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Comparison of the mutations at *Hprt* exon 3 of T-lymphocytes from B6C3F1 mice and F344 rats exposed by inhalation to 1,3-butadiene or the racemic mixture of 1,2:3,4-diepoxybutane

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Abstract

Experiments were conducted to define the spectra of mutations occurring in *Hprt* exon 3 of T-cells isolated from spleens of female B6C3F1 mice and F344 rats exposed by inhalation to 1,3-butadiene (BD) or its reactive metabolite, (±)-diepoxybutane (DEB). *Hprt* mutant frequencies (M*f*s) in BD-exposed (1250 ppm for 2 weeks or 625 ppm for 4 weeks; 6 h/day, 5 days/week) and DEB-exposed (2 or 4 ppm for 4 weeks or 5 ppm for 6 weeks; 6 h/day, 5 days/week) mice and rats were significantly increased over concurrent control values. Mutant T-cell colonies from control and treated animals were screened for mutations in *Hprt* exon 3 using PCR amplification of genomic DNA and denaturing gradient gel electrophoresis, followed by sequence analysis. Exon 3 mutations were found at the following frequencies: 20/394 (5%) in control mice, 56/712 (8%) in BD-exposed mice, 59/1178 (5%) in BD-exposed rats, 66/642 (10%) in DEB-exposed mice, and 51/732 (7%) in DEB-exposed rats. Mutations in exposed animals included base substitutions, small deletions (1 to 74 bp), and small insertions (1 to 8 bp), with base substitutions predominating. Among the types of base substitutions observed in mice, the proportions of G·C → A·T transitions ($p = 0.035$, Fisher's Exact Test) and G·C → C·G transversions ($p = 0.05$) were significantly different in control vs. BD-exposed animals. Given the small number of exon 3 mutants analyzed, there was a high degree of overlap in the mutational spectra between BD-exposed mice and rats, between BD- and DEB-exposed mice, and between BD- and DEB-exposed rats in terms of the sites with base substitutions, the mutations found at those mutated sites, the relative occurrence of the most frequently observed base substitutions, and the occurrence of a consistent strand bias for the most frequently observed base substitutions. The spectra data suggest that adduction of both G·C and A·T bps is important in the induction of in vivo mutations by BD metabolites in exposed mice and rats.

Keywords: Butadiene; Diepoxybutane; *Hprt*; Lymphocytes; Mutational spectra; Mouse; Rat

Abbreviations: BD, 1,3-butadiene; DEB, 1,2,3,4-diepoxybutane; DGGE, denaturant gradient gel electrophoresis; EB, 1,2-epoxybutene; M*f*, mutant frequency; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; RT–PCR, reverse transcriptase–polymerase chain reaction

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1. Introduction

1,3-Butadiene (BD, CAS # 106-99-0), an important industrial chemical used in the manufacture of styrene–butadiene rubber, is a multi-site carcinogen in both mice and rats [1–3]. Mice are more sensitive to BD-induced tumorigenicity than rats, however, the only type of tumor caused by BD exposure in both species is mammary gland neoplasia. Epidemiological studies have found some evidence for a causal relationship between BD exposure and the occurrence of leukemia/lymphoma in exposed populations, but the human data have not been conclusive [4–6]. The species differences in the tumorigenicity of BD in rodents and the equivocal epidemiological data have complicated the assessment of cancer risk in people occupationally exposed to BD.

The genotoxicity studies with BD indicate that the carcinogenicity of this agent is related to its metabolic activation to several DNA-reactive metabolites, including 1,2-epoxybutene (EB; butadiene monoepoxide), 1,2:3,4-diepoxybutane (DEB; butadiene diepoxide), and 3,4-epoxy-1,2-butanediol (butadiene diol epoxide). Two of these three metabolites (i.e., EB and DEB) have been measured in tissues of BD-exposed mice and rats, and the greater carcinogenic susceptibility of the mouse compared to the rat correlates with the circulating levels of these genotoxic metabolites in animals exposed to the same levels of parent compound [7–9]. In vitro mutagenicity studies have also demonstrated that the mutagenic efficiencies of these two epoxide intermediates are much greater than that of butadiene diol epoxide [10]. Nevertheless, the relative contribution of EB and DEB to the mutagenic and carcinogenic actions of BD in mice and rats is still uncertain.

The current report is part of a larger effort to determine if mutation data from mice and rats exposed to BD or directly to its individual epoxide metabolites can be used to define the relative significance of each epoxide to the mutagenicity of the parent compound in each species. The relative contributions of EB and DEB to the mutagenicity of BD was initially assessed by exposing mice and rats to selected concentrations of BD, EB and DEB, and comparing the mutagenic potency of each compound when comparable blood levels of metabolites were achieved [11,12]. For this purpose, a novel mathematical model was developed to derive an estimate of the total mutagenic burden by integrating the area under the 'mutant manifestation' curves for the frequency of 6-thioguanine-resistant (i.e., *Hprt* mutant) T-lymphocytes from the spleens of treated vs. control animals [13,14]. The resulting *Hprt* mutant frequency (Mf) data indicated that at lower BD exposures (≤ 62.5 ppm) DEB is a major contributor to the mutagenicity of BD in mice, while the available metabolism data and Mf data suggested that other metabolites are probably responsible for mutations in BD-exposed rats and for the progressive mutagenic effects at higher exposures to BD in mice [12]. The biological basis and underlying assumptions for this approach to estimating mutagenic response, as well as the strengths and weaknesses of the approach, are discussed systematically elsewhere [14].

In the present study, mutant T-cell clones collected from the mice and rats exposed to BD and DEB [11–13] were examined, by denaturing gradient gel electrophoresis (DGGE) and DNA sequencing, for the type and location of mutations (mutational spectrum) occurring in exon 3 of the *Hprt* gene and the resulting mutational spectra were compared with that of control mice. Under the experimental conditions used for the *rac*-EB inhalation studies (25 ppm for 4 weeks), the mutagenic response in exposed mice and rats was too low to generate mutational spectra data for this metabolite [12]. Also, the mutational spectra data resulting from DGGE analysis of *Hprt* mutants from the current study of BD were compared to preliminary spectra data obtained by reverse transcriptase–polymerase chain reaction (RT–PCR) of *Hprt* mRNA from propagated mutant clones of control and BD-exposed mice [12].

2. Materials and methods

2.1. Chemicals

BD and the racemic mixture of DEB [(±)-DEB; CAS # 298018-0) were purchased from Aldrich Chemical (St. Louis, MO) [11–13]. Other chemicals or reagents for DNA extractions, PCR amplifications, evaluation of PCR efficiency, denaturing gradient gel electrophoresis (DGGE), and DNA sequencing were of the highest grade available from

commercial sources and have been listed elsewhere [14].

2.2. Animal exposures

Animal exposures were described in detail previously [11–13,16]. Briefly, in the first experiment with BD, female B6C3F1 mice and Fischer 344 rats (4–5 weeks of age) were exposed by inhalation to 0 or 1250 ppm BD for 2 weeks (6 h/day, 5 days/week) [13]. In the second experiment with BD, similarly aged mice were exposed to 0 or 625 ppm BD for 4 weeks (6 h/day, 5 days/week) [11]. In experiments with DEB, similarly aged mice and rats were exposed by inhalation to 0, 2 or 4 ppm (±)-DEB for 4 weeks [12], or to 0 or 5 ppm (±)-DEB for 6 weeks (6 h/day, 5 days/week) [16]. The housing of animals conformed with NIH guidelines (NIH Publication no. 86-23, 1985), and all procedures involving the use of animals were approved by the Institutional Animal Care and Use Committees where the exposures and animal experiments were performed. Control and treated animals were necropsied at multiple times post-exposure to measure *Hprt* M*f*s [11–13,16] and to collect mutant T-cell clones for molecular analysis.

2.3. Molecular analysis of mouse and rat genomic DNA for Hprt exon 3 mutations

The procedures to identify and characterize mutations in the exon 3 region of the mouse and rat *Hprt* gene were performed as previously described [14,17], with minor modifications. In brief, genomic DNA from each mutant colony was extracted using a Tween 20/proteinase K digestion mix, aliquots of DNA were used to amplify *Hprt* exon 3 by PCR, mutations in the low- and high-temperature melting domains of exon 3 were identified and purified using DGGE, and DNA eluted from heteroduplex bands excised from DGGE gels was amplified and sequenced.

All of the PCR primers previously developed for the amplification of mouse and rat *Hprt* exon 3 [15,17] were used in this study, except for the 3′-primers used by Mittelstaedt et al. [17] for the first round PCR of an extended exon 3 fragment in the rat (primer 'R308R') and for the second round PCR to analyze the low-temperature melting domain of rat exon 3 (primer 'H3'). The 3′ primer (5′-TAATTA-TACTTACACAGTA-3′) used here, in lieu of R308R and H3, was complementary to the last five bases of exon 3 and the first 14 bases of intron 3 of the coding strand. DNA sequences for the other rat-specific primers that were used in the current study were kindly provided prior to the publication of this information by Mittelstaedt et al. [17].

Exon 3 PCR products from both mice and rats were analyzed for mutation by DGGE as previously described for the mouse [15] except that the low-temperature melting domain products were screened using a 27%–36% denaturant gradient. Instead of extracting DNA [15] or precipitating DNA [17] from PCR reactions to be analyzed by DGGE (both primary gels for mutant identification and secondary gels for purification of mutant/wild type heteroduplexes), 5 μl of 50 μM EDTA was added to 23 μl of the PCR products to inactivate *Taq* polymerase prior to heteroduplex formation. Heteroduplexes that overlapped on primary DGGE gels were subsequently resolved into upper and lower bands using narrower denaturing gradients for both the low-temperature and high-temperature domains. Sets of four bands on a purification DGGE gel (i.e., two sets of heteroduplexes representing a single mutation) were excised and eluted to provide templates for strand-biased PCR [15]. All four strand-bias PCR reactions were run through Centricon 50 concentrators (Amicon, Beverly, MA) to filter out the primers. The filtrates were vacuum dried and resuspended in 20 μl sterile water as templates for single-strand PCR reactions using the appropriate 5′ primer for sequencing in a single direction. Single-strand PCR products were filtered through CentriSep spin columns (Princeton Separations, Adelphia, NJ) and aliquots of these PCR products were then sequenced using the sequenase/dideoxy protocol specified by Applied Biosystems (Foster City, CA). For each mutant, this approach produced a sequence for each of the four heteroduplexes from purification DGGE gels, with two of the sequences (corresponding to a pair of either upper or lower heteroduplex bands) usually having wild type sequence and the other two sequences (corresponding to the opposite heteroduplex bands) having the same mutation in exon 3.

2.4. Statistical analysis

A Fisher's Exact Test for homogeneity of proportions of mutation types in control mice vs. BD- or (±)-DEB-exposed mice was performed. Also, a Fisher's Exact hypothesis test was used to determine if individual types of base substitutions in control and treatment (BD or DEB) groups were significantly different, with the computations performed under the null hypothesis that percents should occur in the same proportions in treatment and control groups. A p-value of < 0.05 was considered significant. The latter test was performed in the fashion of Carr and Gorelick [18], except that Mf was not first corrected to 'mutation frequency' for each mutation type for reasons related to T-cell kinetics in the mouse (discussed in Ref. [14]).

3. Results

3.1. Analysis of mutations in splenic T-cells from control B6C3F1 mice

A total of 394 *Hprt* mutant T-cell colonies from untreated or vehicle-treated B6C3F1 mice were analyzed by PCR, DGGE and DNA sequencing for the presence of mutations in exon 3. These mice were concurrent controls for exposures to various compounds, including BD [13], DEB [12], *N*-ethyl-*N*-nitrosourea [19,20], ethylene oxide [21,22], and cyclophosphamide (CP) [23], and all mice were necropsied as young adults at 12 to 16 weeks of age (see footnotes to Table 2). Twenty *Hprt* mutant colonies (20/394, 5%) contained mutations in exon 3; these mutations were a combination of base substitutions (15/20, 75%), frameshifts (3/20, 15%), one small deletion (5%), and one complex mutation (5%). The 15 base substitutions occurred at 12 different sites, and involved transitions and transversions at both G · C (11/20) and A · T bps (4/20). One base substitution (G · C → A · T at bp 152) was detected in three different mice and another one (G · C → A · T at bp 168) was seen in two different animals. Table 1 presents of a summary of the classes of mutations found in exon 3 of control mice compared with BD- and DEB-exposed mice while Table 2 lists the types and locations of the individual mutations occurring in control mice.

3.2. Analysis of mutations in splenic T-cells from BD-exposed B6C3F1 mice

A total of 712 *Hprt* mutant T-cell clones from mice exposed to 1250 ppm BD for 2 weeks [13] or 625 ppm BD for 4 weeks [11] were screened for mutations in exon 3. *Hprt* mutant frequencies (Mfs) in these BD-treated mice averaged 7.2-fold (ranging from 5- to 11-fold) higher than those in concurrent control mice (i.e., 2.0 ± 0.5 (S.D.) $\times 10^{-6}$) [11,13]. Fifty-six mutations (8%) were determined to reside in exon 3 of exposed mice. The majority of these mutations were base substitutions (47/56, 84%; 21 sites), while the rest were comprised of frameshift mutations (6/56, 11%; six sites), complex mutations (2/56, 4%), and one small deletion (2%) (Tables 1 and 3). The base substitutions were composed of transitions and transversions at both G · C (29/56, 52%; 11 sites) and A · T bps (18/56, 32%; 10 sites). Two different substitutions occurred at three of the mutated G · C bps (i.e., 207, 211 and 219) and at two of the mutated A · T bps (i.e., 217 and 299). The most common exon 3 mutations were G · C → C · G transversions at bp 211 (found in four mice), G · C → C · G transversions at bp 212 (observed in four mice), and A · T → C · G transversions at bp 216 (seen in six mice). Notably, 16/18 of the guanine bases involved in the G · C → C · G transversions found in BD-treated mice were situated on the nontranscribed strand (Table 3), whereas the guanine in the only G · C → C · G transversion identified thus far in a control mouse occurred on the transcribed strand (Table 2). Also, 8/8 of the thymine bases at risk in the A · T → C · G transversions of BD-exposed mice resided on the nontranscribed strand (Table 3), as was true for the only A · T → C · G transversion found in a control mouse. Remarkably, 38% (21/56) of the exon 3 mutations in BD-treated mice were detected in a run of six guanines (bp 207–212); these mutations included one +G insertion, one −G deletion, and substitutions at four of the six guanine bases (Table 3). Finally, there were four sites in exon 3 (bps 151, 207, 299 and 311) at which base substitutions were seen in both control and BD-treated mice (among 17 and 30 mutated sites in each group, respectively), with identical mutations occurring at only two of these mutual sites (Tables 2 and 3).

Table 1
Summary of exon 3 mutations in *Hprt* mutant T-cells clones from control B6C3F1 mice vs. B6C3F1 mice exposed to butadiene or (±)-diepoxybutane

Class of mutation	Number of experimentally observed mutations in the exon 3 region of *Hprt*							
	Control mice		Butadiene-exposed mice			Diepoxybutane-exposed mice		
	Spontaneous mutants	Mutant fx.[a] $\times 10^{-8}$	BD-treated mutants	Mutant fx.[a] $\times 10^{-8}$	BD-induced mutants[b] $\times 10^{-8}$	DEB-treated mutants	Mutant fx.[a] $\times 10^{-8}$	DEB-induced mutants[b] $\times 10^{-8}$
Base substitutions								
G · C → A · T	7 (35%)	3.57	6 (11%)[c]	11.79	8.22	28 (42%)	20.96	17.39
A · T → G · C	2 (10%)	1.02	6 (11%)	11.79	10.77	11 (17%)	8.23	7.21
G · C → T · A	3 (15%)	1.53	5 (9%)	9.82	8.29	2 (3%)	1.50	0
G · C → C · G	1 (5%)	0.51	18 (32%)[c]	35.36	34.85	8 (12%)	5.99	5.48
A · T → C · G	1 (5%)	0.51	8 (14%)	15.71	15.20	8 (12%)	5.99	5.48
A · T → T · A	1 (5%)	0.51	4 (7%)	7.85	7.35	3 (5%)	2.24	1.74
Frameshifts	3 (15%)	1.53	6 (11%)	11.78	10.26	5 (8%)	3.75	2.22
Small deletions	1 (5%)	0.51	1 (2%)	1.96	1.45	1 (2%)	0.75	0.24
Small insertions	0	0	0	0	0	0	0	0
Complex mutations	1 (5%)	0.51	2 (4%)	3.93	3.42	0	0	0
Mutations at bp 207–212[g]	1 (5%)	0.51	21 (38%)	41.8	41.3	22 (33%)	16.5	16.0
Total	20 (100%)	10.2[d]	56[c] (100%)	110[e]	99.8	66 (100%)	49.4[f]	39.2

[a] Mutant fraction = (observed Mf accounted for by the percentage of mutant clones with mutations in exon 3) × (percent of class of mutation).
[b] Estimated induced mutant fraction in exon 3 = (mutant fraction of treated mice) − (mutant fraction of control mice).
[c] Statistical analysis of the occurrence of individual types of base substitutions among control vs. BD-exposed mice show that G · C → A · T and G · C → C · G are the only classes with a significant difference ($P = 0.035$ and 0.05, respectively; Fisher's exact hypothesis test, as used by Carr and Gorelick [18]).
[d] 10.2×10^{-8} = (Average observed Mf in control mice; 2.0×10^{-6}) × (percent of *Hprt* mutant clones with mutations in exon 3; 20/394).
[e] 110×10^{-8} = (Average observed Mf in BD-exposed mice; 14×10^{-6}) × (percent of *Hprt* mutant clones with mutations in exon 3; 56/712).
[f] 49.4×10^{-8} = (Average observed Mf in DEB-exposed mice; 4.8×10^{-6}) × (percent of *Hprt* mutant clones with mutations in exon 3; 66/642).
[g] Note that five clones from one BD-exposed mouse (#609, Table 4) had the same mutation at bp 211 and may represent a clonal expansion of a single mutation.

Statistical analysis of the proportions of mutational types in control vs. BD-exposed mice failed to demonstrate a significant difference in homogeneity ($p = 0.358$), however, when we conditioned on 20 mutations in controls and 49 in BD-exposed mice (as done for the test for homogeneity of proportions) and looked to see if the individual base substitutions were significantly different, the frequencies of G · C → A · T transitions ($p = 0.035$) and G · C → C · G transversions ($p < 0.05$) were significantly different. It should be noted that 5 *Hprt* mutant clones from one BD-exposed mouse (Table 3, #609) had G · C → C · G transitions at bp 211 and may represent a clonal expansion of a single mutation (see Ref. [14]). Nevertheless, the frequency of this mutational type would remain significantly elevated in BD-exposed mice even if these duplicate mutations in mouse #609 were considered mutant siblings and counted as a single mutation. Finally, the mutant fraction [i.e., (observed Mf accounted for by the percentage of mutant clones with mutations in exon 3) × (average percent total exon 3 mutants for a class of mutation)] can be used to estimate the induced mutant frequency within each class of mutation (i.e., mutant fraction in BD-exposed mice−mutant fraction in control mice), and the resulting mutant fraction data for control and BD-exposed mice suggest that BD induces a variety of base substitutions and other mutations in *Hprt* exon 3 (Table 1). Notably, extension of this analysis to the ''fraction of mutations in the run of six guanines'' in exon 3 indicated that BD exposure of mice was associated with an 80-fold

Table 2
Base alterations in *Hprt* exon 3 of splenic T-lymphocytes from control B6C3F1 mice[a]

Base[b] and mutation[c]	Animal number																
	BDC-22	BDC-25	BDC-506	BDC-508	BDC-509	BDC-517	BDC-518	MS-15	MS-18	EC-1	EC-3	ETOC-15	ETOC-21	ETC-11	ETC-15	K-12	Total
145 C → T															1		1
151 C → T											1						1
152 G → T			1		1											1	3
165 G → A									1								1
168 G → A				1			1										2
179 A → G							1										1
207 G → A														1			1
213 C → G	1																1
252 A → G						1											1
288 T → G												1					1
299 T → A													1				1
311 G → A								1									1
135 −G										1							1
191 +A						1											1
243–248 −6 bp		1															1
289 +G												1					1
293 ATTT → CTCCC		1															1
Total	1	2	1	1	1	2	2	1	1	1	1	2	1	1	1	1	20

[a] Control mice, with the indicated prefixes, were the following ages when necropsied: BDC, 12 to 16 weeks [11,13]; MS, EC, ETC and K, 12 weeks [15,19,20,23]; ETOC, 12–16 weeks [21,22].
[b] Numbering of the *Hprt* gene according to Melton et al. [36].
[c] The mutation in the nontranscribed strand is reported.

Table 3
Base alterations in *Hprt* exon 3 of splenic T-cells from B6C3F1 mice exposed by inhalation to butadiene

Base[a] and mutation[b]	Animal number																		
	86	88	89	93	97	103	107	108	114	117	118	119	605	606	607	608	609	610	Total
151 C → T												1							1
164 A → T																		1	1
171 G → A													1	1					2
190 G → T									1										1
197 G → A																		1	1
204 C → G														1			1		2
206 A → G				1															1
207 G → T										1									1
207 G → C														1					1
208 G → T													1						1
211 G → T				1															1
211 G → C													2	1		2	5		10[c]
212 G → C													1	2		1	1		5
214 T → G															1				1
216 T → G			1	1				1			1				1		1		6
217 A → G			1																1
217 A → T													1						1
219 G → A	1																		1
219 G → T																	1		1
221 T → C			1			1													2
223 T → C											1								1
228 T → A								1											1
230 A → T														1					1
299 T → C		1																	1
299 T → G																		1	1
311 G → A																	1		1
187 +G	1																		1
207–212 +G										1									1
207–212 −G							1												1
214–220 −7 bp																	1		1
228 +A							1												1
228–229 TG → C								1											1
229 +A																	1		1
239–244																	1		1
ATTACA → CT																			
247–249 −A					1														1
Total	2	1	3	3	1	1	2	3	1	2	2	1	6	7	2	3	13	3	56

[a] Numbering of the *Hprt* gene according to Melton et al. [36].
[b] The mutation in the nontranscribed strand is reported.
[c] Note that five clones from one BD-exposed mouse (#609, Table 4) had the same mutation at bp 211 and may represent a clonal expansion of a single mutation.

increase in the occurrence of mutations at bp 207–212 compared with control mice (Table 1).

3.3. Analysis of mutations in splenic T-cells from DEB-exposed B6C3F1 mice

A total of 642 *Hprt* mutant colonies from mice exposed to 4 ppm DEB for 4 weeks [12] were assessed for mutations in exon 3. *Hprt* M*f*s in these DEB-treated mice averaged 2.7-fold (ranging from 2- to 3-fold) higher than those measured in control mice [12]. Sixty-six T-cell mutants (10%) were found to contain mutations in exon 3; these mutations included base substitutions (60/66, 91%; 22 sites), frameshifts (5/66, 7.5%; five sites), and one small deletion (1.5%) (Tables 1 and 4). The base substitu-

Table 4
Base alterations in *Hprt* exon 3 of splenic T-lymphocytes from B6C3F1 mice exposed by inhalation to (±)-diepoxybutane

Base[a] & Mutation[b]	29	30	31	32	33	34	35	37	38	39	40	41	42	43	44	45	48	50	55	56	63	64	65	67	68	69	70	71	72	73	74	77	81	Total
143 G → C																	2	1																3
166 G → T																							1											1
G → A												1															1							2
168 G → A																							1	1										2
173 G → A							1																											1
197 G → A					1	1																												2
206 A → T										1																1								2
207 G → T																			1															1
208 G → A											3			2	1	1								1			1			1	2			12
210 G → A										1																								1
211 G → C		1						2																			1							4
G → A																											2							2
212 G → A																														1				1
214 T → A								1																										1
216 T → G											1	1								1							1		2		1			7
219 G → A	1																							1	1								1	4
220 T → C																		1										1						2
221 T → C				1																														1
223 T → C							2						1																		2			5
252 A → G			1																															1
260 G → A		1																																1
273 A → G			1			1																												2
299 T → G																					1													1
302 G → C																						1												1
158 +A																											1							1
187 +A															1																			1
207-212 –G									1																									1
215-216 –AT or																																		
216-217 –TA									1																									1
219 –G													1																					1
222 +A																											1							1
Total	1	2	2	1	1	2	3	3	2	2	4	2	2	2	2	1	2	2	1	1	1	1	2	3	1	1	7	2	2	1	4	2	1	66

[a] Numbering of the *Hprt* gene according to Melton et al. [36].
[b] The mutation in the nontranscribed strand is reported.

tions consisted of transitions and transversions at both G · C (38/66, 58%, 13 sites) and A · T bps (22/66, 33%, nine sites). Two different substitutions were found at two of the mutated G · C bps (i.e., 166 and 211). The most frequently occurring exon 3 mutations in DEB-exposed mice were G · C → A · T transitions at bp 208 (found a total of 12 times in eight different mice), G · C → C · G transversions at bp 211 (observed a total of four times in three different mice), and A · T → C · G transversions at bp 216 (seen a total of seven times in six different mice). Notably, all 28 of the guanine bases involved in the G · C → A · T transitions found in DEB-treated mice were situated on the nontranscribed strand (Table 4), whereas in control mice the guanines in the analogous transitions occurred on both the nontranscribed (5/7, 71%) and transcribed strands (2/7, 29%) (Table 2). Likewise, in DEB-exposed mice, all of the guanine bases at risk in the G · C → C · G transversions and all of the thymine bases at risk in the A · T → C · G transversions were located on the nontranscribed strand. Remarkably, one-third (22/66) of the exon 3 mutations occurring in DEB-exposed mice were found in the run of six guanines at bp 207–212. These mutations included a single-G deletion and base substitutions at five of the six guanines; however, there were no +G insertions found in this stretch of guanines in DEB-treated mice. There were four sites in exon 3 (bps 168, 207, 252 and 299) at which base substitutions were seen

in both control and BD-treated mice (among 17 and 28 mutated sites in each group, respectively), with identical mutations occurring at only two of these sites (Tables 2 and 4). Finally, 12 sites with base substitutions were common to BD-exposed mice (among 21 total sites with substitutions) and DEB-exposed mice (among 22 total sites with substitutions), with eight of these mutual sites having identical mutations (Tables 3 and 4)

Statistical analyses failed to demonstrate a significant difference in the spectra data for control vs. DEB-exposed mice, but the mutant fraction estimates for control and treated animals suggested that DEB exposure was associated with several types of point mutations (Table 1). Notably, DEB exposure was associated with 30-fold increase in the occurrence of mutations at bp 207–212 compared with control mice (Table 1). Furthermore, when the average fold increase in *Hprt* M*f*s in DEB-exposed ($\sim$2.7 over background) and BD-exposed mice ($\sim$7.2 over background) were considered, the frequency of bp 207–212 mutations in DEB- and BD-treated mice appeared to be consistent.

3.4. Analysis of mutations in splenic T-cells from DEB-exposed B6C3F rats

A total of 1168 *Hprt* mutant clones collected from F344 rats exposed to 1250 ppm BD for 2 weeks or 625 ppm BD for 4 weeks [11,13] were evaluated for mutations in exon 3. *Hprt* M*f*s in these BD-treated rats averaged 2.7-fold (ranging from 2- to 3-fold) higher than the M*f* values in concurrent control rats [11,13]. DGGE analysis and sequencing revealed that 59 T-cell mutants (5%) contained mutations in exon 3; these mutations included a combination of base substitutions (41/59, 70%; 24 sites), frameshifts (12/59, 20%; six sites), and small deletions (6/59, 10%; six sites) (Tables 5 and 6). Transitions and transversions were observed at both G · C (23/59, 39%, 15 sites) and A · T (18/59, 31%, nine sites) bps. Two different base substitutions occurred at two of the mutated A · T bps (i.e., 215 and 299) and at one of the mutated G · C bps (i.e., 208). The most common base substitution was an A · T → C · G transversion at bp 216 (seen in four rats). Moreover, 7/9 of the thymine bases in jeopardy in the entire set of A · T → C · G transversions in BD-exposed rats were located on the nontranscribed strand. Among the frameshift mutations, there were three different insertions that were each observed in more than one rat (i.e., +T at bp 140 in two rats, +G at bps 207–212 in two rats, and +T at bp 299/300 in three rats). Interestingly, the set of deletions found in BD-treated rats included two deletions that were substantially larger (−37 and −25 bps) than those usually identified by DGGE [15,19]. Thirteen of the exon 3 mutations (13/59, 22%) detected in BD-exposed rats occurred at the run of six guanines (bps 207–212), and these included the +G insertions and base substitutions at five of the six guanines. Furthermore, the increased occurrence of bp 207–212 mutations in BD-exposed rats appeared consistent with that seen in BD-exposed mice (38% of exon 3 mutations) given the smaller increase in *Hprt* M*f* in the rat (2.7-fold) compared with the mouse (7.2-fold). Finally, 12 sites with base substitutions were common to BD-exposed mice (among 21 total sites with substitutions) and rats (among 24 total sites with substitutions), with nine of these shared sites having identical mutations (Tables 3 and 6).

Table 5
Summary of exon 3 mutations in *Hprt* mutant T-cell clones from F344 rats exposed to butadiene or (±)-diepoxybutane

Class of mutation	Butadiene-exposed rats	Diepoxybutane-exposed rats
Base substitutions		
G·C → A·T	11 (19%)	14 (28%)
A·T → G·C	4 (7%)	6 (12%)
G·C → T·A	7 (12%)	8 (16%)
G·C → C·G	5 (9%)	2 (4%)
A·T → C·G	9 (15%)	8 (16%)
A·T → T·A	5 (9%)	6 (12%)
Frameshifts	12 (21%)	4 (8%)
Small deletion	6 (10%)	2 (4%)
Small insertions	0	1 (2%)
Complex	0	0
Mutations at bp 207–212	13 (22%)	5 (10%)
Total	59 (100%)	51 (100%)

3.5. Analysis of mutations in splenic T-cells from DEB-exposed F344 rats

A total of 732 *Hprt* mutant clones collected from rats exposed to 4 ppm DEB for 4 weeks or 5 ppm

Table 6
Base alterations in *Hprt* exon 3 of splenic T-cells from F344 rats exposed by inhalation to butadiene

Base[a] and mutations[b]	Animal number																							
	287	288	289	291	292	293	294	295	297	298	300	301	302	303	304	307	312	317	320	822	823	824	825	Total
151 C → T													1											1
154 G → A		1						1				1		1										4
156 T → A							1																	1
162 G → C	1																							1
164 A → T																							3	3
165 G → T				1																				1
190 G → C																						1		1
195 C → A															1									1
202 C → A															1									1
207 G → A								1																1
208 G → A																2	1			1				4
208 G → C															1									1
210 G → T																				1				1
211 G → C						1																		1
212 G → T					1																			1
215 A → C							2																	2
215 A → G										1														1
216 T → G	2		1																	1		1		5
219 G → T																					1		1	2
221 T → C																					1			1
277 A → G																							1	1
287 C → G									1															1
296 T → G																1								1
298 A → T																					1			1
299 T → C	1																							1
299 T → G																				1				1
311 G → A								1																1
140 +T											1							1						2
175–180 −6 bp				1																				1
227–237 −8 bp[c]																		1						1
207–212 +G							2												2					4
219–255 −37 bp																						1		1
220 or 221 +T																1								1
243 −C					1																			1
244–249 −6 bp																				1				1
283–308 −25 bp							1																	1
294–297 +T																		1						1
299 or 300 +T															1			1			1			3
300–303 or 301–304 −4 bp											1													1
Total	4	1	1	2	2	1	6	3	1	1	2	1	1	1	4	4	1	4	2	5	4	3	5	59

[a]Numbering of the *Hprt* gene according to Melton et al. [36].
[b]The mutation in the nontranscribed strand is reported.
[c]There are eight possible sites for the eight base deletion between 227 and 237.

DEB for 6 weeks [12,16] were examined for mutations in exon 3. *Hprt* M*f*s in DEB-treated rats were 3- to 4.5-fold higher than the concurrent control values [12]. DGGE analysis and sequencing showed that 51 T-cell mutants (7%) had mutations in exon 3; these mutations included 44 base substitutions (86%; 24 sites), four frameshifts (8%; four sites), two small deletions (4%; two sites), and one small insertion of eight bps (2%) (Tables 5 and 7). Transitions and transversions were found at both G · C (24/51, 47%; 13 sites) and A · T bps (20/51, 37%; 11 sites). Two different base substitutions occurred at two of the mutated G · C bps (i.e., 190 and 308). The most common substitution was an A · T → C · G transversion at bp 216, observed a total of five times in three different rats. Furthermore, 7/8 of the thymine bases involved in the A · T → C · G transversions were located on the nontranscribed strand. Only five muta-

Table 7
Base alterations in *Hprt* exon 3 of splenic T-lymphocytes from F344 rats exposed by inhalation to (±)-diepoxybutane

Base[a] and mutations[b]	Animal number											
	137	138	139	143	144	145	173	174	175	176	177	Total
146 T → C						1						1
148 G → C								1				1
152 G → T							1					1
154 G → A								1				1
164 A → T									3	1	1	5
168 G → T							1					1
179 A → G										1		1
190 G → A		1										1
190 G → C											1	1
191 C → A				1								1
194 T → C		1										1
203 T → G	1	1										2
204 G → A							2		1			3
205 A → T		1										1
208 G → T		1										1
208 G → A										2	1	3
216 T → G	3	1	1									5
217 A → C			1									1
219 G → T		2	1						1			4
221 T → C									1			1
274 T → C										1		1
281 C → T								1				1
285 G → A					1							1
296 T → C				1								1
304 C → T				1								1
311 G → A								2	1			3
174–248			1									1
or 175–249 −74 bp												
175 or 176 −G									1			1
199 −G							1					1
201 −G							1					1
207–212 −G							1					1
280–303										1		1
or 281–304 −24 bp												
283 +8 bp		1										1
Total	4	9	4	3	1	1	7	5	8	6	3	51

[a] Numbering of the *Hprt* gene according to Melton et al. [36].
[b] The mutation in the transcribed strand is reported.

tions (10%) were found in the six consecutive guanines of exon 3 (bps 207–212). The two deletions detected in DEB-treated rats were relatively large (−24 and −74 bps) compared with deletions previously identified using DGGE techniques [15,19]. Finally, eight sites with base substitutions were common to both BD-exposed and DEB-exposed rats (among 24 total sites with substitutions in each group), with all but one of these common sites having identical mutations (Tables 6 and 7). In contrast, there were just five sites at which base substitutions were seen in both DEB-exposed rats and DEB-exposed mice (among 24 and 22 total sites with substitutions in each respective species), with identical mutations occurring at three of these shared sites (Tables 4 and 7).

4. Discussion

Due to the poor growth potential of rodent T-cell in culture, DGGE has been used as a temporary expedient for molecular analyses of exon-specific *Hprt* mutations because it provides an analytical tool for purifying mutant sequences from contaminating wild-type sequences [15,19,24,25]. The disadvantages of the DGGE/sequencing approach are that it is labor intensive and it can only detect base pair substitutions and small deletions or insertions. Thus, the recent development of methods for the substantial expansion of mutant T-cell clones from mice and rats allows the use of a variety of techniques to identify a broader range of DNA lesions over the entire *Hprt* gene of unexposed and chemically exposed animals [24,25].

A major advantage of inspecting propagated *Hprt* mutant T-cell clones for mutations occurring in control and BD-exposed mice is readily apparent when the summaries of mutations identified by RT–PCR analysis of *Hprt* mRNA [11] are compared to the summaries of mutations identified by the DGGE approach in the present study. The most striking feature in the RT–PCR analysis of mutations in a subset of control and exposed mice from the current study (see Ref. [24]) was the occurrence of a significant increase in total deletions of the *Hprt* gene in BD-exposed mice. This result is in keeping with earlier in vitro experiments in DEB- and EB-exposed human lymphoblastoid cells [10,26,27], suggesting that exposure of mice to BD should lead to large deletions as well as point mutations. The finding of 51% exon deletions or total deletions of *Hprt* in cDNA of BD-exposed mice [11] reveals the limited value of in vivo mutational spectra data obtained using approaches that detect basically point mutations, such as DGGE analysis of *Hprt* exon 3 ([28]; present study) and analysis of the *lacI* gene [29,30] in similarly exposed rodents. Nevertheless, the DGGE analysis of mutant clones from BD- and DEB-exposed mice and rats represents a tremendous amount of work and several broad conclusions can be drawn from these data: (i) point mutations at both G · C and A · T bps are induced by BD and (±)-DEB, (ii) a run of six quanine bases in exon 3 (bp 207–212) appears to be a 'hotspot' for induced mutation, (iii) G · C → C · G transversions appear to be an important mutational event in exon 3 of BD-exposed mice, and (iv) there is a strong bias for guanines or thymines to occur in the nontranscribed strand at mutated sites in exposed mice and rats.

The proportions of *Hprt* exon 3 mutations identified by DGGE analysis of mutant clones from BD- and (±)-DEB-exposed B6C3F1 mice were lower than those reported for B6C3F1 mice treated with ethylene oxide (18%) [15,22] and cyclophosphamide (22%) [23]. These observed differences may be largely due to the fact that deletions involving the loss of one or more exons or the loss of the entire *Hprt* gene will go undetected using DGGE. Because DEB is a bifunctional alkylating agent that induces large deletions and DNA rearrangements as well as point mutations in vitro [10,26], the lower fraction of mutations in exon 3 of BD- and DEB-exposed mice may be attributable in part to the relatively increased occurrence of large scale mutations which do not contribute to observable mutations in exon 3. This conclusion is supported by the detection of a significantly increased frequency of large deletions in *Hprt* cDNA of expanded mutant clones from BD-exposed mice compared with controls [11].

The spectrum of mutations observed in *Hprt* exon 3 of T-cells from BD-exposed B6C3F1 mice differed substantially from the limited sequence data from control B6C3F1 mice and from the robust spectra data from B6C3F1 mice treated with CP [23], ENU [19,20] or ETO [15,22]. The limited *Hprt* mutational

spectra data from earlier studies of BD (13 exon 3 mutations) [28] and ethylene oxide (18 exon 3 mutations) in mice [15] suggested that there might be a substantial overlap in the types and locations of mutations produced by these agents, but the similarities are less striking when the *Hprt* mutation data for BD in the current study (56 exon 3 mutations) are compared to an expanded data set for mice exposed to ethylene oxide by i.p. injections or inhalation (57 and 48 exon 3 mutations, respectively) ([15,22]; Walker et al., unpublished data). A more detailed comparison of the mutagenic specificities of BD and ethylene oxide in mice will be presented in a report concerning the mutagenicity of ethylene oxide in the mouse vs. the rat.

In contrast, there was a high degree of overlap in the mutational spectra data between BD-exposed mice and rats, between BD- and DEB-exposed mice, and between BD- and DEB-exposed rats in terms of the sites with base substitutions, the mutations found at those mutated sites, the relative occurrence of the most frequently observed base substitutions, and the occurrence of a consistent strand bias for the most frequently observed base substitutions (although there was no identifiable sequence context that could explain the examples of strand bias observed). For example, half of the mutated sites with base substitutions in BD-exposed mice (12/21 sites) and rats (12/24) were common to both species and 75% of these shared sites had identical mutations (Tables 3 and 6). The same degree of shared sites for base substitutions and identical mutations were found in BD- and DEB-exposed mice (Tables 3 and 4). Furthermore, a large number of the exon 3 mutations (22% to 38%) observed in BD-exposed mice and rats and in DEB-exposed mice occurred in a run of six guanines (bp 207–212). These data alone suggest a high probability that some mutations were induced through similar mutagenic mechanisms in BD-exposed mice, BD-exposed rats, and DEB-exposed mice.

Among the different classes of base substitutions, only $A \cdot T \rightarrow C \cdot G$ transversions appeared to be substantially increased in *Hprt* exon 3 of BD- and DEB-exposed mice and rats, and the majority of these transversions (23/33) were found at bp 216 in multiple animals of all four treatment groups. Remarkably, 30 of 33 thymine bases at risk in $A \cdot T \rightarrow C \cdot G$ transversions in all of the BD- and DEB-exposed animals combined were located on the nontranscribed strand. Thus, bp 216 appears to be a highly mutable site for BD metabolites in the context of mutations produced specifically in *Hprt* exon 3.

The overall occurrence of $G \cdot C \rightarrow C \cdot G$ transversions was significantly increased in BD-exposed mice and appeared increased in DEB-exposed mice, with the majority of these base substitutions observed at bp 211 or 212. Twenty-five of 26 guanines in jeopardy in these $G \cdot C \rightarrow C \cdot G$ transversions were situated on the nonstranscribed strand of *Hprt* exon 3, which may reflect either preferential repair in the transcribed strand of *Hprt* exon 3, which may reflect either preferential repair in the transcribed strand or differential fidelity of leading and lagging strand replication of adducted DNA [19]. On the other hand, there was no increase in the frequency of $G \cdot C \rightarrow C \cdot G$ transversions in the limited set of base substitutions found in *Hprt* cDNA from expanded mutant clones for BD-exposed mice compared with controls [11].

$G \cdot C \rightarrow A \cdot T$ transitions at bp 208 identified by DGGE analysis were the most frequently observed mutation in *Hprt* exon 3 of DEB-exposed mice (i.e., 12 occurrences in eight mice, among 66 exon 3 mutations), indicating that these mutations were likely related to DEB treatment. This mutation also occurred a few times in BD- and DEB-exposed rats (4/59 and 3/51 exon 3 mutations, respectively), but was not seen in BD-exposed mice. Yet, the occurrence of $G \cdot C \rightarrow A \cdot T$ transitions at bp 208 in *Hprt* cDNA of expanded mutant clones from three of five BD-exposed mice, but not in concurrent controls, suggest that this mutation is produced by one or more BD metabolites in the mouse [11].

Two research groups have also conducted studies in BD-, DEB-, and EB-exposed mice to investigate the relative contributions of these two metabolites to mutational specificity of the parent compound in vivo. The work of Cochrane and Skopek [28], using the endogenous *Hprt* gene as a mutational target in mouse T-cells, was a point of departure for the studies reported here. One notable difference between the findings in the earlier mutational spectra studies in BD-, DEB- and EB-treated preweanlings vs. the current inhalation studies in BD- and DEB-exposed mice (and rats) is that the +G frameshift

mutation at bp 207–212 that appeared as a putative mutagenic 'hotspot' for BD and its epoxide metabolites in preweanling mice was seen only once in mice exposed in the current study. Following RT–PCR analysis of expanded *Hprt* mutant clones, however, BD-exposed adult mice (compared with controls) had increased frequencies of +G frameshifts at several sites, including a run of five guanines at bp 333–337 [11]. Thus, some differences should be expected in these *Hprt* mutational spectra studies because of differences in (i) the ages of the mice used, (ii) the routes of treatment (in the case of DEB and EB), (iii) the treatment regimens, and (iv) the sample sizes.

Parallel mutational spectra studies have also been conducted in B6C3F1 *lacI* transgenic mice exposed to BD [29,30], DEB and EB. The mice exposed to DEB or EB for the *lacI* studies were placed in the same exposure chambers as the conventional mice used in the current *Hprt* studies and it is too early to compare the resulting *lacI* and *Hprt* mutational spectra data for these epoxy compounds. The major findings in transgenic mice exposed to 1250 ppm BD for 4 weeks by inhalation, relative to air controls, were increased frequencies of point mutations at A · T bps in bone marrow and spleen and an increased frequency of specific base substitutions at G · C bps (i.e., G · C → A · T transitions at non-CpG sites and G · C → T · A transversions) in spleen. The predominant mutations in BD-exposed transgenic mice were A · T → T · A transversions. Thus, both the *lacI* and *Hprt* mutational spectra data indicate that in vivo exposures to BD induce point mutations at both A · T and G · C bps.

Although considerable knowledge has been gained from the various in vitro and in vivo experiments assessing the mutagenic specificity of BD and the racemic mixtures of DEB and EB, the methods recently developed for propagating mutant T-cell clones from rodents [24,25,31,32] and the initial examination of mutations occurring over the entire *Hprt* gene of control and BD-exposed mice [11], lays the groundwork for a more complete analysis of mutations produced by BD and its metabolites in both the mouse and the rat. Accumulating data suggest potential roles for *meso*-DEB and BD diol epoxide in BD-induced mutagenicity [33–35] and, thus, additional work is needed to determine more definitively the relative contribution of BD metabolites, in various stereochemical configurations, to the mutation patterns in mice and rats. Future applications of methods for detecting and characterizing both large- and small-scale DNA damage in the *Hprt* gene of mice and rats will permit more meaningful comparisons to the point mutations identified in *Hprt* exon 3 and *lacI* of BD-exposed mice and will further delineate the mutagenic mechanisms of this agent in rodents.

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