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Susceptibility to induction of chromosomal damage by metabolites of 1,3-butadiene and its relationship to 'spontaneous' sister chromatid exchange frequencies in human lymphocytes

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Summary. Occupational exposure to butadiene is associated with the occurrence of lymphohaematopoietic cancers. The mutagenicity of butadiene is thought to be mediated by its mono- and diepoxide metabolites, which are capable of binding to DNA. Diepoxybutane is the most potent genotoxic metabolite and is known to produce interstrand DNA cross-links. In order to study individual differences in response to the genotoxicity of diepoxybutane, we devised a human lymphocyte culture system that involves short-term culture of T lymphocytes and measurement of sister chromatid exchange (SCE) and chromosomal aberration frequency as genotoxic end-points. We observed that when lymphocytes from healthy individuals are exposed *in vitro* to 6 μM of diepoxybutane, the number of SCEs induced is distributed bimodally: about 20% of 173 healthy workers studied were twice as sensitive to the induction of SCEs as the remaining 80%. Cells from sensitive individuals also contain four times more diepoxybutane-induced chromosomal deletions and exchanges. Of particular interest is the observation that diepoxybutane-sensitive individuals have higher frequencies of baseline (i.e., uninduced) SCEs. We have now examined the sensitivity of individual lymphocytes to SCE induction by another DNA cross-linking agent (nitrogen mustard) and to monoepoxybutene. The results indicate that lymphocytes sensitive to diepoxybutane-induced SCEs have normal sensitivity to nitrogen mustard and a moderately increased response to the monofunctional agent monoepoxybutene. Measurement of diepoxybutane-induced SCEs is a potential biomarker of sensitivity to the genotoxic effects of butadiene and may be useful in occupational epidemiological studies. Such studies, in combination with measures of butadiene metabolism, could be useful in ascertaining whether the sensitivity is mediated by enzyme polymorphisms.

Introduction

1,3-Butadiene is a widely used industrial chemical and environmental pollutant (Morrow, 1990). It is carcinogenic in rodents (Huff *et al.*, 1985; Melnick *et al.*, 1990) and has been associated with the occurrence of lymphohaematopoietic cancers in man (Landrigan, 1990; Santos-Burgoa *et al.*, 1992). Butadiene is considered to be a pro-carcinogen, in that it must be metabolized by cytochrome P450 mixed-function oxidases to highly reactive metabolites, including monoepoxybutene and diepoxybutane (Malvoisin & Roberfroid, 1979; Arce *et al.*, 1990), which are thought to be the activated forms responsible for DNA binding and mutation induction. Studies in experimental animals have demonstrated marked species variations in the carcinogenic effects of butadiene, which are suggested to be due to genetically determined differences in metabolic activation and detoxification (Kreiling *et al.*, 1987). In humans, little is known of the specific isoforms of P450 responsible for butadiene activation or of variations in other enzymatic pathways (e.g., epoxide hydrolase, glutathione *S*-transferase) that may be involved in the detoxification of its epoxide metabolites. In addition to polymorphisms in xenobiotic metabolism, genetic variations in DNA metabolism or repair may also affect individual cancer risk in humans exposed to butadiene. Although rare in their homozygous forms, genetic deficiencies are known to exist in humans which lead to extreme sensitivities to the genotoxic effects of diepoxybutane (Fanconi, 1967; Cervenka & Hirsch, 1983; Auerbach *et al.*, 1989). In animals, the chromosomal end-point of sister chromatid exchange (SCE) induction has been used to explore species variation in susceptibility to butadiene; the results show that bone-marrow genotoxicity, as reflected by SCE induction, parallels the species differences in the carcinogenicity of butadiene in chronic bioassays (Cunningham *et al.*, 1986). These results also suggest that the SCE response is a valid biomarker for studying human genotoxicity associated with exposure to butadiene. Because chromosomal end-points such as SCE are the net result of metabolic processes and DNA damage, the increased SCE responsiveness could be due to variations in either butadiene metabolism or DNA damage and repair. The risk to individuals for genotoxic and possibly carcinogenic effects will depend on environmental and genetic factors that modify the balance of metabolic activation/detoxification and induction and repair of DNA damage.

Because internal exposure to diepoxybutane may result from environmental exposure to butadiene and lead to genotoxic effects, we have developed an in-vitro method involving cultured peripheral blood lymphocytes to study potential individual variations in the induction of chromosomal damage by diepoxybutane. The goal of such studies is to develop a noninvasive laboratory method that can be used to identify genetic factors that increase genotoxic and carcinogenic risks associated with exposure to butadiene in human populations.

Materials and methods

Study groups

Studies were carried out over a three-year period in four populations of healthy individuals (see references for details of the characteristics of each group). These included 58 healthy workers who were members of the Graphic Communications Workers Union, Local 39 (Wiencke *et al.*, 1991a), 55 female employees of the University of California Medical Center in San Francisco (Wiencke *et al.*, 1991b), 40 members of the Oil, Chemical

and Atomic Workers Union employed in a butadiene production plant and 20 volunteers from the Normative Aging Study. All participants completed a questionnaire and provided a blood sample. The questionnaire elicited demographic data, age, occupation, medical status (including history of cancer), diet, smoking history and prior or current exposure to medications or environmental agents that could affect the SCE assay (e.g., x rays, birth control pills, oestrogens, thyroid hormones, anticancer chemotherapy). Work histories were obtained, and exposure to potential occupational genotoxins evaluated from historical data and the results of industrial hygiene sampling at the work place. None of the subjects had taken medications known to alter SCE frequencies.

Cell culture and cytogenetic studies

Venous blood was drawn into sodium-heparinized Vacutainers. For cell cultures, 0.5 ml of whole blood was added to a final volume of 5 ml of RPMI 1640 tissue culture medium containing 10% fetal calf serum, 0.1 ml of phytohaemagglutinin (DIFCO Laboratories, Detroit, MI), penicillin (100 U/ml) and streptomycin (100 µg/ml) in 1-oz glass prescription bottles. Lymphocytes were treated with 6 µM diepoxybutane ([±]1,3-butadiene diepoxide; Aldrich Chemical Co., Milwaukee, WI), monoepoxybutene or nitrogen mustard at 21 h of culture. Each test compound was diluted in sterile water, and a fresh stock solution was prepared for each experiment. At 24 h of culture, 50 µM of bromodeoxyuridine were added to each culture. Cells were cultured for 72 h at 37.5 °C in 5% CO_2 with 98% relative humidity. Two hours before fixation, Colcemid (2×10^{-7} M final concentration; Ciba Pharmaceuticals, Summit, NJ) was added. Cells collected by centrifugation were exposed for 8 min to 0.075 M potassium chloride at 37 °C to spread the chromosomes and fixed three times in methanol:acetic acid (3:1). The resulting suspension was dropped onto microscope slides and stained differentially by a modification of the fluorescence-plus-Giemsa technique (Perry & Wolff, 1974). The slides were immersed for 15 min in a solution of 5 µg Hoechst 33258 (Riedel-De Haen AG, Hanover, Germany) per ml of Sorensen's buffer, pH 6.8, then washed, dried, mounted with buffer under the coverslip and exposed for 8 min to black light 2 cm from two BLB GE tubes at 55 °C. The slides were then stained for 4 min in a 3% Giemsa solution made in the same Sorensen's buffer. First-division cells in 72-h cultures containing bromodeoxyuridine were identified by their characteristic fluorescence staining pattern and were analysed for chromosomal aberrations. For each subject, 100 cells from an untreated culture and 100 cells from a culture treated with 6 µM diepoxybutane were analysed. The aberrations scored were chromatid and isochromatid deletions and chromatid exchanges. Deletions were scored if the separation of the chromatid was greater than the width of the chromatid; smaller gaps were not scored. All analyses of SCE and chromosomal aberrations were performed on coded slides and scored blindly.

Statistical analysis

The mean number of SCE/cell was calculated by scoring 50 cells per individual for baseline SCEs and by scoring 30 cells for diepoxybutane-induced SCEs. Subjects were defined as diepoxybutane-sensitive when there were > 90 SCEs/cell and as diepoxybutane-resistant when there were < 90 SCEs/cell. The mean baseline numbers of SCEs/cell in resistant and sensitive individuals were compared using Student's *t* test. Linear regression was used to assess the effects of age on the numbers of baseline SCEs/cell and diepoxybutane-

induced SCEs. Analysis of variance was used to assess the relative contribution of factors such as race, smoking and diepoxybutane sensitivity to the variance in SCE scores. Statistical runs were performed using the PC SAS program.

Results

Bimodal response to diepoxybutane *in vitro*

In our initial studies (Wiencke *et al.*, 1991a), we tested for the presence of a dose–response relationship for diepoxybutane in blood lymphocyte cultures and found that the yields of SCEs induced were linearly related to the concentrations of diepoxybutane. Significant differences were observed between several of the individuals studied, and the slope of the dose–response curves varied by more than two fold in some cases. Estimates of the slope, derived from repeated experiments within individuals and carried out at different times, varied by less than 7%, indicating that within a subject the yields of induced SCEs are quite stable. We next compared the numbers of SCEs induced at a single concentration of 6 μM diepoxybutane in 58 newspaper workers (Table 1). The results indicated a bimodal distribution of SCEs induced by diepoxybutane: about 24% (14/58) of the group had > 95 SCEs/cell (diepoxybutane-sensitive), and the remaining 76% (44/58) had < 80 SCEs/cell (diepoxybutane-resistant). The difference between the two modes was 46.7 SCEs/cell. When the background frequencies of SCEs/cell were subtracted from the frequencies of diepoxybutane-treated SCEs for each individual, the difference in the two modes was similar to the uncorrected SCE score, 45.6 diepoxybutane-induced SCEs/cell, indicating that relatively small variations in baseline SCE frequency were not responsible for the differences in diepoxybutane-induced SCEs between the two modes of the SCE distribution. On the basis of this initial distribution, we defined people who are relatively sensitive to SCE induction as having an SCE yield of > 90 SCEs/cell.

Several factors were assessed by analysis of variance for their potential effects on the frequency of diepoxybutane-induced SCEs. Low-level exposure to solvents in some individuals in the group had no effect on diepoxybutane-induced SCE frequencies. Age, alcohol and coffee consumption, familial history of cancer and red and white blood cell counts were also not associated with diepoxybutane-induced SCE frequencies. Smoking was associated with small differences in the frequencies in sensitive and resistant individuals, but was not associated with variations in diepoxybutane-induced SCE scores when the analysis was carried out on the whole group. The relatively small effect of smoking (approximately 5 SCEs/cell) was much less than the difference between the resistant and sensitive groups.

The bimodal nature of the distribution of diepoxybutane-induced SCEs was confirmed in three subsequent studies (Table 1), and the proportions of individuals classified as relatively sensitive to diepoxybutane were similar in each population. In all data sets, the potential effects on the results of factors such as age, sex, smoking and occupational histories were examined, but in no case was any of these factors found to account for the bimodal nature of the distribution of SCE frequencies.

Correlation of sister chromatid exchange induction with chromosomal aberrations

In order to ascertain whether lymphocytes sensitive to SCE induction by diepoxybutane are also more sensitive to the induction of structural chromosomal aberrations, we compared

Table 1. Prevalence of sensitivity to induction of chromosomal damage by diepoxybutane in healthy individuals

Group	No.	No. sensitive	No. resistant	Proportion sensitive
Newspaper workers	58	14	44	0.24
Female employees at the University of California, San Francisco	55	11	44	0.20
Members of the Oil, Chemical and Atomic Workers Union (butadiene production polymerization plant)	40	6	34	0.15
Normative Aging Study volunteers	20	4	16	0.20
Total	173	35	138	0.202

Proportion of the total referent group classified as diepoxybutane-sensitive, 0.202; 95% confidence interval of the estimate, 0.142–0.262

the number of aberrations present in cells from sensitive individuals with those in cells from people relatively resistant to the induction of SCEs by this compound. Chromosomal aberrations measured in first-division cells from nine sensitive individuals and nine relatively resistant individuals are shown in Table 2. Significant increases in the frequencies of diepoxybutane-induced chromatid deletions ($t = 6.39$; $p < 0.001$), isochromatid deletions ($t = 5.95$; $p < 0.001$), chromatid exchanges ($t = 3.87$; $p = 0.001$) and total aberrations ($t = 9.03$; $p < 0.001$) were observed in cells from the sensitive people. Lymphocytes from these people contained 3.5 times more chromatid deletions, 6.2 times more isochromatid deletions and 19 times more chromatid exchanges than those from resistant individuals.

Association between sensitivity to diepoxybutane and baseline frequencies of sister chromatid exchange

A wide variation in baseline SCE frequencies has been reported in lymphocytes from healthy individuals in various studies (Lambert *et al.*, 1982), although most of the variation is unexplained. Cigarette smoking is the largest single component of individual variation, about 20% of the variation being ascribed to this factor. Other factors, such as age and sex, account for smaller and more variable contributions. In order to determine whether sensitivity to the induction of SCEs is correlated with the 'spontaneous' frequency of SCEs, we carried out statistical analyses to assess the potential contribution of the diepoxybutane sensitivity trait to baseline SCEs. Table 3 summarizes the results of the analyses for two different groups (Kelsey *et al.*, 1991a; Wiencke *et al.*, 1991b). In each case, the only factors that significantly affected the results were cigarette smoking and an individual's diepoxybutane sensitivity status. In fact, sensitivity to diepoxybutane *in vitro*, used as a dichotomous variable in the analysis, accounted for a larger portion of the individual variation in SCEs than smoking. These studies indicate that induction of SCEs *in vitro* by diepoxybutane identifies a subgroup of individuals whose lymphocytes have an intrinsically higher frequency of SCEs.

Table 2. Mean numbers of induced chromosomal aberrations in lymphocytes from nine people relatively sensitive and nine relatively resistant to the induction of sister chromatid exchange by diepoxybutane

Group	Mean no. of chromatid aberrations/100 cells			
	Chromatid deletions	Isochromatid deletions	Chromatid exchanges	Total
Resistant (mean SCEs/cell, 60.4 ± 8.7)	6.4 ± 4.7	1.4 ± 1.5	0.2 ± 0.7	8.1 ± 4.9
Sensitive (mean SCEs/cell, 106.8 ± 7.4)	22.4 ± 5.9	9.0 ± 3.5	4.2 ± 3.0	35.0 ± 7.5
Ratio of mean for sensitive:resistant	3.5	6.3	19.2	4.3

See Wiencke *et al.* (1991a); values corrected for background levels of aberrations

Table 3. Analysis of variance and contribution of diepoxybutane (DEB) sensitivity trait to baseline frequencies of sister chromatid exchange in two populations

Population[a]	Source of variation	Degrees of freedom	Percent of variance explained	*F* statistic	*p*
1	Smoking[b]	1	13.7	10.3	0.002
	DEB trait[c]	1	12.3	9.1	0.004
2	Smoking[b]	1	3.4	4.6	0.036
	DEB trait[c]	1	57.6	78.3	0.0001

[a]Population 1, Kelsey *et al.* (1991a); population 2, Wiencke *et al.* (1991a)
[b]Smoking was modelled as a dichotomous variable.
[c]Sensitivity to diepoxybutane was modelled as a dichotomous variable.

Studies with monoepoxybutene and nitrogen mustard

In order to test the specificity of the trait of increased sensitivity to diepoxybutane and whether it could influence the yields of SCEs induced by the monofunctional monoepoxybutene or another DNA cross-linking agent, we treated lymphocyte cultures from people previously identified as sensitive or resistant with different concentrations of monoepoxybutene or nitrogen mustard. The results of the monoepoxybutene experiments are shown in Table 4: a significant increase in SCE induction in diepoxybutane-sensitive individuals was observed at 0.5 and 1.0 mM monoepoxybutene over that in diepoxybutane-resistant individuals. With 2.0 mM monoepoxybutene, cell toxicity was observed and increased variability in SCE yields noted; at this concentration, no significant increase in the frequency of SCE could be observed in diepoxybutane-sensitive individuals. Experiments were also carried out in one sensitive and two resistant individuals using 0, 0.2, 0.4 and 0.6 μM nitrogen mustard, which, like diepoxybutane, produces DNA interstrand cross-links. Linear increases in SCE frequencies were observed for each subject; no significant difference between the sensitive and resistant subjects in SCE yields was observed at any concentration (data not shown). Thus, sensitivity

to induction of SCEs is not specific for DNA cross-linking agents but appears to be associated with both the mono- and bifunctional butane epoxides.

Table 4. Frequencies of sister chromatid exchange (SCE) induced by monoepoxybutene in lymphocyte cultures from people sensitive and resistant to diepoxybutane

Group	No.	Concentration of monoepoxybutene (mM)			
		0	0.5	1.0	2.0
Resistant	6	8.9 ± 1.0	16.0 ± 2.6	28.8 ± 6.9	44.7 ± 6.5
Sensitive	3	9.1 ± 1.1	23.0* ± 3.1	37.4* ± 6.0	55.2 ± 13.0

*Significantly greater ($p < 0.05$) than SCE frequency in resistant subjects
The fold increase in induced SCEs in sensitive individuals is 1.96 and 1.42 at 0.5 and 1.0 mM monoepoxybutene, respectively. A marked delay in cell cycling was observed. The ratio of SCEs in sensitive and resistant individuals was 1.29 at 2.0 mM monoepoxybutene and was not statistically significant.

Discussion

It has been proposed that identification of the host factors that modify individual susceptibility to genotoxic effects, using appropriate biomarkers, can improve the assessment of cancer risks associated with exposures to carcinogens (Perera & Weinstein, 1982; Hulka & Wilcosky, 1988). In the case of enzyme polymorphisms known to influence the metabolism of specific carcinogens, direct measurement of genetic variation at the level of DNA may be the most reliable marker for epidemiological studies. In a previous study, we found that genetic deficiency of glutathione *S*-transferase type μ leads to bimodal induction of SCEs by epoxide substrates of the isozyme; cells from deficient individuals had SCE induction scores that were within the upper of the two modes of the population distribution of SCEs (Wiencke *et al.*, 1990). This observation provides support for the proposal that glutathione *S*-transferase modifies the cellular genotoxicity of specific epoxide mutagens. A technique based on the polymerase chain reaction is now available to detect this polymorphism and can be used in instances of exposures to chemicals the metabolism of which involves glutathione conjugation by the μ isoform.

In the case of butadiene, little is known about the genetic determinants of individual susceptibility to the mutagenic metabolites generated *in vivo*. We have explored the possibility that subgroups of the population may be susceptible to the genotoxicity of diepoxybutane and monoepoxybutene. The results of the studies described here, which included four independent samples from healthy blood donors, indicate that the bimodal distribution of diepoxybutane-induced SCE frequencies is a highly reproducible phenomenon. The fact that the two modes do not overlap within the distribution of SCE scores allowed us to differentiate two groups of people with respect to sensitivity to SCE induction by diepoxybutane: about 20% were classified as relatively sensitive and the remaining 80% as relatively resistant. Susceptibility to induction of SCE by diepoxybutane was not affected by age, sex, diet, alcohol or coffee consumption, cigarette smoking or occupational exposures to chemicals.

A pilot study of twins to investigate familial involvement in the bimodal response indicated that familial factors are associated with sensitivity to diepoxybutane (Kelsey *et al.*, 1991b). In addition, we observed a highly significant relationship between the diepoxybutane sensitivity trait and the frequency of baseline SCEs, which have previously been considered to be 'spontaneous'. It would now appear that many of the baseline SCEs are determined by familial factors associated with sensitivity to diepoxybutane induction of SCEs. The mechanisms responsible for these associations are unknown. Past studies suggest, however, that the factors responsible for the sensitivity to diepoxybutane that we have observed are not likely to be identical to the previously identified genetic factors that are responsible for Fanconi's anaemia, which also involves hypersensitivity to diepoxybutane. The relatively high prevalence of diepoxybutane sensitivity in our study group (20%) argues against a direct relationship with heterozygosity for Fanconi's anaemia, which is found in only 1 out of 200–300 individuals in North America (Swift, 1971). In addition, patients with Fanconi's anaemia are hypersensitive to nitrogen mustard, whereas the trait we have identified is not associated with an increased SCE response to that agent.

Regardless of the mechanisms involved in sensitivity to diepoxybutane, the population frequency of this trait and its association with high baseline SCE frequencies that are apparently unrelated to exposure have substantial implications for the use of SCEs as an end-point in biomonitoring of exposures to chemicals. Because such a large portion of the variation in baseline SCE frequencies is attributable to sensitivity to diepoxybutane, potential differences in SCE frequencies due to other exposures will be obscured if the trait is not considered in analyses. The most likely result of such unexplained variability is bias towards the null hypothesis (Type II error, false negatives). The possibility should also be considered that sensitivity to induction of SCEs by diepoxybutane is associated with a more generalized genomic instability that affects other genotoxic events. Our studies demonstrate a strong correlation between increased susceptibility to diepoxybutane-induced structural abnormalities *in vitro* and the inducibility of SCEs; the most dramatic increases were observed for chromatid exchanges, which can lead to chromosomal translocations in subsequent cell divisions. Consequently, it will be of interest in future studies of exposure to butadiene to assess not only SCE induction in groups sensitive and resistant to diepoxybutane but also structural chromosomal abnormalities, which may be more directly involved in the pathogenetic pathways that lead to malignancy.

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