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Modeling Cell Survival Fraction and Other Dose-Response Relationships for Immunodeficient C.B-17 SCID Mice Exposed to 320-kV X Rays

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Abstract

US homeland security concerns related to potential misuse of γ-ray-emitting radiation sources employed in radiobiological research (eg, shielded cesium-137 irradiators) led to recommendations by the National Research Council to conduct studies into possibly replacing γ-ray irradiators used in research involving small rodent and other models with X-ray instruments. A limiting factor is suitability of the X-ray photon energy spectra. The objective of our research was to demonstrate the suitability of the radiation energy spectrum of 320-kV X rays after filtration (HVL = 4 mm Cu) for in-vivo cytotoxicity studies in immunodeficient C.B-17 SCID mice. By using a previously-published Hazard Function (HF) model to characterize dose-response relationships for in vivo bone marrow and spleen cell survival fractions and also to characterize the acute lethality risk (hematopoietic syndrome mode) we demonstrate that the filtered 320-kV X-ray beam appears suitable for such studies. A key finding for C.B-17 SCID mice when compared to results previously obtained for immunocompetent C.B-17 mice is that the immunodeficient mice appear to be more radioresistant, implicating a possible role of the immune system capacity in radiosensitivity of mammals.

Keywords

Cs-137 γ rays, 320-kV X rays, bone marrow, spleen

Introduction

The world we live in today is challenged by the threat of radiological terrorism. This includes the possible use by terrorists of a radioactivity dispersal device containing radioactive materials such as cesium-137 (^{137}Cs), a γ-emitting radionuclide. While the use of such a device is likely not life-threatening, it could possibly lead to large costs associated with population evacuation, relocation, and area decontamination. There is an ongoing effort to replace ^{137}Cs irradiators used in radiobiological studies with X-ray irradiators with suitable energy spectra. One attractive X-ray irradiator is the X-RAD 320.[1]

For each type of radiobiological study (eg, in vitro, in vivo) of interest that would be conducted using ^{137}Cs γ rays, it is important to consider an alternative X-ray source with a suitable photon energy spectrum.[2-15] Self-shielded ^{137}Cs-chloride irradiators with radioactivity at the International Atomic Energy Agency Category 1 and 2 levels (ie, greater than 27 Ci) are mainly used in 3 major applications[16]: (1) biomedical research involving live cells in culture and small mammals (eg, mice, rats), (2) blood sterilization, and (3) calibration. Cesium-137 chloride was selected for the irradiators because of the radiation photon energy spectra in the different radiation exposure chambers. The radioisotope has a single initial photon energy (0.662 MeV), very long half-life, low cost (relatively), and moderate shielding requirements. After the emitted photons penetrate the irradiator housing materials there is a complex spectrum of photon energy.

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A report by the National Research Council[16] recommended that careful consideration be given to the possibility of replacing ^{137}Cs irradiators with suitable X-ray irradiators with respect to characteristics of the filtered (which removes low-energy, X-ray photons that produce higher local radiation doses than high-energy photons) photon energy spectrum.

Our previous research[12] that relates to the findings discussed here focused on comparing the relative biological effectiveness (RBE) of filtered 320-kV X rays (HVL = 4 mm Cu) as compared to shielded ^{137}Cs γ rays for 2 in vivo endpoints in *immunocompetent* C.B-17 male and female mice after split-dose, whole-body-bilateral exposure: (1) in vivo cytotoxicity to bone marrow and spleen cells evaluated at 1 day post exposure and (2) bone marrow and spleen reconstitution deficits (repopulation shortfalls) evaluated at 6 weeks after radiation exposure. We found that the cell survival fraction (and related cell lethality hazard) dose-response relationships for bone marrow and spleen cells are complex, involving negative curvature (decreasing slope magnitude on semi-log plot of survival fraction as dose increases). The negative curvature can be explained on the basis of a known mixed cell population comprised of hyper-radiosensitive, moderately radiosensitive, and radioresistant cells. The hyper-radiosensitive cells appeared to respond with 50% being killed by an absorbed dose <0.5 Gy.

The X-ray RBE, relative to γ rays, for a loss of bone marrow cells in vivo was found to be > 1, while for a loss of splenocytes it was unexpectedly <1 (explainable based on absorbed doses to spleen being possibly underestimated). Dosimetric research has revealed that absorbed dose underestimation for internal organs of X-ray-exposed mice can be an issue.[2,10]

In contrast to early occurring effects of irradiation, dose-response relationships for reconstitution deficits (ie, shortfall on full recovery) in the bone marrow and spleen of immunocompetent C.B-17 mice at 6 weeks post irradiation were of the threshold type with γ rays apparently being more effective in causing reconstitution deficit.[14] This may relate to radiation doses to spleen being in error and/or to RBE being different for reconstitution deficit than for early (after 24 hours) cytotoxicity response.

The observed differences between 320-kV X rays and Cs-137 γ rays for mouse bone marrow and spleen cells may also relate in part to microdosimetric differences for bone marrow cells and spleen cells,[2,10,13,17-20] which cannot be resolved using macroscopic absorbed dose.[2] Bone marrow cells near bone receive higher local radiation doses (from photoelectrons) than is the case for ^{137}Cs γ rays.[2,10] In addition, at the level of individual cells and for subcellular targets (eg, nucleus), the microdose distribution is inferred to be different for shielded ^{137}Cs γ rays and filtered 320-kV X rays (based on in vitro studies data and *biological microdosimetry* for other cell types).[13] This is consistent with physical microdosimetric principles.[21,22]

Research Aim

Research results reported here relate to determining whether the energy spectrum of filtered photons of the X-RAD 320 irradiator[1] is suitable for simultaneously depleting live cells in bone marrow and spleen of irradiated male and female C.B-17 SCID mice to levels (associated with highest dose of 7 Gy used) *expected to be suitable for bone marrow transplantation studies*. Judgements about successful depletion are based on cell survival levels observed after successful bone marrow engraftment following ^{137}Cs gamma-ray exposure. Bone marrow transplantation was not performed in the new study discussed. The immunodeficient mice were used because immunocompetent C.B-17 mice (as previously used) are no longer available. We assumed that bone marrow and spleen cell radiosensitivity is not influenced by the immune system or gender.

A key finding of our dose-response modeling research using the immunodeficient mice is that *they appear to be more radioresistant than the immunocompetent mice, implicating a possible role of the immune system in radiosensitivity in vivo.*

Materials and Methods

Mouse Strain

Equal numbers of male and female C.B-17 SCID mice were purchased from Taconic and used in this research. The animals were placed in standard rodent housing and provided water containing Baytril (enrofloxacin, 175 mg/L) antibiotic for 1 week prior to irradiation.

Irradiator

The X-RAD 320 Unit[1] from Precision X-Ray, Inc. (Nort Brandford, CT) in conjunction with a GE ISOVOLT 320 TITAN X-ray Unit is a self-contained X-ray-photon source designed for biological and medical research. A cathode generator with a power electronics module and anode generator are used to generate the negative and positive high voltages to excite the X-ray tube. X-ray exposures employed a filter comprised of 0.75-mm tin + 0.25-mm copper + 1.5-mm aluminum (HVL = 4-mm Cu).[1] The PTW UNIDOSE E dosimeter and related Farmer chamber recommended by Precision X-Ray were used for accurate dose measurements rather than relying on the setting of the X-RAD 320 device. Radiation exposure was unilateral-single-dose at a source to surface distance of 50 cm. A fixed dose rate of 1 Gy/min was used. The X-RAD 320 irradiator is housed in a secure area only accessible to approved qualified individuals.

Exposure of Mice

Mice 6-8 weeks old at the start of irradiations were placed in a pie-cage on a rotating specimen turntable designed for X-RAD devices and whole-body exposed to 0.1, 1, 3, 4.5, or 7 Gy of filtered 320-kV X rays. Age-matched, non-irradiated control mice were placed in the pie-cage and mock-irradiated for the same period of time as for the highest dose (7 Gy) group. After irradiation, mice were housed in autoclaved cage setups and provided antibiotic-containing drinking water and irradiated

food. The protocol for animal experiments was approved by Institutional Animal Care and Use Committees (IACUC) of Lovelace Biomedical Research Institute.

Surviving Cells Assessment for Bone Marrow and Spleen

On Day 1 after radiation exposure, bone marrow and spleen were prepared from the irradiated and control mice. Six irradiated mice/dose group (n = 30 mice total for irradiated mice, with half males and half females) were euthanized 24 hours after irradiation and the extent of bone marrow and splenocyte cellularity was assessed. The control mice (n = 6, with half males and half females) were also euthanized 24 hours after *mock irradiation* and assessed for spleen and bone marrow cellularity. Spleen and bone marrow from each of the euthanized mice were harvested, evaluated to determine *live cell counts per unit body weight* (an adjustment for different body sizes to that of a 25 g mouse irrespective of gender).

Evaluating Mortality via the Hematopoietic Syndrome Mode

Mortality via the hematopoietic syndrome mode was assessed based on 30-day lethality among 60 mice (10 for each of 6 exposure groups with equal numbers of males and females) assigned to a 6 weeks follow-up group. Thirty-day lethality has historically been used in mouse studies when assessing expected deaths caused by damage to the hematopoietic system by brief, high-dose-rate, total-body exposure to ionizing radiation.[23] The median lethal dose for 30-day lethality (i.e., $LD_{50/30}$) is monitored in mouse models for radiation countermeasures research related to the hematopoietic mode of death.[24]

Bone Marrow Isolation and Spleen Cell Preparation

The femur was removed from a euthanized mouse and cleaned of soft tissues and muscles. The bones were then cut at both ends and the marrow flushed into 15 ml tubes with RPMI 1640 medium containing 5% FBS. The bone marrow was collected by centrifugation at 500 × g for 10 minutes and re-suspended in 1 ml of ACK lysis buffer (Gibco, A10492-01) at room temperature for 3 minutes to lyse the red blood cells. The debris were removed by addition of 10 ml of PBS and centrifugation at 500 × g for 10 minutes, and the bone marrow cells were resuspended in 3 ml of medium. Single cell suspension of the spleen was prepared by pressing mouse spleen against a 70 μM size cell strainer. After red blood cells were lysed using ACK lysis buffer, the spleen cells were resuspended in 3 ml RPMI 1640 medium. The tissues and cells were placed on ice where applicable during the preparation.

Cell Counting

Cells were stained with Trypan Blue and counted using Countess II automated cell counter (Life Technologies) with default settings, which provided cell counts of dead, live, and total number of cells per ml. The data were used to calculate total counts of bone marrow cells from femurs isolated from mice resuspended in the same amount of medium as described above.

Dose-Response-Modeling Approach

A Hazard Function (HF) model[12] (mathematical construct is specific for high dose rates)[25] was used for evaluating in vivo cell survival fractions. The model has the very useful features that not only can it be applied to cell survival modeling,[12,13] but also to acute lethality modeling as well.[25] For combined exposures to different radiation types or for combining results for different radiation sources (e.g., ^{137}Cs gamma ray and 320-kV X rays), a more complicated form of the HF model can also be employed using RBE-weighted dose and dose rate.[5,25] Changing dose rates from internal radionuclides can also be addressed with the more complicated form.[25]

In the current research we use HF models (specific for high dose-rate exposures) for both cell survival fraction and acute lethality via the hematopoietic mode for 320 kV X-ray-exposed C.B-17 SCID mice. Different *model-parameter and dose-response-function indicators* are used in distinguishing these 2 endpoints. We first present the general HF model with related basic parameters and then introduce new parameter and dose-response function indicators to differentiate between cell killing and acute lethality via the hematopoietic mode as endpoints.

With the HF model and radiation absorbed dose D for the target tissue of interest, the survival probability S(D) (for cells or individuals), associated risk R(D), and hazard function H(D) are related through the following equation:

$$S(D) = 1 - R(D) = e^{-H(D)}. \quad (1)$$

Values of both S(D) and R(D) are limited to the range 0 to 1, while H(D) can take on values from 0 to very large numbers.

The hazard function H(D) as previously modeled and as used here has but 2 parameters: shape parameter v and median effective dose D_{50} which is the radiation dose for a risk of 0.5 (or 50% being affected). For cell killing D_{50} is the dose associated with loss of 50% of the cells. For acute lethality of mice related to the hematopoietic syndrome, D_{50} corresponds to the LD_{50} dose for 50% mortality of irradiated individuals. For mice, the hematopoietic syndrome mortality has been estimated based on lives lost within 30 days after brief, single-dose exposure at a high dose rate, so $LD_{50/30}$ evaluations are used for estimating LD_{50}.[23,24]

The function H(D) is given by the following equation:

$$H(D) = \ln(2)[D/D_{50}]^{v}. \quad (2)$$

When $D = D_{50}$, $H = \ln(2) = 0.6931$. Based on Equation 2, the dose D_x for x% of the cells being killed (when modeling cell survival) or for x% of the irradiated population of animals not surviving (when modeling acute lethality) is given by the following, where H_x is the value of the hazard function H(D) for the effect level of interest:

$$D_x = D_{50}[H_x/\ln(2)]^{1/v}. \quad (3)$$

Because Equation 2 applies to different endpoints (cell killing [*ck*] or acute lethality [*al*]) in this paper, to distinguish these endpoints we substitute vck for v for cell-killing modeling and also substitute D_{50ck} for D_{50}. Corresponding substitutions for acute lethality [*ac*] of animals are val and LD_{50} (estimated based on $LD_{50/30}$ for mice).[23,24] The parameter LD_{50} is formally the historical *median lethal dose*. The mode of acute lethality focused on here, that is the hematopoietic mode, is linked to bone marrow damage.

A benefit of being able to use the HF Model for characterizing dose-response relationships for S(D), R(D), and H(D) for cytotoxicity is that this allows for generating *expected cell survival curves for subpopulations of cells* with differing radiosensitivity distributions. For example, a cell survival curve can be generated for the subpopulation of cells expected to be eliminated by doses up to but not exceeding the LD_{50}. This allows for comparing expected cell survival curves for radiation doses up to acute lethality dose percentiles, eg, LD_{01} (dose expected to eliminate 1% of animals), LD_{20}, LD_{50}, LD_{90}, and LD_{99} (dose expected to eliminate 99% of animals). These curves can also be compared to the modeled cell survival curve for the total cell population.

Characterizing Cell Survival Fraction and Related Dose-Response Relationships

The predicted average cell survival fraction, dose-response for the subpopulation of cells (highly radio-sensitive and moderately radiosensitive) eliminated by absorbed radiation doses up to the LD_{50} is given by the following equation (for D in the range 0 to LD_{50} but not any higher doses):

$$S_{50ck}(D \le LD_{50}) = [S_{ck}(D) - S_{ck}(LD_{50})]/[1 - S_{ck}(LD_{50})]. \quad (4)$$

Again, the subscript *ck* is used to specify the cell killing endpoint, rather than the acute lethality (*al*) endpoint. Also, LD_{50} relates to acute lethality of animals here and not to cell loss specifically, as previously indicated. For any other acute lethality dose percentile LD_x (for x % of the irradiation population of animals eliminated), the more general cell survival faction equation $S_x(D \le LD_x)$ is given by the following equation:

$$S_{xck}(D \le LD_x) = [S_{ck}(D) - S_{ck}(LD_x)]/[1 - S_{ck}(LD_x)]. \quad (5)$$

Equation 5 only applies to radiation doses up to the acute lethality dose percentile LD_x. Previously, vck has been assumed to have the same value for both X rays and γ rays but may differ for different tissue.[12]

For D = 0 Gy, $S_{xck}(D \le LD_x) = 1$, so $S_{xck}(D \le LD_x)$ takes on the value $[1 - S_{ck}(LD_x)]/[1 - S_{ck}(LD_x)]$ which equals 1. For $D = LD_x$, all of the cells in the *subpopulation considered* are expected to be eliminated and this is reflected by the numerator of Equation 5 then being $[S_{ck}(LD_x) - S_{ck}(LD_x)]$, which is of course 0 at the indicated dose LD_x.

Values for LD_x and related uncertainty can be obtained when using Bayesian inference in applying the HF model to data for acute lethality. We have focused on the 3 specific values LD_1, LD_{50}, and LD_{99} for some comparisons and on LD_{01}, LD_{20}, LD_{50}, LD_{90}, and LD_{99} for other comparisons. Of the different dose percentiles, only LD_{50} is a HF model parameter (for lethality of animals) that is evaluated (along with related uncertainty) via our current Bayesian analyzes program. However, the other acute lethality dose percentiles can be (and are) calculated (central estimates) based on Bayesian-analysis posterior means for HF model parameters.

For X-ray cytotoxicity in vivo, $R_{ck}(D)$ corresponds to the ablation function A(D), as used in Gott et al[5] for immunocompetent C.B-17 mice. For characterizing the *average live cell counts C(D)* in bone marrow (or spleen) per unit body weight 1 day after radiation exposure as a function of absorbed dose D, an additional parameter C_0 can be used to represent the average live cell count per unit body weight among un-irradiated control replicates. The expected average live cell count per unit body weight after dose D is given by $C(D) = C_0S_{ck}(D)$. The expected average survival fraction for live cells after animal exposure to dose D is derived from data for C(D) based on the following relationship:

$$C(D)/C_0 = [C_0S_{ck}(D)]/C_0 = S_{ck}(D). \quad (6)$$

Thus, experimental data for C(D) and C_0 can be used to generate data for $S_{ck}(D)$, which is the approach that has been used in this paper. This is how the data in Figure 1 for bone marrow cells and in Figure 2 for spleen cells are related to the corresponding cell survival fraction data (averages) in Figures 3 and 4. Because mice in our study were of differing body weights, results obtained for cell counts per gram body weight, were adjusted to cell counts for a reference 25 g mouse. Thus, results in Figures 1 and 2 relate to 25 g mice. The adjustment has no impact on $S_{ck}(D)$ as evaluated.

Characterizing Acute Lethality Dose-Response Relationships

For modeling acute lethality (*al*) via the hematopoietic mode, where *ck* has been used above for cell killing, *al* is used for acute lethality via the hematopoietic mode for HF model functions and the related parameter v, as previously pointed out. Thus, $S_{al}(D)$, $H_{al}(D)$, and $R_{al}(D)$, and val (for v) are used for survival function, hazard function, risk function, and shape parameter respectfully, for acute lethality modeling. Because acute lethality is the interest, LD_{50} (for 30-day lethality) is used instead of D_{50} as previously pointed out.

Fitting the HF Model to Data for Cell Survival Fraction and Acute Lethality Frequency

The HF-model-based fitted cell survival fraction and acute lethality frequency dose-response curves were obtained via

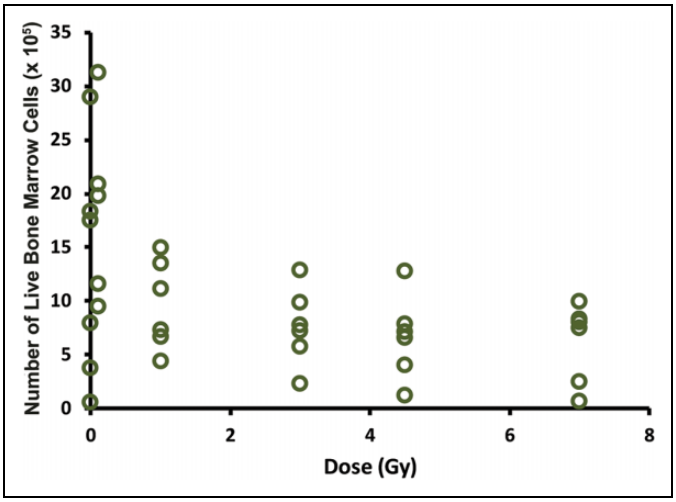

Figure 1. Live bone marrow cell counts ($\times 10^5$) 1 day after 320-kV X-ray exposures of C.B-17 SCID mice. Cell counts were adjusted to a body weight of 25 g (reference mouse) using data for cell count per unit body weight. Each data point represents an individual animal. The dose scale is linear so the 0.1 Gy group is near the 0-dose group (controls). As illustrated, there is large variability in the data. The live cell count data are negatively correlated with radiation dose [$r = -0.466$; $t = -3.02$, p (non-directional) $= 0.00482$, www.vassarstats.net]; thus there is a significant radiation dose dependence. The data are suggestive of a negative curvature dose-response relationship where the magnitude of the slope on a semi logarithm plot decreases with increasing dose. One outlier (lowest data point for controls) was excluded in modeling of the data.

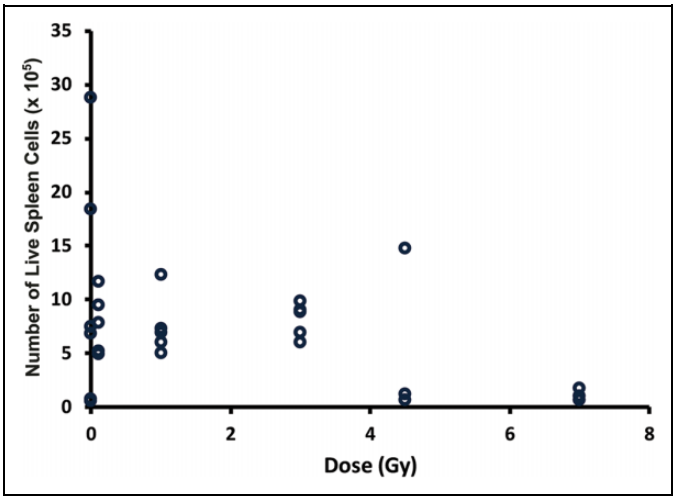

Figure 2. Live spleen cell counts ($\times 10^5$) 1 day after 320-kV X-ray unilateral-single-dose exposures of C.B-17 SCID mice. Cell counts were adjusted to a body weight of 25 g using data for cell count per unit body weight. Each data point represents an individual animal. The dose scale is linear so the 0.1 Gy group is near the 0-dose group (controls). As illustrated, there is some variability in the data and there is a clear dose association. Compared to bone marrow, a smaller number of data points were available at the time of the modeling of the data. A sufficient number of data points were however available for reliable modeling as the model used for characterizing in vivo cell survival fractions has but 2 parameters. One outlier (lowest data point for controls) was excluded in modeling of the data.

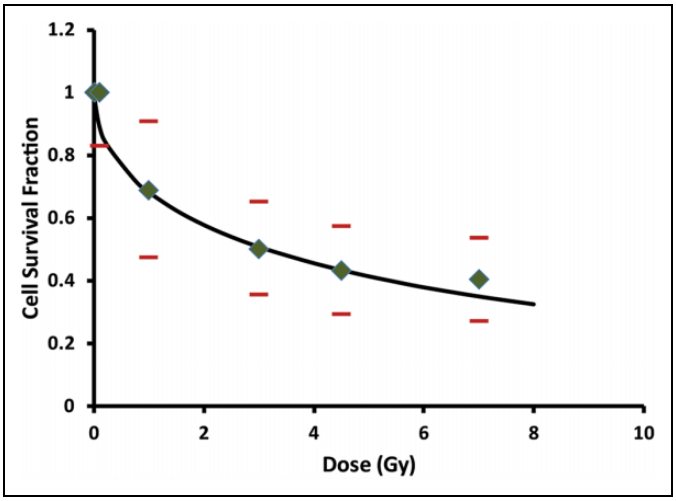

Figure 3. Fitted dose-response curve for the bone marrow cell survival fraction (average) for 320-kV X-ray exposed C.B-17 SCID mice, based on average bone marrow survival fraction data in Table 1 and HF model parameters (means) in Table 2. Error bars for the presented data points are ± 1 standard error.

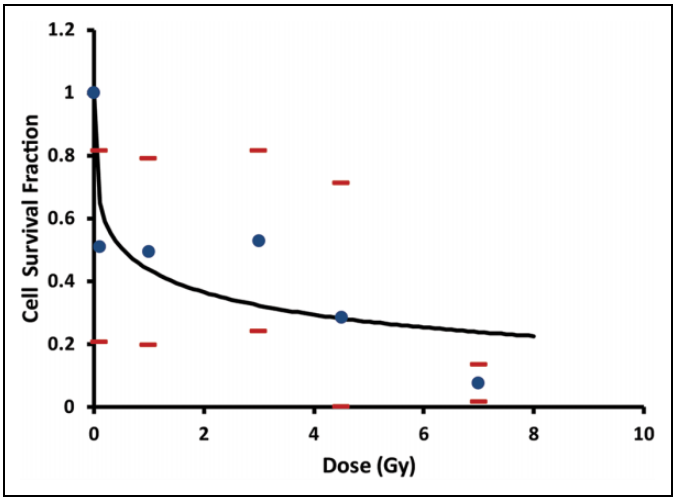

Figure 4. Fitted dose-response curve for spleen cell survival fraction (average) based on average spleen survival fraction data in Table 3 and HF model parameters (means) in Table 4. Error bars for the presented data points are ± 2 standard errors as a smaller number of animals were used for the spleen data than for the bone marrow data.

Bayesian analysis implemented by Markov chain Monte Carlo (MCMC) evaluations,[26,27] which is the same approach as we have previously used for cell survival.[12] A formal description of using Bayesian inference in dose-response-data analysis is provided in the Appendix and is an update of Scott et al.[12] Vector notations used have changed.

With employing MCMC via WinBUGS software,[28] thousands of iterations were used with some early (during the iterations) unreliable data discarded (ie, burn-in data discarded), which helps to get reliable model parameter estimates and related uncertainty (standard deviation and distributions

percentiles). MCMC data autocorrelations were monitored which helped in deciding how many iterations to use. Twenty thousand iterations (single MCMC chain) were judged to be more than adequate based on autocorrelation, probability density plots, and ratios Monte Carlo error/parameter standard deviation <0.05. For the results presented, of the 20000 data points generated per endpoint (e.g., model parameter vck or val), the first 10000 were discarded.

Results

Modeling Results for Bone Marrow Cells

Table 1 shows data for the bone marrow cell survival fraction $S_{ck}(D)$ (an average) that was obtained based on Equation 6. The standard deviations for $S_{ck}(D)$ values were obtained via error propagation evaluations. The related standard errors were obtained from the standard deviation values, based on sample sizes. Results of application of the HF model to the data in Table 1 are provided in Table 2.

A negative curvature dose-response relationship for $H_{ck}(D)$ is implicated by vck <1.0 in Table 2. A negative curvature dose-response relationship for $H_{ck}(D)$ was also found for in-vivo killing of bone marrow cells for whole-body, split-dose exposure of anesthetized immunocompetent C.B-17 mice to 320-kV X rays and Cs-137 γ rays separately.[5,12] This also implicates a negative curvature dose-response relationship for the live bone marrow cells per unit body weight evaluated 1 day after irradiation. Results in Figure 1 are based on cell counts per unit body weight (in grams) converted to corresponding cell counts ($\times 10^5$) for a reference 25-g mouse.

Table 1. Calculated Values (Averages) for $S_{ck}(D)$ and Related Standard Deviation (SD) and Standard Error (SE) for Bone Marrow Cells In Vivo After Unilateral-Single-Dose Exposure of C.B-17 SCID Mice to Filtered 320-kV X Rays, Based on Cell Count Data in Figure 1 Which Is Adjusted for 25 g Mouse.

Dose (Gy)	Number of mice	$S_{ck}(D)$	SD	SE
0	5[a]	1	0.830	0.371
0.1	5	1.224[b]	0.881	0.394
1	6	0.690	0.530	0.216
3	6	0.502	0.365	0.149
4.5	6	0.434	0.346	0.141
7	6	0.404	0.324	0.132

[a]One outlier (lowest data point) in Figure 1 was excluded.
[b]Replaced with upper limit 1 when fitting HF model to the data.

Data for $S_{ck}(D)$ need to be plotted on semi logarithmic scale (vertical axis logarithmic) to see a decreasing slope magnitude with increasing dose that implicates negative curvature. However, $H_{ck}(D)$ can be plotted on linear vs. linear scales to see negative curvature. In this case, the magnitude of the positive slope decreases with increasing dose, which is characteristic of a mixed cell population with hyper-radiosensitive, moderately radiosensitive, and radioresistant cells (as appears to be the case for both bone marrow and spleen of C.B-17 mice).[5,12] *For a negative exponential survival curve, $H_{ck}(D)$ is a linear-no-threshold (LNT) function of the radiation absorbed dose and therefore has no curvature*.

In general, vck <1 always indicates negative curvature for $H_{ck}(D)$, vck = 1 (typical for some in vivo cell survival studies) corresponds to no curvature for $H_{ck}(D)$, and vck > 1 (typical for some in vitro studies) indicates positive curvature (magnitude of slope [positive] *increases* with dose). For vck = n with n > 1, the slope of $H_{ck}(D)$ (ie, $dH_{ck}(D)/dD$) increases as D raised to the n-1 power. For example, for n = 3, the slope at dose D is proportional to D squared.

For acute lethality (eg, via hematopoietic syndrome or gastrointestinal syndrome mode) rather than cell loss as the endpoint of interest, values of val (replaces vck) much larger than 1 can occur and dose-response relationships for $R_{al}(D)$ has an effective threshold (dose below which the *very small risk calculated* has no practical value). Dose-response relationships for $R_{al}(D)$ for val > 2 are sigmoidal (ie, distorted-S shape).[25,29] For low radiation doses, $R_{al}(D)$ and $H_{al}(D)$ have essentially the same value for a given dose D.

The fitted dose-response relationship obtained for cell survival fraction using the information in Tables 1 and 2 for bone marrow cell survival after unilateral exposure of C.B-17 SCID mice to 320-kV X rays is shown in Figure 3. Mean (from Bayesian analysis posterior distribution) HF model parameter estimates were used for the smooth curve. Error bars are ± 1 standard error (SE). As already indicated, the fitted curve was obtained via Bayesian analysis implemented by MCMC evaluations. *Data were weighted by sample size for a given dose level*. The calculated average survival fraction at 0.1 Gy exceeded the upper limit of 1.0 and therefore was set to 1.0.

Modeling Results for Spleen Cells

The average spleen cell survival fraction data used in our Bayesian-inference modeling are presented in Table 3. Modeling results obtained are presented in Table 4 and are the basis for the dose-response curve plotted along with the related

Table 2. Estimates of Hazard Function (HF) Model Parameters D_{50ck} and vck for In Vivo Bone Marrow Cell Survival Fraction After Single-Dose-Unilateral Exposure of C.B-17 Mice to 320-kV X Rays.[a]

Parameter	Posterior distribution mean	SD (Monte Carlo error)	2.5%	50%	97.5%	MCMC sample size used
Median effective dose D_{50ck} (Gy)	3.13	1.03 (*0.0114*)	2.43	4.06	6.44	10,000
Shape parameter vck	0.518	0.19 (0.0025)	0.42	0.71	1.18	10,000

[a]The % values are percentiles of the distribution of the many values obtained via Bayesian inference implemented with Markov chain Monte Carlo (MCMC).

measured cell survival fraction data in Figure 4. Based on the mean value of vck (0.281) in Table 4 and 97.5% (percentile) value (0.597), the dose-response curve for $S_{ck}(D)$ clearly has negative curvature (ie, vck < 1), and this applies also to the dose-response curve for $H_{ck}(D)$. The posterior distribution (from Bayesian analysis) mean value for D_{50ck} (0.531 Gy) for in vivo spleen cells is much smaller than the corresponding value for bone marrow cells ($D_{50ck} = 3.13$ Gy), indicating a *much higher (about 6-fold) radio-sensitivity for spleen cells* of irradiated C.B-17 SCID un-anesthetized mice at the D_{50ck} dose. The negative curvature dose-response relationships for $S_{ck}(D)$ and $H_{ck}(D)$ for both cell types can be explained on the basis of a mixture of cells with different radiosensitivity distributions for both spleen and bone marrow.[30-32] Interestingly, for spleen there is strong evidence for a hyper-radiosensitive subpopulation, where 50% of all cells are eliminated by a dose of approximately 0.5 Gy (500 mGy). This is not the case for bone marrow.

Modeling Results for Acute Lethality

Data for 30-day lethality of 320-kV, X-ray-exposed C.B-17 SCID mice are presented in Table 5 and are presumed to be associated with the hematopoietic syndrome mode. Although there were a relative small number of animals involved, there are but 2 HF model parameters that need to be assessed so that the data are still valuable. Bayesian inference implemented via MCMC was also used for modeling the data. HF Model parameter estimates (posterior distribution means) and related statistics (standard deviation and specific percentiles) are presented in Table 6.

Table 3. Calculated Values (Averages) for $S_{ck}(D)$ for Spleen Cells In Vivo and Related Standard Deviation (SD) and Standard Error (SE) After Unilateral-Single-Dose Exposure of C.B-17 SCID Mice to Filtered 320-kV X Rays, Based on Cell Count Data in Figure 2 Which Is Adjusted for 25 g Mouse.

Dose(Gy)	Number of mice	$S_{ck}(D)$	SD	SE
0	4[a]	1	0.826	0.413
0.1	5	0.509	0.340	0.152
1	5	0.493	0.331	0.148
3	5	0.527	0.321	0.144
4.5	4	0.286	0.423	0.212
7	3	0.0748	0.0524	0.03

[a]One outlier with low abnormal cell count excluded.

Discussion

Studies using small animal models of hematopoietic system cell survival after exposure to X rays or γ rays has been a practice for decades and are important for bone marrow transplantation, medical counter measures, and other study-protocol planning.[32] Previously we performed successful bone marrow transplantation in Cs-137 γ-ray, split-dose exposed (bilaterally) C.B-17 immunocompetent anesthetized mice[5] but not with now-considered "not comparable" effective doses (based on an updated RBE estimate) of 320-kV X rays from the same X-RAD 320 irradiator as used in our present studies. Based on careful consideration of the research data generated, we think the most likely reason for the lack of success for 320 kV X rays was due to the RBE estimate (RBE = 1.3) used in the study design being too large, leading to the absorbed doses used being too small. Based on physical microdosimetry performed by others[2,10] for bone marrow of mice, an RBE ≈ 1 would be expected for filtered (HVL ≈ 4-mm Cu) 320-kV X rays. This suggests that the X-ray doses we used should have been about a factor of 1.3 higher (ie, same doses as used for Cs-137 γ rays).

The dose-response relationship for 30-day lethality in mice has traditionally been used for characterizing the risk of mortality via the hematopoietic-syndrome mode (bone marrow failure related).[23,24] For brief single-dose exposure at a high dose rate, the risk curve for this mode of death has previously been characterized using the HF Model[25,29] and the model was therefore also used in this paper for modeling the risk of acute lethality via this mode for the C.B-17 SCID mice exposed to filtered 320-kV X rays. However, as previously found[12] and also found in this new research, model parameter values for acute lethality are quite different than those for the cell survival fraction in vivo.

The shape parameter for in vitro studies of cell survival fraction may also differ from that for in vivo radiation exposure. Exponential cell survival fraction curves (ie, vck = 1) were previously used for characterizing in vitro cell survival of bronchial epithelial cells (HBEC-13 and HBEC-2) exposed to 320-kV X rays or Cs-137 γ rays.[13] The exponential nature of the survival curves allowed for the introduction of *a new area of dosimetric research, namely biological microdosimetry*.[13] However, this form of microdosimetry is not widely known.

We had planned to repeat our previous X-ray study related to bone marrow transplantation using higher does and C.B-17 immunocompetent mice, but the immunocompetent mice are no longer available as they have been replaced by an immunodeficient (immunocompromised) strain as already indicated.

Table 4. Estimates of Hazard Function (HF) Model Parameters D_{50ck} and vck for Spleen Cell Survival After Single-Dose-Unilateral Exposure of C.B-17 Mice to 320-kV X Rays.[a]

Parameter	Posterior distribution mean	SD (Monte Carlo error)	2.5%	50%	97.50%	MCMC sample size used
Median effective dose D_{50ck} (Gy)	0.531	0.278 (*0.0036*)	0.047	0.538	0.977	10,000
Shape parameter vck	0.281	0.164 (*0.0019*)	0.023	0.271	0.579	10,000

[a]The % values are percentiles of the distribution of the many values obtained via Bayesian inference implemented with Markov chain Monte Carlo (MCMC).

Under the *hypothesis that dose-response relationships for the in vivo cell survival fraction for bone marrow and spleen have similar curvature* (ie, displaying negative curvature) for the immunocompetent and immunodeficient mice, we conducted X-ray dose-response modeling using newly generated (at Lovelace Biomedical Research Institute) bone marrow and spleen cell survival fraction data for C.B-17 SCID mice reported in this paper to test our assumption. As demonstrated in Figures 3 and 4 along with associated central estimates (mean of modeled distribution) of the shape parameter vck (< 1) in Tables 2 and 4, this is indeed the case at 1 day post radiation exposure.

As is revealed in Figure 5 (results based on modeling of cell survival fractions for subpopulations of cells) for 320-kV X-ray irradiated C.B-17 SCID mice, there appears to be a subpopulation of bone marrow cells in vivo that are *radioresistant*. Based on results for the LD_{99} (for 99% acute lethality of the mice), *this radioresistant subpopulation may be as large as about 40% of the total cell population* of the immunodeficient mice. Such a large percentage essentially guarantees a negative curvature dose-response relationship, with much larger doses than 7 Gy being needed to reduce the cell survival fraction to a level that would permit successful bone marrow transplantation based on published results for immunocompetent C.B-17 mice.[5] Regarding the radioresistant cell subpopulation for bone marrow, which cell types are represented cannot be resolved from the data used in our analyzes. For spleen, some cell type enrichment information related to high doses can be derived from findings from a study by others[30] that are discussed in a subsequent paragraph.

It was informative to have considered in Figure 5 the relationship between survival of subpopulations of bone marrow cells and the risk of acute lethality as a function of the radiation absorbed dose of 320-kV X rays and of acute lethality (for mice) dose percentiles LD_1, LD_{50}, and LD_{99}. With the indicated predicted loss of a large number of hyper-radiosensitive and radiosensitive cells at the LD_{01} (for acute lethality of mice) of 1.9 Gy, there is little if any expected increase in the risk of acute lethality via the hematopoietic-syndrome mode. This is not the case for the predicted cell losses at the LD_{50} (for expected acute lethality in 50% of mice) of 3.79 Gy where large numbers of cells of moderate radiosensitivity in addition to hyper-radiosensitive and radiosensitive cells are predicted to be lost. For the LD_{99}, the predicted cell loss is worse (more subpopulations lost) but even so, greater than 35% of bone marrow cells (radioresistant) are expected to survive (Figures 6 and 7) but a somewhat smaller fraction (less than 30%) of radioresistant spleen cells.

Table 5. Thirty-Day Mortality Data for C.B-17 SCID Mice After Unilateral-Single-Dose Exposure to 320 kV X Rays.

Dose (in grays)	Mice	Deaths in thirty days	Thirty-day mortality
0	10	0	0
0.1	10	0	0
1	10	0	0
3	10	0	0
4.5	10	10	1.0[a]
7	10	10	1.0

[a]The value was entered as 0.999 in the MCMC analysis to facilitate estimating the 2 model parameters LD_{50} and val.

Interestingly, regarding the highest 320-kV X-ray dose in Figure 6 (LD_{99} = 5.19 Gy), had a somewhat higher dose (eg, 5.2 Gy) been used in the bone marrow transplantation study performed by Gott et al,[5] where the central estimate of the highest X-ray dose actually used is 4.8 Gy (rounded), successful bone marrow engraftment would be expected as can be inferred from our modeling results in Figure 7. The modeling results (bone marrow survival fractions) in Figure 7 are for the same absorbed doses (LD_{01}, LD_{20}, LD_{50}, LD_{90}, and LD_{99}, respectfully) as in Figure 6 and results for Cs-137 γ rays and 320-kV X rays for immunocompetent mice are also included for comparison. Successful engraftment was achieved after using a ^{137}Cs-γ-ray dose of 5.1 Gy (central estimate) and a dose of 6.6 Gy (central estimate).[5] Based on the study of Gott et al[5] with γ rays, we expect that the bone marrow cell survival fraction would need to be reduced to around 0.15 (15% cell survival) for successful bone marrow engraftment. The 15% survival also relates to the radioresistant subpopulation of cells as can be inferred from Figure 7. *These findings support possible successful use of the X-RAD 320 irradiator along with immunocompetent mice (e.g., immunocompetent BALB/c mice) in bone marrow transplantation studies*.

The notion that bone marrow and spleen cellularity after exposure at the level of the LD_{99} is likely to be comprised of highly radioresistant cells (via cellular enrichment) is supported by findings of Harrington et al[30] for splenocytes in vivo. Their flow cytometric analysis of spleen of C57Bl/6 mice whole-body exposed at 1.15 Gy/min to ^{137}Cs γ rays revealed both a decrease in spleen cellularity (similar to results in Figure 4) and an alteration in the relative composition of constituent spleen cell populations as dose increased. This was interpreted by the researchers to reflect *differential radiosensitivity* along with *selective enrichment* of NK cells (seven-fold) and CD4+ T lymphocytes (3-fold). The enrichment occurred within 1

Table 6. Estimates of Hazard Function Model (HF) Parameters (for 320-kV X-Ray Exposed C.B-17 SCID Mice) for Acute Lethality via the Hematopoietic Mode.[a]

Parameter	Posterior distribution mean	Standard deviation	2.5%	50%	97.5%	MCMC sample size used
Shape parameter val	6.08	2.43	1.52	6.21	9.8	10,000
LD_{50} (Gy)	3.79	0.68	2.54	3.76	5.33	10,000

[a]The % values are percentiles of the distribution of the many values obtained via MCMC analysis.

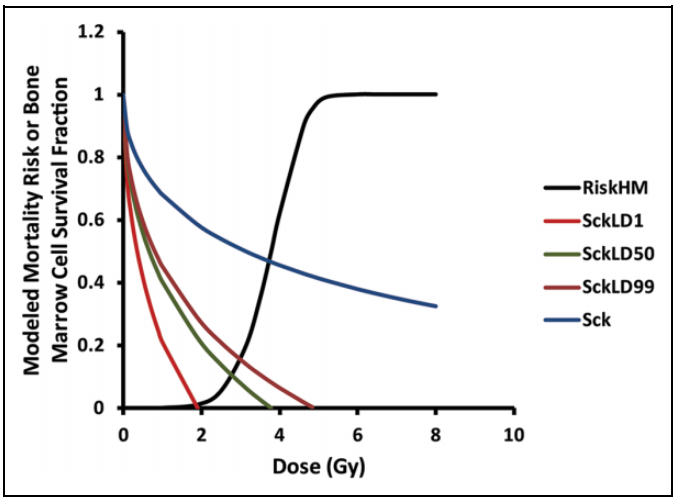

Figure 5. HF-model-based expected bone marrow cells subpopulations survival fraction curves for $S_{1ck}(D \leq LD_1)$, indicated as SckLD1; $S_{50ck}(D \leq LD_{50})$, indicated as SckLD50; $S_{99ck}(D \leq LD_{99})$, indicated as SckLD99; full cell population survival fraction $S_{ck}(D)$, indicated as Sck; and expected risk of lethality $R_{al}(D)$ via the hematopoietic syndrome mode, indicated as RiskHM. Results apply to immunodeficient C.B-7 mice single-dose-unilaterally exposed 320-kV X rays. For the acute lethality risk curve, $LD_1 = 1.9$ Gy, $LD_{50} = 3.79$ Gy, $LD_{99} = 5.19$ Gy.

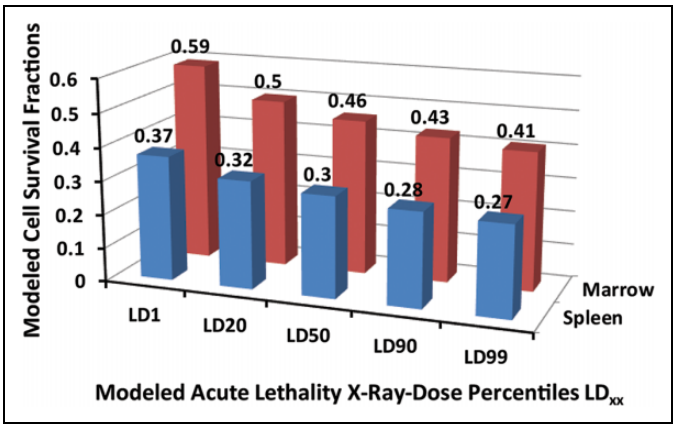

Figure 6. Expected relationship between modeled lethal dose (for animal lethality) percentiles LD_1, LD_{20}, LD_{50}, LD_{90}, and LD_{99} and modeled cell survival fractions $S_{ck}(D)$ (for total cell population) for bone marrow and spleen for 320-kV X-ray-exposed, immunodeffi-cient C.B-17 mice, based on HF models for cell killing and for acute lethality via the hematopoietic mode. Numbers on top of the 3D objects are the expected average cell survival fractions. Values for LD_{xx}: $LD_1 = 1.9$ Gy, $LD_{20} = 3.13$ Gy, $LD_{50} = 3.79$ Gy, $LD_{90} = 4.62$ Gy, and $LD_{99} = 5.19$ Gy.

week after radiation exposure, which is longer follow-up than for our presented data for immunodeficient and immunocompetent C.B-17 mice. Our results relate only to cell survival fractions 1 day after radiation exposure. For a longer period before assessing cell survival, some repopulation of cells could occur.[32]

Our view that successful bone marrow engraftment studies in mice could be achieved using 320-kV X rays is supported by

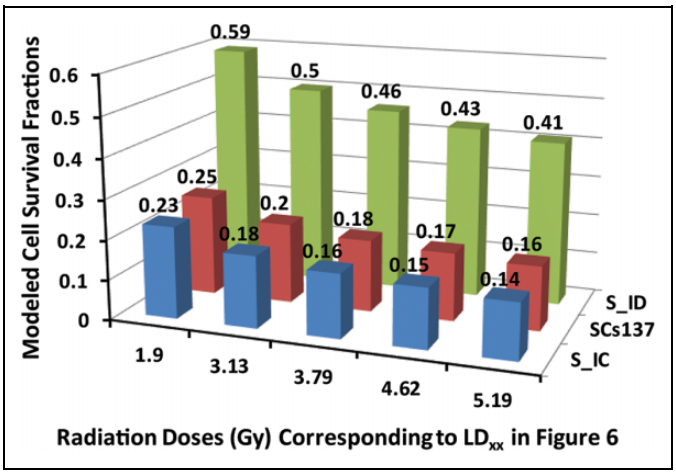

Figure 7. Modeled bone marrow cell survival fractions for immunocompetent (S_IC) C.B-17 mice bilaterally exposed to 320-kV X rays (model parameters from: Scott et al[12]; Gott et al[5]), for immunocompetent (SCs137) C.B-17 mice bilaterally exposed to Cs-137 γ rays (model parameters from: Scott et al[12]; Gott et al[5]), and immunodeficient (S_ID) C.B-17 mice unilaterally exposed to 320-kV X rays. LD_{xx} (for animal lethality) for doses in increasing order are LD_1, LD_{20}, LD_{50}, LD_{90}, and LD_{99}.

results of others[4] who achieved successful bone marrow engraftment in male C57BL/6 mice whole-body exposed via an X-ray source irradiator (Rad Source 2000 X-ray Biologic Irradiator, Rad Source Technologies, Alpharetta, GA). They also achieved successful engraftment using ^{137}Cs γ rays (Irradiator: Gamma Cell 40, MSD Nordion, Ottawa, Ontario, Canada). Based on their research results, it was concluded by the researchers that although both sources ablated endogenous bone marrow sufficiently to allow for successful bone marrow engraftment, there were distinct physiologic responses that should be considered when choosing the best radiation source for such studies. A key observation was that *radiation from the X-ray source used was associated with higher overall morbidity due to opportunistic infection* than was the case for ^{137}Cs γ rays.

Our present study focused on C.B-17 SCID mice while our earlier studies[5] used immunocompetent C.B-17 mice as already indicated. X-ray exposure research was initiated in 2020 by our research team using immunocompetent BALB/c mice. The genetic background of the C.B-17 strain is the same as for the immune competent BALB/c mice. The only difference is that BALB/c mice carry the Igh-1b allele of the C57BL/Ka strain. Preliminary findings are suggestive of negative curvature dose-response relationships for both bone marrow and spleen cells in vivo, similar to findings for immunocompetent C.B-17 mice. Similar radiosensitivities of the immunocompetent C.B-17 mice and BALB/c mice is also suggested, which if validated would support the view that C.B-17 SCID mice may be more radioresistant than immunocompetent C.B-17 mice. However, formal modeling of the BALB/c data would need to be taken up in a follow-on project. Thus, we hope to address the modeling

needs and any additional experimental research needs in a follow-on research project.

Conclusions

We have demonstrated through dose-response modeling with the previously-introduced HF model and Bayesian inference that dose-response relationships for the in vivo cell survival fractions for both bone marrow and spleen cells of 320-kV X-ray exposed C.B-17 SCID mice exhibit negative curvature (vck < 1), explainable based on subpopulations of cells with differing radiosensitivity distributions. Hyper-radiosensitive cells are removed by very low radiation doses (eg, 0.1 Gy) and many radioresistant cells survive even a 70-fold higher radiation doses (ie, 7 Gy). The 7 Gy X-ray dose likely led to enrichment of radioresistant cells.

For acute lethality via the hematopoietic syndrome mode, the dose-response relationship for the lethality hazard $H_{al}(D)$ (and related survival probability $S_{al}(D)$) has positive curvature (shape parameter val > 1) and can be explained on the basis of the mixed subpopulations of cells that are killed by radiation doses above an effective threshold (for animal lethality) and up to near the acute lethality dose percentile LD_{99}. However, the dose-response relationship for the risk $R_{al}(D)$ of acute lethality via the hematopoietic syndrome mode is sigmoidal.

Radiation sources such as 320-kV X rays from an X-RAD 320 irradiator and other irradiators (eg, Rad Source 2000 X-ray Biologic Irradiator) are suitable for in vivo studies of cell survival in mice. While successful bone marrow transplantation in mice has been demonstrated using the Rad Source 2000 X-ray Biologic Irradiator, this is not the case for the X-RAD 320 device. Thus, there is still a need to verify that filtered 320-kV X rays from an X-RAD 320 device can be successfully used in a bone marrow transplantation study using a mouse model.

A key finding for C.B-17 SCID mice when compared to results previously obtained for immunocompetent C.B-17 mice is that the immunodeficient mice appear to be markedly more radioresistant, implicating a possible key role of the immune system capacity in radiosensitivity of mammals. However, confirmation will necessitate additional research.

Appendix

Evaluating Dose-Response Model Parameters Using Bayesian Inference Implemented With MCMC

With the Bayesian approach, dose-response model parameters and the biological effects for data of interest (eg, cell survival fraction, animal lethality risk) are considered as random variables.[12,27,33] If **dat** (bolded) is used to denote the observed *biological effects data vector* and $\boldsymbol{\Omega}$ (bolded) is used to denote a *dose response model parameters vector*, then formal Bayesian inference requires setting up a *joint probability distribution* P(**dat**,$\boldsymbol{\Omega}$) over the quantities in the 2 vectors (in vivo biological effects and model parameters). This joint distribution comprises 2 parts: a prior distribution, P($\boldsymbol{\Omega}$), and a likelihood, P(**dat**|$\boldsymbol{\Omega}$). The vertical bar indicates that the data vector **dat** is conditional on the parameter vector $\boldsymbol{\Omega}$. The joint probability distribution is specified as follows:

$$P(\mathbf{dat}, \boldsymbol{\Omega}) = P(\mathbf{dat}|\boldsymbol{\Omega})P(\boldsymbol{\Omega}) = P(\boldsymbol{\Omega}|\mathbf{dat})P(\mathbf{dat}). \quad \text{(A1)}$$

Both P($\boldsymbol{\Omega}$) and P(**dat**) are *unconditional distributions*. When new observations are available, Bayes theorem is used to update the distribution of the parameters, P($\boldsymbol{\Omega}$|**dat**), given (ie, conditional on) the new observations. The Bayesian updating relationship can be expressed as follows[31]:

$$\begin{aligned} P(\boldsymbol{\Omega}|\mathbf{dat}) &= P(\mathbf{dat}|\boldsymbol{\Omega})P(\boldsymbol{\Omega}) / \int P(\mathbf{dat}|\boldsymbol{\Omega})P(\boldsymbol{\Omega})d\boldsymbol{\Omega} \\ &= P(\mathbf{dat}|\boldsymbol{\Omega})P(\boldsymbol{\Omega})/P(\mathbf{dat}). \end{aligned} \quad \text{(A2)}$$

Analytical solutions to Equation A2 may exist, depending on the complexity of the model being addressed. Analytical solutions do not exist for some complex stochastic models. However, whether or not an analytical solution does exist, the integral ($\int \ldots d\boldsymbol{\Omega}$) can be estimated numerically using Markov chain Monte Carlo (MCMC).[26,28] MCMC methods involve a group of rather sophisticated methods that are often used in challenging Bayesian analyzes. The most common implementation uses what are called the *Metropolis-Hastings algorithm and Gibbs sampling*.

Markov chains provide a framework under which simulation techniques can be implemented to explore various distribution properties, generally related to what is called the *posterior distribution*. Gibbs sampling is iterative and allows the marginal distribution of model parameters (eg, HF Model parameters v and D_{50}) to be directly sampled. The Markov chain component of Gibbs sampling provides the framework by which random samples (e.g., of model parameters) are generated. However, to allow Monte Carlo sampling it is required that the distribution of all model parameters, conditional on all other influential parameters and the data fitted, to be specified. Generally, single or multiple chains are used and *data from the initial portion from a Markov chain sample is discarded so that the effect of the initial values on the posterior inference is minimized*. The discarded data are called *burn-in*. For our MCMC analyzes a single very long chain was used with the first ½ of the chain discarded as burn-in data. Our MCMC evaluations were implemented using WinBUGS Software.[28]

Authors' Note

This article describes objective technical results and analysis. Any subjective views or opinions that might be expressed in the article do not necessarily represent the views of Lovelace Biomedical Research Institute, Sandia National Laboratories, the US Department of Energy, or the U.S. Government.

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