Analysis of hprt mutations occurring in human TK6 lymphoblastoid cells following exposure to 1,2,3,4-diepoxybutyrate

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1,3-Butadiene (BD) is a rodent carcinogen that is bioactivated to at least two genotoxic metabolites, 1,2-epoxybutene (EB) and 1,2,3,4-diepoxybutane (DEB). The mutational spectrum for DEB at hprt in human TK6 lymphoblasts (TK6 cells) was determined and compared with the mutational spectrum from spontaneous mutants. A DEB exposure of 4 μM for 24 h resulted in an average 5-fold increase in the hprt mutant frequency. Hprt mutants for molecular analysis were isolated from TK6 cells exposed to 4 μM DEB for 24 h (51 DEB-induced mutants) and from a set of spontaneous mutants (n = 43) isolated from the same TK6 stock cell cultures. Molecular analyses of hprt mutations were done by reverse transcription–polymerase chain reaction (RT–PCR) of hprt mRNA or exon-specific genomic PCR amplification of hprt followed by sequencing of PCR products. There was an increased frequency of A:T→T:A transversions among the DEB-induced mutants compared to spontaneous mutants (9/51; 18% DEB-induced compared to 2/43; 5% in spontaneous) (one-way Fisher’s exact test; P < 0.05). DEB-induced hprt mutants also had an increased frequency of genomic deletions affecting the 5’ region of hprt (7/51; 14% DEB-induced compared to 1/43; 2% in spontaneous). Therefore, DEB is a mutagenic carcinogen that can induce genotoxicity by large deletions, rearrangements or single base substitution mutations.

Introduction

1,3-Butadiene (BD) is used in the production of synthetic rubber and is regarded as a hazardous air pollutant that is regulated under the 1990 Clean Air Act Amendment (United States Environmental Protection Agency, 1991). BD is carcinogenic in rodents at multiple sites, with mice more susceptible than rats to the carcinogenic effects (Melnick et al., 1990). The International Agency for Research on Cancer (IARC) has classified BD as a Group 2A carcinogen (probable human carcinogen) (IARC, 1992). This 1992 IARC classification for BD was based on sufficient evidence of animal carcinogenicity and limited evidence for carcinogenicity in humans. The epidemiology and toxicology of BD has been recently reviewed (Himmelstein et al., 1997).

BD is bioactivated to at least two genotoxic metabolites, 1,2-epoxybutene (EB) and 1,2,3,4-diepoxybutane (DEB). The bioactivation of EB to DEB occurs in purified human CYP2E1 and CYP3A4 enzyme preparations and in human, mouse and liver microsomes (Seaton et al., 1995). The increased susceptibility of mice compared to rats to BD-induced genotoxicity and carcinogenicity is postulated to be due to differences in BD metabolism. Mice produce higher circulating levels of the genotoxic and carcinogenic metabolites EB and DEB than rats at the same levels of exposure to the parent compound BD (Van Duuren, 1969; Himmelstein et al., 1994, 1997).

DEB is a direct-acting mutagen in bacterial and mammalian cells (de Meester, 1988). Exposure of mammalian cells in vitro to DEB induces an increased frequency of chromosomal alterations and sister chromatid exchange (SCE) (de Meester, 1988; Sasiadek et al., 1991a,b). In human TK6 cells, DEB is mutagenic at the hprt and tk loci at ~100-fold lower concentration than EB (Cochrane and Skopek, 1994a). Southern blot analysis of hprt mutants isolated from DEB-exposed human TK6 cells (Cochrane and Skopek, 1994a) indicated that a substantial fraction of these mutants had a total loss of hprt hybridization (21%). However in that study, the frequency of Southern blot detectable alterations in spontaneous hprt mutants was not reported. These studies in human TK6 cells (Cochrane and Skopek, 1994a), also demonstrated that DEB induced a preferential increase in tk− mutants with the slow growth phenotype that is associated with loss of heterozygosity at tk (Li et al., 1992). DEB also induced deletion events (50-8000 bp) at the rosy locus in Drosophila (Reardon et al., 1987; Gay and Contaminte, 1993). Taken together, these data indicate that a mechanism of genotoxicity induced by DEB is through the induction of large deletions and rearrangements.

In this study, we examined the mutational spectrum for DEB at hprt in human TK6 lymphoblasts (TK6 cells) and compared it to the mutational spectrum obtained from a set of spontaneous hprt mutants isolated from the same stock cultures to identify DEB-induced mutations. These studies extend previous work by others on the mutational spectrum for DEB at hprt in human TK6 cells. Although previous studies have examined DEB mutants for gross alterations detectable by Southern blot analysis (Cochrane and Skopek, 1994a), the present studies were done to identify the spectrum of mutations that occur in mutants isolated from DEB-exposed TK6 cells that have not been previously characterized. By characterizing the types of DNA changes induced by DEB these studies will develop a further insight into genotoxic mechanisms induced by the parent compound BD.

Materials and methods

Cell culture and exposure

Human TK6 cells have been previously described (Skopek et al., 1978; Liber and Thilly, 1982). TK6 cells were cultured in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 60 IU/ml penicillin, 60 μg/ml streptomycin and 10% heat-inactivated equine serum at 37°C in a humidified atmosphere containing 5% CO2.

Prior to exposure, TK6 cells were treated for 2 days with CHAT (cytidine 10−5 M; hypoxanthine 2×10−4 M; aminopterin 2×10−4 M; and thymidine 1.75×10−5 M)-containing medium to reduce the background hprt mutant frequency. After CHAT treatment, the cells were then grown for 1 day in THC medium (thymidine 10−5 M; hypoxanthine 2×10−4 M; and cytidine 10−5 M).
Exposure with DEB (CAS#298-18-0; Sigma Chemical Co, St Louis, MO, USA) was done in capped flasks with complete medium as described above. Ethyl methanesulphonate (EMS; CAS#62-50-0; Sigma) was used as a positive control chemical. Both EMS and DEB were dissolved in dimethylsulphoxide, (DMSO); CAS#67-68-5; Sigma).

Mutant frequency and mutant isolation

Prior to initiating studies on the mutagenicity of DEB, the cytotoxicity of DEB in TK6 cells was assessed following exposure to various concentrations of DEB. TK6 cells (4X10^5 cells/ml in 10 ml) were exposed for 24 h to 0, 2, 4, 6, 8 or 10 μM DEB for 24 h. Solvent control (DMSO) and unexposed background cultures of the TK6 independent cell lines were included with every experiment. An exposure of 200 μM EMS was included with each experiment as a positive control. After exposure, cells were put in fresh medium and plated to estimate relative cell survival by cloning efficiency in 96-well microtitre plates (2 cells/well for DMSO and unexposed cells, 8 cells/well for cells exposed to DEB and EMS). Cultures were counted and diluted daily to 4X10^5 cells/ml for 7–10 days to estimate cytotoxicity by growth curve extrapolation to the day of exposure.

An experiment to determine the hprt mutant frequency (MF) was set up in parallel to collect hprt mutants for molecular analysis of DEB-exposed (4 μM×24 h) TK6 cells. TK6 cells (4X10^5 cells/ml in 50 ml) were exposed to DEB (and EMS as a positive control) as described above. After the phenotypic expression, cells were seeded at 40,000 cells/well in 96-well microtitre plates in the presence of 6-thioguanine (6-TG, CAS#154-42-7) 1 μg/ml and at 2 cells/well without 6-TG to determine cloning efficiency values. The plates were counted for growing colonies 10 days after plating. Cloning efficiencies with and without 6-TG were calculated, assuming a Poisson distribution for colony formation, to determine the hprt mutant frequency.

For collection of hprt mutants for molecular analyses, six 75 ml flasks containing human TK6 cells (4X10^5 cells/ml in 50 ml) were exposed to 4 μM DEB×24 h. After the 24 h exposure, the cells were put in fresh medium and each 50 ml flask was divided into 10×10 ml cultures in 25 ml flasks at 1X10^5 cells/ml for a total of 60 independent flasks. After 8 days of expression time, cells from each flask were plated on two microtitre plates at 40,000 cells/well in 6-TG (1 μg/ml). After 10 days, one clone/plate was chosen and expanded into 10 ml media containing 6-TG (1 μg/ml). To ensure that each mutant to be analysed was independent, cells from each clone (0.5–1X10^5 TK6 cells/ml) were then replated on two plates at low cell density. 0.3 cells/well in 6-TG (1 μg/ml). One colony was chosen per plate and plated in 10 ml media containing 6-TG (1 μg/ml). Following expansion to 0.5–1X10^5 TK6 cells/ml, samples from each mutant were frozen for molecular analysis of hprt. One mutant corresponding to each of the original flasks inoculated immediately after exposure (see above) was used for molecular analysis of DEB-induced mutants. From these independent flasks, a total of 51 mutants were isolated (60 were tested) and 8 were excluded.

For the spontaneous mutational spectrum, spontaneous mutants were isolated by inoculating 10 ml media with 1X10^5 TK6 cells. Following growth to ~4X10^5 TK6 cells/ml, cells were maintained for 7 days and then plated at 40,000 cells/well in 6-TG (1 μg/ml). After 10 days, one clone/plate was chosen and expanded in 10 ml of medium (25 ml flasks) with 1 μg/ml of 6-TG. To ensure that each clone was independent, cells from each flask (0.5–1X10^5 TK6 cells/ml) were then replated on two plates at low cell density, 0.3 cells/well in 6-TG (1 μg/ml). One colony was per clone per plate and in 10 ml media containing 6-TG (1 μg/ml). Following expansion to 0.5–1X10^5 TK6 cells/ml, samples from each mutant were frozen for molecular analysis of hprt. From these independent flasks, a total of 43 mutants were analysed as spontaneous mutants.

Molecular analyses of hprt mutant human TK6 cells

All hprt mutant human TK6 cell clones isolated as spontaneous mutants or from DEB-exposed (DEB-induced) were directly selected by hprt-specific reverse transcription–polymerase chain reaction (RT–PCR) amplification. Mutants that produced a RT–PCR product or an altered RT–PCR product were further analysed by DNA sequencing, or by exon-specific genomic PCR and/or DNA sequencing. Mutants that did not produce an exon-specific PCR product were further analysed by exon-specific PCR amplification of adjacent exons to assess the extent of hprt genomic loss. This iterative approach was used to ultimately determine the molecular basis for each hprt mutant analysed in the present study.

RNA and DNA preparation

For RNA preparation, 5–10X10^6 cells were collected by centrifugation, resuspended in 1 ml TRIzol™ reagent (Gibco BRL, Baltimore, MD, USA) and stored at –80°C. Total RNA was prepared from those tubes according to the manufacturer’s instructions. Briefly, cells were homogenized with repetitive pipetting in the TRIzol™ reagent, chloroform was added and the aqueous phase was collected for isopropanol precipitation of RNA. The total RNA was washed in 75% ethanol and then dissolved in 200 μl water.

To analyse hprt genomic DNA alterations, frozen cell pellets were lysed as described (Fuscoe et al., 1992). A 1.5 ml cell suspension of the cell lysate (0.5–1X10^5 TK6 cells) was washed once in phosphate-buffered saline (PBS) and frozen at –80°C.

Reverse transcription–polymerase chain reaction

Ten μl total RNA solution was used in a final 20 μl reverse transcriptase reaction containing 200 IU Superscript™HIRT (Gibco BRL), 40 IU RNase Out (Promega, Madison, WI, USA), 200 μM of each dNTP (Pharmacia, Piscataway, NJ, USA), RT buffer (10 mM Tris–HCl pH 8.3; 50 mM KCl; 1.5 mM MgCl2) and 50 pmole of a hprt-specific primer (HPR1, see Table I). The RT reaction was incubated in a Perkin Elmer 9600 for 5 min at 25°C, 30 min at 42°C, 5 min at 99°C and then 4°C. For the subsequent PCR amplification of the hprt exons, 80 μl PCR mix (200 μM of each dNTP, Pharmacia), PCR buffer (10 mM Tris–HCl pH 8.3; 50 mM KCl; 1.5 mM MgCl2, 5 IU Taq (Perkin Elmer, Foster City, CA, USA) and 50 pmole of each primer (HPR1 and HPR6) (Table I), was added to the RT tubes. The PCR cycle programme was 5 min at 94°C followed by a two-step cycle: 1 min denaturation at 94°C and 1 min annealing at 60°C for 30 cycles, with the last cycle containing a 2 min extension at 68°C. An aliquot of the RT-PCR products was then analysed on a 1.5% agarose gel. In samples with a poor RT-PCR product, one further PCR amplification was done using 0.5 μl from the first reaction as a template (PCR conditions as above).
amplification and DNA sequencing. The genomic PCR products were further analysed by DNA sequencing to identify mutations in splice donor or acceptor regions. For mutants that did not produce exon-specific PCR products from genomic DNA, exon-specific PCR was used to amplify adjacent exons to assess the extent of DNA loss. For example, mutant 3F61A in Table IV had loss of exons 6, 7, 8 in hprt cDNA and in genomic DNA however, it retained exon-specific genome products from exon 5 and exon 9. Mutants that produced a hprt-specific RT-PCR product with loss of DNA sequences were considered to be hprt internal deletions.

For mutants that did not yield an hprt-specific RT-PCR product visible on an agarose gel, genomic DNA was analysed for the presence of PCR products from hprt exons 1, 2, 4 and 9. Hprt-specific primers complimentary to intron sequences adjacent to each hprt exon were used (Gibbs et al., 1990; Fuscoe et al., 1992). Large deletions were considered to be hprt mutants that did not produce any hprt-specific PCR products from genomic DNA. Mutants with partial 5' hprt deletions were mutants that lacked PCR amplification products from the 5' region of hprt (absence of PCR products for exon 1, exon 1 and 2, or exon 1, 2 and 4). Mutants that did not produce a PCR product for hprt exon 9 or exon 9 and 4 were considered to be hprt mutants with partial 3' deletions.

**Direct sequencing of hprt PCR products**

PCR products (50–100 µl) were purified on Wizard columns (Promega) and eluted with 50 µl of H2O (Sigma). For each purified PCR product 7 µl was used in a DNA cycle sequencing reaction using a Top DyeDeoxy™ Terminator sequencing kit. For cDNA PCR products, Hprt 3, Hprt 6, Hprt-S413 and Hprt-S535 primers were used for DNA sequence analysis (Table I). Sequencing reactions were purified on Centrisep columns and run on a 6% denaturing gel using an Applied Biosystems 373A DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data obtained from each primer were aligned with respect to the wild-type sequence using Factura™ and Autoassembler™ sequence analysis software. Mutations were confirmed by visual inspection of each individual histogram with respect to a wild-type sequence.

**Analysis of the DEB-induced mutational spectrum**

The frequency of all mutational types among the spontaneous and DEB-induced mutants were tabulated as a frequency and percentage. The statistical differences between the mutational types in spontaneous compared to DEB-induced was assessed by a one-way Fisher's exact test using P = 0.05.

**Results**

**Cell survival and mutant frequency**

Two experiments were done to determine the relative percentage survival and hprt MF in TK6 cells exposed to DEB. TK6 cells (4×10⁵ cells/ml in 10 ml) were exposed for 24 h to 0, 2, 4, 6, 8 or 10 µM DEB. Cytotoxicity was assessed by cloning efficiency and growth curve extrapolation. Both methods to assess cytotoxicity indicated that 4 µM DEB×24 h results in ~10% relative survival (data not shown). These data are consistent with those reported in another study (Cochrane and Skopek, 1994a).

In mutagenicity dose–response experiments conducted by others in TK6 cells (Cochrane and Skopek, 1994a), the maximal hprt mutagenic response induced by DEB occurred at 3.9 µM DEB×24 h. Therefore, an exposure×time concentration of 4 µM DEB×24 h was used to conduct a mutagenicity experiment and to initiate cultures for hprt mutant isolation from DEB-exposed TK6 cells. The mutagenicity experiment was done by exposing 50 ml cultures of human TK6 cells at 4×10⁵ cells/ml to 4 µM DEB×24 h. Exposure of TK6 cells to 4 µM DEB×24 h resulted in an average 5-fold increase in mutant frequency above background (Table II). The data reported in Table II are parallel cultures from the DEB exposure of TK6 cells that was used for molecular analysis of isolated mutants described below. Mutants for the hprt spontaneous mutational spectrum were isolated from the same stock cultures to provide a direct comparison to the spectrum of mutations determined in DEB-exposed TK6 cells.

**Table II. Cytotoxicity and mutagenicity at the hprt locus in human TK6 lymphoblasts exposed to diepoxybutane (DEB; 4 µM DEBX24 h)**

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Relative percentage survival</th>
<th>hprt mutant frequency (x 10⁴*†)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK6-1</td>
<td>100</td>
<td>2.6</td>
</tr>
<tr>
<td>TK6-3</td>
<td>100</td>
<td>3.8</td>
</tr>
<tr>
<td>TK6-1</td>
<td>100</td>
<td>2.6</td>
</tr>
<tr>
<td>TK6-3</td>
<td>100</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*TK6-1 and -3 are two subcultures exposed separately. Relative percentage survival is based on separate experiments and is based on the growth curve and cloning efficiencies of exposed and unexposed TK6 cells.

Four independently exposed flasks.

*Ethyl methane sulphonate (200 µM×24 h).

**RT-PCR and agarose gel electrophoresis analysis**

Of 51 hprt mutants isolated from DEB-exposed TK6 cells, 32 resulted in a RT–PCR product visible on a 1% agarose gel. More than half these 32 mutants had an altered migration pattern on the agarose gel, suggesting exon loss or genomic deletion. Of 43 hprt mutants isolated to represent spontaneous mutations, 32 mutants produced an hprt-specific RT–PCR product visible on a 1% agarose gel. Of these 32 mutants, 11 produced an RT–PCR product with an altered migration pattern suggesting exon loss or genomic deletion.

**Deletion screening of hprt mutants by exon-specific genomic PCR**

There were 19 of 51 DEB-induced mutants and 11 of 43 spontaneous mutants that did not produce a RT–PCR product. These mutants were further analysed by exon-specific PCR amplification. Exon-specific PCR amplification of exons 1, 2, 4 and 9 was used to assess genomic hprt for exon loss (Table III). Of 19 DEB-induced mutants, five did not produce any exon-specific PCR products compared to two of 11 spontaneous mutants. These mutants were considered as large deletions. Seven DEB-induced mutants compared with one spontaneous mutant did not produce a PCR amplification product from either exon 1, exons 1 and 2 or exons 1, 2 and 4. These mutations were considered to be partial 5' deletion mutants. Seven DEB mutants compared with eight spontaneous mutants did not produce a PCR product for either exon 9 or exons 4 and exon 9 and were considered to be 3' partial deletion mutants.
in splice donor/acceptor sites that could result in aberrant splicing of the corresponding genomic regions of hprt. Certain PCR products (e.g. mutants with single exon skipping in cDNA) were further examined at the genomic level by exon-specific PCR to determine the mutational basis for the altered hprt cDNA products. Intron primers that would produce a PCR product encompassing the exon(s) absent in the cDNA (and flanking regions) were used to PCR-amplify the corresponding genomic regions of hprt. Certain PCR products (e.g. mutants with single exon skipping in cDNA) were analysed by DNA sequencing and examined for mutations in splice donor/acceptor sites that could result in aberrant splicing of hprt mRNA. Of 13 DEB mutants that showed single exon loss, six were due to base substitution mutations in splice site sequences (Table V). DEB mutant 3B42A had a T→A transversion in the intron 1 donor splice site resulting in the inclusion of 48 bp of intron 1 sequence between exon 1 and 2 in hprt cDNA. DEB mutant 3A31A had a 1 bp deletion in the donor site (Table IV) resulting in exon 8 skipping; while the mutation found in mutant 3A51B, also with exon 8 skipping, was a C→T mutation within exon 8 (Table V). DEB-induced mutant 3F32A had a 46 bp deletion within exon 6 that resulted in exon 6 skipping in hprt cDNA. The remaining DEB mutants with single exon skipping had either genomic deletions of the corresponding exon sequences in DNA or mutations affecting PCR primer annealing sites. Two DEB mutants with exons 2 and 3 absent in the hprt cDNA (3D11B and 3F92A) had genomic deletions of exon 3. The remaining mutants with either 2 or 3 exons absent in hprt cDNA had the corresponding regions deleted from genomic hprt. These mutants were not further characterized to assess breakpoint junctions.

Among the spontaneous mutants, nine had single exon skipping in hprt cDNA. In two mutants with exon 4 skipping in cDNA (2.3-5a2, 2.3-7bl; Table V), point mutations were observed at adjacent base pairs in the splice acceptor region for exon 4. In spontaneous mutant 2.1-6bl, the identical C→T mutation within exon 8 that occurred in DEB mutant 3A51B resulting in exon 8 skipping in hprt cDNA was observed (Table V). Genomic deletions of single exons in five spontaneous mutants resulted in single exon skipping in hprt cDNA (Table IV). A 10 bp genomic deletion in exon 2 resulted in exon 2 skipping in hprt cDNA (mutant 3tla; Table IV). Genomic deletion of exon 3 (mutant 2.3-9a1) resulted in exon skipping of exons 2 and 3 in hprt cDNA and in a mutant with three exons skipped in cDNA (mutant 3k1b), all three exons were absent in genomic DNA. In mutant 1.10A1, an A→T substitution in the splice acceptor site caused a 21 bp deletion of exon 8 at the cDNA level, indicating that a cryptic splice site within exon 8 was likely used (Table V).

Insertions and single base deletions
One DEB mutant had a 4 bp insertion in exon 4; mutant 3C91B had a 4 bp insertion of an ATGG, that is a duplication of the preceding four bases (Table IV).

One DEB mutant (3A31A) had a single base deletion in the splice donor region resulting in exon 8 skipping in hprt cDNA. Two spontaneous mutants had single base deletions (3j1b and 2.1-10a1) resulting in frameshift mutations in hprt cDNA.

Single base substitutions
Single base substitutions in the hprt coding region were identified in 10 out of 51 DEB mutants; eight were transversions and two were transitions (Table V). Single base substitutions were found at intron splice site sequences in six mutants that resulted in aberrant splicing of hprt mRNA (by exon skipping or splicing at presumed cryptic splice sites). Among DEB-induced mutants, there were a total of 11 mutations at A:T base pairs and seven at G:C base pairs; four transitions and 14 transversions (Table V). Among the 43 spontaneous hprt mutants, 22 single base substitutions were observed, 14 at G:C base pairs and eight at A:T base pairs; 13 transitions and nine transversions (Table V).

Statistical analysis of the DEB mutational spectrum compared to spontaneous
Statistical analysis (Fisher's exact test) was done on the frequency of each mutational type determined in DEB-induced

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Table IV. Internal deletions and insertions in of diepoxybutane-induced and spontaneous hprt mutant human TK6 cells

| Mutant | Exon(s) affected | Comments*
|--------|-----------------|-----------------
| 3D92B | 2,6             | genomic deletion of exon 2; exon 6 absent in cDNA |
| 3D11B | 2,6             | genomic deletion of exon 3; exons 2 and 3 absent in cDNA |
| 3F92A | 2,6             | genomic deletion of exons 3 and 6; exons 2 and 5 absent in cDNA |
| 3C21B | 2,6             | genomic deletion of exons 4 and 5; exons 4 and 5 absent in cDNA |
| 3F11B | 2,6             | genomic deletion of exons 4, 5, 6; exons 4, 5, 6 absent in cDNA |
| 3E41B | 5               | genomic deletion of exon 5; exons 5 and 6 absent in cDNA |
| 3F62A | 6               | genomic deletion of exon 5; exons 5 and 6 absent in cDNA |
| 3D61A | 6               | genomic deletion of exon 5; exons 5 and 6 absent in cDNA |
| 3F32A | 6               | genomic deletion of exon 6; exon 6 absent in cDNA |
| 3E61A | 6,7             | genomic deletion of exons 6, 7, 8; exons 6, 7, 8 absent in cDNA |
| 3B91B | 7               | genomic deletion of exons 7 and 8; exons 7 and 8 absent in cDNA |
| 3C81B | 7,8             | genomic deletion of exons 7 and 8; exons 7 and 8 absent in cDNA |
| 3A31A | 8               | genomic deletion of exons 7 and 8; exons 7 and 8 absent in cDNA |
| 3C91B | 4               | Insertion of ATG after hprt cDNA bp 352 TA ATTG GT |
|        |                 | ATTG GT |

Spontaneous mutants

- 3i1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3i1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3j1b  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3k1b  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3l1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3l1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3l1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |
- 3l1a  3, 4, 5  genomic deletion of exons 3, 4, 5; exons 3, 4, 5 absent in cDNA |

*The Edwards et al. (1990) numbering system for the entire gene is given in parentheses.
of the carcinogen BD. DEB is mutagenic in human TK6 cells the mechanisms of genotoxicity induced by DEB, a metabolite of the genotoxic and carcinogenic effects of BD. The present studies examined the in vitro mutational spectrum of DEB to provide the data necessary to assess its mutational potential role in the tumour of DEB to provide the data necessary to assess its genotoxicity and carcinogenic effects of DEB. The present studies were performed to develop a further understanding of the mechanisms of genotoxicity induced by DEB, a metabolite of the carcinogenic BD. DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a). DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a). DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a).

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Discussion

The in vivo biotransformation of BD produces at least two genotoxic metabolites, EB and DEB, that likely mediate many of the genotoxic and carcinogenic effects of BD. The present studies were performed to develop a further understanding of the mechanisms of genotoxicity induced by DEB, a metabolite of the carcinogenic BD. DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a). DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a). DEB is mutagenic in human TK6 cells at concentrations that are 100-fold lower than EB, a proximate genotoxic metabolite of DEB (Cochrane and Skopek, 1994a).
but not provided to compare DEB-induced with spontaneous mutations. In the present studies, hprt mutants representing spontaneous hprt mutations were also analysed from the same stock cultures of human TK6 cells to provide a direct comparison to the mutations observed from DEB-exposed cultures. An attempt was made to analyse all mutants at the DNA sequence level; however, deletion end points in hprt mutants were not determined. Deletions were assessed by the absence or presence of specific hprt exons in the genomic DNA by PCR. Intron/exon junction sites were sequenced in mutants displaying aberrant mRNA splicing (exon loss) to examine donor and acceptor splice site sequence mutations.

The distribution of mutational types determined in DEB-exposed human TK6 cells was statistically compared to the distribution of mutational types determined in spontaneous mutants isolated from the same stock cultures. There was an increased frequency of A:T—>T:A transversions in DEB-exposed human TK6 cells when compared to the frequency of A:T—>T:A transversions in spontaneous mutants. An increased frequency of A:T—>T:A transversions has been observed at the lacI transgene in B6C3F1 lacI transgenic mice exposed to the parent compound BD (Sisk et al., 1994; Recio and Meyer, 1995). A:T—>T:A transversions also occur at hprt in mutant T-lymphocytes from B6C3F1 mice exposed to BD however, the frequency of A:T—>T:A transversions at hprt in T-lymphocytes from unexposed mice (spontaneous mutants) was not reported (Cochrane and Skopek, 1994b). These data indicate that DNA adducts at A:T base pairs may be biomarkers that are relevant to DEB-induced mutagenic events.

A comparison of the sites mutated in the present study with the hprt background base substitutions in the hprt mutation database (Cariello et al., 1992), indicates that most of the positions within the hprt sequence containing base substitutions from the DEB-exposed TK6 cells were different except for two mutations, one at bp 119 and one at bp 449. Only the T—>A at bp 449 was the identical mutation to that observed in a background mutant reported in the database.

A aberrant splicing of hprt mRNA occurs from mutations in sequences that regulate mRNA splicing or deletions of exon regions from the genomic DNA. In two mutants with exon 8 skipping in hprt cDNA (DEB-induced, 3A51B and spontaneous, 2.1-6b1), the aberrant splicing was due to a mutation within exon 8. This mutation was previously observed in human cells and in spontaneous mutants from human TK6 cells (Steingrimsdottir et al., 1992; Lichtenau-Kaligis et al., 1995). In a spontaneous mutant (1.10a), a base substitution mutation in the exon 8 splice acceptor region resulted in the use of a cryptic splice site within exon 8, 21 bp downstream from the beginning of exon 8. The cryptic splicing in exon 8 occurred 2 bp within the base substitution mutation observed in DEB-induced mutant 3A51B and spontaneous mutant 2.1-6b1. These data are consistent with previous studies indicating that sequences within exon 8 have a role in regulating hprt mRNA splicing of exon 8 (Steingrimsdottir et al., 1992).

Exposure of mammalian cells or rodents to DEB induces a number of clastogenic alterations (Tice et al., 1987). DEB induces chromosome alterations in vitro in rodent and in human cells (Perry and Evans, 1975; Dean and Hodgson-Walker, 1979; Sasiadek et al., 1991a,b). In comparative studies of EB and DEB, DEB induces chromosomal alterations at concentrations that are 10–100-fold lower than EB (Sasiadek et al., 1991a,b). In developing rat spermatids exposed in vitro to EB and DEB, only DEB increased the frequency of micronuclei (Sjöblom and Lähdetie, 1996). The i.p. injection of DEB induces micronuclei in splenocytes and germ cells of mice and rats (Xiao and Tates, 1993). Taken together, these data indicate that a characteristic lesion induced by DEB in mammalian cells is the induction of chromosomal alterations.

In the present studies, the frequency of genomic deletions at hprt among DEB-induced mutants was assessed by exon-specific PCR. These analyses indicated that there was an increased frequency of deletions affecting the 5’ region of hprt. In five DEB-induced mutants and in none of the spontaneous mutants, exon-specific genomic PCR indicated the presence of exon 2 but the absence of exon 1. Since the distance between exon 1 and 2 is ~13.3 kilobases, it is apparent that DEB exposure of human cells can induce genomic deletions at hprt that are detectable at the molecular level. The induction of genomic deletions at hprt is consistent with the induction of chromosomal alterations in DEB-exposed mammalian cells.

DEB is a bifunctional alkylating agent that can alkylate DNA at the N7 position of guanine and can induce interstrand cross-links between adjacent guanine bases (Lawley and Brookes, 1967; Tretaykova et al., 1996). While certain DNA adducts have been reported from in vitro DNA reactions with BD metabolites (Citti et al., 1993; Leuratti et al., 1994; Koivisto et al., 1995; Selzer and Elfarra, 1996; Tretaykova et al., 1996), a comprehensive evaluation of DNA adducts from BD exposure in vitro or in vivo is not available. Many DNA adduct studies thus far have focused on adducts resulting from exposure to EB and not DEB. Therefore, invoking a specific adduct(s) for the observed biological effects from exposure to BD and its metabolites, especially for DEB, is premature. Furthermore, it is uncertain how the DNA adducts described thus far can be mechanistically linked to the spectrum of genotoxicity induced by exposure to BD ranging from single base substitution mutations to rearrangements of DNA that are detectable at the cytogenetic level.

Exposure to DEB in human cells results in a wide range of genotoxic events that include large scale rearrangements of DNA to the induction of single base substitutions. The induction of both of these genotoxic events likely contribute to the

**Table VI. A summary of results from the molecular analysis of human hprt mutant TK6 cells**

<table>
<thead>
<tr>
<th>Mutational type</th>
<th>Mutations at hprt in human TK6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spontaneous (%)</td>
</tr>
<tr>
<td><strong>Base substitutions</strong></td>
<td></td>
</tr>
<tr>
<td>Transitions</td>
<td></td>
</tr>
<tr>
<td>AT→GC</td>
<td>3 (7)</td>
</tr>
<tr>
<td>GC→AT</td>
<td>10 (23)</td>
</tr>
<tr>
<td><strong>Transversions</strong></td>
<td></td>
</tr>
<tr>
<td>AT→CG</td>
<td>3 (7)</td>
</tr>
<tr>
<td>AT→TA</td>
<td>2 (5)</td>
</tr>
<tr>
<td>GC→CG</td>
<td>1 (2)</td>
</tr>
<tr>
<td>GC→TA</td>
<td>3 (7)</td>
</tr>
<tr>
<td><strong>Other alterations</strong></td>
<td></td>
</tr>
<tr>
<td>Insertions</td>
<td>0 (&lt;2)</td>
</tr>
<tr>
<td>Genomic hprt deletions</td>
<td></td>
</tr>
<tr>
<td>Partial 5’ loss</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Partial 3’ loss</td>
<td>8 (19)</td>
</tr>
<tr>
<td>Internal</td>
<td>10 (23)</td>
</tr>
<tr>
<td>Large deletions</td>
<td>2 (5)</td>
</tr>
</tbody>
</table>

DEB = 1,2,3,4-diepoxybutane.

*P ≤ 0.05 (one-way Fisher’s exact test).
overall mutagenicity and carcinogenicity resulting from BD exposure. Which of these mutational events, (large deletions or rearrangements or single base substitution mutations) predominates at the low levels of BD exposure normally encountered by humans is uncertain. These and other data indicate that human biomonitoring of BD exposed populations with the hprt mutant T-lymphocyte cloning assay (Ward et al., 1994, 1996; Hayes et al., 1996) should include the molecular analysis of hprt deletions as well as the spectrum of specific base substitution mutations. In addition, relevant biomarkers of BD exposure, particularly DNA adducts, should be able to mechanistically account for the range of genotoxic events that are known to be induced by exposure to BD and its metabolites.

Acknowledgements

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