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Evaluation of frequencies of *HPRT* mutant lymphocytes in butadiene polymer workers in a Southeast Texas facility

Jeffrey K. Wickliffe, Marinel M. Ammenheuser, P. Jene Adler, Sherif Z. Abdel-Rahman, and Jonathan B. Ward Jr.

Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX 77555-1110

Abstract

We examined the frequency of mutant lymphocytes (VFs) in workers ($n = 30$) occupationally exposed to the petrochemical, 1,3-butadiene (BD), using the autoradiographic *HPRT* mutant lymphocyte assay. Current exposures were determined with organic vapor monitors that had a 12-hour method detection limit (MDL) of 2.5 parts per billion (ppb). *HPRT* VFs were analyzed with respect to current exposure estimates, age in years, and occupational longevity (OL; defined as years working in the BD industry at this facility). Current exposures were low (mean 93.5 ppb, median 2.5 ppb) with only one individual's estimate (1,683.5 ppb) exceeding the Occupational Safety and Health Administration's permissible exposure limit of 1,000 ppb. The majority (>50%) of current exposures were below the MDL. *HPRT* VFs were not significantly associated with current exposures ($n = 29$), and they were not significantly associated with age ($n = 29$). *HPRT* VFs were, however, significantly associated with OL ($n = 29$, $R^2 = 0.107$, $p < 0.046$). This result suggests that chronic and/or past, high-level exposures might leave a mutagenic signature that is revealed by the *HPRT* assay, possibly through the retention of mutant, long-term memory T-cells. While it is encouraging that current occupational exposures to BD in this facility do not appear to be increasing the frequency of mutant T-lymphocytes, evidence from workers with a lengthy history in the industry (≥ 30 years in this case) indicates that these individuals likely require additional biomonitoring for possible mutagenic effects resulting from chronic, past exposures.

Keywords

occupational longevity; mutation; 1,3-butadiene

Introduction

Occupational exposure to 1,3-butadiene (BD; CAS # 106-99-0) has steadily declined in the United States over the last 50 years due, in part, to increasingly restrictive Occupational Safety and Health Administration (OSHA) standards. However, BD continues to be produced and used worldwide in the manufacture of synthetic rubbers and plastics. In fact, following a steady decline to a low in years 2000–2001, production and use of BD has risen and is predicted to continue rising for the next several years [Tullo, 2005]. Concern regarding the health of workers exposed to BD stems primarily from epidemiological analyses of workers in the styrene-butadiene rubber industry that demonstrate an increased risk of leukemia correlated with duration and level of exposure to BD [Delzell et al., 2001; Graff et al., 2005; Sathiakumar et al., 2005]. In addition, studies examining rodents exposed

to BD by inhalation have documented that BD is both mutagenic and carcinogenic [Huff et al., 1985; Cochrane and Skopek, 1994; Wickliffe et al., 2003; Meng et al., 2004]. Previous studies examining workers exposed to BD in monomer and polymer plants in Southeast Texas indicated that relatively high, current exposures (i.e. from 150-3,000 parts per billion [ppb] in air) were associated with increased frequencies of mutant peripheral blood lymphocytes [Ward et al., 1996; Ma et al., 2000; Ammenheuser et al., 2001; Ward et al., 2001a; Abdel-Rahman et al., 2001]. The present OSHA standard sets the time-weighted average for BD at 1,000 ppb in air. Studies recently conducted on workers in other countries have not found that exposures to BD were associated with somatic cell mutations [Hayes et al., 1996; Hayes et al., 2001; Albertini et al., 2003; Zhang et al., 2004]. This may have been a function of low levels of exposure that did not appreciably increase the frequency of mutations above background, or that quantifiable mutation detection was beyond the sensitivity of the various assays that were used. There also may have been technical differences in the mutagenicity assays and the procedures used to estimate genetic damage. Finally, potential confounders not influencing the results in the examination of U.S. workers might have played a role in the apparent lack of concordance between all of these studies. Therefore, it remains important to understand whether or not the current permissible exposure limit (PEL) is sufficient in protecting exposed workers who are either producing BD or using BD in the manufacture of synthetic rubbers and plastics.

We examined workers from a plant that manufactures polymerized polybutadiene rubber, as well as styrene-butadiene di-block and tri-block copolymers, for evidence of genotoxicity using the autoradiographic *HPRT* mutant lymphocyte assay. We hypothesized that *HPRT* variant frequencies (VFs) would increase in an exposure-related manner. Workers were monitored for BD exposure, and the association between exposure and *HPRT* mutation was examined. In addition, we examined the relationship between occupational longevity (i.e. years employed in the BD industry) and *HPRT* VFs. We hypothesized that, because historical exposures were substantially and allowably higher based on the evolution of the OSHA PELs, and longevity can result in long-term chronic exposures, *HPRT* VFs would be elevated in workers with longer service in the BD industry.

Materials and Methods

Study population and sample collection

A study using the same design and sampling protocol as our 1998 Ameripol-Synpol study, was conducted at the Firestone Polymers plant in Orange, Texas [Ward et al., 2001b]. The company conducted a parallel exposure assessment using the pumped tube method currently recommended by the National Institute of Occupational Safety and Health (NIOSH) so the two methods could be compared. The company's approach used a standard active sampling method with a calibrated battery powered mini-pump and charcoal tube. The active sampler was worn in the breast pocket on the side opposite our passive organic vapor monitor. Both dosimeters were applied and removed at the same time. The correspondence of the results between the methods was excellent with high concordance of samples above and below the detection limit, as well as a strong correlation between the results for detectable exposure levels ($r^2 = 0.90$, $p < 0.01$). Firestone Polymers uses a unique anionic polymerization technology to produce high quality polybutadiene rubber, as well as di- and tri-block styrene-butadiene copolymers. This process is more efficient, resulting in less unreacted monomer, so lower exposures would be anticipated. In other respects the study design and sampling procedures were the same. Two work shifts were studied in January and April, 1999, and two shifts in August and October, 1999.

Methods of exposure assessment

The laboratory procedures for organic vapor monitor (OVM) analysis have been previously described in detail [Chung et al., 1999]. The general approach for handling the OVMs and calculating analyte concentrations follows the procedures recommended by the 3M Company (3M Co., 1993), but with modifications to the extraction and analysis methods to allow for measurement of the target analytes at low concentrations. Specific modifications included: (1) use of 1.0 ml freshly prepared mixed extraction solvent, a 2:1 v/v mix of acetone (glass distilled: AX0116-1; EM Science, Gibbstown, NJ) and CS₂ (99.9+% redistilled: 42464-1; Sigma-Aldrich, Milwaukee, WI) with 5 µg/ml 4-bromofluorobenzene (Supelco, Inc., Bellefonte, PA) added as an extraction surrogate; (2) desorption by sonication during 40 minutes in an ultrasonic water bath at 15-18° C; (3) analysis of 200-µl extract aliquots with 10 µl of an internal standard mix of 200 µg/ml of 1,4-difluorobenzene and chlorobenzene-d5 prepared in the mixed solvent from a 1000 µg/ml certified standard in methanol (Supelco) by gas chromatography. The analytical conditions are described in full detail in Chung et al., [1999]. Working analytical standards in the range of 0.1 to 10 µg/ml were prepared with the mixed solvent and two certified commercial standard dilutions, containing all analytes except BD (2000 µg/mL in low benzene CS₂; EM Science) and the other containing BD in methanol (2000 µg/ml; Accustandard, Inc., New Haven, CT). OVM extraction efficiencies were determined at room temperature following the 3M-recommended procedure (3M Co., 1993) at mass loadings ranging from 0.1 to 10 µg per compound per badge using the mixed standards. The method detection limits (MDLs) for BD were determined from multiple injections of the lowest-concentration standard solution. Equivalent BD concentrations were calculated, as described by 3M (3M Co., 1993), using their published sampling rates, resulting in a MDL concentration of 2-2.5 ppb for a 12-hour exposure.

Recruitment of Study Subjects

Workers at the facility were made aware of the study by letter, and they were subsequently asked if they would agree to participate. Each worker that volunteered then gave consent using a form that was approved by the human subject Institutional Review Board of the University of Texas Medical Branch (UTMB). Each volunteer then completed a questionnaire providing information on age, ethnicity, gender, history of tobacco use, customary use of alcohol and caffeine-containing beverages, health status and medication use, and work-related information including job title, work locations, and occupational history including years at this particular facility. Exclusion criteria included recent, acute viral or bacterial infection, major chronic illness (e.g. cancer or autoimmune disorder), recent blood transfusion, chemotherapeutic treatment, and employment involving exposure to known or potentially mutagenic chemicals such as polycyclic aromatic hydrocarbons or radiation. In addition, serum cotinine levels were measured as an objective, independent biomarker of tobacco use (described in detail below). Seventy-two individuals were recruited for participation in this study. Fourteen did not wish to provide a blood sample, 1 individual's blood sample was not sufficient, 3 individuals were not included because they met one or more of the exclusion criteria. Serum cotinine measures and *HPRT* mutant lymphocyte assays were conducted for the remaining 54 individuals.

Data from outside reference subjects were obtained from another, independently funded study also approved by the IRB at UTMB. These individuals were subject to the same questionnaire information and exclusion criteria as the volunteers recruited specifically for this study. Outside reference subjects were matched to workers based on age, gender, race, and ethnicity. An insufficient number of older reference individuals were available for comparison to older workers. Finally, because our particular study was not a case-control or

control-exposed design, these subjects should be considered as outside reference subjects representing healthy, unexposed individuals in statistical comparisons.

Lymphocyte and plasma isolation and cryopreservation

A 70 ml blood sample was collected from each worker using sodium heparinized vacuum tubes. The samples were delivered within 3-15 hours to our laboratory at the UTMB where the mononuclear cells and plasma were isolated by density centrifugation on Histopaque (Sigma, St. Louis, MO). The lymphocytes were cryopreserved, as previously described, and stored in liquid nitrogen [Ward et al., 2001a].

A 2 ml aliquot of plasma was frozen and was later analyzed for cotinine concentration, by a radioimmunoassay, in the laboratory of Dr. Helen van Vunakis at Brandeis University, Waltham, MA [van Vunakis et al., 1987]. Subjects with cotinine concentrations ≥ 20 ng/ml of plasma were considered to be active tobacco users. The remaining blood plasma was stored at -20°C for later use in the *HPRT* assay.

The *HPRT* mutant lymphocyte assay

Frequencies of *HPRT* mutant lymphocytes (VFs) were estimated in this study using the autoradiographic assay. This assay was used because of our demonstrated expertise with the method, the sensitivity of the method as compared to the *HPRT* cloning assay, and for comparability with our previous studies. Methods for this assay previously have been described in detail [Ammenheuser et al., 1994; 1997; 1998; 2001; Ward et al., 2001a]. Briefly, $30\text{--}50 \times 10^6$ cryopreserved lymphocytes were thawed, washed, checked for viability by Trypan Blue exclusion, and counted. The cells were suspended in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA) with 20% HL-1 (Cambrex, East Rutherford, NJ), 2% reagent grade phytohemagglutinin (Remel, Lenexa, KS) and 25% autologous plasma. An aliquot of about 5×10^6 cells in 5 ml of growth medium was added to one vented culture flask to provide a labeling index for estimating the ability of an individual subject's lymphocytes to grow in culture. To the remaining cell suspension, 6-thioguanine (TG; Sigma, St. Louis, MO) was added to produce a final concentration of 2×10^{-4} M TG, and the cells, in aliquots of 5×10^6 cells, were added to 4-8 vented flasks (Falcon, No. 3108, Lincoln Park, NJ). All of the flasks were incubated at 37°C for 24 hours, at which time 25 μCi of tritiated thymidine with a specific activity of 6.7 Ci/mM (ICN, Costa Mesa, CA) was added to each flask. After an additional 18 hours of culture, the cells were harvested and nuclei were released with 0.1 M citric acid. The nuclei were washed and resuspended in 0.3-0.4ml of fixative and were stored at 4°C for at least 2 hours. Fixed nuclei were counted, and nuclei from each sample were placed on 18×18 mm coverslips previously affixed to 2-3 microscope slides. The slides were stained with aceto-orcein, dipped (in total darkness) in NTB-2 emulsion (Kodak, Rochester, NY), stored for 2-3 days at 4°C and developed with Kodak D-19.

All slides were coded, to prevent identification of the subjects by the slide reader. They were read with a Nikon light microscope. For the mutant-selection slides, prepared with cells from the TG-containing cultures, all labeled cells were counted. The slide containing an aliquot of cells from the culture without TG had been prepared with cells spread out on the slide. A differential count of 3000 labeled and unlabeled cells was made from this labeling index (LI) slide. The VF was calculated by taking the total number of labeled cells scored from an individual subject's TG slides and dividing this by the total number of cells initially added to the TG slides multiplied by the LI. This denominator (labeling index times total cells) is referred to as the number of evaluable cells. In order to keep the confidence intervals of our VF data as narrow as possible, we used sufficient numbers of cells in our

assays to provide at least 0.5×10^6 evaluable cells, and we usually had from 1 to 3×10^6 evaluable cells.

Statistical analysis

Statistical tests were conducted using the SPSS version 14.0 software for Windows (SPSS Inc.; Chicago, IL). *HPRT* VFs were transformed as in Ammenheuser et al. [1997], and estimates of BD exposure were log transformed prior to statistical analyses. Linear regression was used to analyze the relationship between *HPRT* VFs and BD exposure, *HPRT* VFs and occupational longevity (OL) in years, and *HPRT* VFs and age in years. Because of the high suspected correlation between age and OL, a backward regression model was used to analyze the relationship between *HPRT* VFs and a model containing both age and OL. In this case, age and OL were initially included and the variable with the smallest partial correlation with the dependent variable, VF, was removed first. Student's *t*-tests were used to analyze *HPRT* VFs as a function of measured current exposure to BD and to analyze *HPRT* VFs in young workers compared to age- and gender-matched subjects with no history of occupation in the BD industry (i.e. external or outside reference subjects). Statistical tests were considered significant at $p < 0.05$.

Results

Data for *HPRT* VFs and BD exposure, presented in the accompanying figures, are displayed as untransformed values. All statistical analyses were conducted on transformed data, as described previously. Linear equations resulting from regression analyses were derived from the transformed data.

Of the 54 individuals for which mutation assays were conducted, 18 had serum cotinine levels ≥ 20 ng/ml and 6 had an insufficient number of evaluable cells for valid *HPRT* assays, and these 24 individuals were not included in the statistical tests. This left 30 subjects for statistical analysis. Workers were employed in a variety of areas in the plant, including production areas where exposures are typically higher and recovery areas where exposures are typically low. Average exposures for these areas are presented in Figure 1. Exposure estimates ranged from non-detectable to 1,683.5 ppb (mean = 93.5, median = 2.5). Individuals with no detectable exposure to BD, based on results from their personal OVM, were assigned a 2.5 ppb exposure prior to statistical analyses because this level represents the method's minimum detection limit (MDL). Only six subjects had estimates of current BD exposure that were > 100 ppb. Of these, only one subject had a current exposure estimate above the OSHA PEL of 1,000 ppb. Approximately one-half of the subjects (54%) had no measurable current exposure to BD using our method.

Ages among workers ranged from 23 to 65 years (mean = 48, median = 55). Years spent in the industry (i.e. occupational longevity, OL) ranged from 1 to 39 years (mean = 21.2, median = 30). With the exception of one subject, the distributions of both age and OL in these workers were distinctly bimodal (Figure 2). Age and OL were highly correlated (Pearson's $r^2 = 0.90$, $p < 0.001$). For OL analyses, subjects were assigned to one of two groups based on the average number of years spent in the industry (short-term, < 21.2 years vs. long-term, ≥ 21.2 years). Short-term workers' years in the industry ranged from 1-12 years (mean = 6.4), and long-term workers' years in the industry ranged from 30-39 years (mean = 35).

A significant, positive relationship was found between BD exposure and *HPRT* VFs ($t = 2.222$, adjusted $R^2 = 0.12$, $p < 0.035$; Figure 3). However, as indicated by Mahalanobis' and Cook's Distances (outlier identification, not shown), this is primarily a function of the single subject with the highest exposure estimate (1,683.5 ppb) and a high *HPRT* VF (18.9×10^{-6}).

Therefore, this subject was considered an outlier and was removed from further analysis. After removing this subject, no relationship between exposure and the induction of *HPRT* mutations appeared to exist in the remaining 29 workers ($t = 1.200$, adjusted $R^2 = 0.015$, $p < 0.241$; Figure 4). This analysis included only three individuals with exposure levels estimated to be at or above 150 ppb (271.4, 444.7, and 484.1 ppb). The results from the Student's t -test comparing *HPRT* VFs from subjects with no detectable exposure ($< \text{MDL}$, $n = 16$) to subjects with a detectable level of exposure (2.8-484.1 ppb, $n = 13$) indicated there was no significant difference ($t = -0.014$, $p < 0.989$) between these two groups.

There was no significant relationship between age and *HPRT* VFs ($t = 1.618$, adjusted $R^2 = 0.055$, $p < 0.117$). However, the backward regression model indicated that there was a significant, positive relationship between OL and *HPRT* VFs ($t = 2.090$, adjusted $R^2 = 0.107$, $p < 0.046$) after removing age from the model. The model with both age and OL as predictors of *HPRT* VF was not significant ($p < 0.124$). The results from the Student's t -test indicated there was no significant difference in current exposure ($t = 0.646$, $p < 0.524$) between short-term workers ($n = 14$) and long-term workers ($n = 15$).

Finally, *HPRT* VFs in the six workers with ages less than the average age (48 years) of workers in this study (range 23 to 41 years), who had no detectable current BD exposure, were compared to subjects who were non-smokers with no history of employment in the BD industry and no reported exposure to specific mutagenic agents (i.e. outside reference subjects). These outside reference subjects ($n = 25$) were gender- and age-matched ($t = 0.723$, $p < 0.475$) and were reported to be healthy, non-smokers. The younger individuals who worked in the BD industry had comparable *HPRT* VFs (mean VF = $2.25 \pm 0.40 \times 10^{-6}$, $t = -1.376$, $p < 0.179$) to those individuals not working in the BD industry (mean VF = $1.68 \pm 0.18 \times 10^{-6}$, $n = 25$, Figure 6). A similar analysis was not possible with the older workers in this study (age > 48 , range 55 to 65 years of age) because sufficient *HPRT* VF data on a matched group of external reference subjects for such a test was not available.

Discussion

Estimated levels of exposure to 1,3-butadiene in this Southeast Texas facility were low in comparison with previous studies [Ward et al., 1996; Ma et al., 2000; Ammenheuser et al., 2001; Ward et al., 2001a; Abdel-Rahman et al., 2001; Vodicka et al., 2004]. With one exception, estimated exposures were well below the OSHA PEL of 1,000 ppb, and most were below the technological limits of detection (2.5 ppb). It should be noted that the one subject with a high exposure level (1,683.5 ppb) has a commensurately high *HPRT* VF (18.9×10^{-6}). This is consistent with the results of our previous studies indicating that high levels of exposure BD, those in excess of 1,000 ppb, are significantly associated with high *HPRT* VFs [Ward et al., 1996; Ma et al., 2000; Ammenheuser et al., 2001; Ward et al., 2001a; Abdel-Rahman et al., 2001].

Consistent with the low level of BD exposure in these workers, there was no evidence of mutagenicity associated with exposure, as measured by the *HPRT* autoradiographic assay. Volunteers with no history of occupational exposure to BD had *HPRT* VFs comparable to gender- and age-matched (< 48 years) workers with no detectable exposure to BD in this facility. This indicates that there are no inherent differences between a sample of the general, healthy population and this group of younger workers, at least with regard to frequencies of *HPRT* mutant lymphocytes.

Our study indicates that the number of years employed in the BD industry (i.e. occupational longevity) is significantly associated with increased *HPRT* VFs. While age in years cannot be excluded as a contributing factor, age is not significantly associated with *HPRT* VFs in

this study. According to a study by Ammenheuser et al. [1994], the expected increase in *HPRT* VF attributable to age per year is approximately 0.04×10^{-6} . The older cohort of workers in the current study had an average age of 61 years with the oldest worker being 65 years of age. The average *HPRT* VF in this older cohort was $6.30 \pm 1.31 \times 10^{-6}$. Assuming that an age-related increase in *HPRT* VF follows the linear function reported in Ammenheuser et al. [1994], age alone would be expected to increase the *HPRT* VF from a conservative 2.55×10^{-6} in the youngest worker (age = 23) to approximately 4.07×10^{-6} in an average 61-year-old worker. This lends support to our conclusion that a factor (or factors) other than age alone is responsible for the increased *HPRT* VFs in those workers with a lengthy tenure in the BD industry. Importantly, age was not positively correlated with exposure (Pearson's $r^2 = -0.14$, $p < 0.46$) suggesting that current exposures are not responsible for this result. Therefore, this elevation in *HPRT* VF might be a function of past and chronic exposures of the older workers to substantially higher levels of BD. These past exposures might easily have been 2-3 orders of magnitude higher than those estimated in this study. A fraction of the T-lymphocytes used in the *HPRT* assay are memory T-cells, and there is evidence from a study by Demkowicz et al. [1996], that this subpopulation of T-cells can persist for years after viral stimulation and clonal expansion. Therefore, memory T-cells may well provide a signature of previous mutagenic BD exposures, resulting in an increased frequency of detected mutants in the older workers. It is also possible that some of these older workers have been exposed to mutagenic compounds other than BD through their industrial occupation or in the environment.

In conclusion, we found that current exposures to BD in this particular Southeast Texas facility are low and do not appear to pose a mutagenic risk, as estimated by the *HPRT* autoradiographic assay. Older workers, however, exhibited increased *HPRT* VFs, in comparison to younger workers, that could not be explained by age alone. This suggests that previous, chronic occupational exposures to higher levels of BD (i.e. previous OSHA PELs of 1,000-100,000 ppb prior to 1990) and/or exposures to some other mutagenic agent(s) are responsible for this result. We suggest that older workers in the synthetic rubber industry continue to be monitored and that future studies be designed to further investigate the possible mutagenic effects and potential adverse health outcomes associated with chronic, historical exposure to BD.

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Figure 1.

Levels of 1,3-butadiene (BD; in parts per billion, ppb) that workers ($n = 30$) were exposed to in different areas of the facility as determined by organic vapor monitors with a minimum detection limit of 2.5 ppb. Levels are presented as arithmetic averages $\pm$ standard error of the mean. Work sites were task areas designated by the facility and workers.

Figure 2. Distribution of age (in years) plotted against occupational longevity (in years). Thirty subjects were used for this plot. Occupational longevity was determined by the self-reported number of years working in the BD industry and specifically this southeast Texas facility.

Figure 3.

Linear relationship ($n = 30$), estimated using least squares regression, between BD exposure (in ppb) and the frequency of mutant T-lymphocytes as measured using the *HPRT* autoradiographic assay. Data were transformed prior to analysis (see Methods), but they are presented here as untransformed. A significant, positive relationship exists between the two ($p < 0.035$) but is heavily influenced by one individual with a high exposure and high *HPRT* variant frequency (VF).

Figure 4.

Linear relationship ($n = 29$), with the presumed outlier (see Fig. 3) removed, between BD exposure (in ppb) and the frequency of mutant T-lymphocytes. Data were transformed prior to analysis, but they are presented here as untransformed. No significant relationship exists between BD exposure and *HPRT* VFs in this analysis ($p < 0.241$).

Figure 5.

Linear relationship ($n = 29$) between occupational longevity (in years) and the frequency of mutant T-lymphocytes. *HPRT* VFs were transformed prior to analysis, but they are presented here as untransformed. A significant, positive relationship exists between occupational longevity and *HPRT* VFs ($p < 0.046$).

Figure 6. *HPRT* VFs in young workers ($n = 6$) and age- and gender-matched reference subjects ($n = 25$) with no history of working in the BD industry. A Student's t -test indicated there was no significant difference in *HPRT* VFs between these two groups ($p < 0.179$).