all. The 10 PCR positive samples were found to correspond to subjects 5–12, 14 and 15 (conjugators) and the six which gave no PCR product were those taken from subjects 1–4, 13 and 16 (non-conjugators). These results show that the presence or absence of the 480 bp PCR product is in complete agreement with the observed conjugator phenotype. Furthermore, in PCR analysis of 53 DNA samples from healthy individuals, 62% have given a PCR product. This frequency is also in agreement with the distribution of the conjugator phenotype reported by Peter et al. (1989).

A Southern blot of *Hin*dIII-digested DNA taken from the six British subjects was probed with ^{32}P-labelled, full-length cDNA encoding GSTT1-1 (Figure 4). A single hybridizing band of 7.5 kb is visible in the DNA from the conjugator phenotypes (tracks 1, 2, 4 and 5) but no band was seen in the DNA isolated from the non-conjugators (tracks 3 and 6). DNA digested with *Eco*RI showed a similar pattern, i.e. a single hybridizing band of length greater than 18 kb was seen in conjugator DNA samples but not in DNA from non-conjugators (results not shown). These data show that GSTT1 cDNA is complementary to only one locus at the applied stringency and that the absence of conjugating activity in non-conjugators is coincident with the partial or total lack of this gene. It is noteworthy that conjugating activity does not coincide with the GSTM1 genotype of these individuals (Table 2).

In conclusion, GSTT1 must encode the liver-derived enzyme GST Theta (Meyer et al., 1991) as their N-terminal sequences are identical. In addition, by correlating the GSTT1 genotype with phenotype, we deduce that the GSTT1 locus also encodes the DCM-conjugating activity present in the erythrocyte. Although the activity of GST Theta towards DCM and the methyl halides has not been studied, we note that a report of considerable interindividual variation in DCM-conjugating activity of human liver, with two individuals having no activity whatsoever (Bogaards et al., 1993), is fully consistent with our results.

DISCUSSION

GSTT1-1, rat subunits 5 and 12, and the GST class Theta multigene family

There are multiple members of GST class Theta in both rat and man (Meyer et al., 1991; Ogura et al., 1991; Pemble and Taylor, 1992; Hussey and Hayes, 1992). Hence the single hybridization signal seen in Southern blot analyses of rat and human DNA using subunit 5 and GSTT1 cDNA probes, described by Pemble and Taylor (1992) and in Figure 4 respectively, is unusual given that other GST families hybridize within themselves at high stringency. These restricted hybridization profiles, taken together with the similar substrate specificities of GSTT1-1 and GST5-5, suggest that these may be orthologous genes. However, several primer pairs selected within the highly conserved N-terminal part of the protein did not give clear-cut PCR results (results not shown). This is probably attributable to the presence of other genes of the multigene family such as that coding for a second human class Theta GST (GSTT2-2) described by Hussey and Hayes (1992). Conservation of nucleotide sequence identity within the rat GST Theta family is much higher at the N-terminus than elsewhere in the coding sequences (Pemble and Taylor, 1992) and if a similar phenomenon occurs in the human family then it could result in interference in PCR analyses using primers complementary to the N-terminal region.

Significance of polymorphisms in the human GSTs

There is a well characterized null genotype within the GST Mu multigene family which results in the absence of GST activity in human leucocytes towards the substrate *trans*-stilbene oxide (Board, 1981; Seidegard et al., 1988). This GST activity is conferred by the product of a gene, GSTM1, which is absent in individuals who lack the enzyme. The homozygous null genotype has a frequency within the human population of ~ 50%. Several studies have attempted to link the GSTM1 null polymorphism to a greater risk of lung cancer among smokers but these have yielded variable results (Seidegard et al., 1990; Zhong et al., 1991; Nazar-Stewart et al., 1993; and references therein). From Table 2, it is apparent that the ability of individuals to metabolize alkyl halides does not correlate with the GSTM1 genotype but corresponds to the homozygous null genotype at the GSTT1 locus. In both GSTT1 and GSTM1, the incidence of the null phenotype is 30–40%; however, these loci are not linked because individuals who are GSTT1-null are not necessarily GSTM1-null, and vice versa.

Like the GSTM1 genotype, the GSTT1 polymorphism may be associated with a greater risk of cancer because there is a rationale for such a linkage, although the enzyme may be either deleterious or beneficial depending upon circumstances. For example, a deleterious effect of dihalomethanes is that DCM can cause liver and lung tumours in mice, and concern exists about their risk to humans (IARC, 1986). By expressing rat GST subunit 5 in the Ames tester strain *Salmonella typhimurium* TA1535, Thier et al. (1993) have shown that the *S*-(1-halomethyl) GSH metabolic intermediate from dihalomethanes is mutagenic. Intracellular activation of the dihalomethanes was essential for mutagenicity because addition of GST5-5 externally did not result in mutagenesis (Thier et al., 1993). Therefore, because GSTT1 seems to be the human orthologue of rat GST subunit 5, it is possible that individuals positive for this gene may be at a greater risk of cancer if exposed to man-made chemicals such as DCM. However, this argument does not take into account a possible beneficial effect, namely the occurrence of GSTT1-1 in the erythrocyte. In *in vitro* investigations using whole blood samples of non-conjugators, Hallier et al. (1993) showed that sister-chromatid exchange rates in lymphocytes increased after exposure to methyl bromide, EO and DCM, whereas the rate in blood samples from conjugators stayed constant. These authors proposed that the lower rates of cytogenetic damage in blood samples of conjugators, compared with non-conjugators, are attributable to a reduced toxin concentration. This results from the elimination of the chemicals in the erythrocytes, similar to the detoxification of methyl halides. Hence the erythrocyte can be likened to a sink. Thus, in epidemiological investigations, any effect of the GSTT1 polymorphism may be unpredictable: its presence may activate tissue- or cell-specific mutagens but their precursor concentration in the blood stream would be reduced by the sink effect in the erythrocyte. Furthermore, individuals who are both GSTT1-null and GSTM1-null may be at heightened risk because they lack both enzymes. Thus our characterization of the GSTT1 polymorphism should allow epidemiological studies to assess human health risks more accurately than using animal models.

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