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Lethality Assessments for Acutely Irradiated Cynomolgus Macaques Under Subject-based Care

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Well-characterized animal models of acute radiation syndrome are needed for the development of radiation medical countermeasures to mitigate injury due to acute exposure to high doses of total- or partial-body radiation. Such animal models must reveal a radiation dose- and time-dependent relationship, pathogenesis of injury, and clinical presentation similar to humans. The focus of this study was to investigate clinical responses, principally lethality patterns, of cynomolgus macaques acutely exposed to relatively high doses of total-body radiation. Such investigations are currently relevant due to the limited availability of rhesus macaques, the dominant and preferred macaque subspecies, due to limited supply and their use in other high-priority areas. In this study employing cynomolgus macaques, a preliminary dose-response relationship was determined using three different radiation doses (4.7, 5.8 and 6.5 Gy, n = 24, n = 8/radiation dose) at a dose rate of 0.6 Gy/min. Animals were provided subject-based supportive care excluding blood products and were monitored for 60 days postirradiation for survival, which was the primary endpoint and the secondary endpoint was hematopoietic recovery. The lethality curve suggested $LD_{30/60}$, $LD_{50/60}$, and $LD_{70/60}$ values as 4.8, 5.3, and 5.8 Gy, respectively. The initial results of this study are deemed critical for future efficacy assessments of newly developed medical countermeasures for acute radiation injuries by making use of an alternative subspecies of macaques, namely cynomolgus macaques (*Macaca fascicularis*).

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INTRODUCTION

The increased risk of radiation exposure can be attributed to a possible radiological/nuclear attack as a consequence of warfare between opposing nations. Those exposed to sufficiently high levels of ionizing radiation (IR) are likely to develop acute radiation syndrome (ARS). An array of injuries can manifest after acute IR exposure, depending on the dose rate, absorption, duration, and body area exposed to radiation [total- or partial-body irradiation (TBI or PBI)], and there are numerous clinical indicators of those injuries. ARS is a composite of at least three distinct sub-syndromes, with each being elicited by a given, but broad range of IR exposures; i.e., hematopoietic ARS (H-ARS) occurs at a radiation dose of 1–6 Gy, gastrointestinal (GI-ARS) at 6–10 Gy, and neurovascular (NV-ARS) at greater than 10 Gy (*1*). Currently, there are treatments only for the mitigation of H-ARS. As of today, the United States Food and Drug Administration (U.S. FDA) has approved the use of nine agents for H-ARS (Neupogen, Nypozi, Zarxio, Neulasta, Udenyca, Stimufend, Ziextenzo, Nplate and Leukine) which can be used postirradiation (*2*). Though there are no GI-ARS specific medical countermeasures (MCMs), previous studies have suggested that individuals with this sub-syndrome can respond to these existing treatments administered according to the clinical signs they present (*3–7*).

Presently, there is no FDA-approved agent for ARS that can be used prior to unwanted (nonclinical) irradiation, though there are a few approved prophylaxes for limited indications (e.g., Amifostine, Palifermin) and internalized radionuclides (ThyroShield) (*4*, *8*). Hence, there is a medical need for radioprotectors in the interest of satisfying this need, the effects of radiation leading to the development of ARS and prophylactic drugs to target its symptoms must be investigated. To carry out biomedical research involving exposure to radiation in humans is considered unethical as the side effects of ARS can be severe and may lead to death.

Therefore, the U.S. FDA established the Animal Rule, which requires demonstrating that an agent is efficacious in well-characterized animal models while also shown to be safe in humans (*5*, *6*, *9*, *10*). NHPs serve as a large animal model that provides essential information in understanding how ARS may present in humans and how new drugs may treat this syndrome (*11*).

The consensus among biomedical investigators with a vested interest in MCM development is that the NHP model is the gold standard for advanced, large animal-based, preclinical studies that involve developing efficacy and safety profiles for any given drug under consideration for regulatory approval by the U.S. FDA and its Animal Rule. This consensus is largely due to NHP's homology to humans in many regards including genetic sequence, metabolic pathways, anatomical structures, pathophysiological responses, and long-life span (*11*). Moreover, clinical signs and histopathological characteristics of ARS in NHPs closely resemble those observed in humans. Historically, rhesus macaques have been the primary NHP model for MCM development, but more recently, cynomolgus macaques are being investigated as an alternative (*12*). To characterize this alternative animal model for developing MCMs, one needs to develop a standard lethality curve so that specific, potentially lethal radiation doses can be delivered to animals for testing the efficacy of potential MCMs.

The primary objective of the current study was to determine the lethality curve of male cynomolgus macaques using animals exposed to three different lethal doses of total-body ^{60}Co γ radiation. These animals received subject-based supportive care excluding blood products. Mortality was observed for sixty days after radiation exposure and an approximate dose-response relationship (DRR) was established for this NHP model of interest. These determinations are of paramount importance to investigate the efficacy of potential MCMs in accordance with the Animal Rule set forth by the U.S. FDA.

MATERIALS AND METHODS

Experimental Design

A total of 24 animals were divided into three groups of eight each. The animals in each group were exposed to a specific dose (4.7, 5.8 or 6.5 Gy) of total-body ^{60}Co γ-radiation at a dose rate of 0.6 Gy/min. In the first cohort, we used 6.5 Gy, the $LD_{50/60}$ for rhesus macaques (*13*). However, due to increased mortalities, a step-down approach was used and 5.8 Gy ($LD_{30/60}$) was used for the second cohort. This dose also resulted in significantly higher mortalities and based upon the input of the statistician, a lower dose of 4.7 Gy was used for the third cohort. All animals were monitored for sixty days postirradiation to assess general sequential change in clinical condition, including end-stage morbidity/moribundity. The hematopoietic response analyses were based on complete blood counts (CBC) along with differential cell counts, in addition to other related parameters.

Animals

Twenty-four naïve cynomolgus macaques (*Macaca fascicularis*, males; aged 4.8–6.9 years, body weight 4.9–7.5 kg) were used for the study. All animals used in this study were males, as female animals were unavailable after the COVID-19 pandemic when animal procurement for this study was taking place. All NHPs were procured from Alpha Genesis Inc. (Yemassee, SC) and maintained in an AAALAC International accredited facility. These animals were imported from Mauritius by the vendor. NHPs were quarantined for six weeks prior to the initiation of the experiments. Animals were housed individually in stainless-steel cages in environmentally controlled rooms maintained at 22°C ± 2°C, 30–70% relative humidity, 10–15 air change cycles per h, and a 12:12 h light-dark cycle. Animals were fed a primate diet (RQ Monkey diet, Zeigler Bros. Inc. Gardners, PA) twice daily and received drinking water ad libitum. All the animals received enrichment food (fresh fruits and vegetables, prima treats, peanuts, marshmallows, etc.) once daily, Monday–Friday. They received mirrors, toys, and challenge balls for enrichment. Televisions were used for sensory enrichment 3–4 times a week. All the animals were able to see, hear, and/or touch the conspecifics through the cages. Animals were serologically negative for Macacine herpesvirus 1 (herpes B virus), simian retrovirus (SRV), simian T-cell leukemia virus (STLV), and simian immunodeficiency virus (SIV). The NHPs were either vaccinated for measles or, in the case of previously measles-vaccinated NHPs, tested for the presence of measles antibodies and tested negative for tuberculin (*14*, *15*).

In accordance with the 3Rs principle (replacement, reduction and refinement) set by the IACUC, an unirradiated control group was not needed in this lethality determination study. This was particularly important since this specific study was performed with nonhuman primates and all efforts are made to minimize the use of such animals. Blood samples collected prior to irradiation were compared with samples collected after exposure, eliminating the use of an additional group of healthy animals. Since all animals were not procured at the same time, randomization was not applicable. Animals for each radiation dose were procured gradually based on the availability of animals and resources. All animals were within a specific body weight and age range as specified in the IACUC protocol.

Total-body γ Irradiation

Food was withheld for 12–18 h prior to irradiation; animals were sedated 30–45 min before exposure with intramuscular (im) ketamine (10–15 mg/kg). Once sedated, the animals were placed in restraint boxes to limit movement and to maintain a proper upright sedated position during the irradiation procedure. The NHP limbs were secured to the box using ropes that were tied onto an external cleat. Animals were then transported to the High-Level Cobalt Facility via elevator, and the NHP tattoo identification numbers were verified again by the attending dosimetrist. A 0.1–0.3 mL im booster of ketamine (100 mg/mL) was administered to the NHPs to limit movement while being irradiated if needed. For irradiation, two NHPs were placed on the irradiation platform facing away from each other (*16*). The animals were paired based on their girth dimensions' similarity (±1 cm). Animals that were not within one cm of another animal's girth were irradiated separately. This strategy was used to ensure that each animal received approximately the same radiation dose to the core of the body, irrespective of body weight. All animals were exposed bilaterally to the core of the abdomen to a specific dose of ^{60}Co γ radiation at the rate of 0.6 Gy/min. The radiation field around the NHP was determined to be uniform with a variance of ± 1.5%. For dosimetry, two polymethylmethacrylate boxes housing identical phantoms placed on foam supports were placed on the exposure table. The phantoms were water-filled cylinders, 34.5 cm long, outer diameters 5.08, 6.99, 10.0, 12.5 and 15.1 cm; the diameter of the sleeve matched the diameter of interchangeable core (IC). Two N30013 ion chambers, manufactured by PTW dosimetry (Freiburg, Germany), were used to measure dose rate as described in the American Association of Physicists in Medicine Protocol TG-51 (*17*). Ion chambers and electrometers were calibrated at the National Institute of Standards and Technology (NIST) traceable Accredited Dosimetry Calibration Laboratory (ADCL) of University of Wisconsin as required by TG-51. Dose rate in each phantom was measured four times with relative standard deviation less than 0.4%. During the irradiation procedure, the NHPs were monitored continuously by closed-circuit security cameras. After completion of the radiation exposure,

TABLE 1
Details of Medical Management/Symptomatic Palliative Care

Drug class	Allowed medication or supportive care agents	Indication and/or criterion for administration
Parenteral fluids	Lactated Ringer's Solution (LRS) or LRS with 5% Dextrose; each at 5 ± 2.5 mL/kg body weight via sc route; up to twice daily depending on extent of dehydration. Fluid administration rates and volume change at the discretion of the veterinarian Bottles containing diluted fruit juice or oral rehydration solutions (Prang™, Bio–Serv) provided 10–20 days postirradiation.	Dehydration: Mild to moderate dehydration signs and symptoms include subtle loss of skin elasticity (observed by skin turgor while in restraint chair), decreased urine output, and increased thirst Severe dehydration signs and symptoms: rapid breathing, lethargy, severe loss of skin elasticity, sunken eyes, dry buccal mucous membranes, extremities cool to the touch, decreased body temperature. Treatment options at discretion of veterinarian.
Topical antiseptic	Chlorohexidine oral spray applied once daily while in restraint chair	For treatment of mouth ulcers as observed while in the restraint chair
Anti-emetics	Ondansetron (1–2 mg/kg), im, iv, or po 25 –90 min prior to irradiation and 30 –45 min after irradiation to suppress emesis	Administered pre– and postirradiation to suppress emesis.
Nutritional support	To facilitate the consumption of food postirradiation, the primates were provided with an array of food products that heightened their interest. Such items included biscuits soaked in Ensure, yogurt, dried fruit, popcorn, cereals, pasta, frozen Gatorade cubes, nuts, seeds etc. Fresh foods provided included: apples, bananas, peppers, carrots, celery, oranges and sweet potato. This brief listing is not meant to exclude any drink or food product that was in the consumption of diet designated for the animals.	Animals received daily enrichment and additional items immediately after blood collection. During days 10–20 postirradiation, if biscuits (normal feed) were not consumed, biscuits soaked in ensure were provided at the next scheduled feeding.
Analgesics	Buprenorphine HCL (Hospira Inc. Lake Forest IL) 0.01 –0.02 mg/kg im BID Rimadyl (Carprofen) 15 mg/tab BID, po or 1–5 mg/kg BID, sc	Body temperature >39.4°C (fever) or visual signs of pain as determined by the veterinarian.
Antibiotics	Enrofloxacin (Baytril Bayer HealthCare LLC, Shawnee Mission, KS) 5+/0.25 mg/kg im or sc, twice a day (BID); or 10+/–mg/kg im or sc once daily (QD).	ANC (absolute neutrophil count): Antibiotics was initiated if the absolute neutrophil count was <500/μL and was continued until ANC reached >500/μL.

NHPs were transported back to the vivarium, placed in their resident cages and closely monitored until fully recovered from sedation.

Cage-side Animal Observations

All NHPs were observed pre-irradiation and for 60 days postirradiation with moribundity (survival) being the primary measured endpoint. Daily observations for signs of pain and distress were made at least twice a day (morning and afternoon) by either the husbandry, veterinary technicians, or research staff. Between days 10–20 postirradiation, animals were observed more frequently, i.e., three times a day approximately 8–10 h apart and an on-call veterinary technician/veterinarian was available 24 h a day in the event of an emergency. Animals were observed for signs of radiation sickness or need for further medical management and treatment. All observations were appropriately documented in animals' medical records (*18*).

Medical Management/Symptomatic Palliative Care

The type of supportive care provided was based on CBC analysis and cage-side observations (Table 1). Antibiotics were initiated when the absolute neutrophil count was ≤500 cells/μL and continued until the count reached >500 cells/μL. The primary antibiotic used was enrofloxacin (Baytril, Bayer HealthCare LLC, Shawnee Mission, KS) and the administered dose was 5 mg/kg *im* or subcutaneously (sc) twice a day (BID), or 10 mg/kg administered im or intravenous (iv) once a day (QD). Supportive blood products were not used in this study. However, additional supportive care measures were provided as needed: these measures included rehydration fluids (administered sc), alternate antibiotics, antipyretics, antidiarrheal agents, analgesics, antiemetics, treatment for mucosal ulcers, and enhanced nutritional support.

Blood Collection

Blood draws and related blood cell analyses were carried out on a periodic and sequential basis over the entire experimental period; i.e., −10 days pre-exposure to 60 days postirradiation. All animals were restrained using the pole-and-collar method and placed in a custom-made chair for blood collection. The blood draw was conducted between 08:00 AM and 10:00 AM, 1–3 h after animals were fed. Blood samples were collected by venipuncture from the saphenous vein of the lower leg or brachial vein after the site was cleaned using a 70% isopropyl alcohol wipe. A 3–5 mL disposable luer-lock syringe with a 23-gauge needle was used to collect the desired volume of blood. Blood samples for CBC were collected for a total of 23 timepoints (day –7, –1, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 34, 38, 42, 50, and 60), while samples for serum

biochemistry and cytokine analysis were collected for a total of 8 timepoints (day –7, –1, 1, 2, 6, 14, 22, 38, 50, and 60). At the time of blood collection, vital signs (pulse, blood pressure, weight and body temperature) were also recorded (*19*). Furthermore, the methodology for serum collection for blood biochemistry and cytokine analysis is described elsewhere (*20*). In brief, the blood was placed in serum separating tubes and allowed to clot for at least 30 minutes prior to being centrifuged (15 min, 400 × g). The serum was then aliquoted into specimen tubes which were then stored at –80°C until use.

CBC Analysis

Whole blood cells were counted using a Bayer Advia-120 hematology analyzer (Siemens Medical Solutions, Malvern, PA) (*21*). Twenty blood parameters were analyzed: white blood cells (WBC), red blood cells (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, and absolute counts and percentages for neutrophils, lymphocytes, monocytes, eosinophils, basophils, and reticulocytes (*22*).

Blood Biochemistry Analysis

Serum blood chemistry was analyzed using a Vitros 350 automatic biochemistry analyzer (Ortho Clinical Diagnostics, Markham, ON, Canada) (*23*). A list of the analyzed blood chemistry parameters has been included as supplementary information.

Multiplex Analysis of Cytokines

The serum samples were also analyzed for the presence and concentration of various cytokines. A Luminex 200 analyzer (Luminex Corp, Austin, TX) was used to investigate the cytokines, chemokines, and growth factors in the serum using custom-made multiplex kits (up to 48 plex) (Bio-Rad Laboratories, Hercules, CA). The following cytokines were measured for their concentration in serum: interleukin-8 (IL-8), granulocyte colony-stimulating factor (G-CSF), IL-2 receptor-α (IL-2Rα), macrophage inflammatory protein-1β (MIP-1β), macrophage colony stimulating factor (M-CSF), IL-16, hepatocyte growth factor (HGF), stem cell factor (SCF), IL-9, interferon-γ (IFN-γ), regulated on activation normal T cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), IL-18, leukemia inhibitory factor (LIF), stem cell growth factor-β (SCGF-β), stromal cell-derived factor-1α (SDF-1α), monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor BB (PDGF-BB), GRO-α (growth regulated protein alpha), macrophage migration inhibitory factor (MIF), and TNF-β (*24*). Standard curves for each cytokine were prepared by serial dilution and run in duplicate. Cytokine concentration (pg/mL) was determined by fluorescence intensity and its quantification was performed using Bio-Plex Manager software, version 6.2 (Bio-Rad Inc.) (*25*). We have presented data of specific cytokines induced related to irradiation.

Euthanasia

Euthanasia was conducted in accordance with the approved versions of the IACUC protocol, the Guide, and the 2020 American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (*26*, *27*). Moribundity was used as a surrogate for mortality, and animals were euthanized to minimize pain and distress (*28*, *29*). When an animal reached a state of moribundity, the animal was euthanized. The following parameters were used as guidelines for moribundity: significant weight loss (10%) from baseline; inappetence (complete anorexia for 2 days and deteriorating conditions); minimal or absence of response to stimuli, severe anemia (<13% hematocrit due to acute blood loss or <4 g/dL hemoglobin) and core body temperature below 96.6°F after a period of febrile neutropenia (such as >103°F and <500 neutrophils/μL); weakened/inability to obtain food or water; severe thrombocytopenia (<10,000 platelets/μL) or other signs of severe organ dysfunction with poor prognosis as determined by the veterinarian such as dyspnea or severe cyanosis; sustained vomiting or diarrhea, obstruction, intussusception and peritonitis; renal failure as determined by clinical chemistry and urinalysis; sustained central nervous system depression, seizures, or paralysis of one or more extremities; non-healing wounds, repeated self-trauma, and severe skin infections; and severe organ system dysfunction with poor prognosis. Any single parameter from the above-listed guidelines did not lead to the euthanasia of any animal. The moribund animals were given pentobarbital sodium *iv* (Virbac AH Inc., Fort Worth, TX) using either saphenous or cephalic veins, needle size 20–25 gauge (100 mg/kg, 1–5 mL). In the event that intravenous administration could not be achieved, intra-cardiac injection was performed on the animal. Prior to pentobarbital administration, animals were sedated using ketamine hydrochloride injection (Mylan Institutional LLC, Rockford, IL) (5–15 mg/kg, im). The animals were deeply anesthetized by isoflurane (Baxter Healthcare Corporation, Deerfield, IL) (1–5%) with oxygen at 1–4 liters per minute via mask before administering the intra-cardiac injection. After pentobarbital sodium administration, the animals were examined by assessing the heart auscultation and pulse to confirm the death.

Necropsy and Histopathology

Necropsy was performed on euthanized animals that were moribund during the study period and at the study's endpoint. A total of eleven tissues were collected and maintained in 10% zinc-buffered formalin for analysis. The tissues include the duodenum, heart, ileum, jejunum, large intestine, kidney, liver, lung, spleen, sternum, and urinary bladder. Hematoxylin and eosin-stained slides were prepared for microscopic assessment. Histopathology analysis of these slides was conducted by a board-certified pathologist. Lesions, including hemorrhage, were assigned a number based on their severity. To do this, a semi-quantification 5-point scale was used, with the low scores (1 or 2) representing minimal to mild pathologic changes, whereas the higher scores (4 and 5) indicated more severe pathological changes.

Data Analysis

All statistical analyses were completed using statistical software IBM SPSS Statistics version 29.0.2.0 (Armonk, NY). A probit analysis was performed using the lethality data gathered in this study to establish a lethality curve for ^{60}Co γ radiation in NHPs. Estimates of radiation doses were made for various death percentages based on the estimated probit model parameters. A two-sided Fischer's exact test was performed to assess significant survival differences between the three radiation dose groups. Two additional tests were performed for CBC, blood chemistry, health charts, and cytokine data: a one-way ANOVA with a Tukey post hoc test to assess significant differences between radiation groups (i.e., comparing platelet counts between the three radiation dose groups at day 1), and a repeated measures two-way ANOVA test to assess significant intergroup changes between pre- and postirradiation values (i.e., comparing pre-irradiation platelet counts to platelet counts at day 2, day 3, etc. within each radiation dose group). A minimum of three animals per radiation dose group were required to establish significance at any given time point for the one-way ANOVA test. A P value of <0.05 was considered statistically significant for all tests performed.

RESULTS

Survival

The primary objective of the current study was to determine morbidity/moribundity effects of total-body ^{60}Co γ radiation in male cynomolgus macaques exposed to three potentially lethal doses of radiation while under a standard regimen of medical care, but one that excluded the use of blood products (Table 1). Each radiation dose group had a total of 8 animals (Table 2). All animals under test were monitored clinically on a periodic basis, both prior to (–10 days) and for 60 days after radiation exposure. Special attention was given to assessing

TABLE 2
Survival Percentage

Dose (Gy)	Animal ID	Survival		Significance *P* values
		Day of euthanasia	Percent on day 60	
4.7	FR2835	60		4.7 Gy vs. 5.8 Gy < 0.132, 4.7 Gy vs. 6.5 Gy < 0.041, 5.8 Gy vs. 6.5 Gy < 1.000
	NV1553	60		
	SB1010	60	75	
	FR2794	60		
	UG3438	60		
	FR2676	28		
	FR2785	60		
	FR2835	60		
	NV1710	22		
5.8	CP176	17		
	MB2726	17		
	FR3115	19		
	MB2355	60	25	
	MB2281	18		
	BC2309	20		
	MB2883	27		
	UG3248	60		
6.5	MB2742	28	12.5	
	NR1009	60		
	NV1752	17		
	SB533	24		
	UG1838	16		
	UG3047	21		
	UG3232	18		
	UG3392	18		

each and every animal for clinical signs, symptoms and severities of evolving morbid/moribund conditions. Overall rates of lethality (or conversely 'rates of survival') were determined at the end of the experimental period, 60 days postirradiation).

Survival rates. The observed rate of survival for animals subjected to the three exposure levels, i.e., 4.7, 5.8, and 6.5 Gy, were 75, 25, and 12.5%, respectively. The resulting survival/mortality responses for each radiation dose are shown in Figs. 1 and 2, and Table 2 (4.7 Gy vs. 5.8 Gy, $P < 0.132$; 4.7 Gy vs. 6.5 Gy, $P < 0.041$; 5.8 Gy vs. 6.5 Gy, $P < 1.000$). These noted responses are highly suggestive of a positive correlation between lethality and radiation dose (Fig. 2). The probit plot indicates that the $LD_{30/60}$, $LD_{50/60,}$ and $LD_{70/60}$ values are 4.79 (95% CI 2.15 to 5.36), 5.29 (95% CI 3.96 to 5.89), and 5.79 (95% CI 5.17 to 7.02) Gy. Since there are only three groups, the confidence intervals are wide. The majority of mortalities occurred between days 15 and 30 postirradiation.

Clinical Assessments

Blood responses/evolving cytopenias. Circulating blood profiles were analyzed throughout the course of the study. The data presented in Figs. 3 and 4 include average values for eleven selected blood cell types/parameters. Generally, all of these blood cell parameters showed decreased levels during the first several weeks after irradiation. In those animals that survived for the full duration of the experiment (60 days), these initially suppressed blood values had returned to approximately pre-irradiation levels; i.e., nearly normal blood profiles had been reestablished within the survivors. However, the recovery period appeared to have increased for survivors exposed at higher radiation doses. All groups demonstrated comparable, radiation dose-dependent changes in their blood profiles (CBC counts and differentials), with the exception, however, of blood lymphocyte counts of the 5.8 Gy irradiated group (i.e., lymphocyte counts did not return to pre-irradiation levels by the end of the study).

Data regarding the neutropenic and thrombocytopenic periods are presented in Tables 3 and 4 for all NHPs in each radiation dose group. The mean duration of neutropenia for the 4.7, 5.8, and 6.5 Gy groups was 6, 9, and 11 days, respectively (Table 3). By comparison, the mean duration of thrombocytopenia was 8, 6, and 9 days for animals in these three dose groups (Table 4).

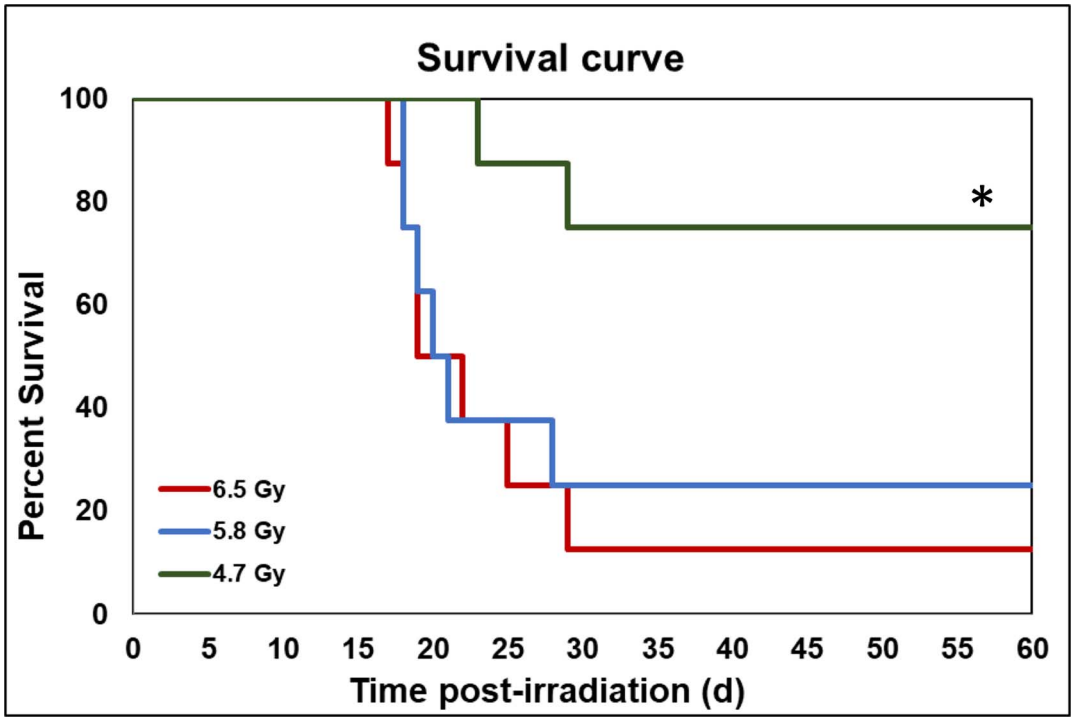
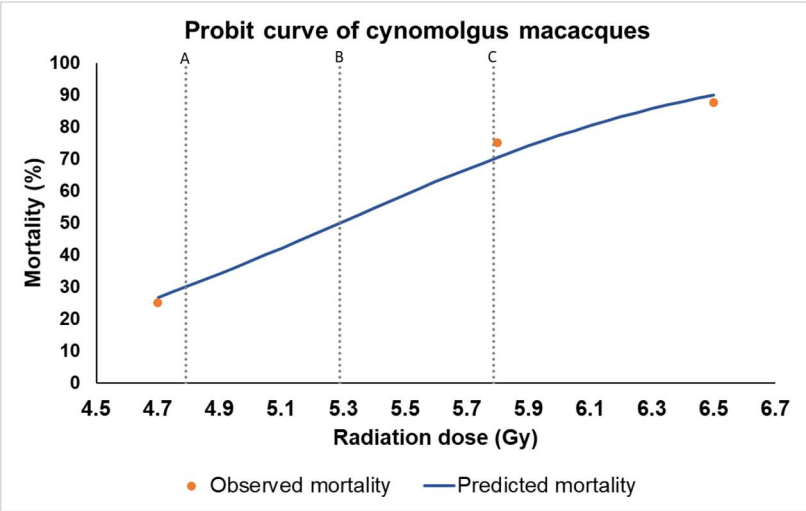


FIG. 1. Survival rates of 24 male cynomolgus macaques exposed to total-body radiation at three different doses: 4.7, 5.8, and 6.5 Gy. NHPs were divided into groups of 8/dose and survival was monitored for 60 days postirradiation. A two-sided Fischer's exact test showed a significant difference in survival outcomes between 4.7 and 6.5 Gy (*). 4.7 Gy vs. 5.8 Gy, $P < 0.132$; 4.7 Gy vs. 6.5 Gy, $P < 0.041$; 5.8 Gy vs. 6.5 Gy, $P < 1.000$.

Relative to the radiation-induced neutropenia, the majority of animals exposed to the lower radiation dose of 4.7 Gy manifested neutropenia (i.e., absolute counts $\leq$500 cells/μL) approximately 18 days postirradiation, with nadirs being reached between 20 to 22 days postirradiation. At the two higher exposure levels (5.8 and 6.5 Gy), neutropenia occurred earlier, approximately 16 days postirradiation with nadirs occurring between days 14 and 24.

Absolute platelet values dropped below 2,000 cells/μL for most of the NHPs in all three radiation dose groups by approximately day 16 postirradiation and nadirs were reached between days 16 and 22. The frequency and severity of thrombocytopenia, increased with rise in radiation exposure levels (Table 4).

The analysis results of the one-way ANOVA with a Tukey post hoc test to assess significant differences between radiation



95 % Confidence Limits for dose			
LD	Estimate (Gy)	Lower Bound	Upper Bound
30	4.791	2.146	5.356
40	5.049	3.121	5.593
50	5.289	3.960	5.887
60	5.530	4.655	6.325
70	5.788	5.169	7.024

FIG. 2. Probit curve of male cynomolgus macaques generated from probit analysis for three radiation doses (4.7, 5.8, and 6.5 Gy) using a ^{60}Co γ-radiation source. The vertical lines denote the following lethal doses: $LD_{30/60}$, $LD_{50/60}$, and $LD_{70/60}$, observed at 4.79 (95% CI 2.15 to 5.36), 5.29 (95% CI 3.96 to 5.89), and 5.79 (95% CI 5.17 to 7.02) Gy, respectively.

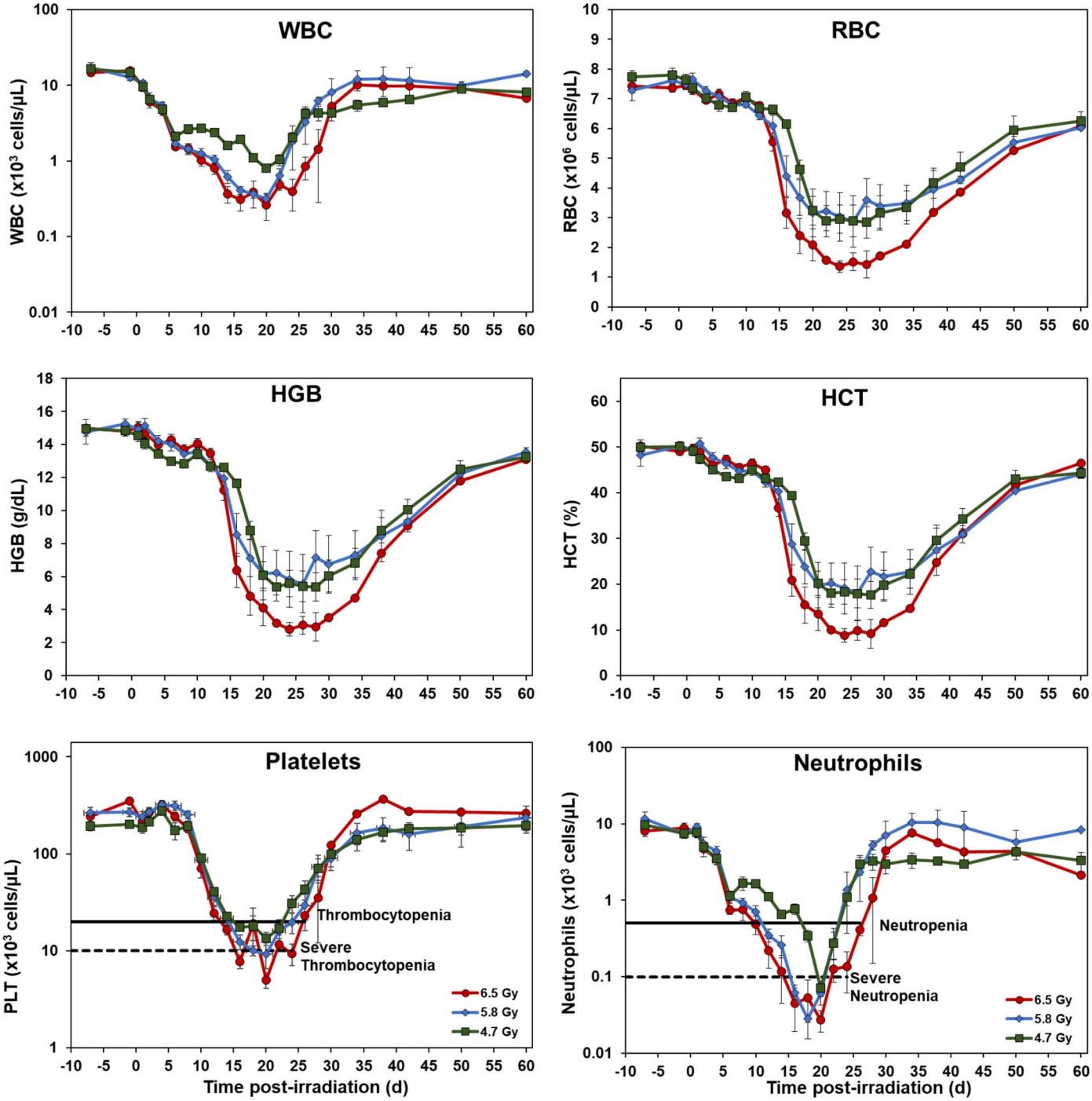


FIG. 3. Complete blood counts of irradiated NHPs exposed to 4.7, 5.8, and 6.5 Gy. The mean counts for white blood cells, red blood cells, hemoglobin, hematocrit, platelets, and neutrophils at each time point for three radiation doses are graphed.

groups and the repeated measures two-way ANOVA test to assess significant intergroup changes between pre- and postirradiation values for various CBC parameters are presented in Supplementary Table S1[1] (https://doi.org/10.1667/RADE-24-00223.1.S1).

Blood chemistries. Blood samples for serum biochemistry analysis were collected pre- and postirradiation at eight selected time points throughout the 60-day study. There were 23 blood chemistry parameters analyzed. While a select number of parameters remained very similar over time and largely independent of the level of radiation exposure (e.g., blood cations/anions-sodium, calcium, chloride), the majority of parameters were clearly affected to varying degrees over the course of the experiment (e.g., amylase,

[1] Editor's note. The online version of this article (DOI: https://doi.org/10.1667/RADE-24-00223.1) contains supplementary information that is available to all authorized users.

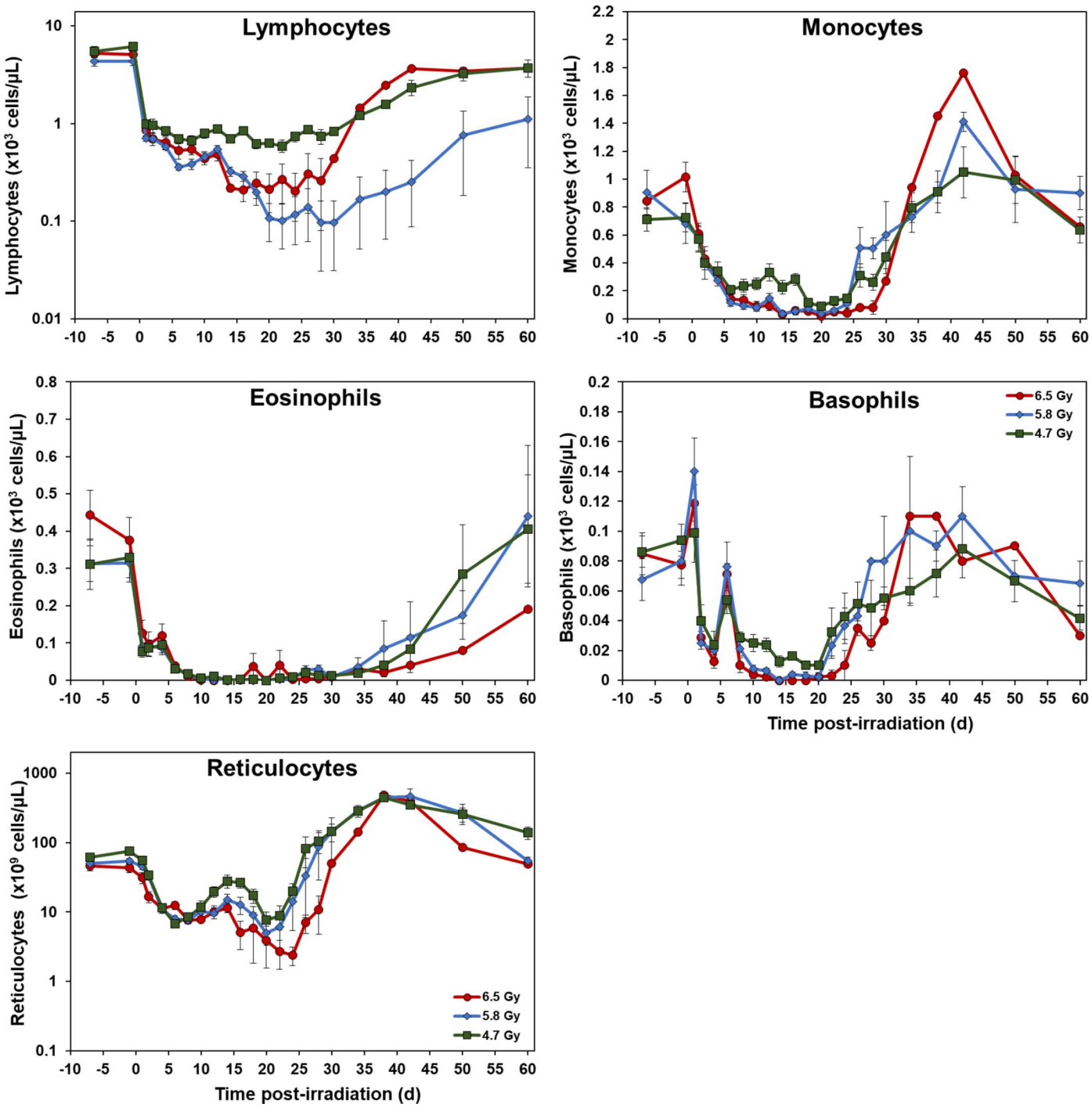


FIG. 4. Complete blood counts of irradiated NHPs exposed to 4.7, 5.8, and 6.5 Gy. The mean counts for lymphocytes, monocytes, eosinophils, basophils, and reticulocytes at each time point for three radiation doses are graphed.

BUN, cholesterol, etc.). Of the latter radiation exposure altered parameters, some occurred early (e.g., GGT, ALT, AST), while others occurred approximately midway into the experiment (e.g., cholesterol, total protein). Many parameters did however exhibit comparable temporal patterns, with the majority returning to near normal, baseline levels by the end of the study (Supplementary Figs. S1–S3; https://doi.org/10.1667/RADE-24-00223.1.S1).

Examples of notable changes in the measured blood elements, i.e., blood chemistry parameters, are as follows: Albumin levels started to decline by day 10 after irradiation and reached a nadir during day 20 or 28 in different cohorts and largely recovered by day 38. Blood levels of cholesterol in all exposure groups started declining by day 10 postirradiation and continued to decline until either day 20 (5.8 Gy) or day 30 in the 4.7 and 6.5 Gy exposure groups,

TABLE 3
Neutrophil-related Parameters for Cynomolgus Macaques Exposed to Total-Body γ Radiation

Dose (Gy)	Animal ID and gender	Survival day	Neutropenia ($\leq 0.5 \times 10^3$ cells/μL)		
			Neutrophil Nadir/(day)[1]	Duration[2]	Recovery $>0.5 \times 10^3$ cells/μL on day[3]
4.7	FR2835	60	0.03 (22)	14–24 (11 days)	26
	NV1553	60	0.03 (20)	18–24 (7 days)	26
	SB1010	60	0.10 (20)	18–20 (3 days)	22
	FR2794	60	0.02 (20)	18–22 (5 days)	24
	UG3438	60	0.06 (20)	18–22 (5 days)	24
	FR2676	28	0.06 (20)	*	Deceased
	FR2785	60	0.08 (22)	20–22 (3 days)	24
	NV1710	22	0.02 (22)	*	Deceased
	Mean[4]	N/A	0.05 ± 0.0105	6 ± 1.23	24 ± 0.61
	Range	N/A	0.02–0.10	3–11	22–26
5.8	CP176	17	0.01 (14)	*	Deceased
	MB2726	17	0 (17)	*	Deceased
	FR3115	19	0.02 (19)	*	Deceased
	MB2355	60	0.03 (18)	16–24 (9 days)	26
	MB2281	18	0.03 (18)	*	Deceased
	BC2309	20	0.01 (20)	*	Deceased
	MB2883	27	0.02 (18)	*	Deceased
	UG3248	60	0.02 (18)	12–20 (9 days)	22
	Mean[4]	N/A	0.018 ± 0.0037	9	24 ± 2.00
	Range	N/A	0–0.03	N/A	22–26
6.5	MB2742	28	0.03 (18)	*	Deceased
	NR1009	60	0.02 (20)	16–26 (11 days)	28
	NV1752	17	0.01 (17)	*	Deceased
	SB533	24	0.01 (24)	*	Deceased
	UG1838	16	0 (16)	*	Deceased
	UG3047	21	0 (21)	*	Deceased
	UG3232	18	0 (14 and 18)	*	Deceased
	UG3392	18	0 (16–18)	*	Deceased
	Mean[4]	N/A	0.0088 ± 0.0040	11	28
	Range	N/A	0–0.03	N/A	28

[1] Numbers outside bracket indicate neutrophil counts, and numbers inside the brackets indicate day postirradiation.
[2] Numbers outside the bracket indicate the duration of neutropenia (first and last day), and numbers inside the brackets indicate a period of neutropenia in days based on the first and last neutropenic days postirradiation.
[3] Numbers indicate the day postirradiation on which animals recovered from neutropenia.
[4] Mean data shown with the standard error of the mean (+SEM).
* The values for these animals are not included for calculating the mean and ranges since these animals were deceased before the end of the cytopenia period.

with subsequent recovery noted thereafter. GGT and total protein levels showed a marked decline between days 10 and 20 and returned to baseline levels by day 60. Phosphorus was observed to have a biphasic pattern, with levels decreasing sharply after irradiation on day 10 before increasing sharply on day 20 to near or above baseline. Thereafter, levels remained slightly below pre-irradiation values through the end of the experiment. The chemistry parameter levels across various time points throughout the study can be viewed in Supplementary Figs. S1–S3 (https://doi.org/10.1667/RADE-24-00223.1.S1). Additionally, the one-way ANOVA with a Tukey post hoc test for assessing significant differences between radiation groups and the repeated measures two-way ANOVA for various blood chemistry parameters are presented in Supplementary Table S2 (https://doi.org/10.1667/RADE-24-00223.1.S1). BUN appears to be elevated at 6.5 Gy, and this may represent a prerenal effect of dehydration.

Vital signs. Vital signs were recorded throughout the study, including heart rate, temperature, blood pressure, and weight. Overall, vital signs were consistent across all groups throughout the study. Blood pressure displayed a slight decrease in all groups starting on day 18, and recovered to baseline values by day 60. Aside from a few isolated instances of decreased heart rate or temperature, all other parameters remained stable through the course of the study for all three radiation dose groups. Vital signs data can be viewed in Supplementary Fig. S4 (https://doi.org/10.1667/RADE-24-00223.1.S1).

TABLE 4
Platelet-related Parameters for Cynomolgus Macaques Exposed to Total-Body γ Radiation

Dose (Gy)	Animal ID and gender	Survival day	Thrombocytopenia ($\leq 20 \times 10^3$ cells/μL)		
			PLT Nadir / (day)[1]	Duration days postirradiation[2]	Recovery PLT $>20 \times 10^3$/μL on day[3]
4.7	FR2835		11 (18)	14-22 (9 days)	24
	NV1553		9 (18)	14–28 (15 days)	30
	SB1010		14 (18)	16–20 (5 days)	22
	FR2794		6 (20)	16–26 (11 days)	28
	UG3438		11 (18)	16–22 (7 days)	24
	FR2676	28	6 (20)	*	Deceased
	FR2785		18 (18)	18–20 (3 days)	22
	NV1710	22	10 (18–22)	*	Deceased
	Mean[4]	N/A	10.63 ± 1.41	8 ± 1.76	25 ± 1.34
	Range	N/A	6–18	3–15	22–30
5.8	CP176	17	6 (17)	*	Deceased
	MB2726	17	4 (16)	*	Deceased
	FR3115	19	6 (18)	*	Deceased
	MB2355	60	9 (20)	16–22 (7 days)	24
	MB2281	18	7 (18)	*	Deceased
	BC2309	20	5 (20)	*	Deceased
	MB2883	27	6 (20)	*	Deceased
	UG3248	60	11 (18)	16–20 (5 days)	22
	Mean[4]	N/A	6.75 ± 0.80	6 ± 1.0	23 ± 1.00
	Range	N/A	4–11	5–7	22–24
6.5	MB2742	28	6 (18)	*	Deceased
	NR1009	60	6 (20)	16–24 (9 days)	26
	NV1752	17	3 (17)	*	Deceased
	SB533	24	4 (20)	*	Deceased
	UG1838	16	5 (16)	*	Deceased
	UG3047	21	3 (20)	*	Deceased
	UG3232	18	4 (18)	*	Deceased
	UG3392	18	7 (16)	*	Deceased
	Mean[4]	N/A	4.75 ± 0.53	9	26
	Range	N/A	3–7	N/A	26

[1] Numbers outside bracket indicate platelet counts and numbers inside the brackets indicate study days postirradiation.
[2] Numbers outside bracket indicate the duration of thrombocytopenia (first and last day) and numbers inside the brackets indicate a period of thrombocytopenia in days based on the first and last thrombocytopenic days postirradiation.
[3] Numbers indicate the day postirradiation on which animals recovered from thrombocytopenia.
[4] Mean data shown with the standard error of the mean (+SEM).
* The values for these animals are not included for calculating the mean and ranges since these animals were deceased before the end of the cytopenia period.

The result of the one-way ANOVA with a Tukey post hoc test for assessing significant differences between radiation groups are presented in Supplementary Table S3 (https://doi.org/10.1667/RADE-24-00223.1.S1). A two-way repeated measures ANOVA could not be performed for 6.5 Gy, as certain data could not be obtained at various time points due to the high mortality rate in this group.

Sequential Change in Blood Serum Cytokines

There were 48 cytokine profiles analyzed in the serum samples collected in this study. Both higher exposure groups, i.e., 5.8 and 6.5 Gy, displayed comparable changes in expressed levels of cytokines over the 60-day course of the experiment (Supplementary Figs. S5–S7; https://doi.org/10.1667/RADE-24-00223.1.S1). There were several notable exceptions, however, e.g., serum levels of M-CSF within the 5.8 Gy group rose significantly during 20–30 days postirradiation, while in the higher exposure group, 6.5 Gy, declined during this period (Supplementary Fig. S5). Still further exceptions were noted during late experimental period (∼30–60 days) in which mean levels of select cytokines (e.g., MIP -1β, SCF, IL-16, etc.) rose within the intermediate dose group, 5.8 Gy, while at the highest dose group, 6.5 Gy, mean values tended to decline (Supplementary Fig. S5). The concentration for several cytokines were observed to be zero or near zero, for all groups on day 20. This is likely not be due to a technical issue as assays were accomplished in several batches and

all samples of day 20 were not analyzed on the same day. Prior to sample analysis, standard calibration controls were used to verify the proper functioning of Luminex system ensuring the accuracy of the cytokine concentrations being reported. Furthermore, we have reported earlier using murine model samples where cytokine responses after irradiation are biphasic (*30*). The one-way ANOVA with a Tukey post hoc test to assess significant differences between radiation groups and the repeated measures two-way ANOVA test to assess significant intergroup changes between pre- and postirradiation data were accomplished for cytokines and values are presented in Supplementary Table S4 (https://doi.org/10.1667/RADE-24-00223.1.S1).

The 4.7 Gy group showed notable differences from the other two radiation dose groups in many of the cytokine profiles, showing less pronounced changes overall. For example, interferon-γ (IFN-γ) levels declined throughout the study and did not show any signs of rebounding. However, and by contrast, in the 5.8 and 6.5 Gy groups, IFN-γ showed a decline after irradiation before having a sharp increase in levels on day 20. After day 20, levels fell again and did not recover (Supplementary Fig. S6; https://doi.org/10.1667/RADE-24-00223.1.S1). Activated, normal T cells expressed and secreted (RANTES) levels were comparable in all three groups. However, the 4.7 Gy group showed much less marked expression patterns. While RANTES levels in the 5.8 and 6.5 Gy groups displayed both sharp decreases and increases after irradiation, the 4.7 Gy group only showed slight decreases and increases throughout the study. Other comparisons in the various cytokine response patterns can be gleaned from the Supplementary Figs. S5–S7. Similarly, stromal cell-derived factor 1 alpha (SDF-1α) levels declined quickly between days 10 and 20 in the 5.8 and 6.5 Gy groups before recovering to levels slightly below baseline. In the 4.7 Gy group, SDF-1α levels had a slight decline on day 20, and slight increases through the rest of the study. Notable changes were also observed in monocyte chemotactic and activating factor (MCAF) levels for the 5.8 and 6.5 Gy groups, with a marked rise from day 10 to 20 (for 5.8 Gy) or day 28 (for 6.5 Gy). For 4.7 Gy, there was no noticeable difference in MCAF levels over the course of the study. Interestingly, tumor necrosis factor-α (TNF-α) levels had greater changes in the 4.7 Gy group compared to the other two doses. The 4.7 Gy group had a much sharper decline from day 10 to 20, and a sharper increase in levels on day 28. In brief, all three radiation groups displayed a similar overall pattern in TNF-α levels in the study.

Histopathology

Histopathological analysis of the eleven selected tissues revealed radiation-induced changes in animals exposed to all three radiation doses (Table 5). Generally, the severity of radiation injury observed in the selected tissues increases with radiation dose.

The major changes in NHPs exposed to 6.5 Gy total-body radiation were noted in the heart, lungs, liver, bone marrow, spleen, and small and large intestines. There were minimal or no changes observed in the kidney and urinary bladder, respectively. Bacterial colonies were observed in the heart, lungs, and liver. Hemorrhages were present in numerous organs, including heart, lungs, liver, sternum, and large intestine. There were also various epithelial and villus changes noted in the intestines. Cellular depletion occurred in the sternum, spleen, and gut-associated lymphoid tissue (GALT) in addition to pneumocyte and liver degeneration.

Notable changes in the 5.8 Gy tissue samples were also observed in heart, lungs, liver, sternum, spleen, and intestines, whereas the kidneys and urinary bladder had minimal changes. Various organs exhibited hemorrhages, including the heart, lungs, sternum, small and large intestines, and urinary bladder. Cellular depletion was characteristic of the bone marrow, spleen, and GALT. The small intestine presented with multiple changes, namely, villus atrophy and reduced crypt mitoses.

For 4.7 Gy irradiated NHPs, severe changes were observed in the spleen and GALT, whereas mild changes were observed in the heart, kidney, and ileum of both moribund animals. Individual changes were observed in the remaining organs: sternum, lungs, liver, duodenum, and jejunum. Cellular depletion was common in the sternum, spleen, small intestine, and GALT. Both the heart and lungs displayed hemorrhages. Bacterial colonies were found in the liver, kidney, and jejunum. Villus atrophy was noted in the small intestine. Interestingly, one of the NHPs irradiated at 4.7 Gy revealed complete loss of all sternal marrow elements, more so than NHPs exposed to the higher doses. The other non-survivor in the 4.7 Gy group revealed less drastic radiation injury in its sternum. Histopathological findings of this specific dose were limited to two non-survivors. This difference can be explained by the relative recovery of the NHPs, where the NHP becoming moribund later showed mild signs of recovery before ultimately becoming moribund and euthanized.

DISCUSSION

Since rhesus macaques are not available for radiation medical countermeasure development work, a suitable animal model is needed for its replacement. Prior experience with cynomolgus macaques in radiation biology studies makes them ideal candidates for further development. (*12*). The objective of this study was to fully characterize the radiobiological responses of cynomolgus macaques to acute, potentially lethal gamma-ray exposure. As such, our results here and elsewhere certainly support the contention that these NHPs are reasonable surrogates for humans subjected unwantedly to acute ionizing radiation and hence, are suitable for supporting the development of MCMs for ARS.

TABLE 5

Radiation-induced Histopathological Changes in Decedent NHPs Exposed to Total-Body γ Radiation

		4.7 Gy		5.8 Gy		6.5 Gy	
Organs	Histological lesions	MED (IQR)	Incidence rate (n = 2)	MED (IQR)	Incidence rate (n = 6)	MED (IQR)	Incidence rate (n = 7)
Sternum	Cellular depletion	2 (0)	1	5 (0)	6	5 (0)	7
	Hemorrhage	3.5 (1.5)	2	3 (0.5)	3	2 (0)	1
Spleen	White pulp depletion	4 (0)	2	4.5 (1)	6	4 (0.5)	7
Liver	Hepatocellular necrosis	2 (0)	1	1.5 (0.5)	2	1 (0)	1
	Bacterial presence	2 (0)	1	0.5 (0.5)	1	1 (0)	1
	Hemorrhage	N/A	N/A	N/A	N/A	3.5 (1.5)	2
	Pigment-laden macrophages	N/A	N/A	N/A	N/A	1 (0)	1
	Mononuclear infiltrate	N/A	N/A	N/A	N/A	N/A	n/a
Lung	Alveolar septal degeneration	N/A	N/A	N/A	N/A	1 (0)	2
	Bacterial presence	N/A	N/A	N/A	N/A	1 (0)	3
	Alveolar hemorrhage	1(0)	1	1 (1)	5	2 (1)	3
	Alveolar edema	2 (0)	1	3 (2)	5	2.5 (1.5)	4
	Increased septal infiltrates	N/A	N/A	2 (0.25)	4	1 (0.75)	6
	Alveolar macrophage aggregates	N/A	N/A	N/A	N/A	N/A	N/A
Heart	Bacterial presence	N/A	N/A	N/A	N/A	1 (0)	1
	Hemorrhage	2 (0)	1	2 (0)	4	2 (0.5)	3
	Mineralization	1 (0)	1	1 (0)	1	1 (0)	1
	Mononuclear infiltrate	N/A	N/A	N/A	N/A	N/A	N/A
Kidney	Tubular regeneration	N/A	N/A	N/A	N/A	1 (0)	1
	Interstitial cellular infiltrates	1 (0)	1	N/A	N/A	N/A	N/A
	Medulla mineralization	N/A	N/A	N/A	N/A	1 (0)	1
	Bacteria	1(0)	1	N/A	N/A	N/A	N/A
Duodenum	Lamina propria infiltrates	N/A	N/A	N/A	N/A	1 (0)	3
	Villus fusion	N/A	N/A	1 (0)	2	1.5 (1)	4
	Villus lacteal dilation	1 (0)	1	N/A	N/A	2 (0)	1
	Reduced crypt mitoses	1 (0)	1	2 (0)	1	2 (1)	2
	Nematode Cyst	N/A	N/A	N/A	N/A	N/A	3
Jejunum	Lamina propria infiltrates	N/A	N/A	N/A	N/A	1 (0)	1
	Villus fusion	1 (0)	1	1 (0)	5	1 (0)	7
	Villi loss	N/A	N/A	N/A	N/A	1 (0)	1
	Reduced crypt mitoses	N/A	N/A	2 (1)	2	1.5 (0.5)	2
	Villus lacteal dilation	1	1	1.5 (0.5)	2	N/A	N/A
	Hemorrhage	N/A	N/A	1 (0)	1	N/A	N/A
	Villus atrophy	1 (0)	1	N/A	N/A	N/A	N/A
	Ulceration	1 (0)	1	N/A	N/A	N/A	N/A
	Bacterial presence	1 (0)	1	N/A	N/A	N/A	N/A
Ileum	Villus lacteal dilation	N/A	N/A	1.5 (0.5)	2	1 (1)	3
	Villus fusion	1 (0)	1	1 (1)	5	1 (0.75)	6
	Villi loss	N/A	N/A	N/A	N/A	1 (0)	1
	Reduced crypt mitoses	2 (0)	1	1 (0)	1	2 (0)	2
	Villus atrophy	1.5 (0.5)	2	3 (0)	1	N/A	N/A
	Hemorrhage	N/A	N/A	1 (0.5)	3	N/A	N/A
Large Intestine	Lamina propria infiltrates	N/A	N/A	1.5 (0.5)	2	1 (0)	2
	Hemorrhage	N/A	N/A	1 (0)	1	2 (0)	1
Urinary bladder	Hemorrhage	N/A	N/A	1 (0)	1	N/A	N/A
GALT	Lymphocyte depletion	5 (0)	2	5 (0)	6	5 (0)	7

Notes. Organs were collected and processed for H&E staining from NHPs that underwent unscheduled euthanasia before the completion of the study (60 days postirradiation). Sections were scored semi-quantitatively on a five-point scale [1 = minimal (≤5% of the area affected); 2 = mild (≤15%); 3 = moderate (≤50%); 4 = marked (≤90%); and 5 = severe (>90%)]. MED (IQR) - Median score (Interquartile Range) (NHPs with a score of 0 were excluded from calculations). Incidence rate indicates a number of animals with lesions out of total number (n) of irradiated NHPs (NHPs with a score of 0 were excluded from calculations). N/A indicates that the histology was within the normal limits. GALT (Gut-associated lymphoid tissue) was present in most of the small and large intestinal sections and was consistently atrophic. One NHP (CP176) had two cysts containing nematode sections attached to the duodenum (this qualitative finding was not scored).

TABLE 6
Comparison of Survival and Complete Blood Count Parameters of Cynomolgus and Rhesus Macaques

Parameter	Radiation dose	Cynomolgus mean (range)	Rhesus mean (range)	*P* values
Percent survival	5.8 Gy	25 (n = 8)	70 (n = 10)	0.153
	6.5 Gy	12.5 (n = 8)	70 (n = 10)	0.025
Neutrophil nadir (day)	5.8 Gy	18 (14–20)	14 (14–18)	<0.001
	6.5 Gy	20 (14–24)	14 (10–17)	0.002
Neutrophil nadir ($\times 10^3$ cells/μL)	5.8 Gy	0.03 (0–0.04)	0.05 (0.01–0.06)	0.089
	6.5 Gy	0.03 (0–0.03)	0.04 (0.01–0.04)	0.142
Platelet nadir (day)	5.8 Gy	20 (16–20)	14 (12–14)	<0.001
	6.5 Gy	20 (16–20)	14 (12–17)	0.002
Platelet nadir ($\times 10^3$ cells/μL)	5.8 Gy	9.25 (4–11)	9.80 (3–33)	0.530
	6.5 Gy	5 (3–7)	9.56 (1–27)	0.361
RBC nadir (day)	5.8 Gy	26 (16–34)	20 (14–28)	0.304
	6.5 Gy	24 (16–28)	18 (12–28)	0.386
RBC nadir ($\times 10^6$ cells/μL)	5.8 Gy	2.87 (1.11–4.09)	2.96 (2.19–3.72)	0.026
	6.5 Gy	1.36 (0.99–1.93)	2.77 (0.4–3.87)	0.001
HGB nadir (day)	5.8 Gy	26 (16–30)	20 (14–22)	0.269
	6.5 Gy	24 (16–28)	20 (12–28)	0.296
HGB nadir (g/dL)	5.8 Gy	5.57 (2.2–8.5)	7.03 (5–8.9)	0.003
	6.5 Gy	2.8 (2.0–4.0)	6.27 (0–9.3)	0.001
HCT nadir (day)	5.8 Gy	26 (16–30)	20 (14–22)	0.233
	6.5 Gy	24 (16–28)	20 (12–28)	0.504
HCT nadir (%)	5.8 Gy	18.0 (8.5–27.1)	22.0 (16.2–28.0)	0.005
	6.5 Gy	8.8 (6.0–13.0)	22.0 (17.9–28.3)	<0.001

Note. A 4.7 Gy dose has not been used in rhesus under identical conditions in our laboratory. Thus, comparative data is not provided.

In previous studies by our group (*6*, *11*, *14*) and others (*3*, *5*, *12*, *16*, *31*, *32*), these two species of macaques respond clinically to acute, potentially lethal ionizing irradiation in quite similar patterns as observed in comparably exposed humans (*33–36*). Specifically, we refer to the commonality of changing clinical and hematological profiles over the initial periods after mid-lethal type exposures. However, what constitutes such "mid-lethal" exposures in basic radiological terms (i.e., dose, dose rate, radiation quality, extent of bodily exposure) can vary, sometimes substantially, in different species (*37*). Common temporal sequences and pathophysiological features of major phases (i.e., prodromal, latency, and manifest illnesses) of ARS, along with specific sub-syndromes (hematological, GI and neurovascular related sub-syndromes) can be clearly recognized, not only between these two species of macaques and humans, but also in other experimental species as well (e.g., canines, porcine, etc.). Take for example the closely similar features of the hematological sub-syndrome between these macaque species: the levels of vital circulating blood elements, in particular blood leukocytes (e.g., lymphocytes and granulocytes), fall precipitously during the first several weeks after acute exposure, reaching low levels (nadirs) that are incompatible for sustained life for sizable numbers of the exposed individuals (*38*, *39*). Without at least a modicum of recovery of these blood compartments, a moribund condition sets in and death ensues. The time-dependent nadirs for both blood neutrophils and platelets are reached within two to three weeks after exposure for rhesus and cynomolgus macaques, respectively (Table 6). Although reports of the blood response profiles of humans exposed to near- or mid-lethal radiation doses are more varied, they still reflect common response patterns seen in experimental animals: specifically, the time-dependent nadirs for blood neutrophils and platelets generally range from two- and four-weeks postirradiation (*36*, *38*).

We observed here that the cynomolgus macaques appear to be more radiosensitive (i.e., relative to mortality rates) compared with rhesus macaques (Table 6) in studies conducted under similar experimental conditions and with the same level of supportive care provided, though experiments with two species were not conducted concurrently in the same study (*14*, *21*, *40*). This finding has relevance in terms of the comparison to the estimated lethality values for humans, in that the greater radiosensitivity of the cynomolgus macaques appears slightly closer to those values estimated for humans (LD_{50} values ranging from 3–4 Gy for humans) (*41*).

It needs to be noted that the latter lethality estimates for humans are not clinically supported; this contrasts the higher values reported here and elsewhere (*42–44*) for acutely irradiated experimental macaques that have generally received at least partial clinical support (standard clinical support, but lacking blood/blood product transfusions) (*12*, *14*, *32*). Considering the latter, it is not unreasonable to suggest that if all

clinical support had been withheld from these experimental animals, the lethality rates would have been substantially higher (or alternatively, LD_{50} values substantially lower). The general "rule of thumb" is that if clinical support is provided to the acutely exposed, the lethality curve will shift approximately 1 Gy to the right; i.e., radioresistance is gained by ~1 Gy (*38*). Assuming the latter assumptions are valid, this would bring the inherent radiosensitivity of the clinically unsupported cynomolgus macaques much closer to that of humans.

Furthermore, the "slope" of the radiation dose-response curve appears somewhat shallower than those previously reported for rhesus macaques (*12*, *23*). However, additional work will be needed to confirm these apparent differences in radiosensitivity between the two types of macaques. The DRR determined using three different radiation doses (4.7, 5.8 and 6.5 Gy, n = 8/radiation dose) of radiation at a dose rate of 0.6 Gy/min, suggested $LD_{30/60}$, $LD_{50/60}$, and $LD_{70/60}$ as 4.79, 5.29, and 5.79 Gy, respectively. Since these lethality values are based on only three doses of radiation, confidence intervals for these values are wide. These animals were provided subject-based supportive care excluding blood products and were monitored for 60 days postirradiation for survival which was the primary endpoint. The majority of mortality occurred 15 to 30 days postirradiation, which was somewhat delayed compared to rhesus (*14*, *23*). The secondary endpoint of this study was hematopoietic recovery. The time of CBC recovery took longer in cynomolgus macaques as compared with rhesus (*13*, *14*, *21*). The nadir for RBC-related parameters (hemoglobin, hematocrit, and reticulocytes) was deeper in cynomolgus compared with rhesus exposed to the same dose of radiation, suggesting a higher radiosensitivity of the hematopoietic system. Furthermore, these differences may be contributing to increased mortality and delayed deaths in cynomolgus macaques relative to those responses of comparably irradiated rhesus (*13*, *14*, *23*). In rhesus, the majority of deaths occur between 10–20 days postirradiation with radiation doses in the H-ARS range while death continues until day 30 in cynomolgus (Fig. 1) (*13*, *14*). Thrombocytopenia is an important predictor of mortality in rhesus macaques, where a longer duration and increased severity was found to be correlated with higher mortality (*45*). We observed similar trend in the current study using cynomolgus macaques.

For the development of MCMs for radiation injury following the U.S. FDA Animal Rule, potential candidates need to be evaluated in suitable large animal models for pharmacokinetics, toxicity, safety, and efficacy in addition to the identification and validation of radiation injury biomarkers, especially for drug dose conversion from preclinical animal models to humans (*9*, *46*). It is well recognized that the US FDA Animal Rule recommends that a large animal model be used (along with complementing small animal models), and also suggests that these animal models should be well-defined, and the mechanism of radiation injury well-understood. These guidelines create logistical challenges to move novel MCM candidates through the various development steps to get regulatory agency approval for their use in humans. The limited availability, high procurement cost, need for trained manpower, and maintenance of a required number of animals for MCM development highlight the expense of such drug development. The cost of NHPs has significantly increased after the COVID-19 pandemic, limiting the number of animals that can be used and the number of experimental groups one can have in any given study.

The cynomolgus macaque has been used in radiobiology research to a lesser extent compared with rhesus. Specifically, several studies have used this animal model to investigate cytokines for H-ARS (*12*, *47–54*). Furthermore, studies have been conducted in cynomolgus macaques using various radiation exposure geometries to induce ARS and imitate non-homogeneous and nonuniform exposures (*12*, *31*, *47*, *48*, *50*, *55–59*). Studies have also been accomplished using mesenchymal stem cells, autologous stem cell transplant, and to derive $CD34^+$ cells (*12*, *57*, *60*, *61*). This animal model has also been used to develop protocols for medical management of H-ARS for high-dose accidental or terrorist nuclear detonation (*62*). Furthermore, studies have been conducted using TBI and PBI models with bone marrow sparing and hematopoietic stem and progenitor cell sparing (*31*, *55*, *56*, *58*, *59*, *63*).

With respect to cytokine induction by acute, potentially lethal irradiation, we did not observe significant differences between the two species (*14*). Similarly, blood chemistry analysis demonstrated only minor differences between the two species of macaques. Furthermore, other vital sign parameters, along with cytokine response profiles, were minimally affected with three different doses of radiation. In brief, though the responses of two species to radiation may differ, they are comparable. This communication demonstrates that the cynomolgus macaque is comparatively more sensitive (Table 6)) to ionizing radiation injury and is a viable, potentially useful large animal model alternative for the evaluation of MCMs since rhesus macaque availability is not very promising for the foreseeable future.

We recognize that there are a few limitations in this study and these limitations are mainly due to the exorbitant high cost of macaques after the COVID-19 pandemic which is related to the changed landscape of such animal import into the U.S. from other countries. There are only three groups of animals with n = 8 in each group for three different doses of radiation. Usually such studies are conducted with more groups. Recently, we have published the lethality curve determination for the rhesus macaques using six different doses of radiation with n = 6 in each group for the same source of radiation (high level ^{60}Co-γ and total-body exposure) (*23*). Unlike our current study, blood products were used as supportive care in this rhesus macaque study. Furthermore, we initiated the current study with 6.5 Gy which is ~$LD_{50/30}$ dose for rhesus but unlike our expectation, cynomolgus was found to have significantly higher

mortality (87%) compared with rhesus at this dose. Next, we opted for a step-down approach and used 5.8 Gy, $\sim LD_{30/60}$ dose for cynomolgus macaque. This dose of radiation was also more lethal in rhesus (75%) compared with rhesus. After deliberation with a statistical consultant, we used 4.7 Gy as third dose. This dose provided 6 survivors out of 8 animals (25% mortality). Keeping in mind cost and limited availability of NHPs at this time, it was not possible to include additional groups. Based on predicted lethality, desired radiation doses will be used for MCM efficacy investigation. In due course of time, additional radiation doses will be used to refine this lethality curve. We did not conduct bone marrow studies with these animals since bone marrow biopsies could have affected the overall survival outcome of the study. We plan to conduct such studies in the future with animals not involved in survival outcome studies, which can ultimately affect the lethality value. Considering the importance of this study for the scientific community interested in understanding the radiation sensitivity of cynomolgus macaques to use in MCM evaluation studies, we decided to publish this work. To some extent, male and female NHPs have different lethality and a probit curve should be developed for both sexes (*14*). In the future, depending on resource availability, we will also try to use females for developing a lethality curve. When this study was initiated, female availability was severely limited.

Lastly, we believe that this study and the results obtained have been a useful, essential exercise in terms of establishing the radiation exposure parameters for the cynomolgus macaque-based model of acute radiation injury, as well as for current and future testing of both efficacy and safety of new pharmaceutical agents designed to counter the medical effects of radiation exposure.

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