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# Acute Effects of Whole-Body Proton Irradiation on the Immune System of the C57BL/6 Mouse

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by

Eric H. Kajioka

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June 1999

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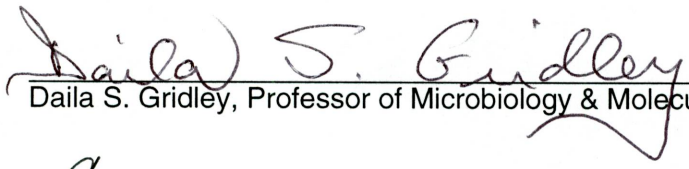

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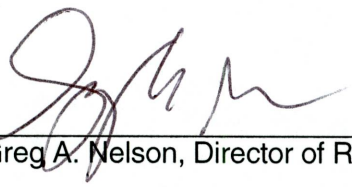

Greg A. Nelson, Director of Radiobiology Program

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**A MANUSCRIPT TO BE SUBMITTED TO: RADIATION RESEARCH**

# ACUTE EFFECTS OF WHOLE-BODY PROTON IRRADIATION ON THE IMMUNE SYSTEM OF THE C57BL/6 MOUSE

Running title: Acute effects of whole-body proton irradiation

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The primary author, Eric H. Kajioka, was responsible for the conception, planning, and implementation of the experiments. The primary author was also responsible for performing the majority of the experiments and wrote the entire manuscript. Assistance and suggestions, when necessary, were provided by Dr. Greg A. Nelson, Dr. Daila S. Gridley, Melba Andres, Jun Li, Dong Kim, Glen Miller, Peyman Haghighat, Bing Chen, Tatyana Timiryasova, and Dr. Michael Pecaut.

## ABSTRACT

## ACUTE EFFECTS OF WHOLE-BODY PROTON IRRADIATION ON THE IMMUNE SYSTEM OF THE C57BL/6 MOUSE

**by**

**Eric H. Kajioka**

The acute effects of proton whole-body irradiation (WBI) on leukocytes, lymphocytes, and hematological parameters in the spleen and blood of C57Bl/6 mice were examined and compared to the effects of photon (gamma) WBI derived from a $^{60}$cobalt ($^{60}$Co) source. Adult, female C57BL/6 mice were exposed to a single dose (3 Gy, 0.4 Gy/min dose rate) of either proton WBI at the Bragg peak, proton WBI at the entry plateau, or $^{60}$Co WBI, and sacrifice intervals were at 1, 4, 7, and 10 days post-WBI. Flow cytometry analysis of the spleen and peripheral blood showed depression in cell counts for all time points when compared to the non-irradiated control group. B (CD19+) and T-cytotoxic/suppressor (CD3+CD8+) lymphocytes were the most radiosensitive, while natural killer (NK1.1+) cells were the most radioresistant. Splenic T cells showed reduced responsiveness to mitogen stimulation for the first four days post-WBI, while splenic B cell responsiveness was reduced at all time periods. Analysis of hematological parameters showed depression of red blood cells, hemoglobin concentration, and hematocrit levels after 4 days post-WBI; platelet counts were low at days 4 and 10. Comparison of the proton and $^{60}$Co-irradiated groups showed few statistically significant differences among the radiation groups at any time point. These data indicate, for the very first time, that cells of the immune system are affected similarly by 3 Gy proton (Bragg peak and entry plateau) and gamma WBI. These findings could have a significant impact on future studies designed to maximize normal tissue protection during and after proton radiation exposure.

## INTRODUCTION

The effects of proton irradiation on the immune system are not well understood. This form of radiation comprises the majority of charged particles found in deep space, and astronauts on extended voyages are expected to receive high levels of exposure to proton radiation (1). Any damage to the highly radiosensitive bone marrow (i.e., the major site of hematopoiesis) and lymphoid organs such as the thymus and spleen could result in increased susceptibility to infections, serious toxicities, or even death. While the effects of photon (cobalt; $^{60}Co$) irradiation on the immune system have been studied to some extent (2-6), much less is known about the effects of proton irradiation. In addition, the biological mechanisms that result in immunosupression, as well as subsequent recovery after whole-body irradiation (WBI) are poorly understood.

The space radiation environment is composed of 4 major categories of particles. The first two, electromagnetic radiation and electrons, are low linear energy transfer (LET) particles which have a low probability of penetrating the shielding of a spacecraft. The other two, protons and heavy-ion particles, are higher LET particles that can penetrate shielding material and damage biological tissues. The different dose-distributions between low and high LET particles have been applied to radiotherapy of cancer patients, and have been reviewed elsewhere (7-11). The occurrence of heavy-ion particles within the space environment is very low. Protons, however, comprise over 80% of deep space radiation particles (1, 12), and while exposure levels for short-term missions are small, longer missions would result in higher levels of exposure that would exceed current safety limits. With the planning of future projects such as the space station, lunar, and Mars missions, understanding the effect of high levels of proton exposure has become a top priority of the National Aeronautics and Space Administration (NASA). In addition, with the cells of the immune system among the most radiosensitive in the body, elucidating the effects of proton radiation on the immune system is essential to understanding the health-related risks associated with deep-space travel.

In general, proton radiation has been found to induce similar effects on certain types of biological tissues when compared to an equivalent dose of $^{60}Co$ radiation. Relative biological effectiveness (RBE) values were derived to calculate the amount of biological damage exerted by different forms of ionizing radiation in relation to photons (i.e., gamma radiation from a $^{60}Co$ source). RBE ratings ranging from 0.8 to 1.5 have been reported in the literature for proton radiation based on experimentation with a variety of cell cultures and animal tissues (13-16). In addition, RBE values between the entry plateau and Bragg peak regions have also varied considerably (13, 14, 17). Since astronauts can be expected to be exposed to both regions of proton radiation, it is important, then, to determine if there are any observable differences between these areas on cells of the immune system.

We report here measurements of immune system damage, functionality, and recovery resulting from proton WBI. Using flow cytometry and hematological analyses, organ weights, leukocyte concentrations, and spontaneous and mitogen-induced blastogenesis assays the effects of proton WBI on C57Bl/6 mice were examined and compared to an equivalent physical dose of $^{60}Co$ radiation.

## MATERIALS AND METHODS

*Animals*

Female C57Bl/6 mice (n=168) were purchased from Charles River Breeding Laboratories, Wilmington, MA at 8 weeks of age and used for testing as young adults two weeks later. The animals were maintained in self-contained filter-top cages (8 mice/cage) in our Animal Care Facility controlled for temperature, humidity, and a 12:12 hr light:dark cycle. Standard rodent food (Purina Lab Chow) and water were provided ad libitum. The mice were observed daily for signs of toxicity (lethargy, bloating, and ruffling of fur) following irradiation. Euthanasia was accomplished by rapid $CO_2$ asphyxiation in compliance with National Institute of Health guidelines. The animal facility is NIH and AAALAC approved. All animal care is under the direction of a licensed veterinarian, and all research protocols involving animals at our institution are approved by the Animal Research Committee prior to initiation.

*External Beam Irradiation*

The mice were placed individually into rectangular plastic boxes (3 cm x 3 cm x 8.5 cm). The walls of the boxes were 1 mm thick with several holes provided in each box for respiration. Eight mice were irradiated simultaneously. The dose delivered to each mouse was 3 Gray (Gy). Whole-body proton irradiation was performed using 250 MeV protons from the proton synchrotron accelerator and a fixed horizontal research beam line. Protons were delivered from the accelerator in 0.3 s long pulses every 2.2 s. The proton beam was enlarged in area using two scattering foils to give a field diameter of 260 mm, 3000 mm downstream of the first foil. One set of mice was irradiated in the entrance part of the beam by placing the surface of the boxes behind a 400 mm by 400 mm polystyrene phantom at a water equivalent depth of 26.4 mm. The surface of the polystyrene phantom was located approximately 3 m from the first scattering foil using a positioning laser. The second set of mice was irradiated behind a polystyrene phantom at a water equivalent depth of 274 mm with a spinning polycarbonate modulator

propeller to generate a uniform dose over a 60 mm wide range of depths. The surface of the phantom was again placed at the positioning laser. Calibration of the dose received by the mice was performed by placing a Markus parallel plate ionization chamber, traceable to the National Institute of Standards and Technology (NIST), at depths corresponding to the center of the mice for each set-up. Measurements were also performed at depths 15 mm shallower and 15 mm deeper than the center of the mice to verify uniformity of dose over the target volume. The ICRU 59 calibration method was used to convert the ionization signal to dose in water. The average dose rate for the modulated set-up was approximately 0.4 Gy/min.

Whole-body $^{60}$Co irradiation was performed using a horizontal beam from an AECL (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada) Eldorado therapy unit. The adjustable collimators were set to project a field size of 320 mm by 320 mm at a distance of 800 mm from the source. The center of the boxes (mice) were placed 1620 mm from the $^{60}$Co source. A 5 mm thick, 170 mm by 170 mm plastic plate was placed immediately upstream of the boxes to place the front surface of the box at a depth of maximum dose. Because of an exponential decrease in dose across the mouse bodies, all boxes were rotated halfway through each irradiation to homogenize the dose across the animals. Calibration of the dose received by the mice was performed using a Capintec Model PRO6-G cylindrical thimble ionization chamber, traceable to the NIST. The chamber was placed at a depth of 20 mm in a 250 mm by 250 mm polystyrene phantom corresponding to half-thickness of the mice plus the build-up plate. The American Association of Physicists in Medicine (AAPM) TG-21 protocol was used to convert the ionization signal to dose delivered in water. The dose delivered to the 250 mm square plastic phantom was corrected to dose to the 150 mm square volume occupied by the mouse-containing boxes by using the tissue-to-air ratio for the two field sizes. The dose rate to the center of the mice was approximately 0.4 Gy/min.

*Mouse Body Weights, Relative Organ Weights, and Leukocyte Counts*

At the time of euthanasia, the mice were weighed and the spleen and thymus were excised and weighed. The organ weight in relation to body weight (ROW: relative organ weight) was calculated as follows: ROW = organ weight (g) x $10^4$/body weight (g). Whole blood was collected in heparin-containing tuberculin syringes by cardiac puncture immediately after euthanasia. Aliquots (20 µl ) of whole blood were mixed with 1.98 ml of ammonium oxalate and thimerosal in Sorenson's phosphate buffer adjusted to 6.8 pH. (Unopette Microcollection System, Becton Dickinson and Co., Rutherford, NJ) to lyse the erythrocytes. The remaining viable leukocytes were counted in a hemocytometer. The spleens were processed into single-celled suspensions, washed, and centrifuged. Erythrocytes in cell pellets were lysed by incubation in 2 ml of lysis buffer ($KHCO_3/NH_4Cl/EDTA$) for 4 min at 4°C. The remaining leukocytes were washed, suspended in 1 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), antibiotics, and 2-mercaptoethanol. Viable cells were counted with a hemocytometer using the trypan blue exclusion method.

*Hematological Measurements*

Whole blood and unlysed spleen suspensions were evaluated using an ABC Vet Hematology Analyzer (Heska Corporation, Waukesha, WI) which was specifically set to analyze hematological parameters in the mouse. The measurements included red blood cell count (RBC;$10^6$/µl), hemoglobin concentration (Hgb; g/dl), hematocrit levels (Hct; % volume of whole blood composed of RBC), mean corpuscular volume (MCV; average volume per RBC in fl), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; the concentration of Hgb in the average RBC in g/dl), and platelet counts (PLT). MCV was calculated by dividing the Hct by

the RBC count and multiplying by 10. MCH was calculated by dividing the Hb concentration by the RBC count and multiplying by 10. MCHC was calculated by dividing the Hb concentration by the Hct and multiplying by 100.

*Spontaneous Blastogenesis*

Aliquots (50 µl) of whole blood and spleen suspensions were mixed with 150 µl of complete RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) and 1 µCi $^3$H-thymidine ($^3$H -TdR; specific activity = 46 Ci/µmol; ICN Biochemicals, Costa Mesa, CA) in 50 µl of medium. The samples were dispensed in triplicate into flat-bottomed wells of 96-well microculture plates and incubated for 4 hr at 37°C in a humidified atmosphere containing 5% $CO_2$. After incubation, the cells were harvested with a multiple-sample harvester and the amount of $^3$H-TdR incorporated into cell DNA was counted in a liquid beta-scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The leukocyte counts/ml and volume of tested blood (50 µl) were used to convert the raw disintergrations per minute (dpm) into dpm/$10^6$ leukocytes.

*Mitogen-induced Splenocyte Proliferation*

The spleens were processed into single-celled suspensions, washed, and centrifuged. Erythrocytes in cell pellets were lysed, by incubation in 2 ml of ACK solution for 4 minutes at 37°C. The remaining leukocytes were then washed and adjusted to a concentration of 2x$10^6$ cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone), antibiotics, and mercaptoethanol. Aliquots (100 µl) of each sample were dispensed in triplicate into flat-bottomed wells of 96-well microtiter plates with phytohemagglutinin (PHA; Sigma), concanavalin A (Con A; Sigma), lipopolysaccharide (LPS; Sigma), and no stimulant. The mitogens were pre-titrated for maximal response. The plates were incubated for 48 hrs in 5% $CO_2$ at 37°C. During the last 4 hr of incubation, $^3$H –TdR (ICN Radiochemicals) was added at 1 µCi/50

µl/well. The cells were harvested using a multiple sample harvester. Filter paper discs with the trapped cells were placed in vials containing 2 ml of scintillation fluid, and the uptake of $^{3}H$ -TdR was quantitated in a beta-scintillation counter. The results are expressed as stimulation index (SI) using the disintegrations per minute (dpm) as follows: SI = (dpm with mitogen – dpm without mitogen)/dpm without mitogen.

*Flow Cytometry Analysis of Spleen and Blood Lymphocyte Populations*

Immunophenotyping of peripheral blood and spleen samples were carried out to quantitate lymphocyte populations using a FACSCalibur™ 4-channel flow cytometer (Becton Dickinson, Inc., San Jose, CA). All monoclonal antibodies (MAb), labeled either with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein (PerCP), were purchased from Pharmingen (San Diego, CA). The MAb were directed against the following markers: CD3 - T cell receptor-associated complex present on all mature T lymphocytes; CD4 - protein on T helper/inducer (Th) cells; CD8 - protein on T cytotoxic/suppressor (Tc) cells, CD19 - protein on B lymphocytes; NK1.1 - protein on NK cells. For the peripheral blood, 100 µl aliquots of diluted samples (1:2 in phosphate buffered saline, PBS) were dispensed into a series of tubes. The fluorescence-labeled MAb were added at 10 µl/tube to give a final concentration of 0.1 µg/ml. Appropriate isotype controls were included. The tubes were incubated in the dark for 15 min on ice to prevent "capping." Acquisition and analysis of 5,000 lymphocyte events/tube was performed using CellQuest™ software version 3.1 (Becton Dickinson). For the spleen samples, 20 µl of the selected 4-color Mab cocktail was added to 50 µl of spleen cells in each tube. The tubes were incubated in the dark at room temperature for 20 minutes. Acquisition and analysis of 10,000 total events/tube was performed using CellQuest™ software version 3.1 (Becton Dickinson). Purity calculations (based on sum of percentages of CD19, CD4, CD8, and NK1.1 positive cells) of the gated lymphocytes in each sample were used to convert the raw

percentages of T, Th, Tc, B, and NK populations to normalized percentages. To obtain absolute counts for each lymphocyte population, the following formula was used: absolute count/ml = leukocyte count/ml x normalized percentage.

*Statistical Analysis*

The results were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test, and the non-parametric Kruskal-Wallis test. These analyses were performed using SigmaStat™ software, version 2.03 (SPSS Inc., Chicago, IL).

## RESULTS

The mice were divided into three separate radiation groups: 1) proton radiation at the modulated Bragg peak (BP); 2) proton radiation at the entry plateau (EP); and 3) $^{60}Co$ gamma radiation ($^{60}Co$). Eight mice from each radiation group were sacrificed every three days after one-day post-WBI. Control mice (n=16) were sacrificed at each time point and recorded values were pooled.

*Body and Organ Weights after Irradiation*

The results (Table 1) show that all of the irradiated mice experienced significant weight loss one-day post-WBI as compared to the non-irradiated controls. However, by day 4, all of the irradiated mice recovered to regain their normal body mass and maintained their weight through day 17. Spleen and thymus weights were lowest on day 4, but recovered to normal weight by day 10. No differences in organ weights or relative organ weights were noted between the different radiation groups.

*Leukocyte Counts and Basal Cellular Proliferation after Irradiation*

White blood cell counts in the spleen and peripheral blood (Table 2) reflect significant damage by day 1 for both groups exposed to proton radiation. Day 4 represents the lowest leukocyte levels, with a 91% loss in the spleen and peripheral blood for the proton (BP)- and proton (EP)-irradiated groups. Partial reconstitution of leukocyte levels for both groups was evident on day 7 and a further increase was seen day 10. However, the cell numbers were still significantly depressed compared to the non-irradiated control levels through days 7 and 10 for all irradiated groups. Leukocyte levels in proton (BP)- and proton (EP)- levels were statistically similar to those seen after $^{60}Co$ irradiation for all time periods.

Figure 1 shows spontaneous blastogenesis of the spleen and peripheral blood when adjusted to $10^6$ cells/ml. In the spleen, $^{3}H$-TdR uptake in the proton (BP)-irradiated group was similar to control levels, but significantly lower than the $^{60}Co$-irradiated group

**Table 1.** Body, spleen, and thymus mass.

| | Body | Organ weight (mg) | | Relative organ weight[a] | |
|---|---|---|---|---|---|
| | weight (g) | spleen | thymus | spleen | thymus |
| Non-Irradiated Control | 21.2+/-0.2[b,c] | 79.0+/-1.9[d] | 65.6+/-2.3[e] | 37.2+/-0.6[d] | 31.0+/-1.2[f] |
| Day 1 | | | | | |
| Proton (BP) | 18.7+/-0.1 | 52.6+/-1.8 | 52.1+/-3.1 | 28.1+/-0.9 | 27.8+/-1.6 |
| Proton (EP) | 18.1+/-0.4 | 49.3+/-1.7 | 54.7+/-2.9 | 27.2+/-0.8 | 30.4+/-2.1 |
| Cobalt | 18.2+/-0.3 | 51.9+/-2.7 | 49.7+/-4.5 | 28.5+/-1.4 | 27.2+/-2.3 |
| Day 4 | | | | | |
| Proton (BP) | 19.6+/-0.3 | 32.0+/-1.0 | 23.5+/-1.5 | 16.3+/-0.5 | 12.0+/-0.8 |
| Proton (EP) | 21.0+/-0.4 | 34.4+/-1.4 | 28.8+/-3.8 | 16.4+/-0.6 | 13.9+/-1.9 |
| Cobalt | 20.0+/-0.4 | 35.9+/-1.0 | 18.0+/-0.3 | 26.8+/-2.0 | 13.3+/-0.8 |
| Day 7 | | | | | |
| Proton (BP) | 21.0+/-0.8 | 43.9+/-2.4 | 51.5+/-2.1 | 20.9+/-0.7 | 24.6+/-1.2 |
| Proton (EP) | 20.7+/-0.5 | 44.2+/-3.3 | 49.8+/-3.2 | 21.3+/-1.4 | 24.0+/-1.5 |
| Cobalt | 21.3+/-0.4 | 47.7+/-2.1 | 51.5+/-4.1 | 22.4+/-0.8 | 24.1+/-1.8 |
| Day 10 | | | | | |
| Proton (BP) | 21.8+/-0.4 | 90.7+/-8.8 | 72.7+/-5.4 | 41.6+/-4.1 | 33.5+/-2.7 |
| Proton (EP) | 20.1+/-0.2 | 70.7+/-3.5 | 64.6+/-2.9 | 35.2+/-1.8 | 32.1+/-1.3 |
| Cobalt | 21.6+/-0.5 | 74.1+/-3.3 | 57.7+/-3.6 | 34.5+/-1.8 | 26.7+/-1.3 |

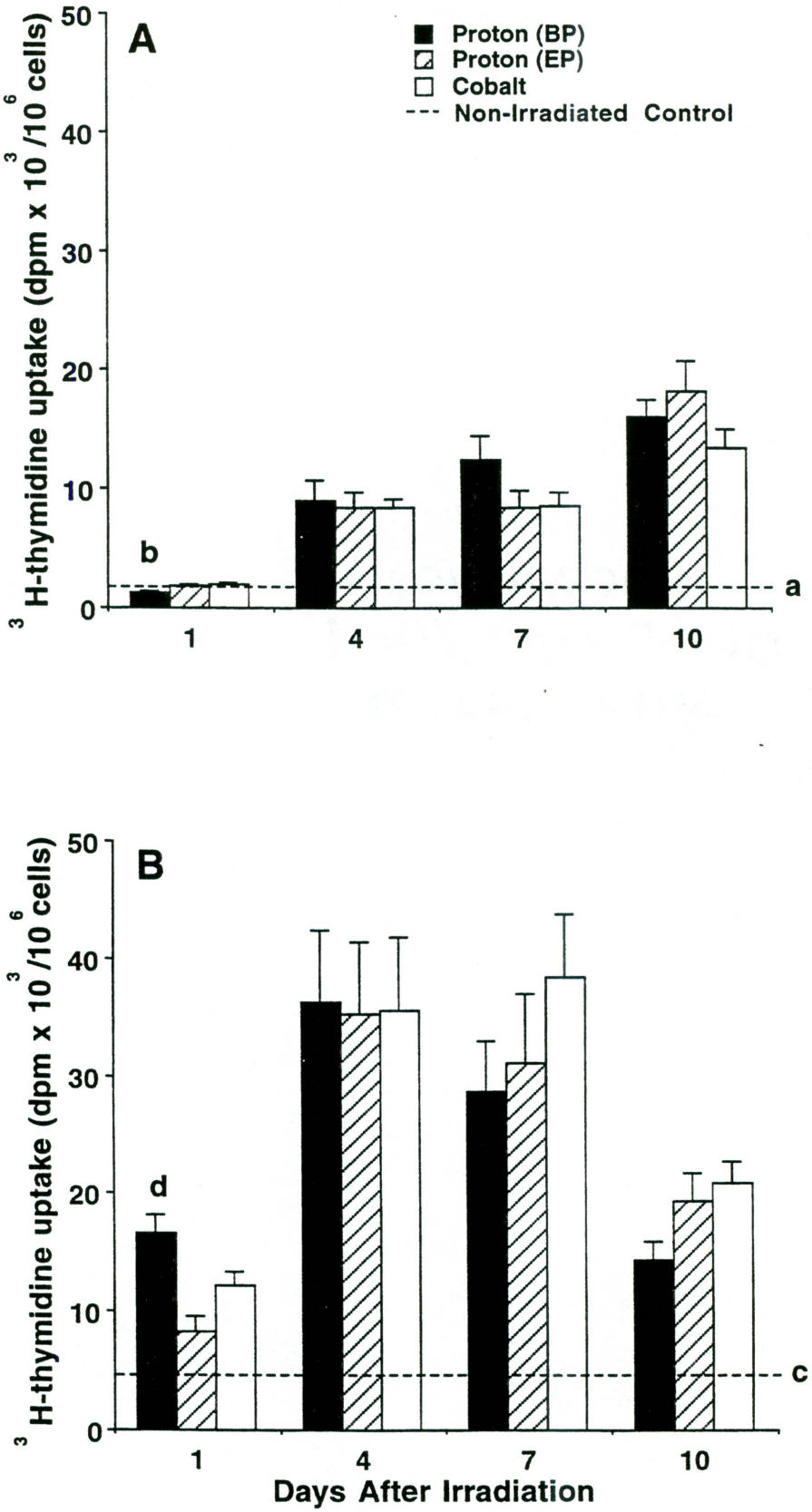
[a]Relative organ (spleen or thymus) weight = organ weight (g) x $10^4$/body weight (g).
[b]Mean +/- S.E.M.
[c]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on day 1; $p<0.001$.
[d]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, and 7; $p<0.001$.
[e]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, and 7; $p<0.01$.
[f]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4 and 7; $p<0.01$.

**Table 2.** Leukocyte and lymphocyte concentrations

| | Leukocytes[a] ($x10^6$/ml) | | Lymphocytes[b] ($x10^6$/ml) | |
|---|---|---|---|---|
| | spleen | blood | spleen | blood |
| Non-Irradiated Control | 80.66+/-5.40[c,d] | 9.89+/-0.52[d] | 55.46+/-3.89[d] | 7.54+/-0.45[d] |
| Day 1 | | | | |
| Proton (BP) | 24.28+/-1.14 | 2.75+/-0.28 | 9.86+/-0.71 | 0.86+/-0.06 |
| Proton (EP) | 17.13+/-1.10 | 4.53+/-0.49 | 6.27+/-0.43 | 1.34+/-0.20 |
| Cobalt | 16.97+/-1.48 | 2.75+/-0.22 | 6.37+/-0.52 | 0.90+/-0.10 |
| Day 4 | | | | |
| Proton (BP) | 7.50+/-0.85 | 1.02+/-0.15 | 3.37+/-0.40 | 0.69+/-0.14 |
| Proton (EP) | 7.06+/-1.00 | 0.80+/-0.15 | 2.98+/-0.42 | 0.45+/-0.06 |
| Cobalt | 7.09+/-0.44 | 0.97+/-0.15 | 3.20+/-0.18 | 0.64+/-0.10 |
| Day 7 | | | | |
| Proton (BP) | 13.22+/-1.69 | 2.97+/-0.42 | 6.77+/-0.91 | 1.26+/-0.17 |
| Proton (EP) | 12.44+/-1.31 | 2.78+/-0.46 | 6.23+/-0.67 | 1.23+/-0.16 |
| Cobalt | 14.44+/-1.19 | 1.81+/-0.25 | 7.48+/-0.62 | 0.95+/-0.12 |
| Day 10 | | | | |
| Proton(BP) | 20.19+/-1.90 | 2.09+/-0.23 | 9.38+/-0.89 | 1.00+/-0.14 |
| Proton (EP) | 16.50+/-1.90 | 2.38+/-0.18 | 7.92+/-0.97 | 1.07+/-0.07 |
| Cobalt | 20.44+/-1.43 | 2.03+/-0.19 | 10.61+/-0.84 | 1.08+/-0.09 |

[a]Leukocytes were counted in a hemocytometer after lysis of erythrocytes. Since thecells from the entire spleen were suspended in 1 ml before counting, these values also represent the number of leukocytes per spleen.
[b]Obtained by flow cytometry analysis using the formula: lymphocytes = (%CD3 + %CD19 + %NK1.1) · (leukocyte concentration).
[c]Mean +/- S.E.M.
[d]Significantly different than proton (BP), proton (EP), and $^{60}Co$ radiation groups on days 4, 7, and 10; $p<0.001$.

**Figure 1.** Spontaneous blastogenesis with time after whole body-irradiation as determined by $^{3}$H-TdR uptake in splenocytes (Panel A) and peripheral blood cells (Panel B). Cells were incubated in a medium containing 1 μCi $^{3}$H-TdR per well for 4 h before counting the amount of radioactivity taken up by cells. Each bar represents the mean +/- S.E.M. a: Significantly lower than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4, 7, and 10 ($p<0.001$); b: Significantly lower than $^{60}$Co radiation group ($p=0.005$); c: Significantly lower than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, 7, and 10 ($p<0.001$); d: Significantly higher than proton (EP) ($p<0.001$) and $^{60}$Co radiation groups ($p=0.036$).


A
Proton (BP)
Proton (EP)
Cobalt
Non-Irradiated Control
$^3$H-thymidine uptake (dpm x 10$^3$/10$^6$ cells)
50
40
30
20
10
0
b
a
1
4
7
10
B
d
c
$^3$H-thymidine uptake (dpm x 10$^3$/10$^6$ cells)
Days After Irradiation

on day 1. By day 4, however, $^{3}$H-TdR uptake was significantly increased, and remained elevated through days 7 and 10. $^{3}$H-TdR uptake by the peripheral blood cells was elevated for all 4 time points compared to the non-irradiated controls. Additionally, on day 1, levels of $^{3}$H-TdR uptake for the proton (BP)-irradiated group was significantly higher than the $^{60}$Co-irradiated group, and nearly 2-fold higher than those for the group exposed to entry plateau protons. No other significant differences were noted between any of the radiation groups in the spleen or peripheral blood.

*Splenic Lymphocytes after Irradiation: Phenotype*

Flow cytometry analysis of mononuclear cell subsets in the spleens from proton (BP)-irradiated mice (Figure 2) show CD3+ (T) cell numbers decreasing from normal levels of 16.12+/-1.08 x$10^{6}$ cells/ml to 1.43+/-0.17 x$10^{6}$ cells/ml by day 4. This corresponds to a 91% loss. CD19+ (B) cells were the most radiosensitive and dropped from normal levels of 35.69+/-2.60 x$10^{6}$ cells/ml to 0.86+/-0.12 x$10^{6}$ cells/ml by day 4 (98% decrease). Examining the CD3+ subsets, CD8+ T-cytotoxic/suppressor (Tc) cells (96% decrease) were slightly more radiosensitive than CD4+ T-helper (Th) cells (90% decrease) by day 4. NK cells were the most radioresistant to proton (BP) with 1.08+/-0.12 x$10^{6}$ cells/ml on day 4 which corresponds to a 70% decrease from normal levels of 3.92 x$10^{6}$ cells/ml. Counts of all the measured lymphocyte subsets increased on day 7 and continued to increase through day 10. However, cell levels still remained severely depressed by day 10 with no subset higher than 28% of normal values. When examining the lymphocyte percentages in the spleens from proton (BP)-irradiated mice (Table 3), CD3+, CD4+ and NK1.1+ cell percentages were significantly higher while CD19+ and CD8+ percentages were significantly lower for all 4 time points.

Splenic mononuclear subsets proton (EP)-irradiated mice showed similar trends as the proton (BP)-irradiated group for both absolute counts (Figure 2) and percentages

**Figure 2.** Lymphocyte populations in the spleen with time after whole-body irradiation. The percentages of each cell type were determined by flow cytometry analysis using fluorescence-labeled monoclonal antibodies against the following: CD3+ (T cells), CD19+ (B cells), CD3+CD4+ (T helper/inducer cells), CD3+CD8+ (T cytotoxic/ suppressor cells), and NK1.1+ (NK cells). The absolute counts were obtained by multiplying the lymphocyte counts/ml by the percentage obtained for each population. Each bar indicates the mean +/- S.E.M. a: Significantly higher than proton (BP), proton (EP), and $^{60}Co$ radiation groups on days 1, 4, 7, and 10 ($p<0.001$).

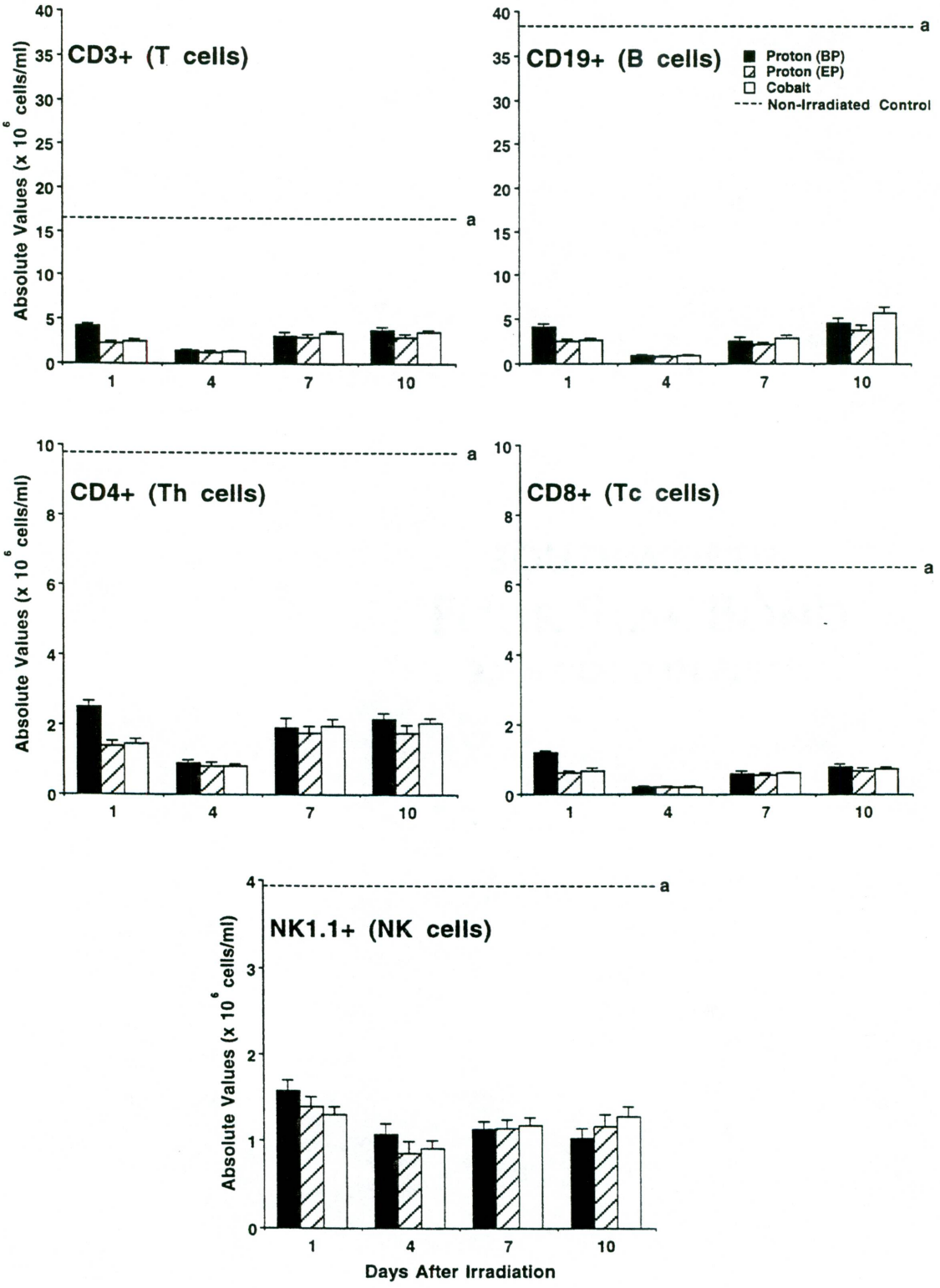

CD3+ (T cells)
CD19+ (B cells)
CD4+ (Th cells)
CD8+ (Tc cells)
NK1.1+ (NK cells)
Absolute Values (x 10^6 cells/ml)
Days After Irradiation
Proton (BP)
Proton (EP)
Cobalt
Non-Irradiated Control
a

**Table 3.** Percentages of splenic lymphocyte populations

| | Lymphocytes[a] (%) | | | | |
|---|---|---|---|---|---|
| | CD3 | CD19 | CD3/CD4 | CD3/CD8 | NK1.1 |
| Non-Irradiated Control | 29.25+/-0.51[b,c] | 64.17+/-0.54[c] | 16.64+/-0.35[c] | 10.94+/-0.23[d] | 6.58+/-0.29[c] |
| Day 1 | | | | | |
| Proton (BP) | 42.95+/-1.78[e] | 40.85+/-1.44 | 25.51+/-0.86[f] | 12.29+/-0.70[g] | 16.19+/-0.94[h] |
| Proton (EP) | 36.92+/-1.14 | 40.98+/-1.85 | 22.43+/-1.06 | 9.87+/-0.35 | 22.11+/-0.96 |
| Cobalt | 37.66+/-0.45 | 40.93+/-1.21 | 22.82+/-0.71 | 10.93+/-0.46 | 20.72+/-1.18 |
| Day 4 | | | | | |
| Proton (BP) | 42.42+/-1.09 | 25.01+/-1.00 | 27.08+/-1.02 | 7.08+/-0.26 | 32.57+/-1.67 |
| Proton (EP) | 43.12+/-0.76 | 27.71+/-1.03 | 27.12+/-0.96 | 7.67+/-0.50 | 29.17+/-1.57 |
| Cobalt | 42.67+/-1.80 | 28.37+/-1.38 | 26.57+/-1.34 | 7.68+/-0.42 | 28.96+/-2.15 |
| Day 7 | | | | | |
| Proton (BP) | 46.60+/-1.25 | 35.53+/-2.00 | 28.39+/-1.06 | 8.77+/-0.54 | 17.87+/-1.36 |
| Proton (EP) | 46.67+/-1.11 | 34.85+/-1.05 | 27.94+/-0.61 | 9.10+/-0.43 | 18.48+/-0.52 |
| Cobalt | 45.48+/-0.56 | 38.47+/-0.93 | 26.35+/-0.42 | 8.55+/-0.38 | 16.04+/-0.76 |

| | | | | | |
|---|---|---|---|---|---|
| Day 10 | | | | | |
| Proton (BP) | 40.79+/-1.15 | 47.72+/-1.99 | 23.19+/-0.74 | 8.55+/-0.38 | 11.48+/-1.38[i] |
| Proton (EP) | 38.55+/-1.14 | 46.43+/-1.51 | 22.64+/-0.79 | 8.91+/-0.59[j] | 15.02+/-0.87 |
| Cobalt | 34.17+/-1.49[k] | 53.51+/-2.02[l] | 19.75+/-0.73[m] | 7.20+/-0.36 | 12.32+/-0.98 |

[a]The percentages of each cell type were determined by flow cytometry analysis using fluorescence-labeled monoclonal antibodies against the following markers: CD3 (T cells), CD19 (B cells), CD3/CD4 (T helper/inducer cells), CD3/CD8 (T cytotoxic/suppressor cells), and NK1.1 (NK cells). Total percentages of lymphocytes were obtained by the raw percentage of leukocytes that are lymphocytes. Percentages for each subset were obtained by dividing the raw percentage of each lymphocyte subset by the total percent of lymphocytes obtained for each population.

[b]Mean +/- S.E.M.

[c]Significantly different than proton (BP), proton (EP), and $^{60}$Co radiation groups on day 1, 4, 7, and 10; $p<0.001$.

[d]Significantly different than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4, 7, and 10; $p<0.001$.

[e]Significantly higher than proton (EP); $p=0.003$: Significantly higher than $^{60}$Co; $p=0.3$.

[f]Significantly higher than proton (EP); $p=0.31$.

[g]Significantly higher than proton (EP); $p=0.003$.

[h]Significantly lower than proton (EP); $p<0.001$: Significantly lower than $^{60}$Co; $p=0.003$.

[i]Significantly lower than proton (EP); $p=0.035$.

[j]Significantly higher than $^{60}$Co; $p=0.025$.

[k]Significantly lower than proton (BP); $p<0.001$: Significantly lower than proton (EP); $p=0.033$.

[l]Significantly lower than proton (BP); $p=0.046$: Significantly lower than proton (EP); $p=0.01$.

[m]Significantly lower than proton (BP); $p=0.04$: Significantly lower than proton (EP); $p=0.019$.

(Table 3). In addition, no significant differences were noted between the absolute cell counts of the proton radiation groups compared to the $^{60}Co$ radiation group.

However, significant differences were found between the percentages of certain lymphocyte populations in the proton (BP), proton (EP), and $^{60}Co$ radiation groups on days 1 and 10 (Table 3).

*Peripheral Blood Lymphocytes after Irradiation: Phenotype*

Flow cytometry analyses of mononuclear cell subsets in the peripheral blood (Figure 3) of proton (BP)-irradiated mice show similar trends of susceptibility to radiation as seen in the spleen. By day 4, CD19+ B cells and CD8+ Tc cells were the most radiosensitive with 98% and 93% decrease in cell counts, respectively. Relative radioresistance of NK cells was also observed with only a 62% decrease seen by day 4. Cell counts of all lymphocyte subsets remained significantly lower than control levels by day 10. However, fluctuations in cell counts were seen with CD3+, CD4+, and CD8+ cells with an increase in levels from day 4 to 7 followed by a decrease in levels on day 10. NK cells recovered to 74% of normal levels by day 10, while CD19+ cells remained depressed at 4% of normal counts. Analysis of lymphocyte percentages (Table 4) in proton (BP)-irradiated animals showed a significantly higher levels of CD4+ and NK cell percentages and significantly lower levels of CD19+ cell percentages for all 4 time points. CD3+ cell percentages were elevated for days 1, 4 and 7. However, by day 10, no differences were noted compared to the normal controls. CD8+ cell percentages showed no significant differences on days 1 and 7, and significantly lower levels on days 4 and 10.

Peripheral blood mononuclear subsets in the proton (EP)-irradiated group again showed similar depression and recovery trends as observed in the proton (BP)-irradiated group, with no significant differences noted between the absolute cell counts (Figure 3). Additionally, no significant differences were noted between the absolute cell

**Figure 3.** Lymphocyte populations in the peripheral blood with time after whole-body irradiation. The percentages of each cell type were determined by flow cytometry analysis using fluorescence-labeled monoclonal antibodies against the following markers: CD3+ (T cells), CD19+ (B cells), CD3+CD4+ (T helper/inducer cells), CD3+CD8+ (T cytotoxic/suppressor cells), and NK1.1+ (NK cells). The absolute counts were obtained by multiplying the lymphocyte counts/ml by the percentage obtained for each population. Each bar indicates the mean +/- S.E.M. a: Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, 7, and 10 ($p<0.001$); b: Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, 7, and 10 ($p<0.05$).

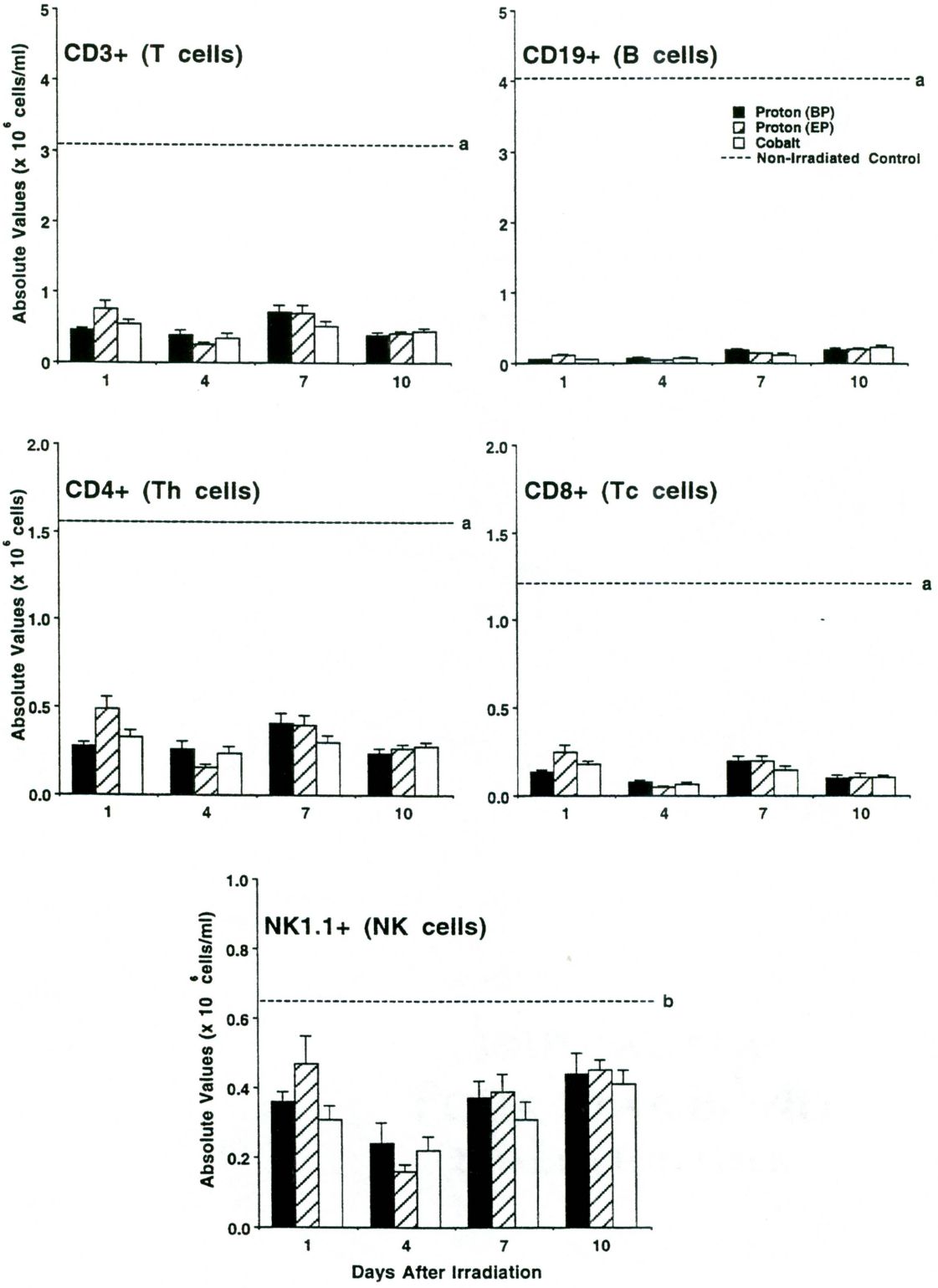

CD3+ (T cells)
CD19+ (B cells)
CD4+ (Th cells)
CD8+ (Tc cells)
NK1.1+ (NK cells)
Absolute Values (x 10^6 cells/ml)
Absolute Values (x 10^6 cells)
Days After Irradiation
Proton (BP)
Proton (EP)
Cobalt
Non-Irradiated Control
a
b
1
4
7
10

**Table 4.** Percentages of blood lymphocyte populations

| | Lymphocytes[a] (%) | | | | |
|---|---|---|---|---|---|
| | CD3 | CD19 | CD3/CD4 | CD3/CD8 | NK1.1 |
| Non-Irradiated Control | 39.20+/-1.20[b,c] | 52.69+/-1.19[d] | 19.90+/-0.78[e] | 15.26+/-0.45[f] | 8.30+/-0.27[d] |
| Day 1 | | | | | |
| Proton (BP) | 52.26+/-1.27 | 6.18+/-0.43 | 32.56+/-0.77 | 16.81+/-0.77 | 41.56+/-1.24 |
| Proton (EP) | 57.30+/-1.85 | 8.31+/-1.14 | 36.90+/-2.14 | 18.97+/-0.98[h] | 34.39+/-1.53 |
| Cobalt | 60.62+/-1.78 | 5.40+/-0.74 | 37.19+/-1.49 | 19.98+/-0.67[i] | 33.98+/-1.30 |
| Day 4 | | | | | |
| Proton (BP) | 56.62+/-1.76 | 10.66+/-1.20 | 38.09+/-1.29 | 12.07+/-0.86 | 32.72+/-1.49 |
| Proton (EP) | 54.98+/-1.29 | 9.76+/-0.47 | 36.31+/-1.16 | 11.85+/-0.67 | 35.26+/-1.28 |
| Cobalt | 55.83+/-2.62 | 10.63+/-0.96 | 37.81+/-1.87 | 11.49+/-0.91 | 33.53+/-2.03 |
| Day 7 | | | | | |
| Proton (BP) | 55.67+/-1.90 | 14.97+/-1.87 | 32.85+/-1.02 | 15.33+/-0.87 | 29.36+/-1.03 |
| Proton (EP) | 55.99+/-1.72 | 12.44+/-1.17 | 32.54+/-1.22 | 16.13+/-0.72 | 31.57+/-1.55 |
| Cobalt | 54.95+/-2.10 | 13.59+/-0.86 | 32.01+/-1.41 | 16.48+/-0.72 | 31.45+/-2.39 |

| | | | | | |
|---|---|---|---|---|---|
| Day 10 | | | | | |
| Proton (BP) | 38.10+/-1.40 | 18.52+/-2.16 | 24.46+/-1.07 | 9.60+/-0.47 | 43.38+/-1.48 |
| Proton (EP) | 38.62+/-1.35 | 18.80+/-0.89 | 25.50+/-1.19 | 10.56+/-1.22 | 42.58+/-1.63 |
| Cobalt | 40.34+/-0.71 | 21.70+/-1.93 | 25.74+/-0.83 | 10.15+/-0.46 | 37.96+/-1.61 |

[a]The percentages of each cell type were determined by flow cytometry analysis using fluorescence-labeled monoclonal antibodies against the following markers: CD3 (T cells), CD19 (B cells), CD3/CD4 (T helper/inducer cells), CD3/CD8 (T cytotoxic/suppressor cells), and NK1.1 (NK cells). Total percentages of lymphocytes were obtained by the raw percentage of leukocytes that are lymphocytes. Percentages for each subset were obtained by dividing the raw percentage of each lymphocyte subset by the total percent of lymphocytes obtained for each population.

[b]Mean +/- S.E.M.

[c]Significantly lower than proton (BP), proton (EP), and $^{60}$Co radiation groups on day 1, 4 and 7; $p<0.001$.

[d]Significantly different than proton (BP), proton (EP), and $^{60}$Co radiation groups on day 1, 4, 7, and 10; $p<0.001$.

[e]Significantly different than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, 7, and 10; $p<0.05$.

[f]Significantly lower than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4 and 10; $p<0.001$.

[g]Significantly higher than non-irradiated control group; $p=0.002$.

[h]Significantly higher than proton (BP) radiation group; $p=0.024$: Significantly higher than non-irradiated control group; $p<0.001$.

counts of the proton radiation groups compared to the $^{60}Co$ radiation group. Similar to spleen percentages, significant differences were noted between the percentages of proton (BP)-, proton (EP)-, and $^{60}Co$-radiation groups on days 1 and 10 (Table 4).

*Response of Spleen Cells to Mitogen Stimulation after Irradiation*

Examination of PHA SI (Figure 4) showed significant depression of $^{3}H$-TdR uptake on days 1, 4, and 10 for the proton (BP)-irradiated group. However, on day 7, no significant difference was found in SI values compared to control levels. Con A SI levels showed significant depression on days 1 and 4 only. In contrast, response to LPS stimulation continued to be depressed by day 10. No significant difference in $^{3}H$-TdR uptake was noted between the proton (BP)- and proton (EP)-irradiated groups and both followed similar patterns. In addition, no significant differences were noted when comparing the proton radiation groups to the $^{60}Co$ radiation group.

*Hematological Parameters of Peripheral Blood after Irradiation*

Significant differences in RBC, Hgb, and Hct counts were not observed until day 4 for the proton (BP)-irradiated group, with respective values at 27, 25, and 28% lower than the non-irradiated control levels (Table 5). MCV and MCHC values also did not significantly change until day 4, but this represented a difference of only 2 and 4%, respectively (data not shown). MCH values did not significantly change over any of the four time points (data not shown). Overall, MCV values ranged from 47.1+/-0.3 to 48.4+/-0.3 fl, and MCHC levels ranged from 32.5+/-0.1 to 34.1+/-0.2 g/dl. Non-irradiated control levels of MCV and MCHC were 48.3+/-0.2 fl and 32.8+/-0.1 g/dl, respectively. MCH levels ranged from 15.7+/-0.1 to 16.1+/-0.1 pg, while non-irradiated control levels were at 15.8+/-0.1 pg. RBC, Hgb, and Hct levels remained significantly depressed on days 7 and 10, while MCV and MCHC levels showed no significant difference by day 10. PLT counts fluctuated, showing significant depression on days 4 and 10, but normal

**Figure 4.** Stimulation index (SI) values of PHA, Con A, and LPS with time after whole-body irradiation. Aliquots (100 µl) of each splenocyte sample were dispensed in triplicate into flat-bottomed wells of 96-well microtiter plates containing either PHA, Con A, LPS, or no stimulant. The plates were incubated for 48 hrs in 5% $CO_2$ at 37°C. Medium containing 1 µCi $^{3}$H-TdR was added to each well 4 h before counting the amount of radioactivity taken up by cells. The results are expressed as SI using the disintergrations per minute (dpm) as follows: SI = (dpm with mitogen – dpm without mitogen)/dpm without mitogen. Each bar represents the mean +/- S.E.M. a: Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, and 10 ($p<0.05$); b: Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1 and 4 ($p<0.05$); c: Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 1, 4, 7, and 10 ($p<0.001$).

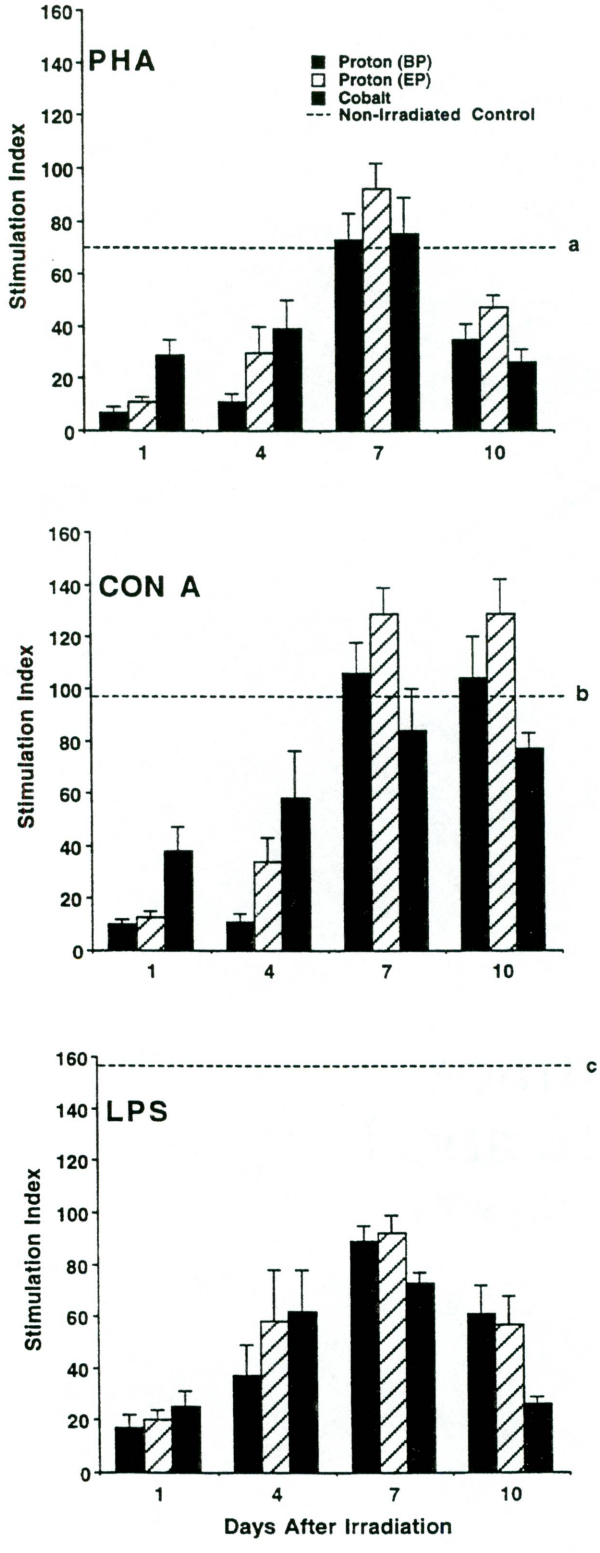

PHA
Proton (BP)
Proton (EP)
Cobalt
Non-Irradiated Control
Stimulation Index
a
CON A
b
LPS
c
Days After Irradiation

**Table 5.** Blood hematological findings[a]

| | RBC | HGB | HCT | PLT |
|---|---|---|---|---|
| Non-Irradiated Control | 9.86+/-0.08[b,c] | 15.6+/-0.1[c] | 47.5+/-0.4[c] | 592+/-16[d] |
| Day 1 | | | | |
| Proton (BP) | 9.76+/-0.13 | 15.5+/-0.1[e] | 46.8+/-0.5[e] | 589+/-18 |
| Proton (EP) | 9.59+/-0.11 | 15.5+/-0.2 | 46.4+/-0.6 | 585+/-16 |
| Cobalt | 9.32+/-0.13[f] | 14.9+/-0.2[f] | 44.6+/-0.6[f] | 623+/-10 |
| Day 4 | | | | |
| Proton (BP) | 7.20+/-0.59 | 11.6+/-0.9 | 34.1+/-2.9 | 317+/-88 |
| Proton (EP) | 7.53+/-0.35 | 12.0+/-0.5 | 35.5+/-1.6 | 271+/-35 |
| Cobalt | 6.62+/-0.54 | 10.7+/-0.9 | 31.2+/-2.6 | 219+/-39 |
| Day 7 | | | | |
| Proton (BP) | 8.36+/-0.13 | 13.1+/-0.2 | 39.7+/-0.7 | 576+/-15 |
| Proton (EP) | 8.56+/-0.13 | 13.5+/-0.2 | 40.9+/-0.6 | 551+/-33 |
| Cobalt | 8.65+/-0.09 | 13.6+/-0.1 | 41.4+/-0.4 | 625+/-19 |
| Day 10 | | | | |
| Proton (BP) | 8.64+/-0.21 | 13.6+/-0.2 | 41.8+/-0.8 | 240+/-31 |
| Proton (EP) | 8.36+/-0.23 | 13.3+/-0.4 | 40.3+/-1.1 | 263+/-14 |
| Cobalt | 8.79+/-0.13 | 13.8+/-0.2 | 42.4+/-0.6 | 284+/-29 |

[a]Obtained from ABC Vet Hematology Analyzer.
[b]Mean +/- S.E.M.
[c]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4, 7, and 10; $p<0.001$.
[d]Significantly higher than proton (BP), proton (EP), and $^{60}$Co radiation groups on days 4 and 10; $p<0.001$.
[e]Significantly higher than $^{60}$Co radiation group; $p=<0.05$.
[f]Significantly lower than non-irradiated control; $p<0.01$.

levels on days 1 and 7. The values for the proton (EP)-irradiated animals followed the same pattern as was observed for the proton (BP)-irradiated group for all parameters.

When comparing the proton-irradiated groups to the $^{60}$Co irradiated group, significantly lower levels of Hgb and Hct were seen on day 1 in the $^{60}$Co irradiated animals when compared to the proton (BP)-irradiated group. In addition, RBC, Hgb, and Hct levels in the $^{60}$Co irradiated animals were also significantly lower than the non-irradiated controls on day 1, while no significant difference from control levels was observed at this time point for the proton groups. No other significant differences were noted between the proton and $^{60}$Co-irradiated groups.

## DISCUSSION

Radiation damage to cells involves complex mechanisms resulting from both primary and secondary radiation-induced events, and different types of radiation particles are able to induce varying results based on their distinct qualitative physical properties. For example, high-LET radiation has been shown to produce more chromosomal aberrations and clustered areas of double stranded-DNA damage than low-LET radiation (18-21). As a result, these high-LET particles have been calculated to have high RBE values. Protons, which are considered a relatively low-LET form of radiation, induce damage primarily by ionization (94% of the radiation dose deposited), rather than through nuclear interactions (6% of the radiation dose deposited) (8). Thus, most proton-induced damage is thought to be similar to that of other types of low-LET ionizing radiation (e.g., photons or gamma rays), and this is reflected in the RBE values for proton radiation remaining close to 1.0 (0.8-1.5) (13, 14, 16). The biological effects of 250-MeV protons on primates were also observed to have RBE values of 1.0-1.1 (15). Additionally, no unique, toxic effects of protons were found, and the effects were similar to other types of ionizing radiation (22). The results of the present study continue to suggest that the RBE of protons (based on acute changes in immunological parameters) is approximately 1.0, since few statistical differences were noted in our study after WBI.

The current consensus is that hematopoietic stem cells differentiate into three major lineage-committed progenitors that yield a) lymphocytes, b) granulocytes, monocytes, and platelets, and c) erythrocytes. In the adult, mechanisms within the bone marrow maintain, regulate, and modulate each pathway in response to demand, although the spleen (as well as the liver) can function as hematopoietic organs in case of bone marrow damage or failure. Our analyses of the spleen and peripheral blood in an *in vivo* system support the previously calculated RBE values, as few differences were found between leukocyte, lymphocyte, and hematological analyses of the proton and $^{60}Co$ radiation groups for the first 10 days post-WBI. Although spleen and thymus masses were back to normal levels by day 10, the leukocyte and lymphocyte counts

were still far below normal. In addition, no analyses of chromosomal aberrations were performed, and the differential effects on lymphocytes at the molecular level resulting from equivalent WBI have yet to be obtained. As such, one can only speculate at this point if differences in damage at the level of DNA exist. Furthermore, since it is known that cells that sustain terminal damage can sometimes undergo multiple replications before losing reproductive capacity, it would be important to examine cell levels beyond the first 10 days in order to more fully characterize immune cell recovery. However, in our study, the degree of depression in lymphocytes as well as their subpopulations was similar in all irradiated groups.

With cells of the immune system being among the most radiosensitive cells in the body, lymphocytes have been suggested as a biological dosimeter for measuring the amount of radiation exposure (5, 23, 24). Lymphocyte subsets showed similar patterns of sensitivity to proton radiation as seen in previous studies following $^{60}$Co exposure (2-5, 25, 26). B cells were extremely radiosensitive, T cells were slightly less radiosensitive, and NK cells showed the least radiosensitivity. The difference in susceptibility between B and T lymphocytes may be related to the fact that most gene rearrangements involved in T cell maturation have already taken place within the thymus by the time of birth, whereas B cell maturation proceeds continuously throughout life in the bone marrow. Within the T lymphocyte population, the Tc (CD8+) cells exhibited greater susceptibility than the Th (CD4+) subset, resulting in a greater than 2-fold increase in the CD4:CD8 ratio in all irradiated groups. However, with the specificity of the adaptive immune response and the conversion of naïve cells into active cells, cellular nuclear damage to B and T cells resulting from proton irradiation could prove to have more of a detrimental effect to immune system functionality than equivalent damage from $^{60}$Co radiation.

The ability of spleen cells to respond to mitogens was markedly depressed following irradiation, especially at the 1 and 4 day time points. However, no statistical difference

in responsiveness was observed among the different radiation groups. Although the selected mitogens are non-specific stimulants, T, Tc, and B cells are the major targets for PHA, ConA, and LPS, respectively. Examination of primary and secondary responses to specific antigens would more fully assess lymphocyte functionality. The effects of proton radiation on dendritic cells, macrophages, and other antigen-presenting cells could also play a major role on adaptive immune responses. NK cells, in contrast to T and B lymphocytes, act in a non-specific manner, and as a result, DNA damage might not be as detrimental to cellular functionality. However, the mechanisms by which radiation affects these cells remain unclear and may be related to differences in DNA protection and repair.

It is known that immune system functionality relies on an intricate balance of activation and suppression. Many immunological disease states (hypersensitivity, autoimmunity, AIDS, etc.) result from imbalance or deficiency of certain immune cell populations. Imbalance of cellular populations can also play a major role in hematopoietic recovery (4), immunoregulation (27), and tumor regression (28). Although no statistical differences were noted for the first 10 days post-WBI, it remains to be seen if the observed changes in cell proportions play a significant role in differences in recovery beyond the 10-day time period or in immune system response to a challenge.

Different total doses and dose rates may also affect immune system responsiveness as DNA repair plays a crucial role in recovery from radiation damage at the cellular level (29-32). Fractionated doses would more closely mimic the space environment. Additionally, although lymphopenia after 3 Gy $^{60}$Co irradiation in monkeys showed no difference between fractionated and acute irradiation, the chromosomal damage was less serious when the dose was fractionated (33). As chromosomal aberrations would be expected to be more prevalent in proton radiation (34, 35), comparison of fractionated doses would provide a more accurate analysis of the immune response in the space environment.

In conclusion, our data provides evidence, for the very first time, that proton (BP) versus proton (EP) WBI at 3 Gy exposure results in little or no difference in the measured immune system parameters during the first 10 days post-irradiation. Additionally, no differences were noted when compared to an equivalent physical dose of $^{60}$Co radiation. These results illustrate that despite the differences in physics and dose distribution between the types of radiation, the biological damaging effects are similar resulting from 3 Gy WBI. It remains to be seen, however, if the increased genetic damage reported in other studies would be found in cells of the immune system, and how these aberrations would directly affect this intricate system, both acutely and over the life span of an organism. The effects of different doses and dose rates on each cell population could also play an important factor in functionality. Further studies are warranted in order to elucidate exact biological mechanisms. These findings may have important implications on future planning of risk assessment of exposure and protection from proton radiation.

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