Lack of Adaptive Response for Micronucleus Induction in Bone Marrow of C57BL/6 and BALB/c Mice Following Low-Dose Gamma Radiation

Proposed running title: Lack of Radio-adaptive Response in Mouse Bone Marrow

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Abstract

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Enhanced cellular DNA repair efficiency and suppression of genomic instability have been proposed as mechanisms underlying the reduction of spontaneous and radiation-induced carcinogenesis in laboratory mice following low-dose radiation exposures. Our previous studies revealed that low-dose irradiation does not generate radio-adpatation by lowering radiation-induced DNA double-strand breaks, as indicated by the number of micronuclei in mouse spleen lymphocytes. These results were extended here by measuring dose and time dependent cytogenetic damage and proliferation changes in bone marrow erythrocyte populations of C57BL/6 and BALB/c mice. In C57BL/6 mice, the induction of micronucleated polychromatic erythrocytes (MN-PCE) was observed at doses of 100 mGy and greater, and supression of erythroblast maturation occurred at doses of 500 mGy and higher. A linear dose response relationship for bone marrow MN-PCE frequencies 24 to 28 hours post-irradiation in C57BL/6

mice was established for doses between 100 mGy and 1 Gy, with departure from linearity at doses ≥ 1Gy. BALB/c mice exhibited increased MN-PCE frequencies following a 20 mGy radiation exposure but did not exhibit radio-sensitivity for MN-PCE frequencies following 2 Gy exposure. Radio-adaptation of bone marrow erythrocytes was not observed in either strain of mice exposed to low-dose priming irradiation (20, 100 mGy or 20 mGy x 4) administered at various times prior to acute 2 Gy irradiation, confirming the lack of radio-adaptive response for induction of cytogenetic damage or suppression or erythrocyte proliferation/maturation in bone marrow of these mouse strains.

Introduction

The classic Linear-No-Threshold (LNT) paradigm (*1*, *2*) asserts that the biological effects of ionizing radiation result from direct targeted damage to nuclear DNA. Stochastic risk estimates from low-dose, low dose-rate exposures (i.e. < 100 mSv) are derived by extrapolation from data obtained for high-dose radiation exposures, with cancer risk viewed as being directly proportional to radiation dose and the absence of a threshold dose for increased risk of cancer. However, LNT postulates are challenged by accumulating experimental evidence that non-(DNA)-targeted effects (NTEs), including radio-adaptive responses, bystander effects, genomic instability, low dose hyper-sensitivity, abscopal effects, and delayed reproductive death, play a crucial role in determining health effects following low dose radiation exposure (*3-10*).

Radio-adaptive responses are a type of NTE manifested as a decrease in either acute detrimental radiation-induced or spontaneous biological health effects following low dose radiation exposure (4, 5, 11-13). Radio-adaptation has been reported for a variety of biological endpoints related to cellular toxicity and genotoxicity *in vitro* (*11*, *13*, *20*, *66*, *74*). However, in relation to human radiation risk estimates, the most valuable experimental studies are those using mouse *in vivo* models. Increased lifespan, enhanced survival following high-dose radiation, decreased spontaneous and radiation-induced carcinogenic potential, and suppression of tumour metastases have been identified as health risks in mice that are modified following chronic or acute low dose radiation (*14-21*). Remarkably, earlier *in vivo* studies carried out in our laboratory showed that low dose gamma-radiation renders mice more resistant to radiation-induced or spontaneous age-related tumorigenesis expressed as increased tumor latency (*14*, *15*, *23*). Enhanced cellular DNA double-strand break (DSB) repair and diminished genomic instability, potentially involving the *Trp53* tumour suppressor gene pathway, have been proposed as mechanisms that govern radio-adaptive responses and suppress carcinogenesis following low-dose ionizing radiation exposures (*15*, *22-28*).

Although radio-adaptive responses are broadly acknowledged, they are by no means universal, and are influenced by a range of factors including radiation quality, dose and dose rate, cell type, tissue type and genetic makeup (*12*, *29*). We previously demonstrated a lack of radio-adaptive response in mouse splenocytes for endpoints related to cytogenetic damage and DNA DSB repair. Mice exposed to low-dose gamma radiation prior to acute high-dose gamma radiation showed no reduction in the numbers of splencoyte DSBs, as measured by γH2AX foci (C57BL/6 mice) (30), or level of clastogenic damage, as measured by the Cytokinesis Block Micronucleus (CBMN) assay (C57BL/6 and BALB/c mice) (*31*). In the present study, analysis was extended to include potential radio-adaptive effects of low-dose gamma radiation on micronuclei (MN) frequencies in bone marrow erythrocytes of C57BL/6 and BALB/c mice.

The *in vivo* rodent micronucleus (MN) assay measures clastogenic and aneugenic damage in immature erythrocytes (polychromatic erythrocytes, or PCEs) and mature erythrocytes (normochromatic erythrocytes, or NCEs) of rodent bone marrow or peripheral blood (*32-35*). In this study, bone marrow MN-PCE frequencies were surveyed following radiation exposures. The polychromatic/normochromatic (P/N) ratio was monitored in parallel as a measure of the erythrocyte proliferation or maturation index. Bone marrow samples in this study were derived from the same mice used to assess CBMN frequencies and cell survival endpoints in spleen tissue in our previous research (*31*), allowing direct comparison of the cytogenetic and cytotoxic effects of low-dose gamma radiation and potential radio-adaptive responses in multiple tissues of radio-resistant and radio-sensitive mouse strains.

Methods

Mice

Female C57BL/6J and BALB/cJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in the specific pathogen-free Biological Research Facility (BRF) at Canadian Nuclear Laboratories (CNL; Chalk River, Canada). Mice were housed in either single or duplex cages (Thoren Caging Systems Inc., Hazelton, PA) and fed ad libitum with Charles River Rodent Chow (Frederick, MD). Experimental protocols received prior approval from CNL's Animal Care Committee and were performed in accordance with the guidelines of the Canadian Council on Animal Care. Experiments were conducted using adult female mice between two to five months of age. Mice were allowed to acclimatize in the BRF for at least two weeks prior to irradiation or sham irradiation. Mice were maintained in the environment with constant temperature (23ºC), air ventilation and 12 h light/dark cycle. Mice were routinely tested for pathogens to confirm their pathogen-free status.

Irradiations

An open beam 60Co-$\square$ source (GammaBeam 150, Hopewell Designs Inc., Alpharetta, GA)) was used to deliver whole body low-dose, low-dose-rate priming doses (20 mGy and 100 mGy at dose rates of 0.5 mGy/min and 10 mGy/min, respectively) and intermediate doses (500 mGy at a dose rate of 10 mGy/min) to mice. For exposures, unrestrained mice in plastic cages were exposed to the beam source. For whole body acute in vivo 1 and 2 Gy irradiations, unrestrained mice individually housed in irradiation vessels were exposed in an enclosed 60Co-$\square$ source irradiator (GammaCell 220, MDS NordionTM Inc., Ottawa, Canada) at dose rates of ~150 mGy/min to 174 mGy/min.

Bone Marrow MN-PCE Frequency and P/N Ratio Determination

Mice were euthanized by cervical dislocation. Cytogenetic analysis of MN in bone marrow PCEs was conducted using standardized procedures (*33, 36, 37*). The femoral bones of each mouse were excised and the bone marrow was aspirated by flushing of the femoral bones with 100 μL of fetal bovine serum (FBS; Sigma-Aldrich®, St. Louis, MO) using a 27 gauge needle and a 1 mL syringe. Marrow cells from each mouse were flushed into a single well of a 96-well microplate. The bone marrow cells were gently mixed and a drop of the resulting cell suspension was then smeared onto a pre-cleaned glass slide to create a monolayer of bone marrow cells. Cell preparations were dried overnight, fixed in 100% methanol (Fisher Scientific, Pittsburgh, PA) and stored at room temperature prior to staining and analysis. Cells were stained for 15 seconds in Acridine Orange (25 μg/mL; Fisher Scientific) dissolved in Gurr Buffer, pH 6.8. Slides were washed three times in Gurr Buffer. Two slides were prepared from each animal. Stained cells were analyzed by fluorescence microscopy (using a 63x oil objective lens) for determination of MN-PCE frequency and differentiation of immature PCEs and mature NCEs for calculation of P/N ratio (PCE/PCE+NCE). One thousand PCEs were scored on each slide (two thousand total PCE per animal) to calculate MN frequency. MN-PCE frequencies were calculated as the total number of MN scored/1000 PCEs x 100 and reported as MN-PCE % (mean pooled value for all mice in treatment cohort ± standard error). Three hundred erythrocytes per slide (six hundred total cells per animal) were scored for determination of the the P/N ratio, calculated as (PCE/PCE+NCE) and reported as P/N (mean pooled value for all mice in treatment cohort ± standard error).

Statistical analysis

For each experimental or control animal group, statistical outliers identified using Grubb's test (significance level 0.05) were excluded from statistical analysis. Statistical comparison of treatment groups was carried out using the unpaired, two-tailed Student's t-test.

Results

Experimental Design and Rationale

We initially characterized dose-response and time-course relationships for MN-PCE frequencies and P/N ratios in mouse bone marrow to i) validate the sensitivity of the assay and ii) choose a proper time for endpoint measurements following a challenging high-dose exposure in the radio-adaptive response experiments. Thus, our study comprised two parts, one part examining dose-responses and kinetics for MN-PCE frequencies and P/N ratios, and a second part investigating radio-adaptive responses using conditions determined in the first part of the study. Low-dose gamma radiation, given alone or as a priming dose prior to a subsequent acute challenging dose, was delivered to either mice or cells using similar dose regimes to those previously used in our laboratory to demonstrate modulation of tumourigenesis in mice (*14, 22*). The time intervals of 4 to 24 hours between priming and challenge doses used in this study were also based on our previous mouse tumourigenesis studies. An overview of the experimental design is presented in Figure 1.

Dose Response for Bone Marrow MN-PCE Frequency and P/N Ratio

The dose-response relationship for C57BL/6 mice was investigated to determine the effects of a range of low to high acute doses of ionizing radiation on the induction of short-term (i.e. 24 to 28 hours post-irradiation) clastogenic damage in PCEs. The mean endogenous MN-PCE frequency measured in un-irradiated C57BL/6 control mice was 0.46 ± 0.045 % (Figure 2A). MN-PCE frequencies did not differ significantly between un-irradiated control and 20 mGy-irradiated animals (Figure 2A). MN-PCE frequencies were increased 2.2, 5.0, 8.7 and 7.3-fold following 100 mGy, 500 mGy, 1 Gy and 2 Gy irradiations, respectively, compared to un-irradiated control animals, with p values < 0.0005 for all irradiated cohorts versus un-irradiated control values (Figure 2A). Maximum MN-PCE frequencies were observed following 1 and 2 Gy radiation exposures (3.98 ± 0.24 % and 3.35 ± 0.21 %, respectively) with no statistical significance between the MN-PCE frequencies at these two doses, indicative of a dose threshold between 500 mGy and 1 Gy for the induction of MN in PCEs. The shape of the dose response curve for excess MN (i.e. above spontaneous levels) between 100 mGy and 1 Gy was best described as by a linear fit (not shown); using regression and correlation analyses the dose-response equation was obtained as follows: $F_{excess\ MN\text{-}PCE\ (\%)} = 3.15D + 0.33$, Rt= 0.99; where D = radiation dose in Gy. Corresponding bone marrow P/N ratios of irradiated C57BL/6 mice were measured to monitor the dose response relationship for suppression of erythrocyte turnover (progenitor proliferation and maturation). The mean endogenous P/N ratio measured in un-irradiated control mice was 0.68 ± 0.027 (Figure 2B). No significant differences from un-irradiated controls animals occurred at doses ≤ 500 mGy. Depression of the P/N ratio by 1.4-fold (p value 0.006) and 1.7-fold (p value < 0.0005) ensued following 1 and 2 Gy irradiations, respectively, compared to un-irradiated control animals (Figure 2B).

MN-PCE frequencies and P/N ratios were also examined for bone marrow of BALB/c mice exposed to acute radiation doses of 20 mGy and 2 Gy (28 and 24 hours post-radiation, respectively). Background control bone marrow MN-PCE frequencies (0.41 ± 0.071 % for BALB/c mice; Figure 2A) and P/N ratios (0.54 ± 0.039 for BALB/C mice; Figure 2B) were not statistically different between the two mouse strains. In contrast to C57BL/6 mice, MN-PCE frequencies were increased 1.7-fold in BALB/c mice irradiated with a single 20 mGy radiation dose in comparison with un-irradiated control animals (Figure 2A), and this increase was of marginal statistical significance (p = 0.042). The P/N ratio in BALB/c bone marrow following 20 mGy irradiation was not statistically different from the control value (Figure 2B). The MN-PCE frequency was increased 7.9-fold (p value < 0.0005 in comparison to un-irradiated mice) in BALB/c mice exposed to 2 Gy radiation, similar in magnitude to the induction of DNA damage observed for C57BL/6 mice following 2 Gy irradiation (Figure 2A). The P/N ratio of BALB/c mice was decreased 1.9-fold in comparison to the endogenous control value following irradiation with 2 Gy (Figure 2B).

Kinetics of Bone Marrow MN-PCE Induction and P/N Ratio Alteration Following 2 Gy Irradiation

Time course experiments were conducted with C57BL/6 mice and a limited number of BALB/c mice to monitor the kinetics of the formation and clearance of MN-PCE and modification of P/N ratios in bone marrow following an acute high dose radiation. Mice were exposed to an acute 2 Gy radiation dose and monitored 18 to 72 hours post-irradiation. Data for MN-PCE frequencies and P/N ratios are shown in Figure 3. MN-PCE frequencies following the 2 Gy radiation dose were highest in C57BL/6 mice 48 hours post-irradiation (9.4-fold above the endogenous control value) and began to decline steeply between 54 and 72 hours post-irradiation (Figure 3A). P/N ratios following 2 Gy irradiation of C57BL/6 mice reached a nadir between 48 and 54 hours post-irradiation and began to recover by 72 hours post-irradiation (Figure 3B) in the same time frame as PCEs with MN began to be cleared from bone marrow of irradiated mice (Figure 3A). The kinetic profile of MN increase in PCEs of BALB/c mice post-irradiation was similar to that of C57BL/6 mice, with the highest MN-PCE frequencies present 54 hours post-irradiation (a 9.2-fold increase above the endogenous frequency) and a steep decline occurring between 54 and 72 hours post-irradiation (Figure 3A). The maximal MN-PCE frequency observed in BALB/c mice at 54 hours post-irradiation (3.8 ± 0.025 %) was similar to the maximal MN-PCE frequencies observed for C57BL/6 mice at 48 hours post-irradiation (4.3 ± 0.225 %). Post-radiation temporal changes in P/N ratios in BALB/c mice were similar (although somewhat lower at most time points) to those found for C57BL/6 mice (Figure 3B).

Radio-adaptive Response for Bone Marrow MN-PCE Frequency and P/N Ratio

Potential radio-adaptive responses occurring *in vivo* in bone marrow of C57BL/6 mice for modulation of MN-PCE frequencies and P/N ratios following acute high-dose radiation were assessed. In the first set of experiments conducted with C57BL/6 mice, a potential radio-adaptive priming dose (20 mGy irradiation with dose rate 0.5 mGy/min or 100 mGy with dose rate 10 mGy/min) was delivered as a single acute dose 4 hours or 24 hours prior to the 2 Gy challenge dose, or as three separate 20 mGy doses (for a total dose of 60 mGy) six, four and two days prior to a 2 Gy challenge dose. MN-PCE frequencies (Figure 4A) and P/N ratios (Figure 4B) were not altered in C57BL/6 mice receiving any of the priming dose regimes tested in comparison to mice receiving the 2 Gy dose only. A single or multiple 20 mGy priming dose(s) was also given to BALB/c mice before a 2 Gy challenge dose to examine potential radio-adaptive responses in the bone marrow of this mouse strain. As observed for C57BL/6 mice, none of the priming dose regimes for the 20 mGy priming dose tested significantly affected MN-PCE frequencies (Figure 4B) or P/N ratios (Table 2) following acute challenge irradiation.

It was feasible to assume that a potential radio-adaptive response to the priming exposures may have been masked by perturbations in the kinetics of 2 Gy - induced MN-PCE frequencies, if such perturbations had occurred. Therefore, in the second set of experiments, a time course spanning 18 to 72 hours post-radiation was conducted with C57BL/6 mice to monitor MN-PCE frequencies and P/N ratios in mice that received a 20 mGy priming dose (delivered 24 hours prior to the challenge dose) in comparison to mice that received the 2 Gy dose alone. In contrast to a radio-adaptive effect, a modest synergistic effect was observed in mice that received a 20

mGy priming dose, with a trend towards modestly increased MN frequencies (1.1 to 1.2-fold in the first 30 hours post-radiation, Figure 5A). Statistical significance between the two experimental cohorts was observed only for the 18 hour time point. No differences in MN-PCE and P/N values were witnessed between mice with and without the priming irradiation between 48 hours and 72 hours following 2 Gy irradiation (Figure 5A and Figure 5B). A trend for a steeper decline in P/N ratio for mice that received a 20 mGy priming dose in relation to those that received only 2 Gy radiation was observed for time points up to 48 hours post-radiation (Figure 5B); however, no statistically significant differences were found for these time points.

Discussion

Radio-adaptive responses increase lifespan and suppress carcinogenesis in animal models, identifying these NTEs as an important consequence of low-dose radiation exposure. However, adaptive responses are highly variable, influenced not only be radiation quality, dose and dose rate, but also varying across cell types, tissues, systems, organisms and individuals (*8, 29, 38, 39*). Further investigation is warranted to define the biological conditions under which radio-adaptive responses do and do not prevail, and to determine underlying associated molecular processes. A multiple endpoint, multiple tissue approach for examining the biological effects of low-dose gamma radiation exposure, including potential radio-adaptive responses, was undertaken in our study. We previously demonstrated a lack of radio-adaptive response in splenocytes of C57BL/6 and BALB/c mice for endpoints related to cytogenetic damage and DNA DSB repair (*30, 31*) and confirmed higher frequencies of radiation-induced MN in splenocytes of BALB/c mice in comparison to C57BL/6 mice (*31*). In this follow-up study, bone marrow tissues from the same mice exposed to various irradiation regimes, including the ones allowing us to testfor radio-adaptive responses, were analyzed to measure the dose response and kinetic profile of MN-PCE induction and depression of erythroblast proliferation/maturation index and to examine cells for the manifestation of radio-adaptive responses in either of these endpoints.

The bone marrow MN-PCE assay measures DNA damage arising from clastogenic or aneugenic insult incurred in progenitor erythroblasts. MN are measured in enucleated daughter PCEs originating from erthythroblasts containing MN that mature into MN-PCEs following nuclear expulsion (*40-42*). Increased bone marrow MN-PCE frequencies are indicative of MN formed in immature, newly formed erythrocytes, during the final cellular divisions, typically from damage incurred within 24 to 48 hours from the sampling time (*32-35*). The frequency of MN-PCEs becomes reduced as the population of marrow PCEs with cytotoxic DNA damage is diluted by the division and maturation of undamaged progenitors. The bone marrow P/N ratio reveals cellular turnover of PCEs and NCEs following short-term cellular damage and recovery post-treatment by replacement of erythrocytes in the marrow with undamaged progenitor cells. The majority of MN present in cells following high-dose radiation exposure are extra-nuclear DNA fragments arising from acentric chromosome fragments originating from unrepaired or misrepaired DSBs (*43*). Previously published studies examining the dose response relationship for acute low-LET ionizing radiation (gamma and X-Rays) and MN-PCEs in bone marrow of laboratory mice support a linear or linear-quadratic function for exposures to low to moderate doses of ionizing radiation (e.g. 0.1 to 0.5 Gy) (*44-50*).

Our measured value of 0.46 % for MN-PCE in bone marrow of un-irradiated adult female C57BL/6 mice was somewhat lower than previously reported results for spontaneous MN levels in marrow of inbred laboratory mice, which are typically reported as between 2 to 3 % (*41*, *51*, *52*). However, assay method (i.e. automated detection by flow cytometry versus manual microscopic scoring), sex, variation between individual mice and age can affect reported outcomes. Our dose response data indicates that a 20 mGy exposure is not sufficiently damaging to increase MN-PCE frequencies in C57BL/6 mice above baseline values. A linear dose response relationship for the induction of C57BL/6 mouse bone marrow MN-PCEs was supported by our experimental data for doses between 100 mGy and 1 Gy. Departure from linearity was observed at doses between 1 and 2 Gy, with saturation observed at doses $\geq$ 1 Gy. This departure from linearity at high doses may be due to a delay in the sampling time required for the detection of the maximum MN-PCE frequency at these doses (as evidenced for the 2 Gy acute radiation exposure kinetic analysis, described below). Acute high doses of radiation $\geq$500 mGy had a cytotoxic effect on bone marrow erythroblasts of C57BL/6 mice, without an observed threshold of maximal effect for doses up to 2 Gy. The 100 mGy radiation exposure, while increasing MN-PCE values in C57BL/6 mice, did not have a cytotoxic effect on bone marrow erythrocyte proliferation, consistent with previous observations (*53*).

In laboratory mice following acute ionizing radiation exposures at doses that do not delay erythroid cell cycle and maturation processes (i.e. doses of $\leq$ 0.5 Gy low-LET radiation), an increase in the frequency of MN-PCEs in bone marrow becomes apparent 5 to 16 hours post-exposure, with the peak of maximum damage occurring 24 to 48 hours following exposure (*44*, *45*, *53-55*). More damaging radiations (> 1Gy) have been shown to prolong the erythroblast cell cycle and, therefore, delay the temporal appearance of radiation-induced MN-PCE in the bone marrow (*44*, *45, 54)*. The loss of linearity at high doses is ascribed to cell cycle effects and under-representation of the cohort of damaged cells in the PCE pool present at the time of harvesting, potentially due to apoptosis of heavily damaged erythroid precursors (*44*, *45*, *48*, *53*, *54*, *56*). In our temporal analysis of radiation-induced DNA damage following a single 2 Gy acute radiation exposure, bone marrow MN-PCE frequencies were highest in C57BL/6 mice 48 hours post-irradiation and began to decline steeply between 54 and 72 hours post-irradiation. Since kinetic analysis was not performed for lower dose exposures, it is not clear whether the maximum peak at 48 hours post-irradiation is delayed by perturbation of the cell cycle, but this is a likely scenario since P/N ratios were depressed as early as 18 hours post-irradiation and did not start to rise again until 72 hours post-irradiation

C57BL/6 mice are considered radio-resistant relative to other strains (*57*, *58*). Radio-sensitive BALB/c mice are less efficient in DSB rejoining due to a polymorphism in the gene encoding DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (*59*, *60*). BALB/c mice are susceptible to developing solid tumors following acute radiation exposures and exhibit increased radiation-induced DNA damage and genomic instability relative to C57BL/6 mice and other strains (*57*, *58*, *61-66*). Splenocyte MN frequencies were found to be significantly higher in BALB/c mice than for C57BL/6 mice following *in vivo* or *in vitro* 2 Gy gamma irradiation in our previous analysis (*31*). Our current study examined the effects of the upper and lower dose ranges (i.e. 20 mGy and 2 Gy) on bone marrow MN-PCE frequencies and P/N ratios of BALB/c mice at 24 to 28 hours post-exposure. MN-PCE frequencies in BALB/c mice were similar to those of C57BL/6 mice following 2 Gy exposure, indicating that BALB/c mouse bone marrow cells were not radio-sensitive in comparison to C57BL/6 mice at this dose. The bone marrow P/N ratios were also similar in the two strains (i.e. a 50 % to 60 % decrease from control values).

Unlike the situation for C57BL/6 mice, where the 20 mGy radiation exposure had no effect on MN-PCE frequencies, a modest but significant increase (1.7-fold) above the spontaneous MN-PCE frequency was observed following 20 mGy irradiation of BALB/c mice, although the P/N ratio of BALB/c mice was not affected. Our data indicate a potentially lower dose threshold for the induction of cytogenetic damage in BALB/c bone marrow erythrocytes than in those of C57BL/6 mice. However, a complete dose response and kinetic induction profile for MN-PCE for intermediate doses of radiation in BALB/c mice is required to fully address strain differences for the bone marrow MN-PCE assay. The kinetic profiles of MN-PCE induction and bone marrow P/N ratio depression in irradiated BALB/c mice were similar to C57BL/6 mice, with MN-PCE frequencies reaching a maximal level at 54 hours and declining between 54 and 72 hours post-irradiation. For both strains of mice, turnover of MN-PCEs was much more rapid than that previously observed for spleen cells (*31*), reflecting the high proliferative index of bone marrow progenitor cells.

Mitigation of high-dose acute radiation-induced DNA damage (reduced frequencies of micronucleated erythrocytes or chromosome/chromatid cytogenetic aberrations) has been documented in bone marrow cells of mice exposed to chronic low-dose rate (*67*) or acute low-dose low-LET gamma or X-ray radiation (*68-70*). Cytogenetic radio-adaptive responses have also been described in bone marrow cells following cross-adaptation experiments in which mice were exposed to non-ionizing radiofrequency (RF) fields (*71*, *72*) or other radio-protective compounds such as melanin (*73*) prior to exposure to acute challenging doses of radiation.

In this study, we examined potential radio-adaptive responses for reduction of radiation-induced cytogenetic damage or protection from radiation-induced suppression of the P/N ratio in bone marrow of C57BL/6 and BALB/c mice. Priming doses of radiation (either single or multiple 20 mGy at a dose rate 0.5 mGy/min or 100 mGy at a dose rate of 10 mGy/min) were delivered to mice before an acute 2 Gy challenge dose. Kinetic analysis of mouse erythropoiesis has shown that progenitor erythroblasts undergo six to seven cell divisions, each lasting 10 to 11 hours, and that 5 to 10 hours elapses following the final mitosis prior to nuclear expulsion (41). Based on what is known regarding the duration of the erythrocyte cell cycle, the priming doses in our study may have targeted the same erythroblast generation (likely for the experiments with a 4 hour interval between priming and challenge doses) or preceding erythroblast generations (likely for the 24 hour time periods and multiple 20 mGy priming regimes) than those cells exposed to the 2 Gy acute radiation dose. A radio-adaptive response manifested as the reduction of MN frequencies in this study could potentially arise due to modulation of the number of cellular DNA double strand breaks, increased DNA repair proficiency, an increase proliferative index, or increased apoptosis of erythroblasts or PCES harboring DNA damage.

The priming radiation dose regimes employed in this study did not affect the levels of MN-PCE or modify P/N ratios following an acute radiation challenge in bone marrow erythrocytes of either mouse strain. In the first 30 hours post-radiation a modest additive effect (a 1.1 to 1.2-fold increase in MN-PCE frequencies) was observed for C57BL/6 mice that received a 20 mGy priming dose in comparison to mice receiving only the acute 2 Gy dose, with statistical significance between the two treatment groups for the 18 hour time point. A trend for lower P/N ratios (although not statistically significant) was also observed for mice that received a 20 mGy priming dose in relation to those that received only 2 Gy radiation for the time period 24 to 48 hours post-radiation. The trend for decreased P/N ratio and increased MN-PCE frequencies in C57BL/6 mice receiving a priming dose indicates that the priming dose may have had a small

effect on impeding the turnover of immature erythrocytes in the bone marrow of mice and the clearance of PCEs with DNA damage in the first 48 hours following a high dose acute radiation exposure. Since the radio-adaptive kinetic analysis was not performed for the BALB/c strain, it cannot be determined if this observation was specific to the C57BL/6 strain. Overall, our results clearly indicate the absence of radio-adaptive responses for radiation-induced cytogenetic damage or radiation-induced suppression of erythrocyte proliferation/ maturation index in bone marrow tissues of irradiated C57BL/6 and BALB/c mice as we failed to observe statistically significant changes in either MN-PCE frequencies or P/N ratios in mice that received low-dose priming radiation exposures.

Summary

The effects of low-dose gamma radiation on spontaneous and radiation-induced levels of clastogenic damage and putative radio-adaptive responses in bone marrow erythrocytes were investigated in radio-resistant (C57BL/6) and radio-sensitive (BALB/c) mice. This study is an extension of our previous study of the cytotoxic effects of low-dose radiation on mouse spleen cells (*31*). Considered together, the results of both studies represent a multiple tissue, multiple endpoint approach to exploring links between the induction and repair of cytogenetic damage in haematopoietic cells, genomic instability and progression to haematological cancer (*74*, *75*). Our results indicate that the threshold for the induction of cytogenetic damage in spleen and bone marrow cells above baseline levels in C57BL/6 mice is between 20 and 100 mGy for gamma radiation. A similar magnitude of induction of MN was observed for C57BL/6 spleen lymphocytes and bone marrow erythrocytes (~10-fold above spontaneous levels) following an acute 2 Gy radiation dose, although faster clearance of damaged cells was observed for rapidly proliferating bone marrow cells. We observed a linear dose response relationship for C57BL/6 bone marrow MN-PCE levels for radiation doses between 100 mGy and 1 Gy, with departure from linearity (saturation) between 1 and 2 Gy, and a linear quadratic dose response relationship for C57BL/6 spleen MN frequencies for doses between 100 mGy and 2 Gy, with no saturation. Our findings support radio-sensitivity of BALB/c mice for DNA damage in spleen lymphocytes and also potentially indicate a lower threshold for the induction of cytogentic damage in BALB/c bone marrow erythrocytes. We failed to observe a radio-adaptive response for radiation-induced MN in either mouse strain in bone marrow erythrocytes or spleen lymphocytes, indicating that acute radiation doses of between 20 mGy and 100 mGy (dose rates 0.5 mGy/min to 10 mGy/min) did not produce radio-protective effects on radiation-induced cytogenetic damage in bone marrow tissues of irradiated C57BL/6 and BALB/c mice and that low-dose radiation exposures leading to reduced carcinogenic potential may not be related to alterations in short-term cytogenetic damage. Our previous study revealed enhanced splenic cell survival in C57BL/6 mice irradiated with a 20 mGy dose prior to an acute challenge dose and a potential slower turnover of cells with DNA damage in the spleen following radiation damage (*31*). The current study revealed no radio-adaptive response in either strain of mice for bone marrow proliferative/maturation index.

Acknowledgements

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Figure Legends

Figure 1. Overview of experimental design.

Bone marrow cells were harvested from femora of C57BL/6 or BALB/c mice following total body *in vivo* LDR (low-dose radiation) or HDR (high dose radiation) exposures to ^{60}Co gamma radiation. For dose response and time-course experiments (A), single acute doses of radiation (20 mGy to 2 Gy) were delivered to mice, and mice were euthanized 18 to 72 hours post-radiation. For radio-adaptive response experiments (B) LDR was delivered as a single or multiple priming dose(s) prior to a subsequent HDR challenge dose, and mice were euthanized 24 hours following the challenge irradiation. The MN-PCE assay was conducted on isolated bone marrow erythrocytes and the ratio of polychromatic: normochromatic erythrocytes (the P/N ratio) was determined.

Figure 2. Dose response for MN-PCE frequencies and P/N Ratios in mouse bone marrow.

Radiation doses were delivered 24 hours (1 and 2 Gy) or 28 hours (20 mGy, 100 mGy and 500 mGy) prior to sacrifice. N = number of animals sampled per treatment group. Data points for C57BL/6 mice (black columns) or BALB/c mice (grey columns) represent the mean value of pooled samples; error bars indicate standard error. * p value < 0.05, ** p value < 0.005 and *** p value < 0.005 for unpaired Student's t-test for irradiated cohorts in comparison to un-irradiated control animals. 100 mGy, 500 mGy and 1 Gy radiation exposures were investigated for the C57BL/6 mouse strain only.

Figure 3. Time Course Analysis of MN-PCE frequencies and P/N Ratios in Mouse Bone Marrow Following 2 Gy Radiation Exposure.

MN-PCE frequencies (panel A) and P/N ratios (panel B) following 2 Gy irradiation of C57BL/6 (square symbols) or BALB/c (circle symbols) mice. Data points represent the mean value of pooled samples; error bars indicate standard error. For C57BL/6 data analysis, six animals were

sampled per treatment group, with the exception of the 24 h time point group comprised of 18 animals for MN-PCE analysis (reproduced from Figure 2) and 5 animals for P/N ratio determination (reproduced from Figure 2). For BALB/c data analysis, two animals were sampled per treatment group, with the exception of the 24 h time point group comprised of 7 animals for MN-PCE analysis (reproduced from Figure 2) and 5 animals for P/N ratio determination (reproduced from Figure 2).Statistical analysis was not performed comparing C57BL/6 and BALB/c treatment cohort samples due to limited number of BALB/c samples for the majority of time points.

Figure 4. Radio-adaptive Response Analysis of MN-PCE frequencies and P/N Ratios in bone marrow of mice.

Single or multiple LDR priming doses of radiation were delivered to C57BL/6 mice (black columns) and BALB/c mice (grey columns) 4, 24 or 48 hours prior to a 2 Gy challenge dose, and mice were euthanized 24 hours post challenge dose. Treatment groups receiving a 2 Gy radiation dose alone served as control groups (reproduced from Figure 2). N = number of animals sampled per treatment group. Data points represent the mean value of pooled samples values; error bars indicate standard error.

Figure 5. Radio-adaptive Response Time Course Analysis of MN-PCE frequencies and P/N Ratios in Bone Marrow of Mice.
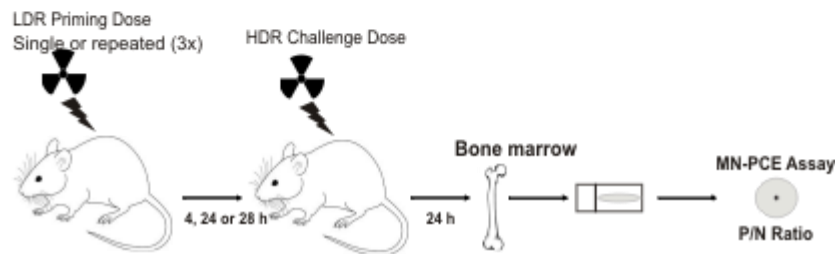
MN-PCE frequencies (panel A) and P/N ratios (panel B) following *in vivo* 20 mGy priming and 2 Gy challenge irradiations (circle symbols) or 2 Gy irradiation only (square symbols; data reproduced from Figure 4). Data points represent the mean value of pooled samples; error bars indicate standard error. Six animals were sampled per treatment group, with the exception of the 24 h time point, comprised of 18 animals for MN-PCE analysis (reproduced from Figure 2) and 5 animals for P/N ratio determination (reproduced from Figure 2) ,and 7 animals for the 2 Gy dose preceded by the 20 mGy priming dose treatment. * p value < 0.05 for unpaired Student's t-test.
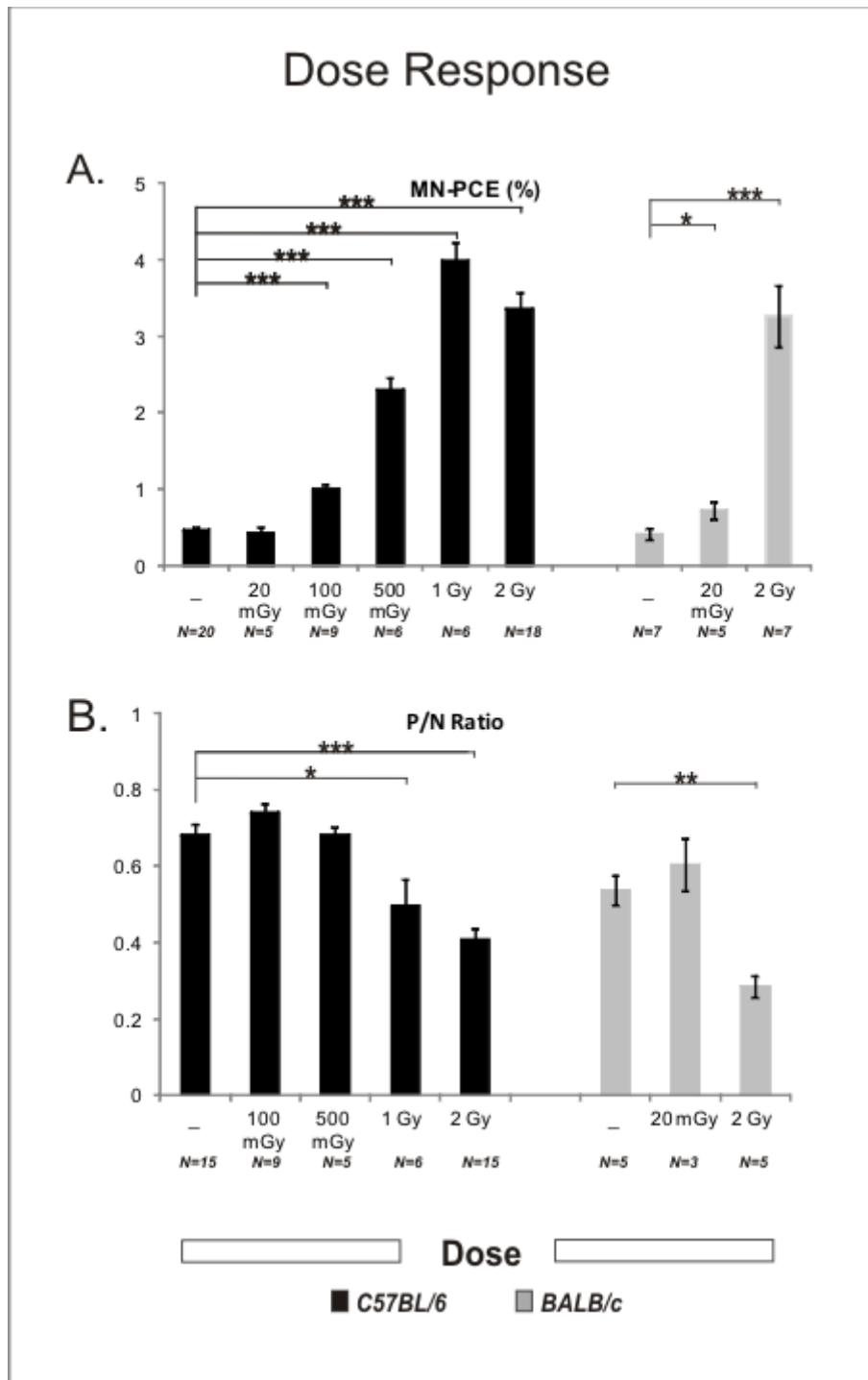
Figures

Figure 1.

Figure 2.

Figure 3.

Radio-adaptive Response

Figure 4.

Figure 5.