Chemico-Biological Interactions xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Hprt mutant frequency and *p53* gene status in mice chronically exposed by inhalation to benzene

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Benzene
Hprt
Loss of heterozygosity
Lymphocytes
Gene mutation
p53 heterozygous mice

ABSTRACT

Hprt mutant frequency and *p53* gene status were assessed in wild-type and *p53* heterozygous (*p53*+/−) mice exposed chronically by inhalation to benzene. Benzene exposures to 100 ppm for 6 h on Monday–Friday, 100 ppm for 10 h on Monday–Wednesday–Friday, or 200 ppm for 5 h on Monday–Wednesday–Friday yielded the same total exposures (concentration × time) of 3000 ppm × h/week. *Hprt* mutations in splenic T-lymphocytes were significantly increased in all benzene groups, ranging from 3.8- to 8.0-fold greater than control values. Wild-type and *p53*+/− mice were equally susceptible to benzene mutagenesis. *Hprt* wild-type and mutant isolates from control and exposed animals were examined for *TCR* gene rearrangements (as markers of *in vivo* clonality) and for loss of *p53* wild-type or mutant alleles. Moderate clonal amplifications were observed among the *Hprt* mutant but not *Hprt* wild-type isolates but was not sufficient to account for the increases in *Hprt* mutant frequencies. Most isolates, whether *Hprt* wild-type or mutant, retained both *p53* alleles in the benzene-exposed *p53*+/− animals (54% and 63%, respectively, for the *Hprt* wild-type and mutants). However, 37% of the *Hprt* wild-type isolates and 46% of the *Hprt* mutant isolates lost the *p53* mutant allele. Only a small percentage of either type of isolate lost the *p53* wild-type allele, and this was always in isolates that that previously lost the *p53* mutant allele. Loss of the *p53* mutant allele was independent of benzene exposure, *Hprt* status, or 6-thioguanine selection. These findings contrast with the *p53* status of thymic lymphomas that had preferentially lost the wild-type *p53* allele in some of these same mice. Possible reasons for loss of the mutant *p53* allele in the *Hprt* mutant and wild-type isolates are discussed.

1. Introduction

Benzene is a major industrial compound that humans are exposed to as a result of a variety of activities in which it is generated, processed, or used [1,2]. Epidemiological studies have established a relationship between benzene exposure and the development of leukemias, and benzene is classified as a known human carcinogen (Group 1) [1,2]. Benzene also causes various cancers in rodent models. In spite of extensive study, the mechanism(s) underlying benzene-induced neoplasia has yet to be established [3].

In the context of environmental and occupational exposures to chemicals like benzene, biomarkers are non-disease indicators of exogenous exposures, biological responses, or susceptibility factors of relevance to disease outcomes such as cancer [4,5]. Measures of *in vivo* mutations at either the gene or chromosome level (aberrations) clearly document non-reversible and heritable alterations in genetic information content in animals or humans, thus, fitting their classification as biomarkers of effect. Current assay techniques allow measures of both gene and chromosome mutations in cancer relevant or "reporter" regions of the genome, the latter serving as surrogates for the former. Among the reporter genes currently used for human biomonitoring are the glycophorin-A gene studied in red blood cells and the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene, the *HLA* genes and the T-cell receptor (*TCR*) genes all measured in T-lymphocytes [4–6]. An emerging assay measures mutations of the phosphatidylinositol glycan-class A (*PigA*) gene in a variety of cell types of hematological origin [6–9]. *Hprt*, *TCR* and *PigA* mutations may also be measured in laboratory rodents, monkeys, and humans, allowing for interspecies comparisons [7–10]. Bacterial transgenes in genetically altered mice provide yet another surrogate marker for cancer gene mutations that might be used to assess effects in various tissues [10].

Abbreviations: CE, cloning efficiency; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; MFs, mutant frequencies; M-F, Monday–Friday; MWF, Monday–Wednesday–Friday; *PigA*, phosphatidylinositol glycan-class A; TCR, T-cell receptor; WT, wild-type.

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0009-2797/$ – see front matter © 2010 Published by Elsevier Ireland Ltd.
doi:10.1016/j.cbi.2009.12.019

The potential role of chromosome and gene mutations in the carcinogenic activity of benzene and its metabolites was recently reviewed [3]. Despite the production of many biologically active metabolites of benzene (i.e., hydroquinone, phenol, catechol, benzoquinone, benzenetriol, benzene oxide, muconaldehyde and muconic acid), evidence for specific DNA reactivity has been scant. ^{32}P-postlabeling showed the induction of a N^2-(4-hydroxyphenyl)-2′-deoxyguansoine-3′-phosphate in HL-60 cells treated *in vitro* hydroquinone and in bone marrow of mice receiving benzene by intraperitoneal injections [11,12]. However, the *in vivo* adducts were at low levels and found only following twice-daily high-dose i.p. exposures. Furthermore, *in vivo* DNA binding studies employing radiolabeled benzene found only low levels of binding without cancer target organ preference [3]. Although *in vitro* studies of reverse gene mutations in prokaryotic systems have been almost universally negative, mammalian cells have given mixed results for both gene (forward mutations) and chromosomal level events [3]. Tests of metabolites, especially hydroquinone, have provided the most consistently positive results. *In vivo*, most of the mutagenicity studies in rodents have been positive, with the majority reporting chromosome level mutations [3]. A study in CYP2E1 deficient mice demonstrated the importance of benzene metabolism in the production of micronuclei [13]. Consistent with this report were the findings of positivity for micronucleus induction both *in vitro* and *in vivo* by hydroquinone, phenol, catecol and benzenetriol and evidence that benzene metabolites may interact in producing chromosomal damage [3]. In two *in vivo* studies of gene level mutations in *lacI* transgenic mice [14,15], benzene-induced alterations in the transgene were almost certainly the result of point-mutations. A single study reported a relationship between subchronic benzene exposure and mutations at the endogenous *Hprt* gene in splenic lymphocytes of mice, as measured using an autoradiographic assay for *Hprt* variants [16]. However, because of discordance with cancer bioassay results and lack of a dose–response effect for *Hprt* variant frequency [16], this study has been considered to be inconclusive [3].

The purpose of the current study was to measure induction of *Hprt* mutations in the splenic lymphocytes of mice, using a more definitive T-cell cloning assay, and to assess the status of the *p53* tumor suppressor gene in T-cells of wild-type (WT) and *p53* heterozygous (*p53*+/−) mice exposed chronically by inhalation to benzene. For this purpose, spleens were obtained from benzene-exposed animals used in a cancer bioassay of thymic lymphoma induction [17,18], allowing a comparison of surrogate gene mutations and actual tumor production in cells of the same origin in the same group of animals. Furthermore, as *p53*+/− mice were used in the bioassay, there was opportunity to compare mutagenesis in these animals as compared to WT. The goal was to obtain information on mutation induction by benzene as well as the use of mutations as mechanistic probes.

2. Materials and methods

2.1. Animals, husbandry, and inhalation exposures

C57BL/6 WT and C57BL/6 *p53*+/− mice (the *p53*+/− construct consists of a partial deletion of intron 4/exon 5 of *p53* with an insertion of a neomycin cassette) were obtained from Taconic Farms (Germantown, NY) at 4–5 weeks of age, and were free of virus titers using standard mouse virus antibody assays. Mice were held for 1–3 weeks, and acclimated to wire caging within the inhalation chamber for 1 week prior to initiation of benzene exposure. At benzene exposure initiation, mice ages ranged from 6 to 9 weeks. Mice were kept in a reverse 12:12-h light/dark cycle; exposure took place during the light cycle. Each exposure or control group was housed in a separate 8 m^3 inhalation chamber, and all mice were individually used in stainless steel wire mesh cages that conformed to federal guidelines. Water (reverse osmosis treated) and commercially available rodent diet were available *ad libitum*, and feed exposed to benzene was discarded following each exposure period. All procedures involving the use of animals were approved by the Institutional Animal Use and Care Committee.

Benzene exposures have been previously described in detail [18]. In brief, WT and *p53*+/− mice were exposed whole-body for up to 38 weeks in 8 m^3 inhalation chambers to target benzene concentrations of 0, 100, or 200 ppm. The benzene-exposed mice consisted of three groups: two at 100 ppm, one at 200 ppm, and one control sham-exposed group at 0 ppm. Start dates for each exposure group were staggered at intervals of 1–2 weeks apart and no exposures were conducted on the weekend. The control group and one group at 100 ppm benzene were exposed for 6 h/day, 5 days/week on Monday–Friday (M–F), while the other group at 100 ppm benzene was exposed 10 h/day, 3 days/week on Monday, Wednesday, and Friday (MWF). The group at 200 ppm was exposed 5 h/day, 3 days/week (MWF). These schedules maintained all benzene-exposed groups at an equal exposure level of 3000 ppm × h/week.

Benzene exposure concentrations were generated by metering known amounts of liquid benzene into a heated j-tube. Benzene vaporized in the j-tube and was carried in a nitrogen stream to the HEPA-filtered chamber air supply where it was diluted to the target concentration. The chamber air supply flowed at ~1800 l/min and was conditioned to 72 F and 50% relative humidity. The control chambers (0 ppm) were operated under similar environmental conditions.

Benzene exposure concentrations were measured with four calibrated infrared spectrophotometers (MIRAN 1A, the Foxboro Co., Foxboro, MA) with one instrument sampling each chamber. The distribution of benzene was checked at nine locations in each chamber prior to initiation of exposure. The environmental conditions were monitored continuously; 30 min averages were recorded and printed daily.

2.2. Isolation and selection of Hprt mutant T-lymphocytes; diagnosis of thymic lymphomas

Due to the staggered state dates for the exposed groups, spleens were collected from benzene-exposed mice for isolation of splenic lymphocytes at 33–37 weeks after initiation of the inhalation study (corresponding to 33, 37, and 34 weeks of benzene exposure to 100 ppm M–F, 100 ppm MWF, and 200 ppm MWF, respectively) [18]. Reagents for cell culture were the highest grade available from commercial sources and have been listed in detail elsewhere [19–21]. At necropsy, spleens were harvested, weighed, and then stored in fresh RPMI 1640 medium on ice for shipment overnight for isolation of splenic lymphocytes. Immediately upon receipt, spleens were 'milked' by piercing with a 20-gauge bent needle and then massaging out the contents in RPMI 1640 medium. The general procedures for washing of isolated cells, growth stimulation of mouse T-cells, selection of *Hprt* mutant T-cell colonies, and calculation of *Hprt* mutant frequencies (MFs) has previously been described in detail [19–21]. Briefly, cells from spleens and thymuses were resuspended in T-flasks with "priming" media (containing rat T-STIM, mouse IL-2, and Con A) overnight at 37 °C and 6% CO_2. Stimulated lymphocytes were then cultured to generate *Hprt* mutant T-cell colonies. U-bottom 96-well microtiter plates were seeded with 4×10^4 cells/well in the presence of 0.5 μg 6-thioguanine/ml in 100 μl/well of fully supplemented medium. Two 96-well plates were seeded with 2 and 4 cells/well plated in the presence of ~3×10^4 lethally irradiated autologous "feeder" cells/well to determine cloning efficiencies (CEs). Plates were scored for colony

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growth with and inverted phase contrast microscope under 20× magnification 8–10 days later.

CEs were calculated based on the Poisson distribution, which allows one to determine the average number of colony-forming units per well (λ), and then *Hprt* MFs were calculated as the ratio of the mean CE in selective media to that in non-selective media. CEs and MFs for experimental groups were expressed as the mean ± the standard deviation (SD). Positive colonies were resuspended and placed in 0.5 ml microcentrifuge tubes. Approximately 10^4 cells were pelleted via centrifugation at 6500 × *g* for 3 min. Growth medium was removed by pipetting and pellets were snap frozen in liquid nitrogen and stored at −70 °C until molecular analyses.

Thymic lymphomas were diagnosed based on impression smears stained with CMS Protocol HEMA 3 stain set (Biochemical Sciences, Swedesboro, NJ); findings were reviewed by a veterinary pathologist. The tumors consisted predominantly of large round cells with lymphoid appearance and blast T-cell characteristics.

2.3. PCR amplification and DNA sequencing of mouse TCR beta gene mRNA and p53 DNA

PCR amplification and DNA sequencing of TCR beta cDNA was carried out directly from pellet lysates. Cell pellets were lysed and reverse transcribed simultaneously using the PerkinElmer reverse transcription kit (#N808-0143). The final reaction component concentrations were as follows: 1× PerkinElmer PCR buffer II, 5 mM $MgCl_2$, 1 mM each dNTP, 2.5 μM oligo dT, 10 units RNase inhibitor, 25 units MuLV reverse transcriptase, and 2.5% NP40 final concentrations. Reaction tubes were placed in a Perkin Elmer 9600 and complete one reverse transcription cycle at 25 °C for 10 min, 42 °C for 15 min, and 99 °C for 5 min (to inactivate the MuLV reverse transcriptase enzyme). Following reverse transcription, a single round of PCR was completed using a constant region primer (C beta 5′-GGA TGG TTG CAG ACA GAA CCC CC) at a final concentration of 0.4 mM and a set of 22 different variable TCR beta primers corresponding to the different V beta genes each at a final concentration of 0.2 mM. The reaction was set up as a two-part reaction using PerkinElmer Ampliwax Beads in a 9600 thermocycler. The final reagent concentrations were 0.2 mM dNTP, 1× Boehringer Mannheim PWO buffer (no $MgSO_4$), 2 mM $MgSO_4$, and 0.025 units/μl PWO polymerase; 5 μl of the reverse transcription reaction was used for the PCR. Samples were run through two initial cycles at 97 °C for 1 min, 67 °C for 3 min, and 72 °C for 1 min; these precycles were followed by 40 cycles at 94 °C for 30 s, 66 °C for 3 min, and 72 °C for 1 min. PCR samples were loaded on a 0.8% agarose gel for electrophoresis, gels were stained with ethidium bromide, and bands were excised from the gel. The products were Gene Cleaned (Bio 101) and sequenced with a C beta primer (5′-GGA GAC CTT GGG TGG AGT CAC). *TCR* DNA sequences were converted to amino acid sequences for presentation and identification of V beta, CDR3 and J beta gene usage.

P53 specific PCR was performed in a Perkin Elmer 9600 Thermocycler. Pellets were lysed in $T_{10}E_1$ with 0.5% tween 20, 0.5% NP40, and 0.1 mg/ml proteinase K for 1 h at 56 °C followed by 10 min at 96 °C. A hot start PCR was carried out with PerkinElmer PCR gems. The final reaction concentrations were 0.3 mM dNTP, 0.3 mM for primer (5′-CAG CAC TGG GGA GGC CAA AGT GGG), 0.3 mM rev. primer (5′-CTT CCA CCC GGA TAC GAT GCT GGG G), 1× Boehringer Mannheim Expand Long PCR Buffer 2, and 0.5 μl Boehringer Mannheim Expand Long PCR enzyme. These primers produced either a single WT *p53* band, mutant *p53* band, or both. The PCR protocol included 2 cycles of 95 °C for 1 min, and 70 °C for 3 min, followed by 40 cycles of 94 °C for 30 s and 69 °C for 3 min. Following PCR, samples were separated on a 0.8% agarose gel, gels were stained with ethidium bromide, and bands were excised from of the gel. Sequencing was performed by direct sequencing of the Gene Cleaned PCR product. A Taq DyeDeoxy Terminator Cycle Sequencing kit (PerkinElmer ABI, Boston, MA) was utilized to amplify the signal for automated sequencing. The new Big Dyes are attached to dideoxy terminators, and mixed with dNTPs, buffer, amplitaq DNA polymerase, 1–5 μl of dsPCR product, and 3.1 pmol/10 μl reaction of sequencing primer in accordance with the directions with one deviation. One-quarter volume reactions (1/4) were used to save on materials and sample without loss of signal quality. The reaction mix underwent 1 round of 25 cycles at 96 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. The products were purified through packed G50 sephadex columns, dried down, resuspended in a formamide-EDTA solution (5:1), vortexed, denatured, and electrophoresed on an ABI automated sequencer Model 373.

2.4. Statistical analyses

Statistical significance of the differences in benzene-exposed groups versus sham-exposed control mice, or one exposure group versus another, was first evaluated via one-way ANOVA and the Holm–Sidak method for multiple comparisons. If the data failed a normality test for homogeneity of variance within each population the one-way ANOVA on Ranks and the Dunn's test were applied. The null hypothesis states that there is no difference between the sham-exposed and individual treatment groups or between differing benzene-exposed groups. Statistical analyses were performed using SigmaStat (SSPSSC, Chicago, Il); p-values < 0.05 were considered significant.

3. Results

3.1. CEs and Hprt MFs in splenic T-cells from control and benzene-exposed mice

The actual exposure concentrations for benzene were determined using periodic samplings of the inhalation chamber atmospheres. The average inhalation chamber concentrations of benzene for the nominal 100 ppm M–F, 100 ppm MWF, and the 200 ppm MWF exposure groups were 100 ± 0.9 ppm, 100 ± 0.7 ppm, and 200 ± 0.6 ppm, respectively. However, based upon physiologically based pharmacokinetic modeling of benzene metabolism and area under the curve for blood levels of a key metabolite hydroquinone [22], the weekly blood dose of hydroquinone was 160, 150, and 79 μM × h/L for the respective exposures to 100 ppm M–F (30 h/week), 100 ppm MWF (30 h/week), and 200 ppm MWF (15 h/week).

Among WT and *p53*+/− mice, there were no significant differences in the average non-selected CEs in splenic T-cells from groups of control and benzene-exposed mice. The means (and ranges) in CEs for each group are as follows: 16.2 ± 6.7% (7.2–29%) in WT control mice versus 17.4 ± 5.2% (11–23%) in *p53*+/− control mice, 12.3 ± 5.2% (3.9–22%) in WT mice versus 14.7 ± 11.3% (6.0–41%) in *p53*+/− mice exposed to 100 ppm benzene M–F, 10.2 ± 4.5% (4.9–16%) in WT mice versus 15.8 ± 6.6% (9.3–24%) in *p53*+/− exposed to 100 ppm benzene MWF, and 17.1 ± 4.3% in WT mice versus 15.3 ± 6.0% (10–21.5%) in *p53*+/− mice exposed to 200 ppm benzene MWF. Thus, there was no evidence of cytotoxic effects based upon CEs in splenic T-cells from chronic benzene exposures of WT or *p53*+/− mice.

Hprt MFs in control and benzene-exposed mice are shown in Table 1. Background MFs in sham-exposed WT and *p53*+/− mice were similar ($1.2 \pm 0.5 \times 10^{-6}$ and $1.5 \pm 1.1 \times 10^{-6}$, respectively) and resembled the spontaneous *Hprt* MF values previously reported in control mice [23–25]. The mutagenic responses among the benzene-exposed groups of mice were elevated in both WT ($p = 0.002$; one-way ANOVA on ranks and Dunn's test due to an

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Table 1
Hprt mutant frequency data for individual mice exposed to chamber supply air or benzene. Groups of mice were exposed for up to 38 weeks to chamber air or benzene using three different exposure regimens that resulted in an equal weekly cumulative exposure (3000 ppm × h/week). T-cells were isolated from spleen and *Hprt* MFs measured as described in Section 2.

Benzene exposure regimen	*Hprt* MF ($\times 10^{-6}$)[a]	
	p53 wild-type (+/+) C57BL/6 mice	*p53*+/− C57BL/6 mice
0 ppm	0.91	0.61
	0.91	0.68
	0.90	1.7
	0.86	3
	0.96	
	1.5	
	2.2	
Mean	1.2 ± 0.5	1.5 ± 1.1
100 ppm 6 h/day, 5 days/week	8.0	21
	7.2	15
	6.7	11
	5.4	11
	4.9	7.6
		6.8
		5.9
		5.6
		4.2
		3.7
Mean	6.4 ± 1.3	9.2 ± 5.4
100 ppm 10 h/day, 3 days/week	19	15
	7.9	11
	7.3	8.2
	4.0	5.6
		3.3
Mean	9.6 ± 6.5	8.6 ± 4.6
200 ppm 5 h/day, 3 days/week	6	11
	5.3	10
	3.7	8.3
	2.8	5.4
		3.3
		3.2
		3.3
Mean	4.5 ± 1.5	6.4 ± 3.4

[a] MF values from four mice in the study were greater than two standard deviations above means for the remaining animals in their respective experimental groups and were considered outliers for calculation of the means and SDs shown above. The outcomes of statistical analyses comparing differences among treatment groups remained the same with or without the inclusion of these four MF values, which included 5.6×10^{-6} in a *p53*+/+ control mouse, 7.2×10^{-6} in a *p53*+/− control mouse, 48×10^{-6} in a *p53*+/+ mouse exposed to 100 ppm benzene MWF, and 88×10^{-6} in *p53*+/− mouse exposed to 100 ppm benzene MWF.

outlier in mice exposed to 100 ppm benzene MWF) and *p53*+/− mice ($p = 0.04$; one-way ANOVA and Holm–Sidak method), with the increases ranging from 3.8- to 8-fold over background depending upon the exposure group. Pair-wise comparisons showed that the increases in *Hprt* MFs were significant for each of the three benzene exposure regimens in both WT and *p53*+/− mice (*p*-values ranging from 0.006 to 0.02) (Table 1). There were no significant differences in the mutagenic effects observed in WT versus *p53*+/− mice at 100 ppm M–F ($p = 0.8$), 100 ppm MWF ($p = 0.3$), or 200 ppm MWF ($p = 0.5$). The observed MF values suggest that the mutagenic responses were greater in mice exposed to 100 ppm benzene than in those exposed to 200 ppm benzene (Fig. 1). In *p53*+/− mice exposed to 100 ppm benzene M–F and 100 ppm benzene MWF, the mean-induced *Hprt* MF (i.e., observed MF value − spontaneous control mouse MF value) was 1.6-fold and 1.4-fold higher than the mean-induced mutagenic response in mice exposed to 200 ppm benzene MWF. However, differences in the observed *Hprt* MFs were not significantly different among mice in the three benzene exposure regimens, possibly due in part to the limited sample size.

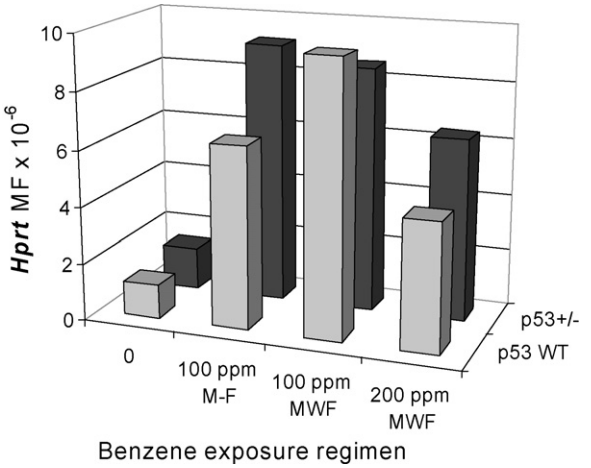

Fig. 1. Frequency of *Hprt* mutations (MF) in splenic T-cells from wild-type (WT) and *p53* heterozygous (*p53*+/−) mice exposed by inhalation to benzene. Mice were exposed for up to 38 weeks to 0 or 100 ppm benzene for 6 h/day on Monday–Friday (M–F), 100 ppm benzene for 10 h/day on Monday, Wednesday, and Friday (MWF), or 200 ppm benzene for 5 h/day on MWF to achieve an equal exposure level of 3000 ppm × h/week. Mutagenic responses in all benzene exposure groups were significantly elevated over spontaneous MF values in control mice, but there were no differences in *Hprt* MFs observed between differing benzene exposure regimens or between benzene-exposed WT and *p53*+/− mice.

3.2. Relationships between Hprt gene status, TCR gene rearrangement patterns, and/or p53 allele status in T-cell isolates or thymic lymphomas from benzene-exposed p53+/− mice

TCR gene rearrangement patterns of T-cell isolates from individual benzene-exposed *p53*+/− mice can be found in Supplementary data to this report; a total of 147 *Hprt* mutant and 86 *Hprt* WT T-cell isolate TCR gene sequences from 16 individual mice are represented. Previous studies have shown that chemical induction of *Hprt* mutations in T-cells of rodents occurs predominately in pre-T-cell pools, with mutant cells then trafficking though the thymus and to the peripheral blood and spleen [26,27]. Therefore, most *Hprt* mutant T-cells *in vivo* are TCR defined during maturation in the thymus [27]. Post-thymic clonal amplifications of mature *Hprt* mutant T-cells (recovered from mouse spleens), as recognized by the recovery of two or more *Hprt* mutant isolates with the identical *TCR* gene hypervariable (CDR3) sequence, were found only in the *Hprt* mutant isolates of benzene-exposed mice. Table 2 lists individual benzene-exposed mice that had one or more sets of *Hprt* mutant T-cell isolates that shared TCR gene identities. Comparison of mouse 642 *Hprt* mutant isolates M9, M11, M15, M17, and M30, which had the same TCR gene sequence, revealed the occurrence of *p53* locus instability as evidenced by progressive loss of alleles in mature T-cells that had undergone post-thymic proliferation.

Table 3 lists the TCR gene sequences found in thymic lymphomas of benzene-exposed *p53*+/− mice as well as spontaneous lymphomas from control mice containing the *p53* transgene. Analyses of the spectra of *p53* genotypes in lymphomas was found to vary from the spectra of *p53* genotypes of peripheral blood T-cell *Hprt* WT and mutant isolates analyzed in this study. For example, loss of the mutant *p53* allele was a relatively frequent event in T-cell isolates, occurring in one or more *Hprt* WT and mutant isolates from 63% to 69% of 16 benzene-exposed mice, respectively (see Table 2 and Supplementary data). In contrast, among 19 T-cell lymphomas from benzene-exposed mice analyzed in this study, loss of mutant *p53* allele occurred in only 11% (2/19) of the lymphomas while both

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Table 2
T-cell receptor (TCR) and *p53* genotypes in *Hprt* mutant splenic T-cell isolates from benzene-exposed *p53*+/− mice. Groups of mice were exposed for up to 38 weeks to benzene using three different exposure regimens that resulted in an equal weekly cumulative exposure (3000 ppm × h/week). Column 1 shows the T-cell isolate name; columns 2–6 give the TCR amino acid sequences; and column 7 shows the *p53* allele status of the isolate. *Abbreviations*: M with a number = mutant isolate number; AA seq = amino acid sequence; WT = *p53* wild-type allele; M = *p53* mutant allele; LOWT = loss of wild-type *p53* allele; LOM = loss of mutant *p53* allele; ND = not determined.

T-cell isolate #	V beta		CDR3	J beta		*p53* genotype
	Family	AA seq	AA seq	AA seq	Family	
Mouse 670 (100 ppm M–F)						
M9	5S2	CASS	RDWGG	DTQYFG	2S5	WT/LOM
M12	5S2	CASS	RDWGG	DTQYFG	2S5	WT/M
M20	8S2	CASGD	ARTMN	TEVFFG	1S1	WT/M
M38	8S2	CASGD	ARTMN	TEVFFG	1S1	WT/M
Mouse 642 (100 ppm M–F)						
M9	5S2	CASSL	LGV	AETLYFG	2S3	LOM/WT
M11	5S2	CASSL	LGV	AETLYFG	2S3	WT/M
M15	5S2	CASSL	LGV	AETLYFG	2S3	LOM/WT
M17	5S2	CASSL	LGV	AETLYFG	2S3	WT/M
M30	5S1	CASSL	LGV	AETLYFG	2S3	LOM/LOWT
M28	16S1	CASSL	GT	NSDYTFG	1S2	LOM/WT
M36	16S1	CASSL	GT	NSDYTFG	1S2	LOM/WT
Mouse 455 (100 ppm MWF)						
M6	6S1	CASS	PGTGGF	EQYFG	2S7	ND
M7	6S1	CASS	PGTGGF	EQYFG	2S7	ND
Mouse 449 (100 ppm MWF)						
M15	4S1	CASS	PTTGG	YEQYFG	2S7	WT/M
M20	4S1	CASS	PTTGG	YEQYFG	2S7	WT/M
M2B	8S3	CAS	GTEN	SGNTLYFG	1S3	WT/M
M3	8S3	CAS	GTEN	SGNTLYFG	1S3	WT/M
M11B	15S1	CGAR	TNFQRKIIF	RS...	1S4	ND
M1B	15S1	CGAR	TNFQRKIIF	RS...	1S4	ND
Mouse 902 (200 ppm MWF)						
M2	11S1	CASSL	DWGG	EQYFG	2S7	LOM/WT
M4	11S1	CASSL	DWGG	EQYFG	2S7	LOM/WT

WT and mutant *p53* alleles were found in 89% (17/19) of the lymphomas (Table 3). However, no loss of the entire WT *p53* gene was observed in tumors from these mice (Table 3), as was found in an occasional Hprt WT or mutant T-cell isolate from the same animals (Table 2 and Supplementary data). Data reported here from lymphomas diverge from the overall results of an earlier study [17] where a WT *p53* allele loss rate of 89% (24/27) was found with retention of the mutant *p53* allele (or transgene) in lymphomas from the same groups of benzene-exposed *p53*+/− mice. Furthermore, there was a concurrent doubling of the mutant *p53* allele in 83% (20/24) of the tumors that had lost the WT *p53* allele in the earlier study [17], which was not observed in the current work (Table 3). The disparity between studies is illustrated in Table 3 by the differences in *p53* genotypes "determined" (column 7) and "reported" (column 8) in tumor numbers 643, 677, 414, 449, 451, and 455. It should be noted that the TCR gene sequences exhibited by the tumors were not found among the WT or *Hprt* mutant isolates recovered from the peripheral T-cell population in our study (Tables 2 and 3, and Supplementary data).

PCR analysis revealed the pattern of allelic loss as seen in Supplementary data and summarized in Table 4. In benzene-exposed *p53*+/− mice (all of which developed T-cell lymphomas), 54% of the *Hprt* mutant isolates retained both *p53* WT and *p53* mutant alleles, 46% lost the *p53* mutant allele, and 6.5% lost both the *p53* WT and *p53* mutant alleles. This distribution of *p53* allele loss can be compared with the distribution observed for the *Hprt* WT isolates from these same animals, where 63% retained both the *p53* WT and *p53* mutant alleles, 37% lost the *p53* mutant allele, and 9.6% lost both the *p53* WT and *p53* mutant alleles. Although relatively few T-cell isolates were analyzed in the sham-exposed *p53*+/− mice, the distribution for retention of both *p53* alleles, loss of the *p53* mutant allele, and loss of both *p53* WT and mutant alleles was 30%, 70%, and 10%, respectively, among the *Hprt* mutant isolates compared with 60%, 40%, and 20%, respectively, for the *Hprt* WT isolates. It is noteworthy that the *p53* WT allele was never lost alone. Therefore, the percentages of isolates that have lost both *p53* alleles are included in the percentages that have lost the *p53* mutant allele.

In *Hprt* mutant and WT T-cell isolates recovered from *p53* WT mice, a small number (~5%) lost both *p53* WT alleles in the absence of benzene exposure while the vast majority of isolates retained both *p53* alleles. Neither benzene exposure nor 6-thioguanine selection influenced frequencies of *p53* allele loss (mutant or WT) in the T-cell isolates.

Lineage analyses were possible for *in vivo Hprt* mutant clonal amplifications found in two different animals (Table 2, mouse 642 T-cell isolates M9, M11, M15, M17, and M30; mouse 670 T-cell isolates M9 and M12). These *in vivo* clones were established on the basis of their *TCR* gene rearrangements and characterized by different *p53* allele retentions among the different isolates for a given *in vivo* clone. Among five *Hprt* mutant isolates with the same TCR sequence in mouse 642, two T-cell isolates retained both the *p53* mutant allele and the *p53* WT allele, two isolates retained only the *p53* WT allele, and one isolate lost both the *p53* WT and the *p53* mutant allele (Fig. 2A). In two *Hprt* mutant isolates with the same TCR sequence in mouse 670, one isolate retained both the *p53* mutant allele and *p53* WT allele while the other isolate had lost the *p53* mutant allele (Fig. 2B). Loss of the *p53* WT allele was an infrequent event and was the last in the sequence of events when it did occur. Of the groups evaluated in these studies, i.e., control versus benzene-exposed *p53*+/− mice, *Hprt* mutant versus *Hprt* WT T-cell isolates, and 6-thioguanine selected versus non-selected cells, no differences were observed in the rate of *p53* mutant allele loss.

4. Discussion

The current finding that chronic inhalation of benzene-induced significant increases in *Hprt* MFs in T-lymphocytes of exposed mice

Please cite this article in press as: R.J. Albertini, et al., *Hprt* mutant frequency and *p53* gene status in mice chronically exposed by inhalation to benzene, Chem. Biol. Interact. (2010), doi:10.1016/j.cbi.2009.12.019

Table 3
T-cell receptor (TCR) and *p53* genotypes of thymic lymphomas from control and benzene-exposed *p53*+/− mice. Groups of mice were exposed for up to 38 weeks to chamber air or benzene using three different exposure regimens that resulted in an equal weekly cumulative exposure (3000 ppm × h/week). TCR data for individual tumors (column 2) in differing exposure groups (column 1), displayed as amino acid sequence of the hypervariable CDR3 region (column 5) and adjacent V beta (column 4) and J beta regions (column 6), are listed along with *p53* allele status as found in the current study (column 7) and in an earlier study reported by Boley et al. [17][a] (column 8). Columns 4 and 6 show the family (in parentheses) and amino acid sequence for the V beta and J beta regions, respectively. Sequence quality is given in degrees sequence heterogeneity in column 3: 1 = a single TCR sequence, 2 = 2 TCR sequences, 3 = mixed TCR sequences but interpretable. *TCR* sequences that could not be interpreted, possibly because of sample contamination by peripheral blood lymphocytes, are not shown. *Abbreviations*: WT = *p53* wild-type allele; M = *p53* mutant allele; MU = mutant (insert) *p53* allele LOWT, loss of wild-type *p53* allele; LOM, loss of mutant *p53* allele; ND, not determined.

Exposure regimen	Tumor #	Sequence quality	V beta	CDR3	J beta	Determined *p53* genotype	Reported *p53* genotype[a]
Control							
	176	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/MU	ND
	227	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/MU	ND
	228	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/MU	ND
	229	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/MU	ND
	230	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	LOWT/LOM	ND
	232	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/MU	ND
100 ppm M–F							
	642	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/M	ND
	643	2	(14s1) CASS	PGDCE	DTQYFG (2s5)	WT/LOM	Loss WT
	646	1	(8s2) CAS	S	QDTQYFG (2s5)	LOWT/LOM	ND
	647	1	(4s1) CASSQ	DRGM	DTQYFG (2s5)	WT/M	ND
	660	1	(8s2) CASG	QGLA	YAEQFFG (2s1)	WT/M	ND
	668	1	(1s1) CASSQ	EGTGL	YEQYFG (2s7)	WT/M	ND
	670	1	(1s1) CASSQ	AWGDN	YAEQFFG (2s1)	WT/M	ND
	677	1	(11s1) CASSL	DGGG	AETLYFG (2s3)	WT/M	Loss WT
	679	1	(10s1) CASS	HDRGG	AETLYFG (2s3)	WT[b]/M	ND
	680	1	(8s2) CASGD	Y	SYEQYFG (2s7)	WT/M	ND
100 ppm MWF							
	402	2	(10s1) CASS	LEGLGAY	TQYFG (2s5)	WT/M	ND
	414	3	(10s1) CAS	RGEGLVRTT	QYFG (2s5)	WT[b]/M	Loss WT
	449	3	(7s1) CASS	YVPNGPGER	REQFFG (2s1)	WT[b]/M	Loss WT
	451	1	(8s3) CASSD	GWTGGF	EQYFG (2s7)	WT/M	Loss WT
	455	3	(?) CAS	TATGE	DTQYFG (2s5)	WT/M	Loss WT
	459	3	(1s1) CASSQ	DGGGV	QDTQYFG (2s5)	WT/M	ND
	461	1	(6s1) CAS	RPTGED	TGQLYFG (2s2)	WT/M	ND
200 ppm MWF							
	902	1	(6s1) CASS	LGQGT	YAEQFFG (2s1)	WT[b]/M	ND
	928	1	(6s1) CASSI	SPQGGG	TGQLYFG (2s2)	WT/M	ND

[b] Greatly decreased intensity WT band.

considerably expands earlier limited evidence [14–16] that benzene causes gene level mutations *in vivo*. Surprisingly, however, WT and *p53*+/− mice were equally sensitive to the mutagenic effects of benzene. Lineage analysis of *in vivo Hprt* mutant clones revealed the frequent loss of the mutant rather than the WT *p53* alleles. This relationship of *Hprt* mutation induction to *p53* status in peripheral blood lymphocytes differs from expectations derived from analyses of the neoplasms induced in the same animals, suggesting a possible difference in the mechanisms underlying induction of surrogate gene mutations in T-cells and induction of T-cell lymphomas.

As noted in Section 1, benzene and/or its metabolites are well known inducers of both structural and numerical chromosome aberrations, but evidence for mutation induction at the gene level has been inconsistent [3]. Studies of reverse mutations in prokaryotic systems from benzene exposures have been almost universally negative. By contrast, forward gene mutation assays in mammalian cells have been positive [3], as have studies of *lacI* mutations in transgenic mice exposed to benzene [14,15].

Results of the present study, using a T-cell cloning assay for measuring MFs, clearly demonstrate that inhalation exposures to leukemogenic levels of benzene induce gene mutations at the endogenous *Hprt* locus in T-cells of mice. The MF and TCR data combined indicate that it was the number of mutations that were responsible for the increases in *Hprt* MFs in T-cells of exposed mice

Table 4
Patterns in loss of *p53* alleles in wild-type (WT) and mutant T-cell isolates from control and benzene-exposed *p53*+/− mice.[a,b].

Benzene exposure	*Hprt* status of T-cell isolates	Retention of both *p53* alleles	Loss of *p53* mutant allele	Loss of both *p53* mutant and WT alleles[c]
No	10 WT isolates	6 (60%)	4 (40%)	2 (20%)
No	10 mutant isolates	3 (30%)	7 (70%)	1 (10%)
Yes	73 WT isolates	46 (63%)	27 (37%)	7 (8%)
Yes	124 mutant isolates	67 (54%)	57 (46%)	8 (6.5%)

[a] The mutant allele is present because of the insertional disruption and partial deletion of one copy of *p53*, to accompany a single functional copy, in creating the *p53* haploinsufficient (+/−) mouse. Groups of mice were exposed for up to 38 weeks to chamber air or benzene using three different exposure regimens that resulted in an equal weekly cumulative exposure (3000 ppm × h/week) (see Section 2).

[b] Lineage analysis is possible for only two *in vivo* in *Hprt* mutant clones. In both, the *Hprt* mutations arose in cells that had retained both *p53* alleles with subsequent loss of the *p53* mutant allele (and loss of the *p53* wild-type allele at a later stage in one case). Note that loss of *p53* wild-type (WT) allele occurred only in clones that first had loss of the *p53* mutant allele.

[c] In this context, loss of both *p53* mutant and wild-type alleles in column 5 is a subcategory of column 4. Therefore, of the 10 wild-type clones obtained from mice that were not treated with benzene, six (60%) retained both *p53* alleles while four (40%) lost the mutant inserted allele. Of the latter four, two (20% of the total of 10 isolates) had also lost the wild-type allele. This is the case for all categories of isolates, i.e., *p53* wild-type and mutant isolates from non-treated and non-treated animals. The percentages shown in columns 3 and 4 total to 100% of the isolates studied.

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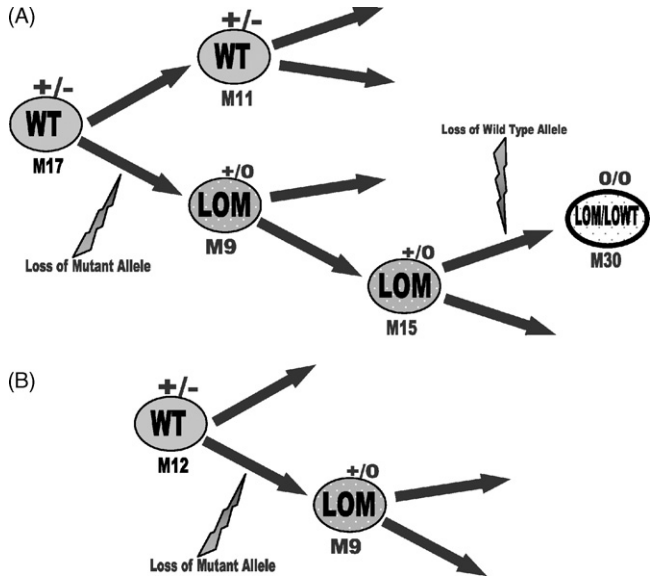

Fig. 2. Progressive loss of *p53* alleles in propagated peripheral blood *Hprt* mutant T-cell isolates. (A) Among five *Hprt* mutant isolates with the same T-cell receptor (TCR) sequence in mouse 642, two T-cell isolates (M17 and M11) contained both the *p53* mutant allele and *p53* mutant allele, two isolates (M9 and M15) retained only the wild-type allele, and one isolate (M30) lost both the *p53*+ WT and mutant alleles. (B) In two *Hprt* mutant isolates with the same TCR sequence in mouse 670, one isolate (M12) contained both the *p53* wild-type allele and the *p53* mutant allele while the other isolate (M9) had lost the *p53* mutant allele. WT = presence of both *p53* wild-type and mutant (transgene) alleles; LOM = loss of mutant allele; LOWT = loss of wild-type allele.

and not simply the number of mutant cells resulting from extensive clonal proliferations. The finding that mutagenic responses induced in *p53*+/− mice exposed for 30 h/week to 100 ppm benzene (M–F or MWF) was ~1.5-fold greater than in *p53*+/− mice exposed for 15 h/week to 200 ppm benzene (MWF) is consistent with the roughly 2-fold higher levels of circulating hydroquinone, the 2-fold higher levels of accumulation of micronuclei [18], and the 2–2.7-fold higher incidence of tumors in *p53*+/− mice similarly exposed to 100 ppm benzene compared to 200 ppm benzene (J. French et al., manuscript in preparation for this special issue). Additional mutagenicity studies performed by our research group employed shorter durations and lower cumulative doses of benzene (i.e., 1, 5, 50 or 200 ppm benzene for 3–12 days delivering total exposures of 72, 360 and 3600 ppm/h over 24 days); MF measurements, which used the T-cell cloning assay, did not reveal significant induction of *Hprt* mutations in T-cells of exposed mice (unpublished data). Thus, it is noteworthy that the benzene concentrations used in current studies were high and the duration of the exposures were long, indicating that large cumulative doses (time × concentration) are required for the observed mutagenic effect. In light of recent research showing that cells can resolve the effects of some exogenous exposures using pathways that have a predominately error-free resolution capacity [28,29], it is not possible to determine whether or not the results of an earlier mutagenesis study of benzene, which used an autoradiographic assay for *Hprt* mutations in T-cells of CD-1 mice exposed to low levels of inhaled benzene (<1.0 ppm) [16], reflects differences in methodology or dose-related expression of error-free resolution pathways.

The present work illustrates how studies of *Hprt* mutations in mouse T-cells designed to reflect the *in vivo* effects of a potential mutagenic exposure also can reveal fundamental biological and toxicological processes, even when these are contrary to expectations. Our studies initially were designed to determine inhalation exposure to benzene induces gene level mutations in mice and, if so, are *p53* WT and *p53*+/− mice differentially susceptible to benzene-induced mutagenesis. If heightened susceptibility to benzene mutagenesis had been observed in the *p53*+/− mice, we also hoped to determine if this was due to clonally restricted genomic instability in the T-lymphocyte population resulting from loss of the *p53* allele. If affirmed, this would then constitute a situation similar to that postulated for the increased susceptibility of *p53*+/− mice to tumor induction, which is presumed to result from genomic instability resulting from loss of the *p53* WT allele in the tumor progenitors [17,30,31].

Our study design was then based on the concept that mutation induction would be more efficient in the *p53*+/− mice than in the WT animals and that this would be because the loss of WT *p53* allele in *in vivo* clones of cells rendered them genomically unstable. We found, however, that the *p53*+/− and homozygous WT mice were equally susceptible to benzene-induced mutations. Lineage analysis of *Hprt* mutant isolates from *p53*+/− animals revealed the basis for this finding; there were very few clones that had lost the *p53* WT allele and, when WT allele loss was observed, it was always preceded by loss of the *p53* mutant allele. Lineage analysis was possible for only two *Hprt* mutant clones derived from the *p53*+/− animals. In both cases, the *Hprt* mutations arose in cells that had retained both *p53* alleles. Loss of both *p53* WT alleles was observed rarely in *Hprt* mutant isolates from the *p53* homozygous WT animals, which may have been an infrequent event in the cells of all animals or may represent infrequent PCR failures in analysis, representing artifacts. This phenomenon indicates that in at least some clones the *Hprt* mutations were the first somatic events to have occurred. In any case, whether real or artifactual, loss of the WT *p53* allele is too infrequent an event to confer a difference in mutagen susceptibility between the *p53*+/− and homozygous WT mice. These findings are inconsistent with the notion that *p53* haploinsufficiency with only a single functional *p53* allele renders cells hypermutable because of a dosage effect [17,30,31].

The frequent and asymmetric loss of the mutant *p53* allele in the *Hprt* mutant clones derived from the *p53*+/− animals initially was unexpected. However, the suggestion that mammalian cells must possess mechanisms to protect against invasion by foreign DNA was made almost 20 years ago [32,33]. This observation is relevant to the current study because the mutant *p53* allele in the *p53*+/− mice used here consists of a partial deletion of *p53* intron 4/exon 5 with an insertion of a neomycin cassette containing bacterial DNA [34]. Sophisticated analyses of transgenic mice have revealed that *lacI* transgenic inserts containing bacterial DNA are eliminated from somatic cells, reducing the numbers of such inserts from expectations in heterozygous animals [35]. That this is a consequence of the bacterial DNA content of the inserts was demonstrated by 'mammalianizing' the insert DNA which totally blocked their elimination. Our analyses of *Hprt* mutant and WT isolates from the *p53*+/− mice, with lineage analyses when possible of *in vivo Hprt* mutant clones, allowed a definitive demonstration of the elimination of bacterial DNA from mammalian genomes. Studies with transgenic animals that involve introduction of foreign DNA must be interpreted with this in mind.

The *p53*+/− animals examined for benzene mutagenesis were also used in cancer bioassays, with the production of lymphomas in >80% of the mice [17]. Nearly 90% of the tumors were reported to have lost the WT *p53* allele with retention of the mutant allele. The methods (in this earlier study [17]) used included multiplex PCR of the WT and mutant *p53* alleles plus characterization of heterozygosity or its loss (LOH) using arrays of closely linked microsatellite markers of mouse chromosome 11. We had the opportunity to study some of the tumors with the methods employed in our work and found retention of both *p53* alleles in the majority of instances. There were, however, occasional reductions of the WT allele band and single instances of loss of the *p53* mutant allele or of both *p53* alleles. Loss of the mutant *p53* allele in the tumors occurred

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in animals where this also was encountered in the lymphocyte isolates recovered from the mutation studies. Our failure to detect loss of only the *p53* WT allele in these thymic lymphomas may have been a technical problem due to infiltration with peripheral blood lymphocytes into the tumor sample. Our study of the tumors also included determinations of *TCR* gene rearrangements in this material. We did detect predominantly monoclonal patterns in most but, again, could not rule out infiltrating peripheral blood lymphocytes producing occasional mixed patterns. None of the *TCR* rearrangement patterns detected in the tumors were detected in the analyses of either *Hprt* WT or mutant isolates recovered from the *Hprt* mutation assays. It is noteworthy that identical *TCR* gene rearrangement patterns were sometimes observed for different tumors induced in different animals. This intriguing observation may indicate that there are certain cells that are susceptible to progression to thymic lymphoma or, alternatively, that these patterns represent infiltrating peripheral blood lymphocytes that are drawn to the tumors as an immunological response. As these findings were peripheral to the purposes of the study, they must be followed up before definitive statements can be made.

Our studies in the *Hprt* mutant and WT T-lymphocyte isolates from mutation assays described here indicate that the isolated loss of the *p53* WT allele in heterozygous mice is a rare event in mature T-cells. Assuming that the thymic lymphomas in the *p53*+/− mice are mono- or oligo-clonal, and have truly lost this WT *p53* allele, suggests that something different must be occurring in the intrathymic environment, perhaps in immature non-differentiated T-cells. The mechanisms of WT allele loss are unknown, although it has been suggested that this is due to the clastogenicity of benzene in exposed animals. The consequences of loss of *p53* function in the tumor progenitor cells are also unknown. Studies reported here failed to find a loss of the WT *p53* allele in mature T-cells of the heterozygous mice and, therefore, shed no light on this phenomenon. We have, however, learned something about benzene mutagenicity and the persistence in mammalian cells of *p53* mutant alleles containing bacterial DNA.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

We acknowledge Drs. Laura N. Healy and Brian Wong for their work on the benzene inhalation study, and Drs. Janice Nicklas and Dale Walker for helpful comments used in revising this report. The inhalation study in benzene-exposed mice, from which materials for the present report were obtained, was performed at the Chemical Industry Institute of Toxicology, now one of the cornerstone institutes at The Hamner Institutes for Health Sciences. The current work was supported, in part, by grants from the Department of Energy (to RJA) and the Health Effects Institute (to VEW).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbi.2009.12.019.

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Please cite this article in press as: R.J. Albertini, et al., *Hprt* mutant frequency and *p53* gene status in mice chronically exposed by inhalation to benzene, Chem. Biol. Interact. (2010), doi:10.1016/j.cbi.2009.12.019

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Please cite this article in press as: R.J. Albertini, et al., *Hprt* mutant frequency and *p53* gene status in mice chronically exposed by inhalation to benzene, Chem. Biol. Interact. (2010), doi:10.1016/j.cbi.2009.12.019