Final Report

Project Title: **Induction of Genomic Instability *in Vivo* by Low Doses of ^{137}Cs Gamma Rays**
Project ID: 0007367
Award Register #: ER63311

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Introduction

The overall goal of this project is to determine if low doses (below or equal to the level traditionally requiring human radiation protection, *i.e.* less than or equal to 10 cGy) of low LET radiation can induce genomic instability. The magnitude of genomic instability was measured as delayed chromosome instability in bone marrow cells of exposed mice with different levels of endogenous DNA-dependent protein kinase catalytic subunit (DNA-PKcs) activity, *i.e.* high (C57BL/6J mice), intermediate (BALB/cJ mice), and extremely low (*Scid* mice). In addition, at early time points (1 and 4 hrs) following irradiation, levels of activation of nuclear factor-kappa B (NF-kappa B), a transcription factor known to be involved in regulating the expression of genes responsible for cell protection following stimuli, were measured in these cells.

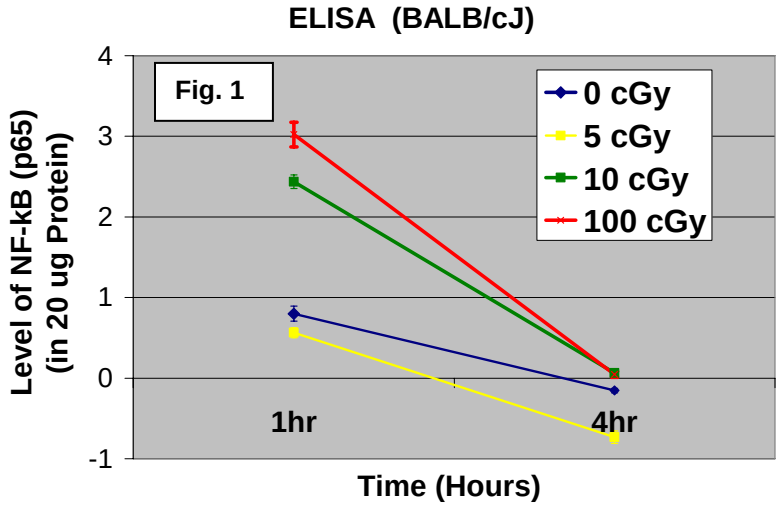
Bone marrow cells were collected at different times following irradiation, *i.e.* 1 hr, 4 hrs, 1 month, and 6 months. A total of five mice per dose per strain were sacrificed at each time point for sample collection. As a result, a total of 80 mice from each strain were used. The frequency and the type of metaphase chromosome aberrations in bone marrow cells collected from exposed mice at different times following irradiation were used as markers for radiation-induced genomic instability. A three-color fluorescence *in situ* hybridization (FISH) protocol for mouse chromosomes 1, 2, and 3 was used for the analysis of delayed stable chromosomal aberrations in metaphase cells. All other visible chromatid-type aberrations and gross structural abnormalities involving non-painted chromosomes were also evaluated on the same metaphase cells used for scoring the stable chromosomal aberrations of painted chromosomes. Levels of nuclear factor-kappa B (NF-κB) activation were also determined in cells at 1 and 4 hrs following irradiation (indicative of early responses).

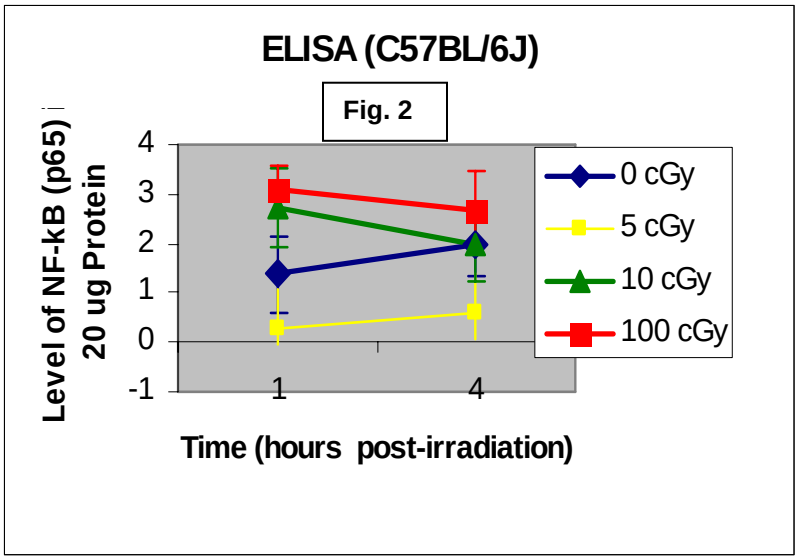
Results

1. Activation of NF-κB We used the NF-κB/p65 Transcription Factor Enzyme-linked Immunosorbance Assay (ELISA kits from Active Motif North America Inc. ,Carlsbad, CA) for detecting NF-κB activation.

With respect to the BALB/cJ (radiosensitive) and C57BL/6J (radioresistant) mice, our results indicated a significant increase in levels of activated NF-κB dimers in lysates of BM cells isolated from both mouse strains at 1 hr post-exposure to 10 and 100 cGy (shown in Figs. 1 and 2 below). These

elevated levels in the BM then precipitously declined by 4 hrs post-exposure of radiosensitive BALB/cJ mice but persisted in the BM cells of exposed radioresistant C57BL/6J mice.





The data also show higher inter-individual variations in the C57BL/6 J mice. The results obtained from exposed BALB/cJ mice have been published *[Rithidech et al 2005 Radiat Environ Biophysics 44(2):139-143]*. The data present, for the first time, evidence for NF-κB activation as an early response to low-dose exposure *in vivo*, at levels as low as those requiring human radiation protection (*i.e.* 10 cGy of ^{137}Cs γ rays). On the other hand, the lack of measurable levels of NF-κB activation in bone marrow cells collected from mice exposed to 5 cGy of ^{137}Cs γ rays was detected in both BALB/cJ and C57BL/6J mice.

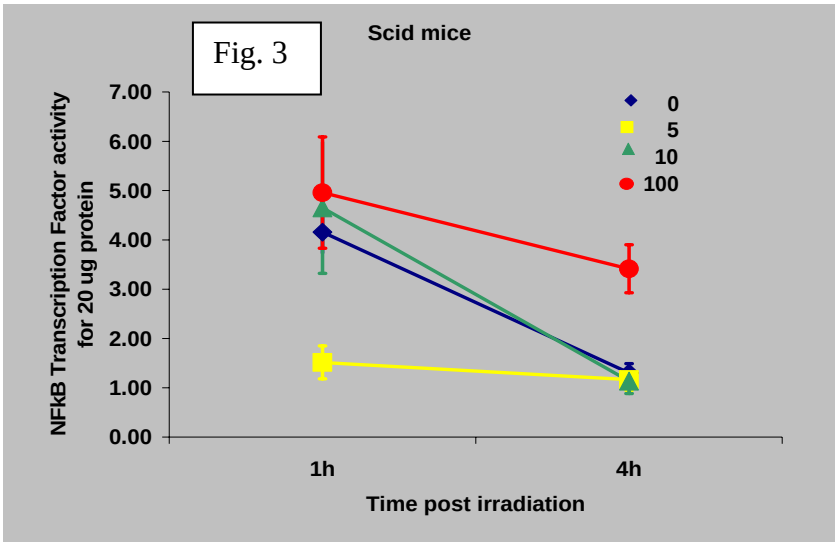
The finding of NF-κB activation at 1 hr in response to 10 and 100 cGy (but not 5 cGy) of ^{137}Cs γ rays suggests a different molecular mechanism in early response to this very low dose (5 cGy) of radiation as compared to those in response to 10 and 100 cGy. If this difference does exist, it may reflect a difference in manifestation of subsequent biological effects between exposure to very low dose radiation (5 cGy) and to higher doses (10 and 100 Gy) of ^{137}Cs γ rays.

It also is possible that 5 cGy of ^{137}Cs γ rays activates other members of the NF-κB family (*i.e.* NF-κB/p50 and NF-κB/p52) which would not be detected by the antibodies specific for mouse NF-κB/p65

used in our study. Activation of NF-κB/p50 subunit following *in vivo* exposure to ionizing radiation has been shown previously**.** However, it also is possible that transduction pathways other than NF-κB activation are induced by 5 cGy of ^{137}Cs γ rays. Nevertheless, our observation on a lack of NF-κB/p65 activation by a single dose of 5 cGy of ^{137}Cs γ rays makes it important to characterize further the comprehensive patterns of protein expression profiles of *in vivo* response to very low dose radiation. Such information ultimately would aid in a better assessment of health risks from exposure to low or high doses of ionizing radiation.

However, although our study is the first to provide evidence for *in vivo* activation of NF-κB induced by irradiation of BALB/cJ and C57BL/6J mice with low does of ^{137}Cs γ rays, followed by subsequent strain-specific post-exposure diminution, it is limited to the analysis of cells collected at only two early time points following irradiation. As a result, the extent to which this differential response contributes to the difference in sensitivity to radiation induced genomic instability (see section **2. Chromosomal Damage and Genomic Instability**) between these two mouse strains remains unclear.

The results from Scid mice (Fig. 3) were inconsistent with those obtained from C57BL/6 or BALB/cJ mice. Unexpectedly, levels of activated NF-κB in BM cells collected from Scid mice exposed to 0, or 10 or 100 cGy at 1 hr post-irradiation were relatively higher than those observed in C57BL/6 or BALB/cJ mice exposed to similar doses of radiation. As mentioned above, Scid mice have extremely low endogenous DNA-PK activity. Therefore, one possible explanation for the detection of high levels of activated NF-κB in Scid mice is that kinase activity of DNA-PK does not play a role in initiating the activation of NF-κB in cells following irradiation. However, similar to the results obtained from BALB/cJ and C57BL/6J mice, levels of activated NF-κB detected in BM cells of Scid mice exposed to 5 cGy of ^{137}Cs γ rays were lower than those observed in samples collected from mice exposed to 0, or10 or 100 cGy. At 4 hrs post-irradiation, however, no activated NF-κB was found in any group of mice, except in BM cells collected from mice exposed to 100 cGy.

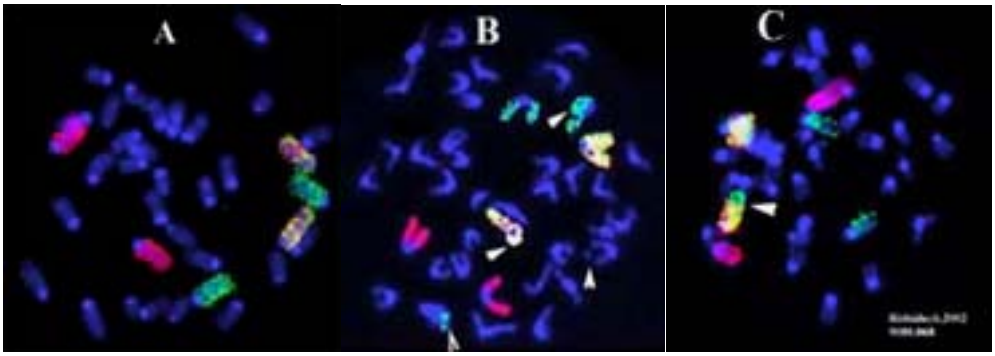


We further examined the expression pattern of mRNAs which have previously been identified as being under transcriptional control by NF-κB in the spleen isolated from BALB/cJ or C57BL/6J mice as a function of radiation dose and time post-irradiation In BALB/cJ mice, significant increased levels of expression of several genes were detected as early as 1 hr post-irradiation in samples from mice exposed to a high dose level (100 cGy). In contrast, high levels of gene expression in samples collected from mice exposed to 5 or 10 cGy were not observed until 4 hrs post-irradiation. The results indicate a delay in expression of genes in samples from

mice exposed to low doses of ^{137}Cs γ rays. Expression of the anti-apoptotic genes Bcl2a1a and Bcl2l was not increased in samples collected from mice exposed to the high dose level (100 cGy), but was increased in samples collected from mice exposed to low doses of ^{137}Cs γ rays (5 and 10 cGy). The results suggest a different molecular mechanism in response to high or low doses of low LET radiation of bone marrow cells exposed *in vivo*. In addition, significant levels of expression of three genes coding for cytokines (Il10, Il11, and Pdgfb) were also found in sham-control samples at both 1 and 4 hrs following irradiation, possibly reflecting an innate immune response of mice to stress triggered by the brief restraint during sham exposure. In contrast to our findings in the BALB/cJ mice, however, when we examined the splenic tissues of irradiated C57BL/6J mice, we observed no significant increase in expression of anti-apoptotic genes, regardless of radiation dose and the subsequent post-exposure interval (1 and 4 hrs after irradiation). Rather, there was a trend of increased expression of genes involved in apoptosis (such as Fas-L, p53) in spleen cells collected at 1 hr from mice exposed to 100 cGy. Although only a single transduction pathway was examined, the data suggest that, at a similar dose of radiation, up-regulation of genes involved in apoptotic pathways is more likely to occur in radiation-resistant C57BL/6J mice as compared to radiation-sensitive BALB/cJ mice.

2. <u>Chromosomal Damage and Genomic Instability</u> All slides were coded before scoring. We used three-color FISH of mouse chromosomes 1, 2, and 3 to analyze stable chromosome aberrations. With our three-color chromosome painting protocol, chromosomes 1 are yellow (or a speckled mixture of red/green) while chromosomes 2 are green and chromosomes 3 are red. All other chromosomes are blue (Figs. **4A, B**, and **C** below): **(A)** Representative of a normal metaphase cell:**(B)** Representative of a metaphase cell with multiple chromosome aberrations (arrows) collected from a mouse at 1 month following *in vivo* exposure to 10 cGy of ^{137}Cs gamma rays: Chromatid deletion on chromosome 1 (yellow) without acentric fragment; Chromatid deletion on chromosome 2 (green) with acentric fragment; and chromatid break on non-painted chromosomes (blue) with acentric fragment and **(C)** Representative of a metaphase with translocation between mouse chromosomes 1 and 2 (arrow). As mentioned earlier, all other chromatid- or chromosome-type aberrations and gross structural abnormalities involving non-painted chromosomes (in blue colors) were determined simultaneously in the same metaphase cells used for scoring aberrations in the painted chromosomes.

<u>Fig.4</u>



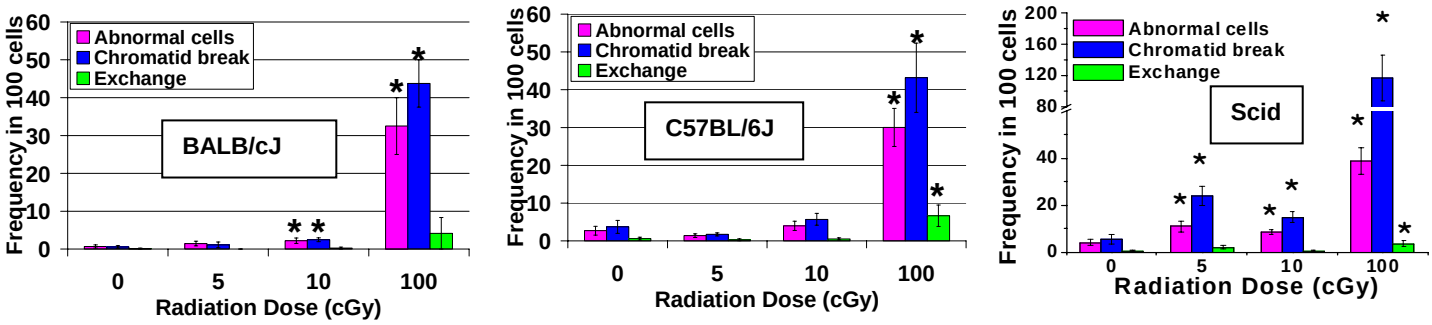
All chromosomal fragments were scored as one break, regardless of whether they appear as double or single fragments with clear displacement of the broken segment. The number of cells with chromosomal damage also was recorded. To determine the number of initial breaks at the early harvest times (1 and 4 hrs post-irradiation), a single chromatid fragment was scored as one chromatid break. However, since one iso-chromatid break results from the breakage of two chromatids within a chromosome, two chromatid breaks were scored to represent one iso-chromatid break.

Prior to statistical analysis, the average square root transformation (ASQT) was applied to the aberration frequency measured in each mouse to achieve reasonable normality and reasonably homogeneous inter-animal variability within treatment combination groups. Following the application of the ASQT, each animal's rate of cell damage per 100 cells scored was obtained. Analysis of variance methods appropriate for two factor studies (post-exposure time and radiation dose in this case) was used to evaluate the resulting data for each strain, along with a regression test for a positive linear dose related trend. For each strain, one factor was time post exposure and the other factor was radiation dose level.

A dose-dependent increase ($p<0.001$) in number of cells with chromosomal damage (breaks and exchanges) was detected as a function of dose at early time (1 and 4 hrs) post- irradiation in all three strains of mice (Figs. 5a and 5b). At these early times, more severe damage was observed in exposed Scid mice (approximately 2- to 3-fold higher than those in exposed BALB/cJ and C57BL/6J mice). At late times (1 and 6 months post-irradiation), there were significant decreases in the levels of chromosomal damage in all three strains of mice as compared to those observed at early times (Figs. 5c and 5d). However, the residual levels of persistent chromosomal damage appeared to remain elevated for up to 6 months in BM cells collected from BALB/cJ and Scid mice only but not in the C57BL/6J mouse. In contrast, a statistically significant reduction ($p<0.05$) in the frequencies of abnormal cells (Fig. 5c, indicated by a pink arrow) and chromosome breaks (Fig. 5d, indicated by a red arrow) was found in BM cells collected at 1 and 6 months post-irradiation from C57BL/6J mice exposed to 5 cGy. Similarly, a statistically significant reduction ($p<0.05$) in the frequency of chromatid breaks was detected in BM cells collected from BALB/cJ mice exposed to 5 cGy at 6 months post-irradiation (Fig. 5d, indicated by a blue arrow). Mechanisms responsible for these selective strain-specific decreases are unknown.

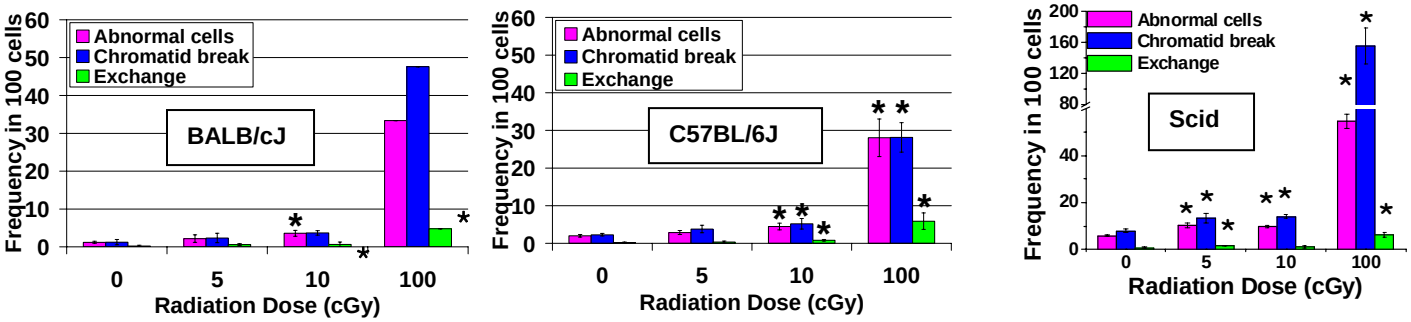
Our data indicated a statistically significant increase ($P<0.05$) in all types of chromosomal damage in bone marrow cells collected at 6 months following irradiation from Scid mice exposed to 10 cGy of radiation, indicating the induction of genomic instability. In BALB/cJ mice, however, a trend of persistent elevation in all types of chromosomal damage was found in cells collected at 6 months post-irradiation with 10 cGy, although this increase was not statistically significant. It is clear, however, that there is a statistically significant increase ($p<0.05$) in the frequency of chromosome aberrations in cells collected at these late times from BALB/cJ or Scid mice exposed to 100 cGy. In contrast, no increase in any type of chromosomal damage (including the number of abnormal cells) was detected in BM cells collected from C57BL/6J mice at these late times post-irradiation regardless of radiation dose, suggesting no *in vivo* induction of sustained genomic instability in the C57BL/6J mouse by the radiation doses used in our study. The differences in response to radiation among the three strains of mice used in our study may reflect differences in repair systems, removal of damaged cells by apoptosis or the cell turnover that removes damaged cells from the populations.

Fig. 5a: 1 hr post-irradiation



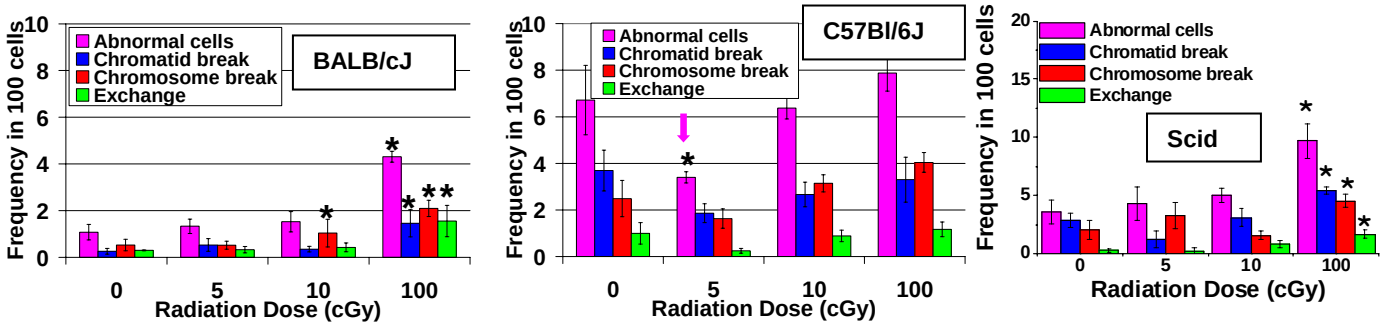
* = statistically significant, $p<0.001$

Fig. 5b: 4 hr post-irradiation



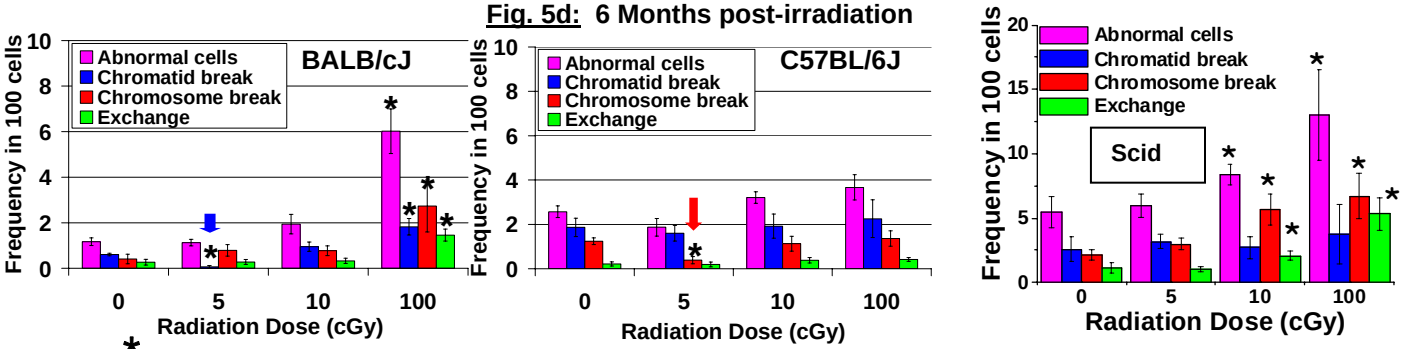
* = statistically significant, $p<0.001$

Fig. 5c: 1 Month post-irradiation



* = statistically significant, $p<0.05$

Fig. 5d: 6 Months post-irradiation



* = statistically significant, $p<0.05$

Overall, the data indicated no evidence for the induction of genomic instability following *in vivo* exposure to 5 cGy of ^{137}Cs γ rays in all strains of mice used in our study. Moreover, our data showed that there was no increase in the frequency of chromosomal damage in the group of mice without the signal of activated NF-κB at any time point included in our study. Instead, a reduction of damage was found in this group of mice. In summary, the new set of data obtained from our laboratory indicates a link between high levels of NF-κB activation early after irradiation and radiosensitivity. At doses higher than 5 cGy, the magnitude of radiation-induced NF-κB activation and radiosensitivity is dose-dependent.

We are preparing manuscripts based upon these data for possible publication in peer-reviewed journals.