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Influence of *GSTM1* and *GSTT1* genotypes on sister chromatid exchange induction by styrene in cultured human lymphocytes

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Glutathione *S*-transferases M1 (GSTM1) and T1 (GSTT1) are polymorphically expressed in humans; about 47% and 13% of Finns lack the GSTM1 and GSTT1 activity due to homozygous deletion of the respective genes (null genotypes). We previously observed that *GSTT1* null genotype was associated with increased induction of sister chromatid exchanges (SCEs) by a metabolite of styrene, styrene-7,8-oxide, in human lymphocyte cultures, while *GSTM1* genotype had no effect. In the present study, we examined the potential effect of these genotypes on SCE induction by the parent compound styrene. Seventy-two hour whole-blood lymphocyte cultures from 24 healthy human donors, representing all different combinations of these genotypes, were examined. In agreement with our earlier findings, styrene was an efficient inducer of SCEs in cultures of all donors. In two separate experiments, the mean number of SCEs/cell induced by 1.5 mM styrene was 1.55 times ($P = 0.011$) or 1.34 times ($P = 0.015$) higher in subjects lacking both *GSTM1* and *GSTT1* than in subjects having both genes. Donors null for only one of the genes showed intermediate SCE induction by styrene. At 0.5 mM styrene, no clear differences in SCE rates among the genotypes were seen. Our results suggest that the concurrent lack of the *GSTM1* and *GSTT1* genes increases the genotoxic effects of styrene in human cells. The discrepant findings obtained for the importance of *GSTM1* genotype in modulating the genotoxic effects induced by styrene-7,8-oxide and styrene may reflect a difference between a direct treatment with styrene-7,8-oxide and its formation from styrene in the cells. Although glutathione conjugation is a minor route in styrene detoxification in human liver *in vivo*, individual sensitivity associated with *GSTM1* and *GSTT1* null genotypes may be important locally in blood circulation and in blood-forming organs.

Introduction

Styrene (ethenylbenzene; CAS No. 100-42-5) is one of the most important monomers worldwide, used, e.g., in the production of plastic resins and styrene-butadiene rubber (1,2). Styrene is an important chemical toxicologically, due to its genotoxicity and suspected carcinogenicity, and due to the fact that styrene exposure levels continue to be high in reinforced plastics industry (1–3).

Styrene-7,8-oxide, formed from styrene in the liver through monooxygenation by cytochrome P-450 (CYP) 2E1, 2B6, 1A2, and possibly other isozymes (4,5), is considered to be the principal reactive and genotoxic intermediate of styrene in humans (1,2). Styrene-7,8-oxide is detoxified by microsomal epoxide hydrolase producing phenyl ethylene glycol which is further metabolized to yield the main urinary metabolites, mandelic acid and phenyl glyoxylic acid, used in biomonitoring of styrene (6). A minor detoxification route for styrene-7,8-oxide *in vivo* involves conjugation with reduced glutathione, as catalyzed by glutathione *S*-transferases (GSTs); the glutathione conjugates are acetylated and are excreted in the urine as styrene-specific mercapturic acids (7).

Styrene was converted to styrene-7,8-oxide also in human whole-blood lymphocyte cultures, which appeared to be the basis for the clear genotoxicity of styrene in this *in vitro* system (8). In contrast with hepatic metabolism, styrene-7,8-oxide formation in whole-blood was observed to be largely dependent on erythrocytes and oxyhemoglobin (9–11), so that removal of erythrocytes from the lymphocyte cultures greatly reduced the genotoxicity of styrene (12,13). The genotoxic activity remaining after erythrocyte removal probably reflected styrene oxidation in the lymphocytes themselves (12); this reaction may be mediated by CYP2E1, the primary form of cytochrome P-450 found in human lymphocytes (14).

As epoxide hydrolase activity appears to be low in human blood cells (15,16), while clear GST activity is found both in leukocytes and erythrocytes (17–19), GSTs are expected to play a more important role in styrene-7,8-oxide detoxification in the blood than in the liver (20,21). Blood cultures could thus serve as a model for the toxic effects of styrene and styrene-7,8-oxide in blood and blood forming organs and possibly other extrahepatic tissues.

In this context, it is of great interest that major GSTs such as GSTM1 found, e.g., in leukocytes, and GSTT1, the principal erythrocytic GST, are polymorphic (17,19,22). In Finns, GSTM1 and GSTT1 activities are absent from 47% and 13% of the population, due to homozygous deletions of the corresponding genes (23). The decreased detoxification capacity associated with homozygous deletion (null genotype) of *GSTM1* and *GSTT1* genes may increase individual sensitivity to styrene-7,8-oxide and other genotoxins.

Accordingly, we earlier showed that subjects with the *GSTM1* null genotype are more sensitive than *GSTM1* positive individuals (with at least one undeleted copy of *GSTM1*) to *in vitro* SCE induction by two epoxides, 1,2-epoxy-3-butene and *trans*-stilbene oxide (24–26). On the other hand, the *GSTT1* null genotype resulted in increased sensitivity to the genotoxicity of 1,2:3,4-diepoxybutane and 1,2-epoxy-3-butene (20,27–32). Moreover, *GSTT1* null donors were more sensitive than *GSTT1* positive individuals to SCE induction by styrene-7,8-oxide (21), while *GSTM1* genotype appeared to have no effect (24).

Abbreviations: CYP2E1, cytochrome P-450 2E1; GSTM1, glutathione *S*-transferase M1; GSTs, glutathione *S*-transferases; GSTT1, glutathione *S*-transferase T1; SCE, sister chromatid exchange.

Table I. Sister chromatid exchanges (SCEs) and replication indices (RI) in lymphocytes after a 48 h *in vitro* treatment (started 24 h following culture initiation) with styrene (in acetone) in 72 h whole-blood cultures of human donors with different *GSTM1 and GSTT1* genotypes (1st experiment)

Genotype	Control (acetone)		Styrene (0.5 mM)		Styrene (1.5 mM)	
Donor age, gender[a]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]
GSTM1 +/GSTT1 +						
29, f	7.9 ± 0.1	2.4 ± 0.0	11.6 ± 1.1	2.4 ± 0.0	15.7 ± 2.3	2.3 ± 0.0
31, f	8.0 ± 0.3	2.4 ± 0.0	9.3 ± 0.0	2.3 ± 0.1	15.3 ± 3.1	2.3 ± 0.0
50, f	8.9 ± 1.2	2.4 ± 0.0	13.7 ± 0.8	2.4 ± 0.0	15.4 ± 0.4	2.3 ± 0.0
44, m	7.8 ± 0.4	2.4 ± 0.0	12.4 ± 0.2	2.4 ± 0.0	18.9 ± 3.7	2.4 ± 0.1
44, m	7.8 ± 0.3	2.4 ± 0.0	15.6 ± 0.2	2.3 ± 0.0	17.9 ± 2.5	2.3 ± 0.0
Mean (SD)	8.1 (0.5)	2.4 (0.0)	12.5 (2.4)	2.4 (0.1)	16.6 (1.7)	2.3 (0.0)
GSTM1–/GSTT1 +						
30, f	9.3 ± 1.2	2.4 ± 0.0	11.5 ± 2.4	2.4 ± 0.0	16.9 ± 3.1	2.3 ± 0.0
44, f	9.1 ± 0.9	2.5 ± 0.0	13.5[c]	2.3	17.5 ± 2.1	2.4 ± 0.2
51, f	9.5 ± 0.3	2.4 ± 0.0	12.3 ± 0.9	2.4 ± 0.0	21.5 ± 1.4	2.3 ± 0.0
37, f	7.7 ± 0.3	2.4 ± 0.0	11.5 ± 1.1	2.4 ± 0.0	21.0 ± 2.4	2.3 ± 0.0
51, f	7.2 ± 0.1	2.4 ± 0.0	9.8 ± 1.6	2.4 ± 0.0	19.3 ± 2.7	2.3 ± 0.0
Mean (SD)	8.6 (1.0)	2.4 (0.0)	11.7 (1.3)	2.4 (0.0)	19.2 (2.0)	2.3 (0.0)
GSTM1 +/GSTT1–						
44, f	8.1 ± 0.7	2.4 ± 0.0	11.0 ± 1.7	2.4 ± 0.0	18.7 ± 3.1	2.2 ± 0.1
36, m	8.3 ± 0.6	2.4 ± 0.0	11.5 ± 1.7	2.4 ± 0.0	18.6 ± 0.9	2.3 ± 0.0
59, m	7.2 ± 0.1	2.4 ± 0.0	10.2 ± 0.6	2.4 ± 0.0	17.4 ± 2.0	2.3 ± 0.0
43, m	7.6 ± 0.1	2.4 ± 0.0	12.1 ± 0.6	2.4 ± 0.0	21.2 ± 0.1	2.3 ± 0.0
53, m	8.1 ± 0.1	2.4 ± 0.0	9.9 ± 0.0	2.4 ± 0.0	17.9 ± 2.1	2.3 ± 0.0
Mean (SD)	7.9 (0.5)	2.4 (0.0)	10.9 (0.9)	2.4 (0.0)	18.8 (1.5)	2.3 (0.0)
GSTM1–/GSTT1–						
38, m	8.5 ± 0.1	2.4 ± 0.0	11.5 ± 1.5	2.4 ± 0.0	23.0 ± 2.2	2.3 ± 0.0
47, f	7.3 ± 0.3	2.4 ± 0.0	11.5 ± 1.1	2.4 ± 0.0	21.1 ± 0.5	2.3 ± 0.0
51, f	8.3 ± 0.1	2.5 ± 0.0	12.4 ± 0.7	2.4 ± 0.0	18.0 ± 2.4	2.3 ± 0.0
30, f	7.7 ± 0.5	2.4 ± 0.0	12.1 ± 0.9	2.4 ± 0.0	24.1 ± 4.1	2.3 ± 0.0
47, f	7.8 ± 0.2	2.4 ± 0.0	13.1 ± 2.1	2.4 ± 0.0	19.7 ± 0.4	2.3 ± 0.0
Mean (SD)	7.9 (0.5)	2.4 (0.0)	12.1 (0.7)	2.4 (0.0)	21.2 (2.5)	2.3 (0.0)

[a]m, male; f, female.
[b]SE between the means of two duplicate cultures.
[c]25 cells scored for SCEs and 100 cells for RI from one culture.

The aim of the present study was to characterize the possible role of the *GSTM1* and *GSTT1* genotypes in modulating individual SCE levels in response to styrene exposure in human whole-blood lymphocyte cultures.

Materials and methods

Peripheral blood samples (5 ml) were collected from 24 healthy volunteers, seven with *GSTT1+/GSTM1+* genotype, five with *GSTT1–/GSTM1+* genotype, seven with *GSTT1+/GSTM1–* genotype, and five with *GSTT1–/GSTM1–* genotype. The blood donors represented both sexes and different ages, and were all current nonsmokers (Table I). The *GSTM1* and *GSTT1* genotypes were determined by techniques based on polymerase chain reaction (PCR), as described earlier (23).

Whole-blood lymphocyte cultures, duplicate for each treatment and donor, were set-up in air-tight glass injection bottles (20 ml) containing 0.3 ml of whole blood and 6.0 ml of culture medium with previously defined constitution (20). Styrene (99%; Fluka, Buchs, Switzerland; dissolved in acetone, 99.5%, Merck, Darmstadt, Germany) was added into the cultures 24 h after initiation by microsyringes at a volume of 10 µl at 0.5 mM or 1.5 mM final concentrations. Control cultures received 10 µl of acetone. The cultures were incubated at 37°C for a total culture time of 72 h, and 2 h before harvest metaphases were arrested by adding 85 µl of Colcemid solution (10 µl/ml, final concentration 0.13 µg/ml; GIBCO BRL, Life Technologies, Paisley, Scotland). After hypotonic treatment, fixation, and coding for a blind analysis, the cell suspensions were dropped onto microscope slides and stained by a modification of the fluorescence-plus-Giemsa technique (12,20,33).

For each culture, one microscopist scored SCEs in 25 second division cells, summing up to a total of 50 cells per donor and treatment. In addition, the frequency of 1st (M_1), 2nd (M_2), 3rd (M_3) division metaphases was evaluated from 100 cells/culture for replication index (mean number of replications completed by the scored metaphases). The replication index (RI) was calculated as: $RI = (M_1 + 2 \times M_2 + 3 \times M_3)/100$.

The genotype effect was evaluated after subtracting the respective acetone control value from each SCE frequency obtained with styrene treatment. The effects of the treatment and genotype on the number of SCEs per cell and replication indices were analyzed statistically by the *t*-test (two tailed).

Results and discussion

The results of the SCE analysis of styrene-treated lymphocyte cultures are presented in Tables I and II for the two separate experiments. In accordance with our previous studies with styrene (8,12), a statistically significant increase ($P < 0.001$) in SCEs was obtained in all donors by both concentrations of styrene and in both experiments; the overall SCE response to styrene was slightly lower in experiment 2 than experiment 1.

The effect of *GSTM1* and *GSTT1* genotypes was evaluated from SCEs induced by styrene; the induced SCE values were calculated by subtracting each donor's control SCE level from the individual mean SCE frequency obtained by the styrene treatment (Figure 1). Among the *GSTM1* null*/GSTT1* null donors, the mean number of SCEs/cell induced by 1.5 mM styrene was, in comparison with *GSTM1* positive/*GSTT1* positive individuals, 1.55 times higher in the 1st experiment (13.26 versus 8.56; $P = 0.011$) and 1.34 times higher in the 2nd experiment (7.52 versus 5.60; $P = 0.015$). Donors null for only one of the genes showed intermediate SCE induction,

Table II. Sister chromatid exchanges (SCEs) and replication indices (RI) in lymphocytes after a 48 h *in vitro* treatment (started 24 h following culture initiation) with styrene (in acetone) in 72 h whole-blood cultures of human donors with different *GSTM1* and *GSTT1* genotypes (2nd experiment)

Genotype	Control (acetone)		Styrene (0.5 mM)		Styrene (1.5 mM)	
Donor age, gender [a]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]	Mean no. SCEs/cell ± SE[b]	Mean RI ± SE[b]
GSTM1+/GSTT1+						
44, f	7.6 ± 0.2	2.4 ± 0.1	9.7 ± 0.1	2.4 ± 0.0	12.0 ± 2.1	2.5 ± 0.0
31, f	7.6 ± 0.3	2.5 ± 0.1	9.8 ± 0.3	2.4 ± 0.1	14.2 ± 0.6	2.3 ± 0.0
44, m	7.3 ± 0.1	2.4 ± 0.1	9.9 ± 1.5	2.5 ± 0.0	13.9 ± 2.4	2.4 ± 0.2
44, m	7.1 ± 0.2	2.3 ± 0.2	11.0 ± 1.5	2.3 ± 0.0	12.5 ± 0.4	2.5 ± 0.2
51, f	9.4 ± 0.4	2.4 ± 0.0	12.1 ± 0.2	2.4 ± 0.0	14.4 ± 1.1	2.3 ± 0.0
Mean (SD)	7.8 (0.9)	2.4 (0.1)	10.5 (1.0)	2.4 (0.1)	13.4 (1.1)	2.4 (0.1)
GSTM1–/GSTT1+						
31, f	9.4 ± 074	2.3 ± 0.0	10.3 ± 0.4	2.3 ± 0.0	15.1 ± 0.4	2.4 ± 0.0
44, f	7.2 ± 0.2	2.5 ± 0.0	11.1 ± 0.5	2.4 ± 0.0	13.5 ± 1.8	2.4 ± 0.0
34, f	7.3 ± 0.2	2.4 ± 0.1	10.7 ± 0.2	2.6±0.0	14.4 ± 1.0	2.4 ± 0.0
37, f	7.1 ± 0.1	2.4 ± 0.0	11.4 ± 0.8	2.4 ± 0.1	13.5 ± 1.5	2.5 ± 0.0
51, f	7.4 ± 0.3	2.5 ± 0.1	8.6 ± 0.1	2.5 ± 0.0	15.2[c]	2.4
Mean (SD)	7.7 (1.0)	2.4 (0.1)	10.4 (1.1)	2.4 (0.1)	14.3 (0.8)	2.4 (0.0)
GSTM1+/GSTT1–						
44, f	7.5 ± 0.6	2.4 ± 0.0	9.6 ± 0.3	2.4 ± 0.0	14.3 ± 1.8	2.5 ± 0.1
36, m	7.2 ± 0.4	2.4 ± 0.0	10.8 ± 0.9	2.4 ± 0.1	15.5 ± 0.1	2.5 ± 0.0
59, m	7.9 ± 0.3	2.4 ± 0.0	10.6 ± 0.8	2.5 ± 0.0	14.8 ± 3.4	2.4 ± 0.0
53, m	8.1 ± 0.1	2.5 ± 0.0	10.2 ± 0.0	2.3 ± 0.0	13.9 ± 0.3	2.3 ± 0.1
Mean (SD)	7.7 (0.4)	2.3 (0.1)	10.3 (0.5)	2.4 (0.1)	14.6 (0.7)	2.4 (0.1)
GSTM1–/GSTT1–						
38, m	7.8 ± 0.4	2.5 ± 0.0	12.9 ± 0.6	2.5 ± 0.0	15.9 ± 0.8	2.5 ± 0.0
47, f	9.2 ± 0.8	2.4 ± 0.0	11.8 ± 0.2	2.5 ± 0.0	16.8 ± 0.7	2.3 ± 0.0
51, f	9.5 ± 1.1	2.3 ± 0.1	11.7 ± 2.1	2.3 ± 0.1	15.3 ± 0.2	2.3 ± 0.0
30, f	8.1 ± 0.1	2.6 ± 0.1	10.3 ± 0.3	2.6 ± 0.0	16.3 ± 1.0	2.3 ± 0.0
47, f	8.2 ± 0.3	2.4 ± 0.0	10.4 ± 0.5	2.4 ± 0.1	16.1 ± 0.9	2.3 ± 0.0
Mean (SD)	8.6 (0.7)	2.4 (0.1)	11.4 (1.1)	2.5 (0.1)	15.9 (0.7)	2.3 (0.0)

[a]m, male; f, female.
[b]SE between the means of two duplicate cultures.
[c]25 cells scored for SCEs and 100 cells for RI from one culture.

but the difference to the *GSTM1* positive/*GSTT1* positive failed to reach statistical significance in the 2-tailed *t*-test. No clear difference between the genotypes was detected at 0.5 mM styrene.

Our results indicate that subjects lacking one or both of the studied *GST* genes have increased sensitivity to the genotoxic effects of styrene, as measured by SCE induction in human whole-blood lymphocyte cultures. This finding agrees with the role of GSTs in the detoxification of styrene-7,8-oxide (7). The genotype effect was observed only at the higher (1.5 mM) of the two concentrations tested, which suggested that the protective effect of GSTM1 and GSTT1 is important mainly at high exposure levels. 1.5 mM styrene did not, however, affect the proliferation rate of the lymphocytes, and was thus not overly toxic.

Our findings on *GSTT1* genotype are in agreement with our earlier study which showed that *GSTT1* null individuals are more sensitive than *GSTT1* positive individuals to SCE induction by styrene-7,8-oxide (21). On the other hand, our results on *GSTM1* genotype disagree with our previous experiment which showed no differences in styrene-7,8-oxide-induced SCEs between *GSTM1* null and *GSTM1* positive genotypes (24). One explanation for the discrepancy may be that treatment with styrene and styrene-7,8-oxide may not be directly comparable, since in styrene-treated cultures styrene-7,8-oxide is formed gradually during the incubation, inside the cells. Although much of the SCEs induced by styrene treatment in lymphocytes seems to be explained by styrene-7,8-oxide originally generated in erythrocytes (12), styrene-7,8-oxide formed inside the lymphocytes by CYP2E1 may be more readily detoxified by lymphocytic GSTM1 than styrene-7,8-oxide coming from outside the lymphocytes. On the other hand, erythrocytic GSTT1 is probably more important in detoxifying styrene-7,8-oxide outside the lymphocytes. These factors might explain why the *GSTM1* genotype effect is observed in cultures treated with styrene but not in those treated with styrene-7,8-oxide (21,24).

The influence of *GSTM1* genotype on styrene-7,8-oxide genotoxicity was supported by studies in various human cell lines, characterized for their GSTM1 status by genotyping, reverse transcriptase, and immunochemistry (34). When treated with styrene-7,8-oxide, GSTM1-deficient cell lines showed higher induction of 6-thioguanine resistant mutants and lower cell growth than GSTM1-proficient cell lines.

Two recent studies of styrene-exposed reinforced plastics workers suggested that GSTM1 has a role in the metabolism of styrene in humans *in vivo* (35,36). *GSTM1* polymorphism was the most significant parameter influencing the concentration of styrene-specific mercapturic acids in the urine. In comparison with *GSTM1* null subjects, urinary phenylhydroxyethyl mercapturic acid concentration in *GSTM1* positive subjects was 2 times higher (corresponding to 20 or 50 p.p.m. time-weighted average ambient styrene level) in one study (35) and 5–6 times higher in the other study (36). *GSTT1* polymorphism did not significantly affect the excretion of the mercapturic acids. This would suggest that GSTM1 is more important than GSTT1 in the hepatic metabolism of styrene.

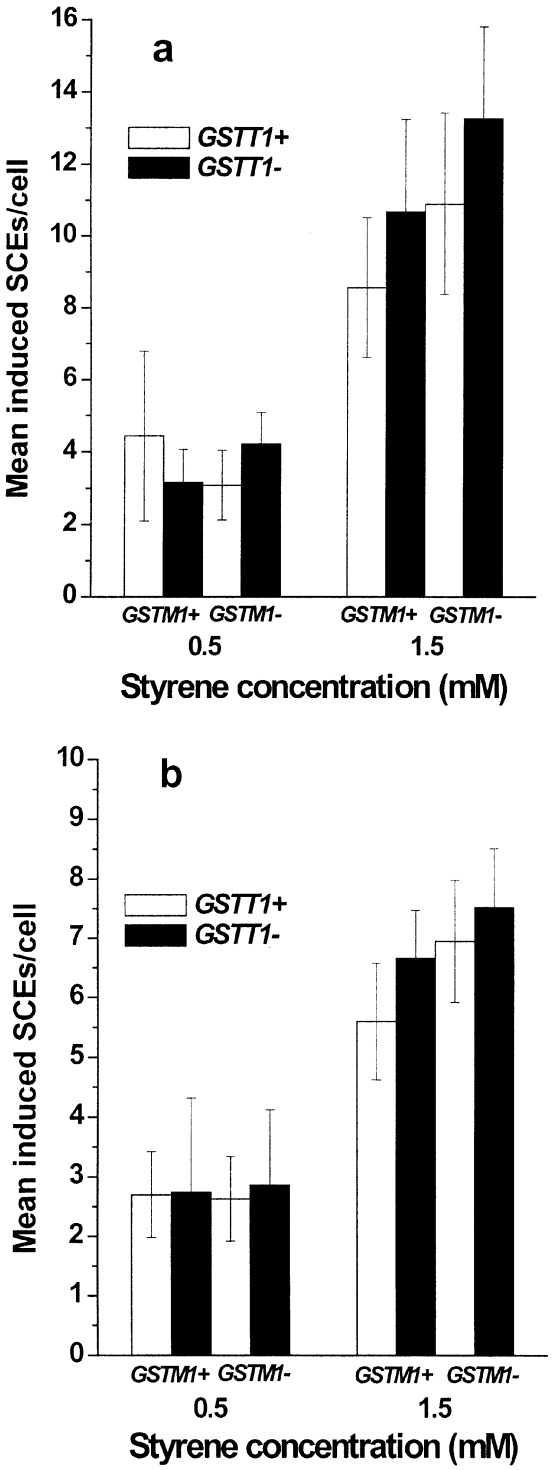

Fig. 1. Mean number of sister chromatid exchanges (SCEs) per cell (± SD; 50 cells scored per donor and treatment) induced by a 48-h *in vitro* treatment with styrene in 72 h whole-blood lymphocyte cultures of human donors with different *GSTT1*/*GSTM1* genotypes. Results from two experiments (**a** and **b**) are shown. The baseline SCE frequency (mean number SCEs/cell from acetone-treated cultures) has been subtracted from the results. RI, a measure of cell cycle delay, did not decrease significantly either at 0.5 mM or 1.5 mM concentration of styrene, and no effect of the *GST* genotypes on RI could be observed.

Two studies examined the influence of *GSTM1* and *GSTT1* genotypes on biomarkers of genotoxicity in styrene-exposed reinforced plastic workers and unexposed controls (37, 38). Vodicka *et al.* (37) reported no effect of *GSTM1* or *GSTT1* genotypes on chromosomal aberrations, somatic mutations, or DNA single-strand breaks among 40 exposed workers and 18 controls (data were not shown). Laffon *et al.* (38) observed no significant differences in the level of leukocyte SCEs, micronuclei, or DNA breakage, or cell proliferation between eight *GSTM1* null and six *GSTT1* positive reinforced plastics workers, although the difference between the exposed and controls was somewhat higher among the *GSTM1* null than *GSTM1* positive subjects for both SCEs and DNA damage; the effect of *GSTT1* genotype could not properly be evaluated, as there were only two null subjects.

It is thus unclear if *GSTM1 and GSTT1* polymorphisms could modulate the genotoxic or other adverse effect of styrene in exposed workers. In general, it is poorly understood how the short-term *in vitro* exposure to high levels of styrene compares with long-term occupational exposure to low levels of styrene. It is not known if erythrocytes could activate styrene in humans *in vivo*. The oxyhemoglobin-mediated reaction appears to require a relatively high styrene concentration – much higher than those encountered in blood of occupationally exposed people (1). On the other hand, peripheral blood contains 20 times more erythrocytes (rich in oxyhemoglobin) per ml than whole-blood cultures. Activation in erythrocytes and GST-mediated detoxification could, in principle, play a role in blood and blood-forming organs. This might have relevance, considering that there is some evidence suggesting an excess of hematopoietic and lymphatic malignancies in workers occupationally exposed to styrene (1, 2).

In conclusion, the present study shows that *GSTM1* null and *GSTT1* null individuals have increased sensitivity to the genotoxic effects of styrene in whole-blood lymphocyte cultures, suggesting that both GSTM1 and GSTT1 are involved in the metabolic detoxification of styrene. Although glutathione conjugation appears to be a minor route in styrene metabolism in human liver *in vivo*, individual sensitivity associated with the lack of *GSTM1 and GSTT1* genes may be important locally in blood circulation, blood-forming organs, and other extrahepatic tissues.

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