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# Strain-related Differences and Radiation Quality Effects on Mouse Leukocytes: Gamma-rays and Protons (with and without Aluminum Shielding)

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**Abstract.** *Increasing evidence indicates that radiation-induced genomic instability plays an important role in the development of cancer. However, radiation quality and genetic background can influence the outcome. The goal of this study was to quantify radiation-induced changes in lymphocyte populations in mouse strains known to differ in susceptibility to genomic instability (C57BL/6, resistant; CBA/Ca, susceptible). The effects of whole-body exposure to γ-rays and protons, with and without aluminum shielding, were compared. Total radiation doses of 0, 0.1, 0.5, and 2.0 Gy were delivered and subsets of mice from each group were euthanized on days 1 and 30 after exposure for spleen and bone marrow analyses. In the spleen on day 1, lymphocyte counts were decreased ($p<0.05$) in C57, but not CBA, mice irradiated with 2 Gy. By day 30 in the C57 strain, counts were still low in the group exposed to 2 Gy shielded protons. Some strain- and radiation-dependent differences were also noted in percentages of specific lymphocyte populations (T, B, NK) and the CD4:CD8 ratio. In bone marrow, percentages of stem/progenitor cells ($CD34^+$, $Ly\text{-}6A/E^+$, $CD34^+Ly\text{-}6A/E^+$) were generally highest 1 day after 2 Gy irradiation, regardless of strain and radiation type. Based on dUTP incorporation, bone marrow cells from C57 mice had consistently higher levels of DNA damage on day 30 after irradiation with doses less than 2 Gy, regardless of quality. Annexin V binding supported the conclusion that C57 bone marrow cells were more susceptible to radiation-induced apoptosis. Overall, the data indicate that leukocytes of CBA mice are less sensitive to the effects of high-linear energy transfer radiation (shielded protons) than C57 mice, a phenomenon consistent with increased possibility for genomic instability and progression to a malignant cell phenotype after sublethal damage.*

Astronauts on space missions are exposed to various types of radiation above levels found on Earth, especially during periods of high solar activity. Exposure to galactic cosmic rays (GCR) and solar particle events (SPE) are a major health risk (1). The GCR spectrum is dominated by protons and high Z energetic (HZE) particles such as iron ions that exert considerable biological effects even at low fluence (2). Flight crews can also be exposed to substantial levels of protons and electrons from trapped radiation belts and SPE.

The energy spectrum of protons in space peaks at around 1 GeV, making effective radioprotection a major concern. Although efforts are underway to identify chemical and biological radioprotectants, at this time shielding is the most likely countermeasure that will be used to minimize radiation damage during extended deep space missions. Shielding, however, can result in numerous secondary particles, including neutrons, which have greater biological effectiveness than the incident space radiation hitting the shield (3, 4). Calculations of GCR doses using different shielding conditions have shown that the biological effect is dependent upon the biophysical model and endpoint evaluated (5, 6). Much more data is needed to more accurately characterize the biological impact of shielding.

In addition to acute effects of radiation that occur within a few cell divisions, studies have shown that longer-term changes occur in the surviving cells leading to pronounced chromosomal instability (7-9). In some studies, an unstable

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genotype has been found in cells that have not been directly hit by radiation, *i.e.* known as the 'bystander effect' (10). However, genetic factors, as well as microenvironment, can have a significant impact on radiation-induced genomic instability. Response of the hematopoietic system is especially important in regaining homeostasis and maintenance of effective immune defense against unstable aberrant cells, some of which have potential to progress to the malignant state (11). Indeed, many studies indicate a strong association between genomic instability and progression to cancer. In some cases, even 'normal' lymphocytes of cancer patients have been reported to exhibit signs of genomic instability after therapeutic irradiation (12-14). Since the number of proton therapy facilities are rapidly increasing worldwide, a better understanding of radiation-induced changes in the context of variable genetic background is important not only to crew members on space missions, but also to patients receiving proton therapy.

In the present study, mice known to have variable responses to ionizing radiation, C57BL/6 and CBA/Ca, were exposed to γ-rays and protons, with and without aluminum shielding. Especially intriguing are reports that these two mouse strains differ in susceptibility to radiation-induced acute myeloid leukemia (AML): C57 mice are AML resistant, while CBA mice are AML susceptible (15, 16). Cell responses in two body compartments, spleen and bone marrow, were evaluated at early and late timepoints post-exposure.

## Materials and Methods

*Animals*. Male C57BL/6J (n=38; stock no. 000664) and CBA/CaJ (n=30; stock no. 000654) mice, hereafter referred to as C57 and CBA, were purchased from The Jackson Laboratories (Bar Harbor, MA, USA) at 8 weeks of age and acclimated for 1-2 weeks prior to study initiation; standard vivarium conditions were maintained throughout. C57 and CBA strains were selected because they are known to be resistant and susceptible, respectively, to radiation-induced genomic instability (17, 18). The animals were routinely observed for signs of toxicity following irradiation and euthanized on days 1 and 30 post-exposure. Rapid $CO_2$ euthanasia was performed in compliance with guidelines of the National Institute of Health (NIH) and the panel on Euthanasia of the American Veterinary Medical Association. The study was approved by the Institutional Animal Care and Use Committee.

*Radiation procedures and shielding*. Immediately prior to total-body γ-irradiation, the mice were placed individually into 1 mm thick, rectangular plastic boxes (30×30×60 $mm^3$ for C57 mice and 30×30×85 $mm^3$ for CBA mice due to their larger size) with holes to allow free exchange of air. The total doses delivered under each radiation condition were 0 Gy, 0.1, 0.5, and 2.0 Gy. Dose rates were 1.5 Gy/min (γ-rays) and 0.8 Gy/min (protons and protons/shielded).

Gamma-irradiation was performed using a vertical beam from an AECL Eldorado unit (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada) containing a $^{60}Co$ source. The boxes were rotated halfway through each exposure to homogenize the dose across mouse bodies. Calibration of the γ-radiation dose was performed using a Capintec Model PRO6-G cylindrical thimble ionization chamber, as specified by the National Institute of Standards and Technology (NIST).

Proton beam irradiation was performed using 250 MeV monoenergetic protons from the synchrotron accelerator in the Proton Treatment and Research Center at Loma Linda University Medical Center (LLUMC). The beam was delivered in 0.3 s pulses every 2.2 s. For some of the groups, a sheet of type 6061-T6 aluminum with an area density of 2.7 $g/cm^2$ was placed between the mice and the proton beam; the shield thickness was approximately 5.6 cm. Calibration of the dose was archived using a NIST-traceable Markus™ parallel plate ionization chamber in a polystyrene phantom.

*Spleen and bone marrow collection*. Procedures for the spleen have been described in detail in our previous publications (19, 20). Briefly, spleens were excised at the times of euthanasia, processed into single-celled suspensions, and erythrocytes were lysed with cold lysing buffer. Spleen samples from each of the mice were evaluated individually. The bone marrow cells were obtained by removing the epiphysis and metaphysis at the proximal and distal ends of the femur and flushing cells out of the marrow cavity by distal insertion of a 21-gauge needle attached to a syringe containing medium. Since the data reported here were part of a much larger study with focus on chromosomal abnormalities in hematopoietic cells (to be reported separately), bone marrow from 6-10 femurs (3-5 mice/strain/dose/timepoint) were pooled and used for the analyses reported here.

*Analysis of splenic lymphocyte populations and progenitor cells in bone marrow*. The spleen cells (12 μl aliquot/sample) were first evaluated using a Vet ABC Hematology Analyzer (Heska Corporation, Waukesha, WI, USA) to obtain lymphocyte counts. The analyzer enumerates cells based on electrical impedance generated by their passage through a calibrated micro-aperture. All cells with a volume ranging from 30 fl to 460 fl were included. Immunophenotyping was performed on both spleen and bone marrow cells using a FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA) as previously described (21, 22). Fluorescein isothiocyanate-(FITC), R-phycoerythrin-(PE), allophycocyanin-(APC), and peridinin chlorophyll protein-(PerCP) conjugated monoclonal antibodies (MAb) (Becton Dickinson) were employed to identify specific lymphocyte populations using a direct staining technique.

For the spleen, cells positive for cluster of differentiation 45 ($CD45^+$; clone 30-F11) *versus* side scatter gate were used to identify leukocytes. An antibody mixture containing CD3/CD8/CD45/CD4 was used to identify total $CD3^+$ T-cells and the $CD3^+CD4^+$ T-helper (Th) and $CD3^+CD8^+$ T-cytotoxic (Tc) subsets for both strains of mice. Antibody against the B220 marker was used to identify B-cells in both strains; anti-NK1.1 and anti-PanNK were used for quantification of natural killer (NK) cells in C57 and CBA mice, respectively, due to differences in marker expression between the two strains. These two mixtures contained the following: CD3/NK1.1/CD45/B220 and CD3/PanNK/CD45/B220. Analysis was performed using CellQuest™ software version 3.1 (Becton Dickinson) on 10,000 acquired events.

For progenitor/stem cells in the bone marrow, a CD45 *versus* side scatter plot was used to separate lineages based on cytoplasmic

complexity. As bone marrow cells mature, they express increasing density of CD45. Lymphocytes generally exhibit the brightest CD45 staining and are low on side scatter (non-granular). A gate was placed on the population of leukocytes with blast-like characteristics, *i.e.* low on side scatter and dim CD45 compared to mature cells such as lymphocytes. Within this gate, cell populations positive for two hematopoietic stem cell markers, lymphocyte antigen 6 complex, locus A and E (Ly-6A/E; clone E13-161.7) and CD34 (clone RAM34), were examined. The three quantified populations were $CD34^+$, Ly-$6A/E^+$, and $CD34^+$Ly-$6A/E^+$.

*DNA damage based on dUTP incorporation by bone marrow cells.* Cells from each group were pooled and analyzed for DNA breaks using the terminal deoxynucleotidyl-transferase dUTP nick end labeling (TUNEL) method for detecting DNA fragmentation as previously described (23). Briefly, 0.5ml of $1X10^6$ cells/ml of suspended cells were dispersed to microfuge tubes, centrifuged (1,500 rpm for 10 min), washed in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), centrifuged again, and the pellet resuspended. Cells were fixed and permeabilized in 70% (v/v) ice-cold ethanol for 15 min, and rehydrated in PBS for 5 min. DNA strand breaks were labeled using an APO-BrdU Kit (Pharmingen, San Diego, CA, USA). The resuspended pellet was incubated overnight at room temperature (RT) with DNA Labeling Solution (TdT enzyme, reaction buffer, Br-dUTP, and distilled water). The labeled cells were then washed and incubated with FITC-labeled anti-BrdU antibody for 30 min at RT, and counter-stained in propidium iodide (PI) (50 μg/ml) /RNAse (200 μg/ml) for 20-30 min. Cells were then washed and mounted onto microscope slides using Permafluor and glass coverslips. Quantitative analysis of FITC-BrdU labeled DNA strand breaks was conducted using a laser scanning cytometer (LSC; CompuCyte, Cambridge, MA, USA) configured with an Olympus BX50 base (23). To measure DNA damage, scanning parameters were adjusted by signal intensity to create a contour of the PI nuclear label. To quantify the cells that were both red and green, the gating parameters were set to contour on red (PI) (y-axis) and to sum (integrate) the green fluorescence intensity (FITC-BrdU) (x-axis). Green fluorescent intensity reflected a quantitative measure of DNA damage, as exposed 3' ends, in the irradiated and control cells. To confirm the gating parameters, cells in selected gates were inspected visually through a CCD camera connected to the microscope. Optimized protocol and display settings were stored as configuration files and utilized for all samples in these experiments. An average of 5,000±500 cells were scanned per slide.

*Annexin V binding assay for apoptotic bone marrow cells.* To assess the proportion of bone marrow cells in early stages of apoptosis, an FITC-annexin V labeling procedure (Pharmingen) was used with modifications as described (23). The cells were incubated with FITC-conjugated annexin V for 45 min at RT, centrifuged, and washed with wash buffer. The cells were then fixed in –20˚C 70% ethanol for 15 min, rehydrated in PBS, and counterstained with PI and RNAse for 30 min. After additional washing and centrifugation, the cells were spread onto microscope slides and glass coverslips were applied to protect the cells. The slides were dried flat in the dark and scanned on the LSC. Quantitative analysis of annexin V labeling was similarly carried out as described above for FITC-BrdU labeling. All of the cell nuclei were labeled red (PI) which allowed them to be located and counted by the LSC. The PI measurements were placed on the y-axis as integrated fluorescence. The x-axis was set to measure green (FITC-annexin V) integral fluorescence. The optimized protocol and display settings were confirmed visually by the microscope camera and stored for subsequent analysis of all cells in these experiments. The average number of cells scanned per slide was 2,500±500.

Table I. *Lymphocyte counts in spleens from both strains of mice on days 1 and 30 post-irradiation. Counts ($\times 10^6$/ml) were obtained using an automated hematology analyzer. P-values were obtained from comparisons within each mouse strain and timepoint.*

| Group | C57BL/6 | | CBA/Ca | |
|---|---|---|---|---|
| | Day 1 | Day 30 | Day 1 | Day 30 |
| 0 Gy control | 9.1±2.0 | 9.6±2.2 | 7.3±0.3 | 4.7±0.4 |
| γ-Rays | | | | |
| 0.1 Gy | 6.8±1.0 | 11.2±0.8 | 4.1±0.3 | 5.8±0.3 |
| 0.5 Gy | 6.2±0.3 | 9.1±0.8 | 5.2±0.9 | 5.2±0.2 |
| 2.0 Gy | 1.8±0.7[a] | 8.9±0.7 | 2.7±0.5 | 4.4±0.1 |
| Protons | | | | |
| 0.1 Gy | 4.8±0.7 | 13.8±1.2[c] | 4.7±0.3 | 6.1±0.2 |
| 0.5 Gy | 3.2±0.6 | 10.8±1.1 | 4.2±0.2 | 5.9±0.3 |
| 2.0 Gy | 1.8±0.3[a] | 6.9±3.2 | 2.5±0.3 | 3.6±0.2 |
| Protons/shielded | | | | |
| 0.1 Gy | 5.1±1.1 | 3.7±0.5 | 6.0±0.4 | 7.3±0.3 |
| 0.5 Gy | 8.6±3.0[b] | 3.6±0.4 | 4.7±0.3 | 6.6±0.6 |
| 2.0 Gy | 1.0±0.2[a] | 3.6±0.2[d] | 2.7±0.4 | 5.4±0.1 |

Mean±SEM (n=3-7 mice/group for each strain at each timepoint). $p<0.05$ *vs.* [a]0 Gy; [b]2 Gy γ-rays, protons, protons/shielded and 0.5 Gy protons; [c]0 Gy and 2 Gy protons; [d]0.1 Gy γ-rays, protons and 0.5 Gy protons.

*Statistical analysis.* Spleen data obtained from individual mice were evaluated using one-way analysis of variance (ANOVA) and Tukey's pairwise multiple comparison test. Comparisons were made for both mouse strains at each timepoint, between non-irradiated control groups and each irradiated group. $p<0.05$ was considered to be significant. SigmaStat™ software version 2.03 (SPSS Inc., Chicago, IL, USA) was used in these analyses.

## Results

*Lymphocytes in spleen.* As shown in Table I, lymphocyte counts were depressed on day 1 in C57 mice exposed to 2 Gy, regardless of radiation regimen ($p<0.05$ *vs.* 0 Gy). Interestingly, the proton/shielded group irradiated with 0.5 Gy had higher counts compared to the 0.5 Gy proton group, as well as all three groups irradiated with 2 Gy ($p<0.05$). In contrast, although the lowest lymphocyte numbers were noted in all 2 Gy groups, there were no statistically significant differences in the CBA strain at the day 1 timepoint. By day 30 (Table I), the C57 mice irradiated with 0.1 Gy protons had higher lymphocyte counts compared to the group irradiated with 2 Gy protons; the 2 Gy

Table II. *Percentages of major lymphocyte populations in spleens from both strains of mice on day 1 post-irradiation. Percentages were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry; 5,000-10,000 events were acquired and analyzed/sample.*

| Group | C57BL/6 | | | CBA/Ca | | |
|---|---|---|---|---|---|---|
| | T-Cells | B-Cells | NK Cells | T-Cells | B-Cells | NK Cells |
| 0 Gy control | 28.6±1.8 | 68.3±1.6 | 3.1±0.3 | 35.9±2.6 | 59.1±2.9 | 5.1±0.4 |
| γ-Rays | | | | | | |
| 0.1 Gy | 30.1±1.2 | 66.3±1.1 | 3.6±0.3 | 40.9±1.7 | 55.1±1.9 | 4.0±0.2 |
| 0.5 Gy | 31.5±0.7 | 64.0±0.8 | 4.6±0.2[a,b] | 38.6±1.3 | 57.0±1.4 | 4.4±0.2 |
| 2.0 Gy | 35.9±1.9 | 56.5±1.3[a] | 7.6±0.7[a,b] | 44.1±0.5 | 49.9±0.8 | 6.0±0.4 |
| Protons | | | | | | |
| 0.1 Gy | 34.6±2.0 | 62.7±2.0 | 2.8±0.2[a,c] | 39.8±0.9 | 57.2±0.9 | 3.0±0.1[a,b] |
| 0.5 Gy | 35.6±2.0 | 61.2±2.0[a] | 3.2±0.3 | 42.3±1.2 | 55.2±1.2 | 2.5±0.1[a,d] |
| 2.0 Gy | 33.7±1.7 | 62.4±2.0 | 3.9±0.9 | 38.1±0.9 | 56.9±1.2 | 5.0±0.6 |
| Protons/shielded | | | | | | |
| 0.1 Gy | 32.6±2.4 | 62.8±1.9 | 4.5±0.5 | 37.9±0.1 | 57.8±0.8 | 4.4±0.2 |
| 0.5 Gy | 28.4±1.2 | 68.2±1.2 | 3.3±0.2 | 44.2±1.3[a] | 53.0±1.1 | 2.7±0.2[a,d] |
| 2.0 Gy | 32.8±0.9 | 60.1±0.8[a] | 7.1±1.0[a,b] | 48.1±4.4[a] | 46.6±3.9[a] | 5.3±0.6 |

Mean±SEM (n=3-7 mice/group for each strain). $p<0.05$ *vs*. [a]0 Gy; [b]protons at the same dose; [c]protons/shielded at the same dose; [d]γ-rays at the same dose.

Table III. *Percentages of major lymphocyte populations in spleens from both strains of mice on day 30 post-irradiation. Percentages were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry; 5,000-10,000 events were acquired and analyzed/sample.*

| Group | C57BL/6 | | | CBA/Ca | | |
|---|---|---|---|---|---|---|
| | T-Cells | B-Cells | NK Cells | T-Cells | B-Cells | NK Cells |
| 0 Gy control | 28.2±1.3 | 68.1±1.4 | 3.7±0.3 | 35.5±1.3 | 59.5±1.3 | 5.0±0.2 |
| γ-Rays | | | | | | |
| 0.1 Gy | 27.3±0.6 | 69.0±0.4 | 3.7±0.3 | 37.4±0.4 | 59.8±0.4 | 2.8±0.1 |
| 0.5 Gy | 28.7±1.7 | 67.7±1.7 | 3.7±0.1 | 38.7±1.6 | 56.9±1.7 | 4.4±0.3 |
| 2.0 Gy | 25.2±0.7 | 71.3±0.7 | 3.5±0.1 | 32.5±1.3 | 62.2±1.2 | 5.4±0.3 |
| Protons | | | | | | |
| 0.1 Gy | 26.0±0.5 | 71.0±0.6 | 3.0±0.2 | 38.8±3.2 | 57.3±2.9 | 3.9±0.3 |
| 0.5 Gy | 26.4±0.7 | 70.7±0.8 | 3.0±0.2 | 39.7±0.9 | 56.7±0.9 | 3.7±0.1 |
| 2.0 Gy | 28.8±6.3 | 68.0±6.7 | 3.2±0.4 | 39.5±1.1 | 55.9±1.3 | 4.6±0.1 |
| Protons/shielded | | | | | | |
| 0.1 Gy | 31.3±0.9 | 65.1±1.3 | 3.6±0.5 | 40.3±1.3 | 55.9±1.1 | 3.8±0.3 |
| 0.5 Gy | 29.1±2.8 | 67.5±3.2 | 3.4±0.4 | 38.8±0.9 | 56.6±0.7 | 4.6±0.2 |
| 2.0 Gy | 28.1±0.6 | 68.6±0.4 | 3.2±0.3 | 37.0±2.9 | 58.2±3.0 | 4.8±0.1 |

Mean±SEM (n=3-7 mice/group for each strain).

proton/shielded group had lower counts *versus* that exposed to 0.1 Gy γ-rays and the groups exposed to either 0.1 Gy or 0.5 Gy protons. There were no significant differences in lymphocyte counts associated with radiation dose or regimen in the CBA strain on day 30.

*Specific lymphocyte populations in spleen*. The proportions of the three major lymphocyte populations (T, B, and NK cells) are presented in Table II. The flow cytometric data showed that on day 1 the C57 strain in the 2 Gy γ-ray, 2 Gy proton/shielded and 0.5 Gy proton groups had a lower B-cell percentage compared to 0 Gy controls ($p<0.05$). At that same timepoint, CBA mice in the 0.5 Gy and 2 Gy proton/shielded groups had a high percentage of T-cells, whereas the B-cell counts were low in the 2 Gy proton/shielded group ($p<0.05$ *vs*. 0 Gy). The most affected, however, were the NK cells. The C57 spleens

from the 0.5 Gy and 2.0 Gy proton and 2 Gy proton/shielded groups had a high percentage of NK cells, whereas the 0.1 Gy proton group had a low percentage ($p<0.05$ *vs*. 0 Gy). In contrast, the CBA mice in the 0.1 Gy and 0.5 Gy proton and 0.5 Gy proton/shielded groups had a low NK percentage ($p<0.05$ *vs*. 0 Gy). Several additional differences among groups in NK percentage were noted for both strains at the early timepoint. By day 30, proportions of all three lymphocyte types were similar to 0 Gy for both mouse strains (Table III).

*CD4:CD8 T cell ratio in spleen*. These data are shown in Table IV. On day 1 post-irradiation, the C57 mice in all three proton-irradiated groups had a higher CD4:CD8 ratio compared to those not irradiated ($p<0.05$). The 0.1 Gy and 0.5 Gy proton groups also had higher ratios than their respective counterparts receiving the same dose of shielded protons ($p<0.05$). There was no radiation effect on the ratio for CBA mice on day 1. By day 30, the balance of CD4 and CD8 cells in spleens from irradiated C57 mice was similar to those unirradiated, regardless of radiation condition. For CBA mice, however, a very different pattern was noted. There was no radiation effect on the CD4:CD8 ratio on day 1, but a significant increase was present on day 30 in all three groups that received 2 Gy ($p<0.05$ *vs*. 0 Gy). Several additional differences between the 2 Gy and other groups receiving lower doses were also noted.

Table IV. *CD4:CD8 T cell ratio in spleens from both strains of mice on days 1 and 30 post-irradiation. Ratios were based on flow cytometry using fluorescence-labeled antibodies; 5,000-10,000 events were acquired and analyzed/sample. P-values were obtained from comparisons within each mouse strain and time point.*

| Group | C57BL/6 | | CBA/C[a] | |
|---|---|---|---|---|
| | Day 1 | Day 30 | Day 1 | Day 30 |
| 0 Gy control | 1.49±0.07 | 1.43±0.08 | 2.16±0.07 | 2.37±0.07 |
| γ-Rays | | | | |
| 0.1 Gy | 1.59±0.07 | 1.55±0.05 | 2.10±0.04 | 2.35±0.04 |
| 0.5 Gy | 1.48±0.07 | 1.59±0.08 | 2.24±0.06 | 2.45±0.08 |
| 2.0 Gy | 1.65±0.20 | 1.96±0.07 | 2.33±0.02 | 3.56±0.10d |
| Protons | | | | |
| 0.1 Gy | 1.95±0.07[a] | 1.37±0.08 | 2.36±0.07 | 2.31±0.12 |
| 0.5 Gy | 1.84±0.03[b] | 1.66±0.06 | 2.43±0.13 | 2.59±0.04 |
| 2.0 Gy | 2.00±0.15[c] | 2.13±0.13 | 2.40±0.07 | 3.39±0.10[e] |
| Protons/shielded | | | | |
| 0.1 Gy | 1.42±0.10 | 1.46±0.03 | 2.42±0.05 | 2.40±0.08 |
| 0.5 Gy | 1.38±0.08 | 1.63±0.04 | 2.46±0.05 | 2.60±0.12 |
| 2.0 Gy | 1.56±0.03 | 2.31±0.21 | 2.40±0.07 | 3.19±0.30[f] |

Mean±SEM (n=3-5 mice/group for each strain at each time point). $p<0.05$ *vs*. [a]0 Gy and 0.1 Gy protons/shielded; [b]0.5 Gy protons/shielded; [c]0 Gy; [d]0 Gy, 0.1 Gy and 0.5 Gy γ-rays; [e]0 Gy, 0.1 Gy and 0.5 Gy protons; [f]0 Gy and 0.1 Gy protons/shielded.

*Progenitor/stem cell populations in bone marrow*. Table V shows the percentage of stem/progenitor cells in bone marrow on day 1 post-irradiation. For C57 mice, the highest percentages of $CD34^+$, Ly-6A/$E^+$ and $CD34^+$Ly-6A/$E^+$ cells were consistently seen in the 2 Gy-irradiated groups. Although this was generally true also for the CBA strain, the highest percentage of $CD34^+$ cells occurred in the 0.1 Gy γ-irradiated group, *i.e*. more than 6-fold above that at 0 Gy. By day 30, the percentages were relatively close to normal (Table VI). There was, however, a noteworthy exception: CBA mice in the proton/shielded groups had percentages of $CD34^+$ cells that were 4- to 5.5-fold higher than in the non-irradiated controls.

*Radiation-induced DNA damage assessed by dUTP incorporation*. DNA damage as assessed by the dUTP-incorporation methodology indicated that in C57 mice, 24 h after exposure, 2 Gy γ-rays, protons, or shielded protons produced relatively high levels of DNA damage in bone marrow cells (Figure 1). This effect was still evident 30 days post-exposure with all three types of radiation. In addition, at this later timepoint, there was a clear dose-dependent increase in DNA damage in γ-irradiated C57 mice.

The DNA damage profile of CBA bone marrow cells at 24 h post-exposure was, in general, similar to that of C57, with the greatest increase in damage observed with 2 Gy shielded protons (Figure 1). However, by 30 days, this profile of damage was remarkably different from that obtained at 24 h. The lowest doses (0.1 and 0.5 Gy) of γ-rays, protons, and shielded protons produced the greatest percentage of DNA damage (20-42%). Interestingly, at 30 days with all three radiation types, there was consistent reduction in the percentage of damage after 2 Gy compared to the two lowest doses used. The level of damage induced with 2 Gy was different in proton-shielded CBA mice relative to non-irradiated controls.

*Radiation-induced apoptosis based on annexin V binding*. Apoptosis induced in bone marrow cells isolated from whole-body irradiated C57 and CBA mice was assessed as the level of annexin V binding (Figure 2). Apoptosis measurements were normalized to those of non-irradiated control levels, and reflect differences from normal background levels. Annexin binding was increased relative to non-irradiated controls in both strains of mice 24 h following exposure to any of the three types and doses of radiation, with the largest increase in apoptosis measured after 2 Gy. Comparison of C57 and CBA apoptotic indices revealed that an early apoptotic response was present at a higher percentage in C57 bone marrow cells, approximately 22% more than in CBA cells. Moreover, at 24 h in C57 cells, there were clear dose-dependent increases measured with γ-rays, protons, and shielded protons. Interestingly, the linear dose response measured in C57 samples after shielded

Table V. *Percentages of stem/progenitor cells in bone marrow on day 1 post-irradiation. Percentages were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Samples from each group were pooled prior to analysis.*

| Group | C57BL/6 | | | CBA/Ca | | |
|---|---|---|---|---|---|---|
| | $CD34^+$ | $Ly\text{-}6A/E^+$ $CD34^+$ | $Ly\text{-}6A/E^+$ | $CD34^+$ | $Ly\text{-}6A/E^+$ | $CD34^+$ $Ly\text{-}6A/E^+$ |
| 0 Gy control | 1.23 | 0.50 | 0.15 | 0.27 | 0.07 | 0.08 |
| γ-Rays | | | | | | |
| 0.1 Gy | 0.34 | 0.23 | 0.09 | 1.78 | 0.06 | 0.11 |
| 0.5 Gy | 0.47 | 0.25 | 0.16 | 0.98 | 0.06 | 0.22 |
| 2.0 Gy | 1.39 | 1.22 | 0.50 | 0.34 | 0.44 | 2.43 |
| Protons | | | | | | |
| 0.1 Gy | 0.26 | 0.38 | 0.07 | 0.36 | 0.05 | 0.08 |
| 0.5 Gy | 0.40 | 0.41 | 0.09 | 0.30 | 0.04 | 0.13 |
| 2.0 Gy | 1.31 | 1.13 | 0.50 | 0.82 | 0.18 | 0.15 |
| Protons/shielded | | | | | | |
| 0.1 Gy | 0.56 | 0.43 | 0.23 | 0.24 | 0.15 | 0.17 |
| 0.5 Gy | 0.57 | 0.37 | 0.22 | 0.19 | 0.28 | 0.15 |
| 2.0 Gy | 0.89 | 0.95 | 0.44 | 0.60 | 0.33 | 1.22 |

Table VI. *Percentages of stem/progenitor cells in bone marrow on day 30 post-irradiation. Percentages were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Samples from each group were pooled prior to analysis.*

| Group | C57BL/6 | | | CBA/Ca | | |
|---|---|---|---|---|---|---|
| | $CD34^+$ | $Ly\text{-}6A/E^+$ | $CD34^+$ $Ly\text{-}6A/E^+$ | $CD34^+$ | $Ly\text{-}6A/E^+$ | $CD34^+$ $Ly\text{-}6A/E^+$ |
| 0 Gy control | 0.34 | 0.27 | 0.09 | 0.08 | 0.94 | 0.16 |
| γ-Rays | | | | | | |
| 0.1 Gy | 0.27 | 0.20 | 0.08 | 0.09 | 0.27 | 0.09 |
| 0.5 Gy | 0.18 | 0.16 | 0.04 | 0.07 | 0.28 | 0.06 |
| 2.0 Gy | 0.71 | 0.34 | 0.16 | 0.11 | 0.38 | 0.09 |
| Protons | | | | | | |
| 0.1 Gy | 0.38 | 0.24 | 0.09 | 0.11 | 0.05 | 0.03 |
| 0.5 Gy | 0.30 | 0.23 | 0.06 | 0.11 | 0.07 | 0.06 |
| 2.0 Gy | 0.28 | 0.22 | 0.05 | 0.09 | 0.33 | 0.04 |
| Protons/shielded | | | | | | |
| 0.1 Gy | 0.39 | 0.18 | 0.10 | 0.32 | 0.02 | 0.10 |
| 0.5 Gy | 0.23 | 0.13 | 0.06 | 0.42 | 0.05 | 0.10 |
| 2.0 Gy | 0.48 | 0.29 | 0.16 | 0.44 | 0.03 | 0.11 |

protons at 24 h was also evident in CBA cells at 24 h. By 30 days post-exposure, proton-irradiated C57 samples continued to display elevated levels of apoptosis, but the increase was less compared to that measured at 24 h. Similarly to C57 cells at 30 days, the persistent increase in apoptosis in CBA cells was lower in magnitude, with the exception of 2 Gy shielded protons which continued to exhibit a 13% increase.

## Discussion

The most striking differences among groups included the low lymphocyte count in spleens from C57 mice 1 day after exposure to 2 Gy, regardless of radiation regimen. By day 30, the count was still low in the C57 mice that had received 2 Gy shielded protons. The low counts, however, do not necessarily mean genomic damage is present in the surviving cells. Chang *et al*. found no difference in the number of chromosomal aberrations in splenic lymphocytes from mice exposed to protons compared to those exposed to aluminum-shielded protons (24). In our study, the decrease in lymphocytes was reflected primarily in the B-cell population, which is well known to be especially radiosensitive. Although relatively low B-cell percentages were also noted for the CBA mice in the 2 Gy groups, significance was obtained only with

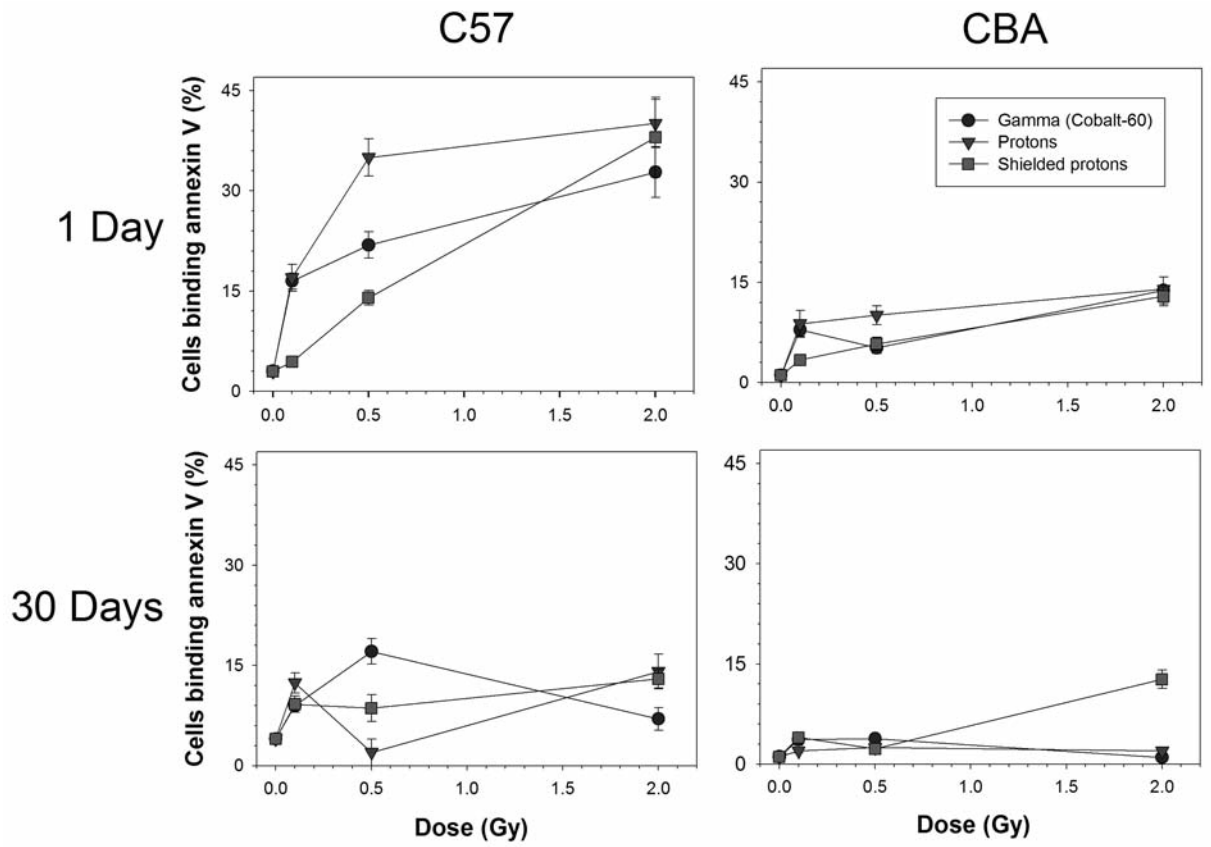


Figure 1. *DNA damage based on percentage of bone marrow cells incorporating dUTP. The data were obtained from samples pooled/group using the TUNEL method that included (FITC)-labeled anti-BrdU antibody and analysis using laser scanning cytometry. Each point represents an average of 5,000±500 cells scanned per slide.*

shielded protons and only at the early timepoint. Interestingly, the CD4:CD8 T-cell ratio was high on day 1 in all C57 mice irradiated with protons, regardless of dose, whereas the ratio was high in CBA mice on day 30 in all three groups that had received 2 Gy. We have previously found that C57 mice exposed to low-dose protracted γ-rays followed by 2 Gy simulated SPE protons have low B-cell numbers and a high CD4:CD8 ratio that was primarily due to sustained reduction in $CD8^+$ T-cells; an increase in the percentage of $Foxp3^+$ T-regulatory cells was also noted (25). Since $CD8^+$ T-lymphocytes can directly kill aberrant cell types and $Foxp3^+$ T-cells are immunosuppressive, radiation-induced alteration in the normal balance of these populations may increase the risk for carcinogenesis. The importance of these cells, as well as others relevant to immune system control of malignant cells, have recently been discussed (26, 27).

Since radiation-induced genomic instability and progression to cancer such as AML has been linked to hematopoietic progenitors (28) and differences in target cell frequency between the two strains are unlikely to be a significant factor (29), we evaluated the status of cells in the bone marrow. Percentages of $CD34^+$, Ly-6A/$E^+$, and $CD34^+$Ly-6A/$E^+$ stem/progenitor cells were generally highest one day after 2 Gy irradiation, regardless of strain or radiation type. This suggests that the rapid depletion of leukocytes in the circulation after 2 Gy exposure resulted in activation of mechanisms needed for reconstitution. CD34 is a surface glycoprotein found on pluripotent hematopoietic cells which functions in cell-to-cell adhesion, thus facilitating migration (30). Ly-6A/E (also known as stem cell antigen 1 and 2, *i.e.* Sca-1, Sca-2) is associated with lymphocyte progenitors, especially the $CD4^+$ T-helper cells that secrete regulatory cytokines important in both innate and adaptive immune responses (31). Ly-6A/E is also expressed on activated T-cells and resting memory T-cells (32). The most dramatic increase in the $CD34^+$Ly-6A/$E^+$ cells occurred in CBA mice on day 1 after exposure to 2 Gy γ-rays, *i.e.* there was a 30-fold increase above that of the respective 0 Gy controls (Table V). In our previous studies, in comparison of 56Fe-irradiated C57 and CBA mice, numerous strain-related differences were noted, including a significantly higher percentage of stem/progenitor cells in bone marrow from the CBA mice (21, 22).

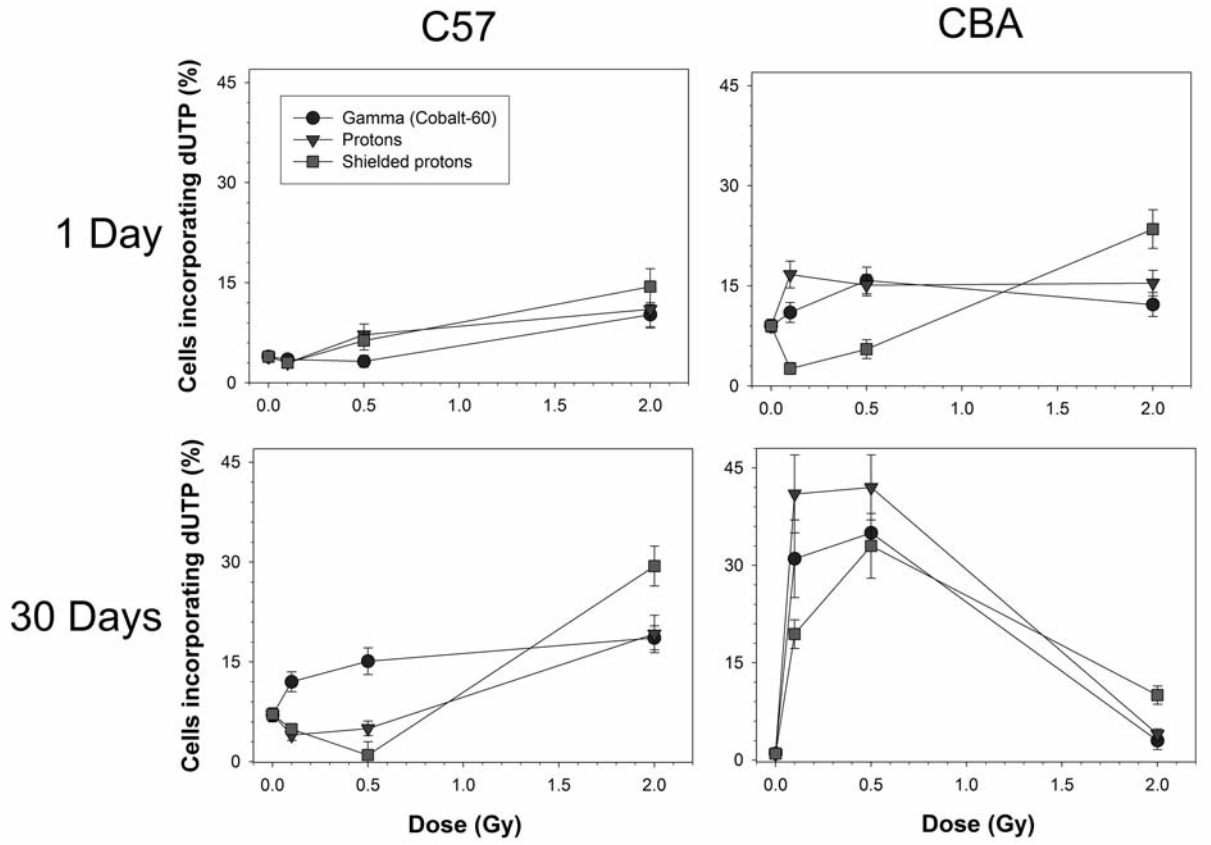


Figure 2. *Apoptosis determination in bone marrow cells based on the level of annexin V binding. The data were obtained from samples pooled/group using an FITC-annexin V labeling procedure and fluorescence intensity was measured by laser scanning cytometry. Each point represents the average of 2,500±500 cells scanned per slide.*

In the present investigation, we also demonstrated that at 30 days after irradiation, bone marrow cells from CBA mice exhibited consistently higher levels of DNA damage at doses less than 2 Gy, regardless of radiation quality. CBA mice also had lower levels of apoptosis at these doses. In general, the level of apoptosis in CBA mice was lower than that of C57 mice at a given timepoint. Therefore, C57 bone marrow cells were found to be more susceptible to radiation-induced apoptosis at early and later timepoints following exposure. The lower doses used here are considered sublethal doses and as such, these findings are consistent with the possibility for increased genomic instability in sublethally damaged cells. Consistent with previous observations, our current findings confirm that leukocytes of CBA mice are less sensitive to the effects of high-LET radiation (shielded protons) than C57 mice, a phenomenon consistent with increased possibility for genomic instability in sublethally damaged cells.

In a previous study, we proton-irradiated C57 and CBA mouse bone marrow cells kept outside or shielded within USA and Russian spacesuit helmets (33). Total doses ranging from 0.5 Gy to 2 Gy were used; analyses were performed on days 2 and 7 post-exposure. The data showed that proton radiation did induce transmissible chromosomal/genomic instability in hematopoietic cells in both strains of mice, regardless of shielding, but there were no significant differences related to strain. However, dramatic differences between the two strains were noted in levels of secreted transforming growth factor-β1 (TGF-β1), an immunosuppressive cytokine that can greatly influence immune and other cell responses (34). The level of TGF-β1 was 8-fold higher in the CBA compared to C57 mice on day 7 after 0.5 Gy irradiation of the shielded cells. In some of the samples there was a trend wherein the increased level of TGF-β1 was coincident with an increased level of cytogenetic aberrations. Thus, it seems possible that increased capacity for TGF-β1 production could facilitate carcinogenesis and reduce ability to eradicate aberrant cell populations after radiation exposure in a shielded environment.

Collectively the data presented here demonstrate great differences in splenic and bone marrow cell responses to the various radiation regimens, as well as differences between the two mouse strains. The findings are consistent with the

premise that survival of sublethally damaged hematopoietic cells may contribute to the relatively high prevalence of radiation-induced AML in CBA mice compared to the C57 strain. However, it should also be noted that the difference in radiation-associated AML between the two strains could be related to differences in macrophage response to tissue damage. After *in vivo* irradiation, the M2-like macrophages of C57 mice exhibit enhanced anti-inflammatory activity, whereas the M1-like macrophages of CBA mice retain their pro-inflammatory phenotype (35). Production of unstable oxygen radicals due to the pro-inflammatory effects of the M1-like macrophages in CBA mice may increase the amount of DNA damage, and thus facilitate development of AML. Furthermore, based on at least some of our measured parameters, the data indicate that exposure to high-LET radiation (shielded protons) is likely to have more profound effects compared to low-LET forms of radiation. The need to better understand the actions and interactions of immune cell populations regarding genomic instability and escape from immune surveillance against aberrant cell phenotypes remains high. The data from this study provides information that warrants further investigation.

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