Induction and time-dependent accumulation of micronuclei in peripheral blood of transgenic p53+/− mice, Tg.AC (v-Ha-ras) and parental wild-type (C57BL/6 and FVB/N) mice exposed to benzene by inhalation

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In this study, we determined the induction and time-dependent accumulation of micronuclei in the peripheral blood of transgenic C57BL/6 p53 +/− mice (p53+/− mice), FVB/N Tg.AC v-Ha-ras mice (Tg.AC mice) and their isogenic parental strains, FVB/N and C57BL/6 following inhalation exposure to benzene. Our objective was to determine the impact of p53 heterozygosity in p53+/− mice and the v-Ha-ras transgene in Tg.AC mice on micronuclei induction following exposure to inhaled benzene. A flow cytometric technique that distinguishes micronucleated red blood cells (MN-RBC) from micronucleated reticulocytes (MN-RET) was used. Mice were exposed to 0, 100 or 200 p.p.m. benzene using three different exposure regimens that resulted in an equal weekly cumulative exposure (3000 p.p.m. × hours) to benzene: 100 p.p.m. for 6 h/day, 5 days/week; 100 p.p.m. for 10 h/day, 3 days/week; and 200 p.p.m. for 5 h/day, 3 days/week. Significant elevations of MN-RBC and MN-RET were observed from 1 week exposure in all of the benzene-exposed groups that increased in a time-dependent manner for up to 13 weeks exposure. Fewer MN-RBC and MN-RET were induced in the 200 p.p.m. benzene exposure group than in mice exposed to 100 p.p.m. The reduction in the frequency of MN-RBC in the 200 p.p.m. × 5 h benzene exposure group is probably due to metabolic saturation resulting in a lower bone marrow dose (concentration × time) than in the 100 p.p.m. exposure groups. No differences were observed in the frequency of MN-RBC or MN-RET in Tg.AC compared with the FVB/N isogenic controls. At certain time points the frequency of micronuclei was less in the heterozygous p53+/− mice than determined in the wild-type C57BL/6 isogenic parental strain. These results indicate that the heterozygous state in p53+/− mice, but not the v-Ha-ras transgene in Tg.AC mice can influence the induction of micronuclei by benzene.

Introduction

Transgenic mouse models that contain alterations of specific genes known to be involved in human cancers are being proposed as useful short-term carcinogenicity bioassays and as alternatives to the conventional chronic rodent bioassays (Tennant et al., 1995, 1996; Gulezian et al., 2000). Two transgenic mouse tumor models, one carrying an activated form of the ras oncogene v-Ha-ras (Tg.AC mice) and another with an inactivated copy (haplinsufficiency) of the p53 tumor suppressor gene (p53+/− mice), are being evaluated for their responses to a number of carcinogenic agents (Robinson, 1998). Preliminary findings of neoplastic responses of these mouse models to chemical carcinogens investigated by the National Toxicology Program (NTP) have been summarized (Eastin et al., 1998). Although limited to the few chemicals tested, these initial findings indicate that p53+/− mice were sensitive to genotoxic carcinogens and that the Tg.AC mice were sensitive to certain non-genotoxic carcinogens, each with an accelerated time-to-tumor onset (Tennant et al., 1996; Eastin et al., 1998).

In contrast to many genotoxic chemical carcinogens, benzene is typically inactive in most in vitro and in vivo mutagenicity bioassays but does produce chromosome aberrations and sister chromatid exchanges both in vivo and in vitro (Dean, 1985; Smith, 1996). Skin painting studies using benzene in Tg.AC mice induces an increased incidence of papillomas (Spalding et al., 2000). In p53+/− mice exposed to benzene by inhalation (100 p.p.m.), a high incidence (>80%) of thymic lymphomas is induced at 36 weeks exposure (Recio et al., 2000). Benzene-induced thymic lymphomas have a high incidence of chromosome 11 loss of heterozygosity, probably mediated by DNA strand break-induced genetic recombination (Boley et al., 2000).

The micronucleus assay is widely used to screen chemicals for genotoxic activity resulting from chromosome breaks or gains and losses in mice (Heddle, 1973; Schmid, 1975; Hayashi et al., 1994; Criswell et al., 1998). Chronic benzene exposure by inhalation is known to induce genotoxicity and hematotoxicity in the bone marrow that is dependent upon the exposure regimen used, sex and the specific strain of mouse (Barak et al., 1985; Luke et al., 1988a, 1988b; Tice et al., 1989). Although micronucleus formation in mice following inhalation exposure to benzene has been well described (Choy et al., 1985; Luke et al., 1988a, 1988b; Farris et al., 1996), the potential modification of this response by the p53 heterozygosity in p53+/− mice and the v-Ha-ras oncogene in Tg.AC is uncertain. Our goal in this study was to investigate the induction and accumulation of micronuclei in p53+/− mice and Tg.AC mice and their isogenic parental strains, C57BL/6 and FVB/N respectively, following chronic inhalation (33–38 weeks) exposure to benzene.

Materials and methods

Animals

C57BL/6, C57BL/6 p53+/− mice, FVB/N and FVB/N Tg AC, were obtained from Taconic Farms (Germantown, NY) at ~4–5 weeks of age, held for 1–3 weeks and acclimated to wire caging within the inhalation chamber 1 week prior to initiation of benzene exposure. At the initiation of benzene exposure mice ages ranged from 6 to 9 weeks. Water (reverse osmosis-treated) and commercially available rodent diet were available ad libidum, and feed exposed to benzene was discarded following each exposure period. Mice were

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kept in a reverse day–night cycle (lights on at 1 am, off at 1 pm); exposure took place during the light cycle. Each exposure or control group was housed in a separate 8 m³ inhalation chamber, and all mice were individually housed in stainless steel wire mesh cages. The institutional Animal Use and Care Committee of the Chemical Industry Institute of Toxicology (CIIT) approved all conditions and animal use.

Experimental design

This study used three benzene exposure groups: two at 100 ppm, one at 200 ppm, and one control unexposed group at 0 ppm. Start dates for each exposure group were staggered at intervals of 1–2 weeks apart and no exposures were conducted at the weekend. The control group and one group at 100 ppm benzene were placed in whole-body 8 m³ inhalation chambers for 5 days/week 6 h/day (Monday to Friday, M–F), while the other group at 100 ppm benzene was exposed based on an every-other-day schedule: 3 days/week (Monday, Wednesday, Thursday, Friday, MWF) 10 h/day. The group at 200 ppm was exposed based on the every-other-day schedule: 3 days/week 5 h/day (MWF). This schedule maintained all benzene-exposed groups at an equal total exposure level of 3000 p.p.m./h/day (Figure 1). To determine micronuclei in peripheral blood, animals (n = 3–4) were bled at exposure weeks 1, 5, 9, and 13, then after ~6 months exposure.

Blood collected for the final determination of micronuclei in mice was done over a single week for logistical considerations. Due to the staggered start dates for the exposed groups, this corresponded to weeks 33–37.

Inhalation exposures

Animals were exposed for up to 38 weeks in 8 m³ inhalation chambers to target benzene concentrations of 100 p.p.m. or 200 p.p.m. Benzene concentrations were generated by metering known amounts of liquid benzene into a heated j-tube. The benzene vaporized in the j-tube and was carried in a nitrogen stream to the HEPA-filtered 8 m³ chamber air supply where it was diluted to the target concentrations. The 8 m³ chamber air supply flowed at ~1800 l/min and was conditioned to ~72 °F and 50% relative humidity. The unexposed control chambers (0 ppm) operated under similar environmental conditions. The environmental conditions were monitored continuously; 30 min averages were recorded and printed daily.

Analysis of benzene exposures

Benzene exposure concentrations were measured with four calibrated infrared spectrophotometers (MIRAN 1A, The Foxboro Co., Foxboro, MA) with one spectrophotometer sampling each chamber. Voltages from the spectrophotometer corresponding to the benzene exposure concentration were transmitted to the Andover Infinity Building Automation System (Andover Controls Corp., Andover, MA), logged and printed in a daily report. The distribution of benzene was checked at nine locations in each of the exposure chambers prior to the initiation of exposure.

The average exposure concentrations (± standard deviation, SD) of benzene for the 100 p.p.m. M–F, 100 p.p.m. MWF and the 200 p.p.m. MWF exposure groups were 100 p.p.m. (±0.9), 100 p.p.m. (±0.7) and 200 p.p.m. (±0.6), respectively. Concentrations of benzene during distribution checks varied by <8%. The average temperatures ranged from 71.1 to 72.8 °F and the average relative humidity from 49 to 57% for the four exposure groups.

Micronucleus assay

The micronucleus assay was based on the procedures of Dertinger et al. (1996). Micronucleus frequencies were determined on three to four mice per time point and 10,000 cells were analyzed from each mouse. Sample preparation and micronucleus evaluation were performed as described in a beta-version test kit of MicroFlowPlus™ Mouse Micronucleus Assay Kit (Stratagene, La Jolla, CA). Approximately 50 μl of blood, collected from the retro-orbital plexus during isoflurane (IsoFlo™, Abbott Laboratories, Chicago, IL) anesthesia, was suspended in 300 μl Hank's balanced salt solution (Sigma Chemical H6648, St Louis, MI), with 50 U/ml heparin (Sigma Chemical H3393). The suspension was injected directly into 2 ml cold methanol (approximately −80°C), struck sharply to dissociate aggregated cells and stored at −80°C until analysis. To prepare cells for analysis, tubes were struck sharply several times when removed from the freezer, and 10 ml of bicarbonate-buffered saline (0.9% NaCl + 5.3 mM sodium bicarbonate) were added. The cells were isolated by centrifugation and stained according to the manufacturer’s recommendations. Cells were incubated in a working solution of fluorescein isothiocyanate (FITC) conjugated antibody and RNase and resuspended in propidium iodide (PI) for analysis.

Flow cytometric analysis of micronucleated red blood cells (MN-RBC) and micronucleated reticulocytes (MN-RET)

The flow cytometric analyses were carried out on a FACS Vantage flow cytometer (Becton Dickenson, San Jose, CA). The laser was tuned to provide 488 nm excitation with UV set at 20 mW as the regulation beam. The FL1 photomultiplier tube was used with a filter (DF 530/30) for the green (FITC) signal, and FL2 was used for the red (PI) signal using a 580 LP filter. Malaria-infected mouse blood was used as a reference standard for consistently defining micronucleus analysis windows, as well as establishing proper PMT voltages and compensation on a daily basis (Dertinger et al., 1999). Micronuclei were identified in PI positive cells, and reticulocytes (RET) were identified as CD71 (transferrin receptor)-FITC positive cells. A solution of 1% Cloroxy™ bleach with 50 mM NaOH in distilled water was passed through the sample line for ~1 min between each sample.

Statistics

Multivariate analysis of the MN-RBC, MN-RET and RET frequencies was used to determine whether there were global interactions between time points, groups and genotypes. As a global interaction was present between these parameters, data sets were individually evaluated with a univariate two-way analysis of variance (ANOVA) using contrasts of least square means to determine treatment effect within each time point. Statistical significance was determined at a P = 0.05 Bonferroni-corrected for the appropriate number of comparisons. The absolute numbers and percentages from the various parameters were presented as mean values ± SD.
Benzene-induced micronuclei in Tg.AC/p53 +/- mice

Fig. 2. (A) Total micronuclei in peripheral blood of C57BL/6 p53+/- and C57BL/6 p53+/- mice. The frequency of MN-RBCs increased with time up to 13 weeks. The p53+/- mice had fewer MN-RBCs compared with the C57BL/6 at the time points indicated. Error bars indicate SD. Closed symbols, p53+/+-; open symbols, p53+/-; open/closed squares, 0 p.p.m. (B) Total micronuclei in peripheral blood of FVB/N and Tg.AC mice. The frequency of MN-RBCs increased among all groups with time for up to 13 weeks. MN was significantly elevated in all benzene groups in all weeks compared with controls. Error bars indicate SD. Closed symbols, FVB/n; open symbols, Tg.AC; open/closed squares, 0 p.p.m. *P < 0.01.

Results

Total micronuclei

After 1 week benzene exposure, total MN-RBC frequencies had increased in all exposure groups and in all genotypes compared with the unexposed 0 p.p.m. controls. There was a cumulative increase in MN-RBC frequency with time up to 13 weeks exposure. At ~6 months exposure, there was no increased MN-RBC frequency above the level determined at 13 weeks (number of weeks at the 6 month time point differ because of staggered start dates). All genotypes had fewer MN-RBC when exposed to 200 p.p.m. MWF as compared with 100 p.p.m. MWF or M-F (Figure 2A and B). There was no difference in the frequency of MN-RBC between the wild-type FVB/N and Tg.AC mice. Among the benzene-exposed groups, p53+/- mice had significantly fewer MN-RBC than the isogenic C57BL/6 controls at one or more time points (Figure 2B).

Micronucleated reticulocytes

MN-RET were significantly increased with time by benzene exposure above the unexposed 0 p.p.m. controls in all genotypes. Technical errors at the week 5 time points in the 100 p.p.m. MWF exposure groups prevented accurate determination of MN-RET. At week 1 in the 200 p.p.m. MWF group, the low MN-RET results may reflect recent cytotoxic impact of benzene on the bone marrow since the cells were collected following that day’s exposure. MN-RET occurred at a higher frequency in the FVB/N background mice (wild-type and Tg.AC mice) as compared with the C57BL/6 background mice (wild-type and p53+/- mice) (Figure 3A and B).

Reticulocytes

In the FVB/N background mice (wild-type and Tg.AC mice), total RET (CD-71 positive cells) were decreased in the 100 p.p.m. M-F group compared with unexposed controls. In the C57BL/6 background mice (wild-type and p53+/- mice) there was no difference in total RET in benzene-exposed mice compared with unexposed controls. Total RET were increased above controls in all genotypes exposed to benzene 100 p.p.m. MWF (Figure 4A and B). Tg.AC mice had a higher frequency of RET in the 200 MWF group compared with the FVB/N animals in the same exposure group (Figure 4B).

Discussion

This study is based on the analysis of peripheral blood to detect micronucleus formation following inhalation exposure to benzene in p53+/- mice, Tg.AC mice and their isogenic parental strains (C57BL/6 and FVB/n, respectively). In addition, to investigate the impact of daily versus every-other-day exposures, three different exposure protocols that exposed the mice to the same weekly exposure of 3000 p.p.m.×h/week were used. The mice in the 100 p.p.m. exposure groups, either
100 p.p.m. M–F or MWF, were exposed to benzene for 30 h/week, while the mice in the 200 p.p.m. exposure group were exposed to benzene for 15 h/week.

Benzene exposure was associated with an increase in MN-RBC frequency in a time-dependent manner for up to 13 weeks exposure. At some point between 13 weeks and ~6 months, the micronucleus frequency leveled off. The frequency of MN-RBC reported here is similar to that determined previously at 100 p.p.m. benzene exposure for up to 8–14 weeks (Luke et al., 1988a, 1988b; Farris et al., 1996).

In this study, exposure of mice to 200 p.p.m. benzene for 5 h/day MWF resulted in fewer MN-RBC than exposure to 100 p.p.m. in all four genotypes examined. These results are in contrast to the increased frequency at 200 p.p.m. compared with 100 p.p.m. benzene reported by Farris et al. (1996), in which there was a concentration-dependent increase in MN-RBC. However, in that study, mice were exposed to 100 or 200 p.p.m. benzene for 6 h/day M–F. These findings suggest that the length of exposure, as well as the pattern of exposure (intermittent versus daily), are more critical to the accumulation of chromosomal damage than the actual benzene concentration.

Benzene must first be metabolized to form the major toxic metabolites hydroquinone, catechol and trans-trans-muconic acid so as to be hematotoxic and/or carcinogenic (Smith, 1996). In the present study, irrespective of the strain, mice exposed to 100 p.p.m. had a greater frequency of benzene-induced micronuclei than those exposed to 200 p.p.m. One possible explanation for the reduced MN-RBC frequency in the 200 p.p.m. group compared with the 100 p.p.m. groups is that the metabolism of benzene at 100 p.p.m. is saturated and that exposure levels >100 p.p.m. do not significantly increase the levels of toxic metabolites. In this case, the mice in the 200 p.p.m. groups would be exposed to a similar total amount of toxic metabolites but for a shorter exposure time (15 h/week), compared with either of the 100 p.p.m. exposure groups (30 h/week). This suggests that the bone marrow dose (concentration×time) of benzene toxic metabolites would be less in the 200 p.p.m., 5 h/day, MWF exposure group than in the 100 p.p.m. exposure groups. These data indicate that at levels of benzene where metabolism is saturated, the length of benzene exposure is more significant than the exposure concentration. Physiologically-based pharmacokinetic modeling of internal dosimetry is in progress to assess levels of hydroquinone (a clastogenic metabolite of benzene) in the bone marrow for each exposure regimen (Cole et al., 2000). These data are needed to define the role of internal dose in the micronuclei responses observed for each exposure regimen used in the present study.

The observation that short-duration, high-level exposure is less toxic than lower, long-term exposure has been previously reported: inhalation of 3000 p.p.m. benzene for 8 days was less damaging than 300 p.p.m. for 80 days (Cronkite et al., 1989).

Haploinsufficiency of p53 resulted in an equivalent or reduced frequency of micronuclei compared with the C57BL/
6 isotype parental controls. This finding was unexpected. Due to the role of p53 in genotoxic stress response, we originally hypothesized that the heterozygous state of p53 in the p53+/− mouse strain would allow for increased survival of chromosome-damaged erythrocyte precursor cells, resulting in an increase in circulating micronucleated erythrocytes. The possibility of compensatory up-regulation of DNA damage response networks, DNA repair or altered regulation of apoptosis is currently under investigation in our laboratory. Another possibility for the reduced MN-RBC frequency in the p53+/− animals is small differences in benzene metabolism rates between the genetically altered p53 model and the parental strain. However, the similar reticulocyte frequency in the p53+/− and C57BL/6 mice in all exposure groups would suggest that the bone marrow dynamics of erythrocyte production during benzene exposure are similar in these two strains. Although statistically significant, the differences in MN-RBC frequency may not be biologically significant. However, we can conclude that the p53+/− mouse is not more sensitive to benzene genotoxicity, as assessed by MN-RBC formation, than in the C57BL/6 wild-type. This finding is consistent with previous reports that demonstrated no differences in mutation frequency or spectrum in the p53+/+ and p53−/− genotypes in any of the tissues examined (Buettner et al., 1997).

The frequency of MN-RETs was reduced in all genotypes at a single time point: 1 week 200 p.p.m. MWF. The week 1 data for the 200 p.p.m. MWF group may have resulted from collecting blood on the same day as exposure. For logistical reasons the other collection time points for the MWF groups were on non-exposure days (Tuesday or Thursday). Since MN-RET represent recently formed micronuclei, the cytotoxicity of 200 p.p.m. benzene would reduce the formation and release increase in circulating micronucleated erythrocytes. The possibility of compensatory up-regulation of DNA damage response networks, DNA repair or altered regulation of apoptosis is currently under investigation in our laboratory. There was an increased frequency of RET in the 100 p.p.m. MWF group as compared with the 100 p.p.m. M−F group among all mouse genotypes examined. We hypothesize that this reflects recovery in the bone marrow between exposures in the intermittent MWF exposure. Why the 200 p.p.m. MWF group did not have the same increase in RET is uncertain, but this may be due to reduced toxicity relative to the 100 p.p.m. MWF group.

The increased frequency of RET in the Tg.AC mice as compared with the FVB/N parental strain in the 200 p.p.m. MWF group may reflect activation of the transgene in the spleens of these animals and a resulting proliferation of erythroid precursor cells. In mice, most erythroid expansion as a result of destruction-related anemia occurs in the spleen (Broudy et al., 1996). Since the v-Ha-ras transgene is normally expressed in the bone marrow of the Tg.AC mice (Trempus et al., 1998) and the frequency of RET in the control (0 p.p.m. benzene) Tg.AC animals was not significantly different to the
FVB/N isotype controls, whether increased expression of the transgene in the hematopoietic tissue would increase the frequency of circulating RET is uncertain. There was a reported association, however, between the expression of the v-Ha-ras transgene in the spleens of Tg.AC mice with oncogenic ras protein-induced proliferation of erythroidopoietic cells and the development of erythroleukemia (Trempus et al., 1998).

The data represented here are from high-level exposures to benzene (100 or 200 p.p.m.). Subtle differences in micronucleus formation due to the contribution of genetic alterations in these mice may be more significant at lower exposure concentrations. A study defining the shape of the dose–response curve for MN formed using these animals would be useful to determine dose-dependent differences in response between strains and between the wild-type and transgenic mice.

In summary, we demonstrated that high-level discontinuous exposure to 200 p.p.m. benzene resulted in fewer MN–RBC than 100 p.p.m. benzene at equal cumulative weekly exposures. The reduction in the frequency of MN–RBC in the 200 p.p.m.×5 h benzene exposure group is probably due to metabolic saturation resulting in a lower bone marrow dose (concentration×time) than in the 100 p.p.m. exposure groups. No differences were observed in the frequency of MN–RBC or MN–RET in Tg.AC compared with the FVB/N isogenic controls. At certain time points, the frequency of micronuclei was less in the heterozygous p53+/− mice than determined in wild-type C57BL/6 isogenic parental strain. These results indicate that the heterozygous state in p53+/− mice, but not the v-Ha-ras transgene in Tg.AC mice can influence the induction of micronuclei by benzene.

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