

Variability in Human Sensitivity to 1,3-Butadiene: Influence of Polymorphisms in the 5'-Flanking Region of the Microsomal Epoxide Hydrolase Gene (EPHX1)

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The carcinogenic effects of 1,3-butadiene (BD), a mutagenic chemical widely used in the manufacture of synthetic rubber, are likely initiated through its epoxide metabolites. In humans, these epoxides are detoxified predominantly by hydrolysis, a reaction mediated by the microsomal epoxide hydrolase (mEH; EPHX1) enzyme. It appears reasonable to hypothesize that BD-exposed individuals possessing lower mEH detoxification capacity may have elevated risk of adverse health effects. The interindividual levels of mEH enzymatic activity vary considerably, and polymorphisms in the mEH gene may contribute to this variability. In addition to the well-studied coding region polymorphisms encoding Tyr113His and His139Arg substitutions, seven other polymorphic sites in the 5'-flanking region of the mEH gene have been reported. These polymorphisms appear to differentially affect mEH gene transcriptional activities. The 5'-flanking region polymorphisms exist in two linkages, the –200 linkage (–200C/T, –259C/T, –290T/G) and the –600 linkage (–362A/G, –613T/C, –699T/C), whereas the –399T/C polymorphism exists as an independent site. Because these polymorphisms may affect total mEH enzymatic activity, we hypothesized that they influence the mutagenic response associated with occupational exposure to BD. We genotyped the 5'-region of the mEH gene in 49 non-smoking workers from two styrene-butadiene rubber facilities in southeast Texas and evaluated the linkage patterns against results obtained from an autoradiographic *HPRT* mutant lymphocyte assay, used as a biomarker of genotoxic effect. In the study population, 67% were exposed to low BD levels, <150 parts per billion, and 33% were exposed to >150 ppb. We used the observed *HPRT* mutant (variant) frequency (VF) in the studied population and a 4-way first-order interaction statistical model to estimate parameters that describe the influence of exposure, genotypes and the interaction between the two on the *HPRT* VF in the target population. The background (baseline) VF, defined as

the VF ($\times 10^{-6}$) \pm S.E.M. at low levels of BD exposure (<150 ppb) where all the genotypes under study are homozygous wild-type, was estimated to be 4.02 ± 1.32 . Exposure to >150 ppb of BD alone resulted in an estimated increase in VF of 3.42 ± 2.47 above the baseline level. Inheritance of the variant ATT allele in the –600 linkages resulted in an estimated increase in VF of 3.39 ± 1.67 above the baseline level. When the interaction between BD exposure and the ATT allele in the –600 linkage group was considered, a statistically significant positive interaction was observed, with an estimated increase in the VF of 10.89 ± 2.16 (95% CI = 6.56–15.20; $p = 0.0027$) above baseline. These new data confirm and extend our previous findings that sensitivity to the genotoxic effects of BD is inversely correlated with predicted mEH activity.

Key Words: polymorphism; epoxide hydrolase; EPHX1; *HPRT*; butadiene; genetic susceptibility; biomarkers; biomonitoring.

INTRODUCTION

1,3-Butadiene (BD) (CAS No. 106–99–0) is an important industrial chemical used to manufacture synthetic rubber and other polymers. It is also a common air pollutant found in auto emissions and cigarette smoke (IARC, 1992). Since the discovery that BD is a potent carcinogen in mice (Melnick *et al.*, 1990), BD has been the subject of intense research. The International Agency for Research on Cancer (IARC) has classified BD in category 2A or “A probable carcinogen in humans” (IARC, 1992). More recently, the U.S. National Toxicology Program (NTP) classified BD as a human carcinogen (NTP, 2001). In recent years, the goals of research on BD have expanded to characterize its carcinogenic risk to humans and to understand the relationships between its metabolism and the mechanisms of its toxicity leading to carcinogenicity.

It is becoming increasingly clear that the carcinogenic and mutagenic effects of BD are due to the formation of the epoxide metabolites, butadiene monoepoxide (BDO), butadiene diepoxide (BDO₂), and butadiene diolepoxide (BDO diol) (summarized by Jackson *et al.*, 2000). The levels of these metabolites

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are most likely mediated by the balance between the oxidation of BD by cytochrome P450 and its detoxification by conjugation with glutathione by the glutathione-S-transferases, GSTM1 and GSTT1, and hydrolysis by microsomal epoxide hydrolase (mEH) (Duescher and Elfarra, 1994; Seaton *et al.*, 1995). In humans, these BD epoxides are detoxified predominantly by hydrolysis, a reaction mediated by the mEH (EPHX1) enzyme (Jackson *et al.*, 2000). The mEH enzyme is expressed in most tissues, but it is found at especially high levels in the liver, the primary site of detoxification reactions. Available data (Kitteringham *et al.*, 1996) suggest that there is a 2- to 10-fold range in the activity of hepatic mEH in humans, and that total hepatic mEH activity is closely associated with levels of the mEH protein (Hasset *et al.*, 1997).

Several polymorphisms (sequence variations occurring at a frequency of more than 1% in the general population) have been characterized in the human mEH gene, with certain polymorphisms resulting in amino acid substitutions in the respective mEH protein (Hasset *et al.*, 1994, 1997). Those studied most frequently are two common mEH coding-region polymorphisms. One is a polymorphism in exon 3 encoding a change in the tyrosine residue 113 (Tyr113) to histidine (His113), while the other occurs in exon 4 and encodes an A → G transition resulting in replacement of the histidine residue at position 139 (His139) with an arginine (Arg139) (Hasset *et al.*, 1994, 1997). *In vitro* expression analyses indicated that the corresponding proteins varied as much as 65% in native activity (Hasset *et al.*, 1994), changes that are likely the result of altered protein stability rather than altered enzymatic activity (Lauenzana *et al.*, 1998). Although these polymorphisms appear biologically and clinically relevant by themselves, they do not account for the complete spectrum of variation in mEH activity within the population.

For a number of years, our laboratory has evaluated occupational exposure to BD in workers in the synthetic rubber industry using the *HPRT* mutant lymphocyte assay as a biomarker of mutagenic effect (Abdel-Rahman *et al.*, 2001, 2003; Ammenheuser *et al.*, 2001; Ma *et al.*, 2000; Ward *et al.*, 1994, 1996, 2001). Because of the possible importance of the mEH enzyme in detoxifying the reactive epoxides of BD (BDO and BDO₂) in humans, we have recently focused on examining the potential role of mEH genetic polymorphisms in human sensitivity to occupational BD exposure (Abdel-Rahman *et al.*, 2001, 2003). Our previous studies indicated that mEH coding region polymorphisms were associated with increased sensitivity to the genotoxic effects of BD. We observed a significant association between elevated frequencies of mutations in the *HPRT* reporter gene and mEH polymorphisms in individuals occupationally exposed to >150 ppb of BD. Recently, we substantiated these findings in a genetically disrupted mEH mouse model (Wickliffe *et al.*, 2003). Our results with the mEH knock-out mouse, using the *HPRT* cloning assay, are in agreement with our human studies, demonstrating that in both systems a perturbation of mEH

activity increased sensitivity to the genotoxic effects of BD (Wickliffe *et al.*, 2003).

The molecular basis for variation in mEH activity and the effect of such variability on BD-induced genetic damage are not fully characterized. Polymorphisms in the promoter region of the human mEH gene were characterized previously, and were shown to differentially regulate gene transcriptional activity *in vitro* (Raaka *et al.*, 1998). These genetic 5'-flanking region polymorphisms may impart an additional contributing factor to the range of functional mEH variability existing in human populations. Because these polymorphisms affect the level of mEH gene transcription by an average of ~30% (Raaka *et al.*, 1998) and, accordingly, may affect levels of total mEH enzymatic activity, it is possible that they may play a significant role in human susceptibility to the genotoxic effects associated with exposure to BD. Here we report that certain of the 5'-flanking region mEH polymorphisms positively increase the mutagenic response associated with occupational exposure to BD.

MATERIALS AND METHODS

Study population, sample collection, and BD exposure assessment.

Forty-nine non-smoking workers from two styrene-butadiene rubber (SBR) plants in southeastern Texas were enrolled in this study. Workers in both facilities work 12-h shifts with a 28-day cycle of rotating shifts. The details of the procedures for recruitment, collection of epidemiological data, and inclusion and exclusion criteria for participation in the study were discussed in detail previously (Abdel-Rahman *et al.*, 2001, 2003). Prior to recruitment, all study subjects were asked to sign an informed consent form that had been approved by the UTMB Institutional Review Board.

Exposure to BD was assessed during three shifts in a 28-day work period by collecting breathing zone air samples with 3M 3520 Organic Vapor Monitors (OVMs) (3M, St. Paul, MN), as discussed in detail in previous publications (Abdel-Rahman *et al.*, 2001, 2003; Ammenheuser *et al.*, 2001; Ward *et al.*, 2001). Based on our previous studies with this population, two levels of BD exposure were identified in the SBR plants investigated. Accordingly, for subsequent analysis of the effect of the mEH polymorphisms under study, the population of workers ($n = 49$) was classified into two groups, a high-exposure group ($n = 16$) and a low-exposure group ($n = 33$) (Abdel-Rahman *et al.*, 2001, 2003). The high-exposure group was exposed to average BD exposures of >150 ppb, whereas the lower-exposure group was exposed to average BD exposures <150 ppb. From each study subject, a blood sample (approximately 65 ml) was obtained at the end of a 28-day work cycle. A code number was assigned to each sample. The lymphocytes and plasma were isolated from the whole blood by density gradient centrifugation with Histopaque (Sigma, St. Louis, MO). A 2-ml aliquot of plasma was frozen for subsequent determination of cotinine concentration by radioimmunoassay (Van Vunakis *et al.*, 1993) for confirmation of non-smoking status. The lymphocytes were washed, frozen, stored in liquid nitrogen, and later thawed and assayed for the determination of exposure-associated genetic damage by means of the *HPRT* mutant lymphocyte assay (Ammenheuser *et al.*, 1991, 1997). About one million cells were used for isolation of DNA, in a non-organic extraction procedure (Abdel-Rahman *et al.*, 1994) followed by mEH genotype analysis. The overall investigation was performed with samples that were coded such that the identity or workplace activity of the subjects was not revealed.

Determination of exposure-associated genetic damage. The autoradiographic *HPRT* mutant lymphocyte assay was used for the determination of

genetic damage associated with BD exposure. The increase in *HPRT* variant (mutant) frequency (VF) was used as a biomarker for BD-induced genetic damage. The method for this assay has been described in detail previously (Ammenheuser *et al.*, 1991, 1997).

Analysis of polymorphisms in the 5'-flanking region of the *mEH* gene. In the present study, polymorphisms in seven previously identified polymorphic sites in the 5'-flanking region of the *mEH* gene were determined in genomic DNA from the study participants by direct sequencing of polymerase chain reaction (PCR) products. To generate amplicons for sequencing, 10 to 30 ng of genomic DNA was added to PCR reactions containing 1× ammonium buffer (supplied with *Taq* enzyme), 2.5 nM dNTP and 1U of *Taq* DNA polymerase (GeneChoice, Frederick, MD), and primers at a final concentration of 0.5 μM. The PCR conditions were as follows: one cycle at 95°C for 2 min, 34 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final cycle of 72°C for 2 min. Polymerase chain reactions were then treated with a mixture of exonuclease I (5 U) and shrimp alkaline phosphatase (2 U) to remove free nucleotides and primers. Reactions were incubated at 37°C for 45 min, and the enzymes were deactivated at 85°C for 15 min. A range of 8–33 ng of PCR amplicon was added to sequencing reactions, which were performed with the CEQ DTCS-Quick Start kit (Beckman Coulter, Fullerton, CA). Sequences were generated with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analysis was performed with Mutation Surveyor DNA Variant Analysis Software (SoftGenetics Inc., State College, PA). Primer sequences used for PCR of the *EPHX1* promoter region were: 5'-GGT CTA GGA ATT GTC AAG CGC CCA G-3', forward primer, and 5'-TGT GAG AGA GGC AGG GCA GGC TGC-3', reverse primer, which generated a PCR fragment 825 bp in length. After their purification, the PCR products were sequenced with a 5'-TGG AGT AAG AGG AAA GCA AAG GTT-3' forward primer, and 5'-CCA GGT GTA TCA CCC AGG-3' reverse primer. Each PCR product was completely sequenced on both DNA strands, and each instance of DNA polymorphism was validated by repeated DNA sequence analysis on both strands. Specifically, we assessed the following polymorphic sites: -200C/T, -259C/T, -290T/G, -362A/G, -399T/C, -613T/C, and -699T/C. Two linkages, each containing three polymorphic sites that had been reported previously (Raaka *et al.*, 1998) were re-confirmed in the present study. These two linkages occur as follows: the -200/-259/-290 alleles exist as either the C/C/T combination or the T/T/G combination, whereas variants at -362/-613/-699 occur as either A/T/T or G/C/C. These combinations are subsequently referred to as the "-200 linkage" (-200, -259, -290) and the "-600 linkage" (-362, -613, -699). The seventh polymorphic site, -399, was independent.

Statistical analysis. A general linear model was used to estimate expected *HPRT* VFs for subsets of the population being studied. The choice of the final model was based on the results of a preliminary analysis in which a simple additive model was used. The observed VFs in the studied population were used to estimate model parameters for effects of exposure and genotypes. The parameters of the final model were useful for estimating the influence of exposure, genotypes, and the interaction between the two on the *HPRT* VF in the target population. A general linear, purely additive, 4-way analysis of variance (ANOVA) exploratory model, described by the equation $VF = C + \text{Exp} + g_1 + g_2 + g_3 + \text{Error}$, and a more advanced 4-way first-order interaction model, described by the equation $VF = C + \text{Exp} + g_1 + g_2 + g_3 + (\text{Exp} g_1) + (\text{Exp} g_2) + (\text{Exp} g_3) + \text{Error}$, were used to determine the effect of the polymorphisms tested on the *HPRT* VF. In these models, "C" is the constant, or background (baseline) level, representing the average VF when all other parameters are at their control level [*i.e.*, genotypes g_1 , g_2 , and g_3 , corresponding to the -200 linkage, the -600 linkage, and the -399 site, respectively, are all homozygous wild-type and the exposure is at the low level (<150 ppb)]. The "Error" in the equation, also called *random error*, represents the effect of all other factors not accounted for by the model that may influence the VF. In these equations, exposure (Exp) was tested at two levels (high or >150 ppb and low or <150 ppb), and each polymorphism was tested at two levels: presence of the variant allele for the tested polymorphisms (in the homozygous or heterozygous state) or absence of such polymorphism.

Homozygous and heterozygous carriers of the variant alleles were combined together to increase the statistical power, as was done in other studies (Abdel-Rahman *et al.*, 2001, 2003; Affatato *et al.*, 2004; Duell *et al.*, 2000; Hou *et al.*, 2002; Sturgis *et al.*, 2000). In the 4-way interaction model, "Exp g " is the interaction term for exposure and polymorphisms at one site at a time, when the other two linkage sites are at their control level (homozygous wild-type). The use of the additive, and subsequently the 4-way, interaction model, allowed for the determination of the parameters (*i.e.*, genotypes, exposure, and the interaction between the two) that significantly affect the *HPRT* VF. Statistical significance was assessed by generating 0.95 level confidence intervals (CIs) corrected for the number of comparisons via the Bonferroni correction (0.95 family level CIs) for the change in mean VF between groups, corresponding to a 0.05 family level of significance for testing purposes.

RESULTS

The demographic make-up of the study population was presented in detail in our earlier publications (Abdel-Rahman *et al.*, 2001, 2003). The subjects who volunteered for our study were predominantly middle-aged men, with a few older individuals and several under the age of 30. The mean (\pm S.E.M.) levels of BD exposures were 2244.2 (\pm 749.1) ppb in the high-exposure group ($n = 16$) and 18.4 (\pm 5.5) ppb in the low-exposure group ($n = 33$). Of the 33 individuals in the low-exposure group, 16 had BD exposures below the detection limit (2.5 ppb). Additional information on the study population and correlations between the frequencies of *HPRT* mutant lymphocytes and exposure to BD are given in our previous publications (Abdel-Rahman *et al.*, 2001, 2003; Ward *et al.*, 2001).

The distribution of *mEH* genotypes and the frequencies of the wild-type and the variant alleles among the BD-exposed workers are presented in Table 1. The wild-type alleles (those that were most prevalent) were the TTG, GCC, and C alleles for the -200, the -600 linkages, and the -399 independent site, respectively. There were no homozygous individuals with the variant CCT allele for the -200 linkage. The variant allele frequencies (CCT for the -200 linkage, ATT for the -600 linkage, and the T allele for the -399 site) were 0.092, 0.214, and 0.296, respectively. Overall, there was no significant difference in the genotype or the frequency distribution of the alleles between the BD high-exposure and low-exposure groups.

In this study, we used the refined linear model to estimate the average effect of exposure to BD alone and the average effect of each of the polymorphisms in the sites under study, in the presence of high or low BD exposure, in relation to the frequency of *HPRT* mutant lymphocytes (VF). The values of the parameters in the models for the contribution of exposure and the three groups of polymorphic variants and the interactions between them on the VF were estimated based on the measured VFs of the individuals in the study. Differences were only considered to be statistically significant if the family p value (p value corrected for the number of comparisons) was <0.05. Individual p values for comparisons between the groups were not considered in this study. This conservative statistical evaluation allowed for more robust conclusions to be made

TABLE 1
Polymorphic sites, allelic frequency, and genotype frequency
in the 5'-flanking region of the mEH gene among
butadiene-exposed workers^a

Group	Genotype (allele)	Allele frequency	Genotype frequency
-200 linkage			
Total population (<i>n</i> = 49)			
	-200T/T, -259T/T, -290G/G (TTG allele) ^b	0.908	0.816
	-200T/C, -259T/C, -290G/T		0.184
	-200C/C, -259C/C, -290T/T (CCT allele) ^d	0.092	ND ^c
High-exposure group (BD >150 ppb, <i>n</i> = 16)			
	-200T/T, -259T/T, -290G/G (TTG allele) ^b	0.937	0.875
	-200T/C, -259T/C, -290G/T		0.125
	-200C/C, -259C/C, -290T/T (CCT allele) ^d	0.063	ND ^c
Low-exposure group (BD <150 ppb, <i>n</i> = 33)			
	-200T/T, -259T/T, -290G/G (TTG allele) ^b	0.894	0.788
	-200T/C, -259T/C, -290G/T		0.212
	-200C/C, -259C/C, -290T/T (CCT allele) ^d	0.106	ND ^c
-600 linkage			
Total population (<i>n</i> = 49)			
	-362G/G, -613C/C, -699C/C (GCC allele) ^b	0.786	0.633
	-362G/A, -613C/T, -699C/T		0.306
	-362A/A, -613T/T, -699T/T (ATT allele) ^d	0.214	0.061
High-exposure group (BD >150 ppb, <i>n</i> = 16)			
	-362G/G, -613C/C, -699C/C (GCC allele) ^b	0.719	0.563
	-362G/A, -613C/T, -699C/T		0.312
	-362A/A, -613T/T, -699T/T (ATT allele) ^d	0.281	0.125
Low-exposure group (BD <150 ppb, <i>n</i> = 33)			
	-362G/G, -613C/C, -699C/C (GCC allele) ^b	0.819	0.667
	-362G/A, -613C/T, -699C/T		0.303
	-362A/A, -613T/T, -699T/T (ATT allele) ^d	0.181	0.030
-399 independent			
Total population (<i>n</i> = 49)			
	-399C/C (C allele) ^b	0.704	0.531
	-399 C/T		0.347
	-399 T/T (T allele) ^d	0.296	0.122
High-exposure group (BD >150 ppb, <i>n</i> = 16)			
	-399C/C (C allele) ^b	0.688	0.531
	-399 C/T		0.347
	-399 T/T (T allele) ^d	0.312	0.122
Low-exposure group (BD <150 ppb, <i>n</i> = 33)			
	-399C/C (C allele) ^b	0.712	0.545
	-399 C/T		0.333
	-399 T/T (T allele) ^d	0.288	0.121

^a49 non-smoking subjects.

^bHomozygous wild-type.

^cNot detected.

^dHomozygous variant.

regarding the effects of the studied polymorphisms on the *HPRT* VF using a relatively small sample size.

With the preliminary purely additive model, we were able to determine the main effects on the *HPRT* VF of each of the parameters under study individually. These were the main effect of exposure on the VF and the main effect, individually, of the -200 and the -600 linked polymorphisms and the -399

polymorphism. Using the refined 4-way first-order interaction model, we were also able to determine the first-order interactions between exposure and the polymorphisms under study on the VF (*i.e.*, the interaction between exposure and polymorphisms in one site at a time, when the other two polymorphic sites were homozygous wild-type). Because of the small sample size in the present study, we could not reliably determine any other interactions.

The background (baseline) VF, defined as the VF ($\times 10^{-6}$) \pm S.E.M. at low levels of BD exposure (<150 ppb), where all the genotypes under study are at their control levels (homozygous wild-type), was estimated to be 4.02 ± 1.32 . As shown in Figure 1A, when we estimated, individually, the relative contribution of each of the studied parameters to the observed VF, we found that exposure alone resulted in an increase in the estimated VF of 3.42 ± 2.47 above the baseline level. The CCT polymorphic allele in the -200 site and the ATT polymorphic allele in the -600 site, when evaluated individually without considering the possible effects of exposure, resulted in an estimated VF increase of 1.11 ± 1.77 and 3.39 ± 1.67 above the baseline level, respectively. A decrease below the VF baseline level was estimated with the T polymorphic allele in the independent -399 site (VF = -1.09 ± 1.55) (Fig. 1A).

As shown in Figure 1B, interaction between exposure and the individual polymorphisms indicated a positive, but not statistically significant, interaction in the case of the CCT allele in the -200 linkage group, resulting in an increase in the estimated VF of 9.98 ± 4.03 above the baseline level (4.02 ± 1.32). When the interaction between exposure and the ATT allele in the -600 linkage group was considered, a statistically significant positive interaction was observed, where an increase in the VF to 10.89 ± 2.16 above baseline was estimated (*p* value = 0.0027, corrected for the number of comparisons, with 95% CI = 6.56-15.2). The estimated decrease in VF associated with the C allele in the -399 site in the interaction model was estimated to be -1.54 ± 2.46 below the baseline level. This possible protective effect, however, was not statistically significant.

DISCUSSION

Several human population studies investigating the sensitivity of workers to the potential genotoxic effects of BD have focused on the role of polymorphisms in the detoxifying glutathione-S-transferase (GST) M1 and T1 enzymes (Hayes *et al.*, 2000; Meng *et al.*, 1999; Sram *et al.*, 1998), and, more recently, the mEH gene (Abdel-Rahman *et al.*, 2001, 2003; Zhang *et al.*, 2004). In view of the evidence indicating that the mEH hydrolytic pathway is the predominant detoxification pathway for BD-reactive intermediates in humans (Jackson *et al.*, 2000; Swenberg *et al.*, 2001), our recent efforts have evaluated mEH genetic polymorphisms as potential modifiers of individual susceptibility to the mutagenic response

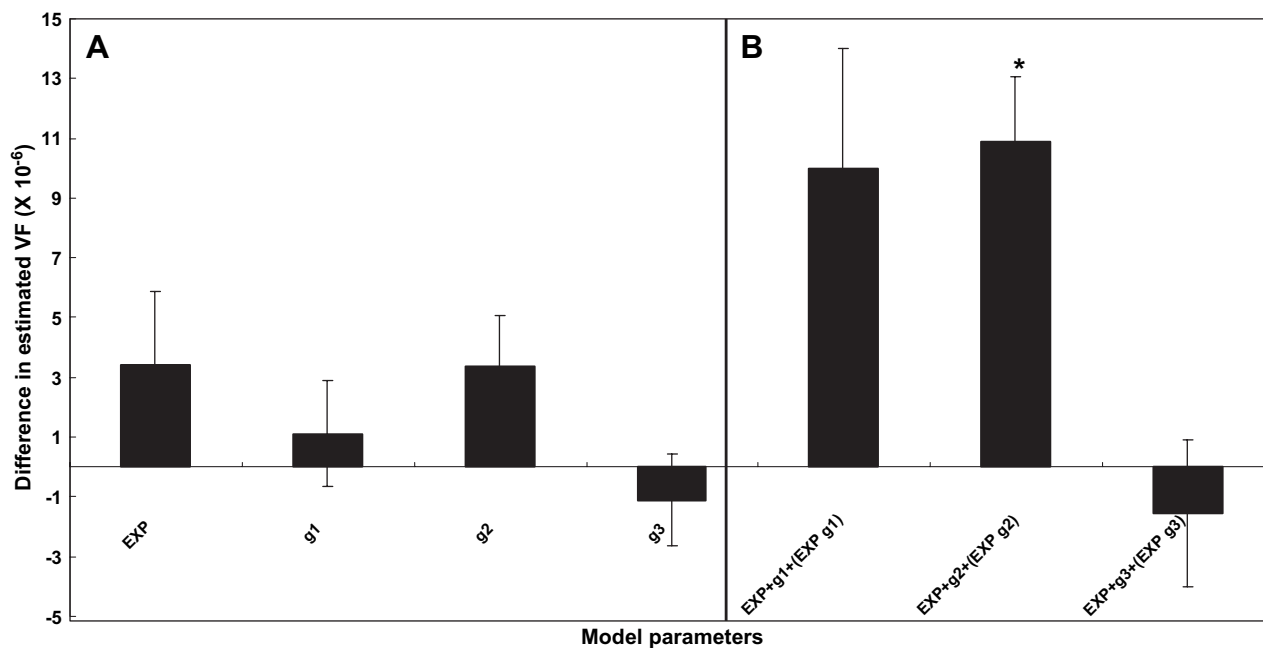


FIG. 1. (A) Estimated change in VFs from background (baseline) level^a, computed using the general linear purely additive model, as it relates to BD exposure (Exp) alone, polymorphisms in g1, g2, and g3 alone (corresponding to the presence of the homozygous or heterozygous variant alleles in the -200 linkage, -600 linkage, and the -399 site, respectively). (B) Estimated change in variant frequencies (VFs) from background (baseline) level^a, computed using the 4-way first-order interaction model, as it relates to the interaction between exposure (Exp) and the presence of the polymorphisms in g1, g2, and g3 (see A, above). "Exp g" is the interaction term for exposure and polymorphisms at one site at a time, when the other two linkage sites are at their control level (homozygous wild-type). *Statistically significant (p value corrected for the number of comparisons = 0.0027). ^aThe background (baseline) VF, defined as the VF ($\times 10^{-6}$) \pm S.E.M. at low levels of BD exposure (<150 ppb), where all the genotypes under study are at their control levels (homozygous wild-type), was estimated to be 4.02 ± 1.32 .

associated with occupational exposure to BD (Abdel-Rahman *et al.*, 2001, 2003; Wickliffe *et al.*, 2003). Our human studies in that area indicate that the coding polymorphisms in exon 3 and exon 4 of the mEH gene likely play an important role in this process. Our animal studies with knock-out mice lacking a functional mEH enzyme, in which we used the cloning *HPRT* assay (Wickliffe *et al.*, 2003) corroborate our findings in humans and indicate that perturbation in mEH activity increases sensitivity to the genotoxic effects of BD and its reactive metabolites.

Because polymorphisms in exon 3 and exon 4 in the mEH gene do not account for the complete spectrum of variation influencing mEH activity, in the present report we extended these investigations to examine the influence of other mEH polymorphisms occurring at seven loci in the 5'-promotor region of the gene. Previously, *in vitro* transcription activation studies conducted in the human hepatoma cell line HepG2 indicated that certain of these polymorphic loci differentially regulate gene transcription (Raaka *et al.*, 1998). These *in vitro* studies provided the rationale for our hypothesis that genetic variations in the 5'-flanking sequence of the mEH gene are additional factors contributing to individual susceptibility to the mutagenic response associated with occupational BD exposure.

In the present investigation, we observed a significant ($p = 0.0029$) positive interaction between exposure to BD and the ATT allele in the -600 linkage of the mEH gene in the 5'-

promotor region. In gene expression studies conducted *in vitro*, two of the three variants in the -600 linkage, specifically the A substitution at site -362 and the T substitution at site -613, were each capable of reducing transcriptional activity in HepG2 cells by an average of approximately 30%, whereas the T substitution at the -699 site did not alter levels of gene expression (Raaka *et al.*, 1998). Therefore, it appears plausible to assume that the presence of the A and the T nucleotides at sites -362 and -613 in the -600 linkage not only reduce transcription of the mEH gene but that, in turn, the presence of these nucleotides result in a lowered level of functional mEH protein production in the liver (Liang *et al.*, 2005). Because liver metabolism is a major contributor to xenobiotic clearance, in the presence of BD exposure reduced functional expression of mEH would be predicted to lead to a reduction in an individual's capacity to detoxify epoxide derivatives of BD and a corresponding increase in the frequency of mutations within the *HPRT* reporter gene. The positive interaction between exposure and polymorphisms in the -600 linkage that we observed in the group of workers exposed to >150 ppb of BD, but not in workers with very low or undetectable exposure, supports this assumption. Our result is in line with the biological concept that a reduced rate of detoxification would only be important if significant exposure to BD had occurred.

An interesting point that warranted further analysis is the issue of whether the polymorphisms in the 5'-promotor region

under study are independent of the two other polymorphisms in exon 3 and exon 4 that we had previously studied (Abdel-Rahman *et al.*, 2001, 2003). We conducted chi-squared analyses that test for independence to determine whether any of the 5'-promotor region polymorphisms are associated in a predictable manner to either the exon 3 or exon 4 polymorphisms. Our analyses indicate that none of the polymorphisms in the promotor region appear to be associated with either the mEH variant in exon 3 or the variant in exon 4 (family *p* value for chi-squared tests >0.05). Thus it can be concluded that no single site accounts for the functional mEH activity, but that the polymorphisms in the 5'-promotor region and the coding region polymorphisms in exons 3 and exon 4 act in concert to determine an individual's mEH activity status.

The data presented here confirm and extend our previous findings that sensitivity to the genotoxic effects of BD is inversely correlated with predicted mEH activity. These results increase our confidence that polymorphisms that affect the function of mEH may play a significant role in human sensitivity to adverse effects from exposure to BD. In support of our data, a recent study conducted in the Slovak Republic, investigating the role of the coding polymorphisms in exons 3 and 4 of the mEH gene in modulating genotoxicity, found increased frequencies of chromosome aberrations in BD-exposed tire plant workers with the low-activity polymorphism (Vodicka *et al.*, 2004). This recent study and our findings, however, stand in contrast to other biomonitoring studies of butadiene-exposed workers (one in China and two in the Czech Republic) where *HPRT* mutant lymphocyte assays were used (Albertini *et al.*, 2003; Hayes *et al.*, 1996, 2000; Tates *et al.*, 1996). Furthermore, in the Chinese population, Zhang *et al.* (2004), using the *HPRT* cloning assay, recently investigated the effect of exon 3 and exon 4 polymorphisms in mEH on *HPRT* mutant frequency after BD exposure, but they did not observe a relationship between these genetic polymorphisms and mutations in the *HPRT* gene.

It is still not clear why, in all of our previous studies conducted in Texas (Ammenheuser *et al.*, 2001; Ma *et al.*, 2000; Ward *et al.*, 1994, 1996, 2001), we have consistently observed an increased *HPRT* VF in BD-exposed workers, and have continued to observe a modifying effect of mEH polymorphisms on the frequency of mutant lymphocytes (Abdel-Rahman *et al.*, 2001, 2003), whereas other studies have not. We believe, however, that issues related to study design, and differences between the United States and other countries in factors such as diet, lifestyle, general health status, and exposure to other genotoxic substances in the ambient environment may be contributing factors. One example of such a difference is the issue of the inclusion of smokers in the studies conducted in other countries. Because smoking can be a major confounder in investigations involving the *HPRT* assay (Ammenheuser *et al.*, 1997), only non-smokers were included in our investigation. In contrast, apparently more than 75% of the men in the BD-exposed group in the Chinese study

population were smokers (Zhang *et al.*, 2004). Although reduced mEH activity is expected to be associated with increased genetic damage in BD-exposed workers due to the potential for reduced detoxification of the reactive BD metabolites, in smokers polymorphisms that decrease mEH activity may actually play a protective role. Accordingly, smokers who have polymorphisms that decrease mEH activity might be expected to have reductions in *HPRT* mutant frequency when compared to smokers with genotypes conferring high mEH activity. This prediction appears consistent with the findings of Benhamou *et al.* (1998) and the results of an epidemiological meta-analysis reported by Lee *et al.* (2002), indicating that inferred low-activity mEH genotypes may provide a protective effect for smoking-related lung cancer. Additional factors that may also contribute to the differences observed among studies investigating the possible genotoxic effects of BD are discussed in more detail in our previous publications (Albertini *et al.*, 2003; Ammenheuser *et al.*, 2001; Ward *et al.*, 2001).

In summary, our data extend and support our previous findings that the mutagenic response associated with BD exposure is inversely correlated with predicted mEH activity. The results from these investigations are consistent with predictions from *in vitro* assays indicating a lowered transcriptional activation potential associated with certain 5'-flanking region mEH polymorphisms. Together with our earlier reports, these findings further substantiate the role of mEH as a modifier of individual genotoxic response to BD exposure and indicate that 5'-regulatory region genetic polymorphisms may independently contribute, along with structural region polymorphisms, to modifying susceptibility to butadiene and other chemicals that are metabolized through this enzymatic pathway.

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