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Mitigating Effects of 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) on Hematopoietic Acute Radiation Syndrome after Total-Body Ionizing Irradiation in Mice

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Acute radiation syndrome (ARS) occurs as a result of partial- or whole-body, high-dose exposure to radiation in a very short period of time. Survival is dependent on the severity of the hematopoietic sub-syndrome of ARS. In this study, we investigated the mitigating effects of a lipid molecule, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), on the kinetics of hematopoietic cells, including absolute neutrophil count (ANC), red blood cells (RBCs) and platelet counts, in mice after gamma-ray total-body irradiation (TBI). Male and female BALB/c mice (11 weeks old) received a $LD_{70/30}$ dose of TBI. PLAG significantly and dose-dependently attenuated radiation-induced mortality ($P = 0.0041$ for PLAG 50 mg/kg; $P < 0.0001$ for PLAG 250 mg/kg) and body weight loss ($P < 0.0001$ for PLAG 50 and 250 mg/kg) in mice. Single-fraction TBI sharply reduced ANC within 3 days postirradiation and maintained the neutropenic state (ANC $<$ 500 cells/μl) by approximately 26.8 $\pm$ 0.8 days. However, administration of PLAG attenuated radiation-induced severe neutropenia (ANC $<$ 100 cells/μl) by effectively delaying the mean day of its onset and decreasing its duration. PLAG also significantly mitigated radiation-induced thrombocytopenia ($P < 0.0001$ for PLAG 250 mg/kg) and anemia ($P = 0.0023$ for PLAG 250 mg/kg) by increasing mean platelet and RBC counts, as well as hemoglobin levels, in peripheral blood. Moreover, delayed administration of PLAG, even at 48 and 72 h after gamma-ray irradiation, significantly attenuated radiation-induced mortality in a time-dependent manner. When compared to olive oil and palmitic linoleic hydroxyl (PLH), only PLAG effectively attenuated radiation-induced mortality, indicating that it has a distinctive mechanism of action. Based on these preclinical observations, we concluded that PLAG has high potential as a radiation countermeasure for the improvement of survivability and the treatment of hematopoietic injury in gamma-ray-induced ARS. © 2019 by Radiation Research Society

INTRODUCTION

Acute radiation syndrome (ARS) is a broad term used to describe a range of signs and symptoms that occur after an entire or large portion of the body is exposed to a high dose of ionizing radiation (*1*). ARS has been traditionally divided into three sub-syndromes, each with a specific dose threshold for the appearance of clinical symptoms: hematopoietic sub-syndrome (H-ARS, 1–6 Gy), gastrointestinal sub-syndrome (GI-ARS, 6–8 Gy), and neurovascular sub-syndrome (>10 Gy) (*2–4*). However, because the damage caused by radiation is not confined to one isolated system, irradiated patients require immediate intensive care to minimize damage to other systems (*4*).

Over several decades, the U.S. Department of Health and Human Services (specifically, National Institutes of Health and the Biomedical Advanced Research and Development Authority) has sought to investigate potential radiation countermeasures that are safe, easily administered and effective at reducing adverse health effects that occur after radiation exposure (*5–7*). Despite these ongoing efforts, as of 2015, only Neupogen® (granulocyte colony-stimulating factor; G-CSF) and Neulasta® (pegylated G-CSF) have been approved by the U.S. Food and Drug Administration (FDA) as radiation countermeasures for the treatment of H-ARS (*8–11*). Recently, Leukine® (granulocyte-macrophage colony-stimulating factor; GM-CSF) was approved by the FDA as a radiomitigator to increase survival and to facilitate the recovery of white blood cells in adults and pediatric patients acutely exposed to a sub-lethal dose of radiation (*12*).

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Hematopoietic organs are highly susceptible to the cytotoxic effects of radiation, resulting in immune suppression including neutropenia, thrombocytopenia, and/or anemia (*13–15*). Patients with neutropenia often experience reduced defense against infection and inflammation, consequently followed by mortality due to sepsis (*16*). Low platelet counts in peripheral blood confer a greater risk of bleeding and delayed wound healing (*17*). The contributory mechanisms of radiation-induced immune suppression are complex. These involve the death of immune cells, blocking of cell maturation and differentiation and failure of bone marrow to produce immune cells (*18*). A deeper understanding of the mechanisms by which radiation damages the immune system is necessary for the development of radiation countermeasures that boost immunity after exposure.

The chemically synthesized lipid molecule, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), also known as EC-18, is identical to a major constituent found in the antlers of sika deer (*19, 20*). Previously, the therapeutic efficacy of PLAG has been demonstrated in chemotherapy-associated hematopoietic dysfunction (*21*). In a published clinical study, PLAG reduced the incidence of gemcitabine-induced neutropenia in patients with unresectable pancreatic cancer (*22*). In addition, we have previously demonstrated that PLAG exerts a synergistic effect with PEGylated G-CSF (pegfilgrastim) in the treatment of chemotherapy-induced neutropenia by regulating neutrophil extravasation (*23*). Based on the effect of PLAG on neutrophil modulation, we sought to determine whether it also had therapeutic efficacy as a radiation countermeasure for H-ARS, after exposure to a sub-lethal dose of gamma radiation in BALB/c mice. In this study, survival rate, body weight reduction, peripheral counts of neutrophils, platelets and RBCs, and hemoglobin levels were examined over a 30-day period.

MATERIALS AND METHODS

Animals

Specific-pathogen-free male and female BALB/c mice (10 weeks old) were obtained from Koatech Co. (Pyongtaek, Republic of Korea). Upon receipt, five mice per cage were housed in a specific-pathogen-free facility and acclimatized for one week under consistent temperature and 12:12 h light-dark schedule. All animals were fed a standard mouse diet with water was available *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and were performed in compliance with the ''Guide for the Care and Use of Laboratory Animals'' by the National Research Council and Korean national laws for animal welfare.

Gamma-ray Irradiation

Male and female BALB/c mice (11 weeks old) were placed in single chambers of a lead-shielding irradiation apparatus and received a single uniform total-body dose of gamma-ray irradiation from a ^{60}Co source (JL Shepherd & Associates, San Fernando, CA) at an exposure rate of 0.833 Gy/min. Dose rates were measured using the EPD Mk2+ electronic dosimeter (Thermo Scientific™, Waltham, MA).

Administration of PLAG, Olive Oil and Palmitic Linoleic Hydroxyl Glycerol (PLH)

PLAG was obtained from Enzychem Lifesciences Corp. (Jecheon, South Korea) and re-suspended in sterile phosphate buffered saline (PBS). For the dose experiment, mice received PLAG [50 or 250 mg/kg, oral (p.o.) administration] or vehicle (sterile PBS; 0.1 ml/mouse, p.o.) beginning 24 h postirradiation and continuing daily to day 30. For the time experiment, mice received PLAG (250 mg/kg, p.o.) either immediately postirradiation (+0 day) or at 24 h (+1 day), 48 h (+2 days) or 72 h (+3 days) postirradiation. and continuing daily to day 30. For the comparative experiment, olive oil (Sigma-Aldrich® LLC, St. Louis, MO) and PLH (Enzychem Lifesciences Corp.) were re-suspended in sterile PBS. Mice were orally administered 250 mg/kg PLAG, 250 mg/kg olive oil, 250 mg/kg PLH or vehicle beginning 24 h postirradiation and continuing daily to day 30.

Assessment of Body Weight

The body weights of mice (10/10 male/female; 20 mice per group) were measured daily for 30 days. The results are expressed as body weights normalized to initial state. Since the mice died from radiation injury, the number of mice that were weighed was different as the study progressed.

Assessment of Survival and the Kinetics of Blood Cells in Peripheral Blood

Mice (20/20 male/female; 40 mice per group) were monitored at least twice daily for survival for 30 days. For assessment of blood cell kinetics, the mice were divided into two cohorts of 20 mice/cohort based on blood collection time. Blood collection schedules were as follows. Cohort 1 collection was performed on days 1, 5, 10, 15, 20 and 27; cohort 2 collection was performed on days 3, 7, 12, 17, 22 and 30. Approximately 30–40 μl of whole blood was collected from the orbital sinuses using EDTA-free capillary tubes (Kimble Chase, Rockwood, TN) and collection tubes containing K3EDTA (Greiner Bio-One International, Kremsmünster, Austria). Since the mice died from radiation injury, the number of blood samples taken from the mice was different as the study progressed. The blood cells were counted and classified by complete blood count (CBC) analysis using a Mindray BC-5000 auto-hematology analyzer (Shenzhen Mindray Biomedical Electronics, Guangdong Sheng, China). The numbers of blood cells were recorded at the appointed dates for 30 days. The mice surviving after the end of experiments underwent euthanasia by CO_2 inhalation followed by cervical dislocation.

Statistical Analyses

Dose-dependent mortality was examined over 30 days using logistic regression. For statistical analysis of hematologic and body weight data, one-way ANOVA followed by Tukey-Kramer post hoc test was performed using GraphPad Prism version 8.0 (LaJolla, CA). P values <0.05 were considered statistically significant, and the results were expressed as the mean ± SD. Paired log-rank (Mantel-Cox) test was used to analyze the survivability and the duration of neutropenia, thrombocytopenia and anemia between control and PLAG-treated groups.

RESULTS

Radiation Dose-Response Relationship (DRR) and Determination of $LD_{XX/30}$

The mortality rate of irradiated mice positively correlates with radiation dose (*24*). Prior to the PLAG efficacy test, we first investigated the relationship between gamma-ray

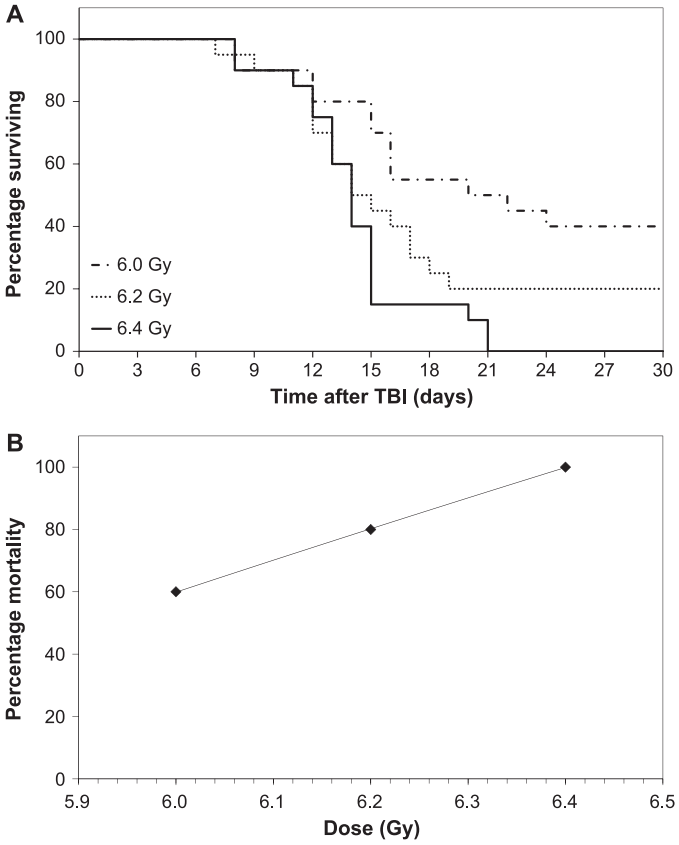

FIG. 1. Survival rates and logistic regression probability of 30-day mortality in BALB/c mice after TBI. Mice (n = 20 per group, 10 males and 10 females) received gamma-ray doses ranging from 6.0 to 6.4 Gy gamma-rays. Panel A: Kaplan-Meier survival curves show the proportion of mice surviving at each time point for each radiation dose. Panel B: Radiation dose-response relationship (DRR) using a probit model. Survival at day 30 was analyzed for each radiation dose and is shown as percentage mortality on the y-axis.

dose and lethality of mice to determine the lethality dose (LD) of gamma-ray irradiation during the 30-day survival observation. Figure 1A shows Kaplan-Meier survival curves of BALB/c mice irradiated at various doses of ^{60}Co gamma rays; increasing radiation dose significantly decreased the overall survival time. The mean survival time (MST) of decedents for each radiation dose cohort ranged from 13.69 to 15.30 days, with the overall MST of decedents across all dose cohorts being 14.38 days (Table 1). Figure 1B shows the radiation dose-response relationship (DRR) using a probit model. Thirty-day survival was calculated at each radiation dose and is shown as percentage mortality on the y-axis. Based on the probit model in Fig. 1B, we determined $LD_{XX/30}$ with 95% confidence intervals around each dose. The $LD_{30/30}$, $LD_{50/30}$, $LD_{70/30}$ and $LD_{95/30}$ values were 5.45, 5.85, 6.11 and 6.35 Gy, respectively (Table 2). The established $LD_{70/30}$ in this experiment was applied in subsequent experiments to determine PLAG efficacy.

TABLE 1
Thirty-day Mortality of BALB/c Mice after Gamma-Ray Irradiation

		Survival time of decedents (days)	
Radiation dose (Gy)	Mortality	MST ± SE	Median
6	12/20 (60%)	15.30 ± 4.98	15.5
6.2	16/20 (80%)	13.69 ± 3.26	13.5
6.4	20/20 (100%)	14.15 ± 3.48	14

Administration of PLAG Attenuates Mortality and Body Weight Loss in Irradiated Mice

We investigated whether administration of PLAG increases the survivability of mice after receiving $LD_{70/30}$ (6.11 Gy) TBI. Exposure alone resulted in the death of 68.5% of the animals in the vehicle control group over the 30-day observation period, with an average survival time of 21.2 days among the decedents (Fig. 2A, Table 3). Therefore, the survival rate of the vehicle control group was 32.5%. Conversely, administration of PLAG (50 and 250 mg/kg) significantly enhanced 30-day survival to 60% ($P = 0.0041$) and 85% ($P < 0.0001$), respectively (Fig. 2A). Moreover, the average survival time of the decedents with PLAG 50 and 250 mg/kg treatment increased to 24.3 and 27.8 days, respectively (Table 3). Based on these observations, PLAG has therapeutic potential for improving survivability and increasing the average duration of life in gamma-ray-induced ARS.

Figure 2B shows the effect of PLAG on changes in the body weights of irradiated mice over a 30-day observation period. Weight loss was examined in a different batch from that of survival and hematological analyses. The $LD_{70/30}$ gamma-ray dose resulted in substantial decreases in body weight in the mice. Eighty percent of mice in the vehicle control group receiving irradiation alone had more than 10% body weight loss, and 40% had extreme weight loss, defined as body weight reduction of 20% or more. Only 35% and 15% of mice in the groups receiving PLAG 50 and 250 mg/kg experienced severe radiation-induced weight loss, respectively (Table 4). This observation indicates that PLAG is very effective in mitigating body weight loss in gamma-ray-induced ARS.

TABLE 2
Estimated Radiation Dose in BALB/c Mice after Gamma-Ray Irradiation

$LD_{XX/30}$	LD estimate (Gy)	Lower 95% CI (Gy)	Upper 95% CI (Gy)
$LD_{30/30}$	5.44	5.17	5.65
$LD_{50/30}$	5.85	5.68	5.99
$LD_{70/30}$	6.11	6	6.21
$LD_{95/30}$	6.35	6.27	6.42

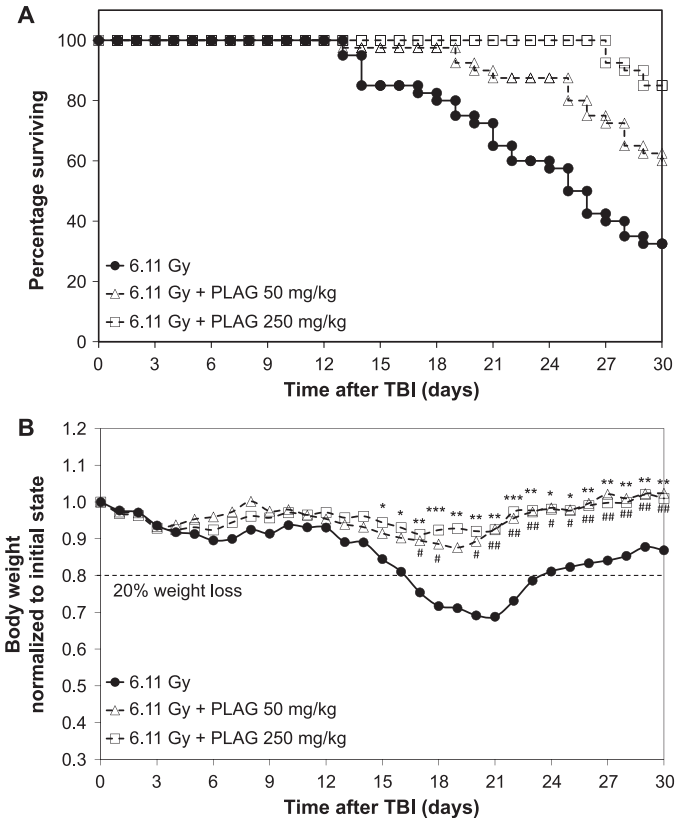

FIG. 2. PLAG increased survival rates and mitigated body weight loss in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 40 per group, 20 males and 20 females) received the $LD_{70/30}$ dose (6.11 Gy) of gamma rays, and were administered 50 or 250 mg/kg of PLAG once a day starting the day after irradiation and continuing until the final day of observation. Panel A: Survival was monitored for 30 days. $P = 0.0041$, 6.11 Gy + PLAG 50 mg/kg vs. 6.11 Gy; $P < 0.0001$, 6.11 Gy + PLAG 250 mg/kg vs. 6.11 Gy (log-rank test). Panel B: Body weights were measured daily for 30 days. Data are presented as mean. $^{\#}$6.11 Gy vs. 6.11 Gy + PLAG 50 mg/kg; *6.11 Gy vs. 6.11 Gy + PLAG 250 mg/kg. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.005$.

Administration of PLAG Mitigates ANC Loss in Peripheral Blood of Irradiated Mice

Hematological nadirs are known to be closely associated with the decreased survival from ARS (*25*). Using CBC analysis, we investigated whether enhanced survivability by PLAG results from increases in nadir values. A single-dose of TBI ($LD_{70/30}$, 6.11 Gy) rapidly diminished the absolute neutrophil counts (ANC) within 3 days after gamma-ray irradiation (Fig. 3A). In particular, the administration of PLAG 50 and 250 mg/kg significantly attenuated radiation-induced depletion of ANC in mice in a dose-dependent manner (Fig. 3A and B). The mean first day of severe neutropenia (ANC $<$ 100 cells/μl) in control and PLAG 50 and 250 mg/kg-treated groups was 3.8 ± 0.3, 5.7 ± 0.6 and 8.5 ± 1.0 days, respectively (Table 5). Although PLAG did not protect irradiated mice from experiencing severe neutropenia, it significantly reduced the duration of severe neutropenia (Table 5). In addition, the group treated with PLAG 250 mg/kg exhibited a significant increase in the mean nadir of ANC, from 20.5 ± 2.2 cells/μl to 49.0 ± 4.9 cells/μl after irradiation (Table 6). These observations show that administration of PLAG has a remarkable effect in preventing gamma-ray-induced ANC depletion.

TABLE 4
Effect of PLAG (Daily Administration) on Occurrence and Severity of Body Weight Loss of Irradiated Mice

	≥10% Body weight loss		≥20% Body weight loss	
Treatment	N	%	N	%
Control	16	80%	8	40%
PLAG 50 mg/kg	11	55%	7	35%
PLAG 250 mg/kg	3	15%	3	15%

Administration of PLAG Mitigates Platelet Loss in Peripheral Blood in Irradiated Mice

Low platelet counts increase bleeding risk (*26*). Thrombocytopenia is a condition in which peripheral blood platelet counts are below 100,000 platelets/μl (*17, 27, 28*). Single-dose gamma-ray TBI (6.11 Gy) rapidly reduced mean platelet counts to below 50% of the baseline within 5 days, and below 100×10^3 cells/μl within approximately 9 days postirradiation (Fig. 4A). Administration of PLAG did not significantly prevent radiation-induced peripheral platelet depletion. However, administration of PLAG 250 mg/kg significantly reduced the duration of thrombocytopenia from 13.1 ± 1.1 to 6.7 ± 0.5 days ($P < 0.0001$) (Table 5). In addition, mice treated with PLAG 250 mg/kg exhibited a remarkable increase in the mean nadir of platelet counts, from 27.6 ± 1.9 × 10^3 cells/μl to 50.1 ± 3.2 × 10^3 cells/μl, after gamma-ray irradiation, and significantly reduced the mean number of days to platelet recovery (≥100,000 cells/μl), from 22.8 ± 1.1 to 16.8 ± 0.4 cells/μl (Table 6). Based on this observation, PLAG administration is very effective for recovering gamma-ray-induced depletion of platelet counts.

TABLE 3
Dose Effect of PLAG (Daily Administration) on Survivability and Average Life Duration of Irradiated Mice

	No. of mice that survived/total	Survivability	Survival time of decedents (days)		Log-rank test P*
			Mean ± SE	Median	
Control	13/40	32.5	21.2 ± 1.0	21	
PLAG 50 mg/kg	24/40	60	24.3 ± 1.2	25.5	0.0041
PLAG 250 mg/kg	34/40	85	27.8 ± 0.4	27.5	<0.0001

TABLE 5
Mean First Day and Mean Duration of Severe Neutropenia (ANC < 100 cells/μl), Thrombocytopenia (PLT $<100 \times 10^3$ cells/μl) and Anemia (HGB <12 g/dL) in Control and PLAG-Treated Mice Exposed to 6.11 Gy

Treatment	Mean first day severe neutropenia[a] (±SE, range)	Mean duration of severe neutropenia in days (±SE, range)	Mean first day thrombocytopenia[a] (±SE, range)	Mean duration of thrombocytopenia in days (±SE, range)	Mean first day anemia[a] (±SE, range)
Control	3.8 ± 0.3 (3–7)	14.2 ± 1.0[b] (8–19)	9.8 ± 0.1 (7–10)	13.1 ± 1.1[e] (7–20)	9.5 ± 0.6 (3–12)
PLAG 50 mg/kg	5.7 ± 0.6 (3–10)	11.7 ± 1.1[c] (2–19)	10.0 ± 0.0 (10–10)	12.8 ± 0.8[f] (7–17)	10.4 ± 0.7 (5–15)
PLAG 250 mg/kg	8.5 ± 1.0 (3–17)	7.3 ± 0.8[d] (2–15)	10.1 ± 0.1 (10–12)	6.7 ± 0.5[g] (3–12)	12.1 ± 0.5 (10–15)
Two-sided *P* values (control vs. PLAG 50 mg/kg)	0.0058	0.075	0.6766	0.832	0.3629
Two-sided *P* values (control vs. PLAG 250 mg/kg)	0.0001	<0.0001	0.6766	<0.0001	0.0023

Note. The durations of severe neutropenia, thrombocytopenia and anemia do not include data from deceased animals unless recovery occurred to that level prior to death.

[a] Includes all animals; [b]n = 15; [c]n = 19; [d]n = 20; [e]n = 13; [f]n = 19; [g]n = 20.

Administration of PLAG Mitigates the Reduction of RBC Counts and Hemoglobin Levels in Peripheral Blood in Irradiated Mice

In addition to reduced ANC and platelet counts, single-dose gamma-ray TBI ($LD_{70/30}$, 6.11 Gy) resulted in reduction of RBC counts and hemoglobin over the 30-day observation period (Fig. 5A and C). Anemia is a condition in which RBC count and hemoglobin level are decreased in peripheral blood, defined as a hemoglobin level of less than 13 g/dl in men and of less than 12 g/dl in women (*29, 30*). Administration of PLAG 250 mg/kg significantly prevented radiation-induced reduction of RBC counts and hemoglobin on days 17 and 22 (Fig. 5B and D). Moreover, administration of PLAG 250 mg/kg significantly reduced the duration of anemia from 15.5 ± 0.8 to 9.7 ± 0.7 days ($P < 0.0001$) (Table 5). Mice treated with PLAG 250 mg/kg exhibited a remarkable increase in the mean nadir of RBC counts, from 3.0 ± 0.3 × 10^6 cells/μl to 4.0 ± 0.2 × 10^6 cells/μl after gamma-ray irradiation, and significantly advanced the mean number of days to recovery of RBCs (≥6.3 × 10^6 cells/μl) from 26.9 ± 0.8 to 24.1 ± 0.7 days (Table 6). These results indicate that PLAG has a remarkable effect in attenuating gamma-ray-induced anemia.

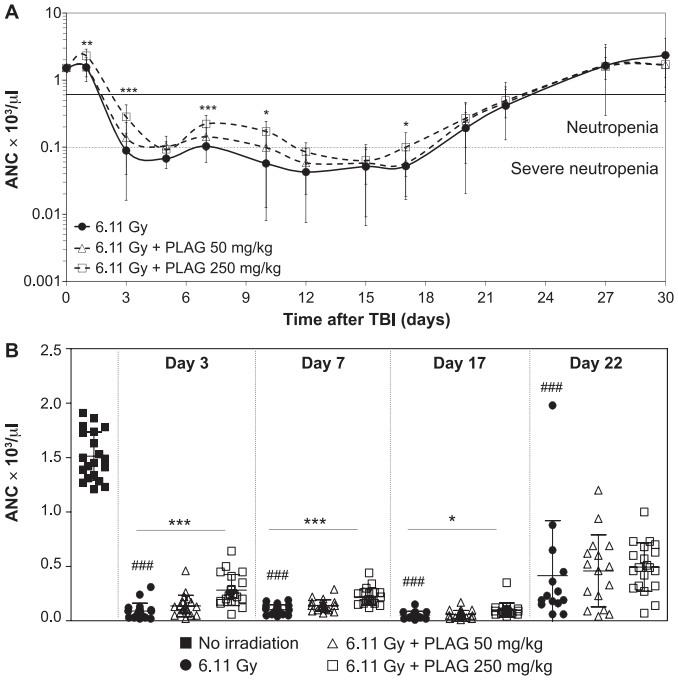

FIG. 3. PLAG mitigated ANC depletion in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 20 per group, 10 males and 10 females) received the $LD_{70/30}$ dose (6.11 Gy) of gamma rays and were administered 50 or 250 mg/kg of PLAG once a day starting the day after irradiation. Panel A: Effect of PLAG administration on the kinetics of ANC after 6.11 Gy irradiation for 30 days. Panel B: Dots indicate individual ANC data for days 3, 7, 17 and 22. #No radiation vs. 6.11 Gy; *6.11 Gy vs. 6.11 Gy + PLAG 250 mg/kg. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.005$.

Time Effect of PLAG Administration on Survivability of Irradiated Mice

Currently, there are no radiation countermeasures approved by the FDA that are effective for alleviating radiation-induced damage when administered later than 48 h postirradiation (*31*). Therefore, we investigated how 24-h, 48-h and 72-h delayed administration of PLAG after irradiation influences survival rate for 30 days. In this experiment, using single-stage, TBI of BALB/c mice at a dose of 6.11 Gy gamma rays ($LD_{70/30}$) caused the death of 65% of the animals in the vehicle control group within 23 days, with the average life span of decedents being 19.2 days, as shown in Figure 6A and Table 6. The survival rate in the vehicle control group was 35%. Survival percentages among irradiated mice receiving PLAG at +0, +1, +2 and +3 days were 80%, 70%, 55% and 55%, respectively. Moreover, the average life spans of decedents that received PLAG at +0, +1, +2 and +3 days were 25.0, 23.7, 18.1, and 22.3 days, respectively (Table 7). This observation indicates that PLAG has therapeutic potential for improving survivability dependent on the starting time of administration in gamma-ray-induced ARS conditions, even if it is administered late in time postirradiation.

TABLE 6
Mean Nadir and Mean Number of Days to Recovery of ANC, Platelets and RBC in Control and PLAG-Treated Mice After 6.11 Gy Irradiation

Treatment	Nadir of ANC[a] (cells/μl)	Mean no. of days to recovery: ANC ≥500/μl (± SE, range)	Nadir of platelets[a] (10^3 cells/μl)	Mean no. of days to recovery: platelets ≥100,000/μl (± SE, range)	Nadir of RBC[a] (10^6 cells/μl)
Control	20.5 ± 2.2	26.8 ± 0.8[b] (22–30)	27.6 ± 1.9	22.8 ± 1.1[e] (17–30)	3.0 ± 0.3
PLAG 50 mg/kg	25.5 ± 4.1	24.4 ± 0.6[c] (20–27)	29.1 ± 1.3	22.8 ± 0.8[f] (17–27)	2.7 ± 0.1
PLAG 250 mg/kg	49.0 ± 4.9	24.7 ± 0.7[d] (20–30)	50.1 ± 3.2	16.8 ± 0.4[g] (15–22)	4.0 ± 0.2
Two-sided *P* values (control vs. PLAG 50 mg/kg)	0.294	0.0334	0.5262	0.9666	0.3266
Two-sided *P* values (control vs. PLAG 250 mg/kg)	<0.0001	0.0698	<0.0001	<0.0001	0.0032

Note. ANC, platelets and RBC recovery parameters do not include data from deceased animals unless recovery occurred to that level prior to death.

[a] Includes all animals; [b]n = 13; [c]n = 19; [d]n = 20; [e]n = 11; [f]n = 17; [g]n = 19.

Effect of Administration of PLAG, Olive Oil and PLH on Survivability and Body Weight Loss in Irradiated Mice

PLAG is a lipid molecule, containing 883 kcal/100 g, with palmitic and linoleic acid esterified at the first and second position of the glycerol backbone and acetyl acid at the third position. We investigated whether the therapeutic efficacy of PLAG results from the uptake of additional calories, compared to olive oil, which contains 884.1 kcal/100 g, and whether the PLAG acetyl moiety contributes to improved survivability, compared to its unacetylated form, PLH. The chemical structures of PLAG and PLH are shown in Fig. 7A. A similar set of experiments was simultaneously performed to analyze the time effect of PLAG administration. The survival rate of the vehicle control group was 35%. The survival percentages of irradiated mice receiving PLAG, olive oil or PLH 250 mg/kg were 70%, 25% and 40%, respectively (Fig. 7B). Moreover, the average life spans of the decedents that received PLAG, olive oil or PLH 250 mg/kg were 23.7, 17.9 and 18.8 days, respectively (Table 8). This observation indicates that the acetyl moiety of PLAG might be attributed to improved survivability in gamma-ray-induced ARS conditions, and the mitigating effect of PLAG is not result of the uptake of additional calories.

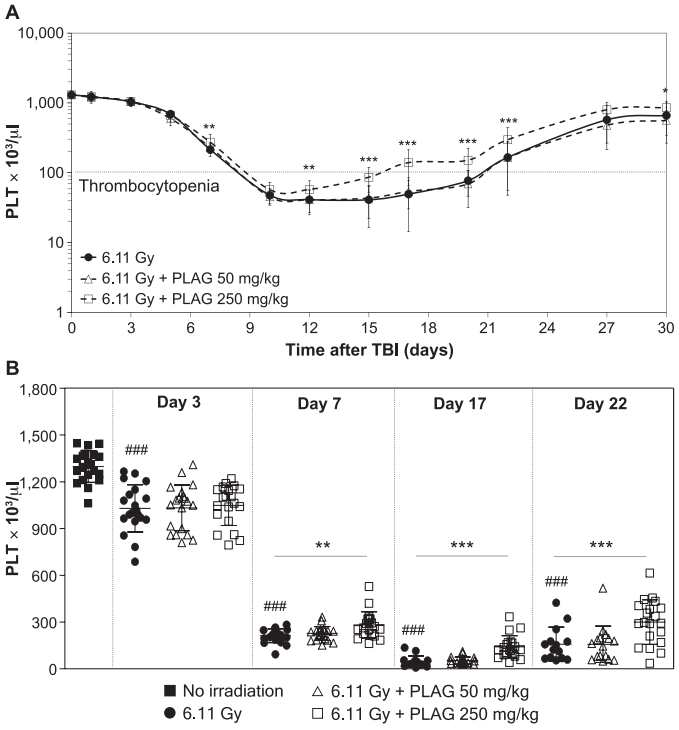

FIG. 4. PLAG mitigated the depletion of platelet counts in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 20 per group, 10 males and 10 females) received the $LD_{70/30}$ dose (6.11 Gy) of gamma rays and were administered 50 or 250 mg/kg of PLAG once a day starting the day after irradiation. Panel A: The effect of PLAG administration on the kinetics of platelet counts after 6.11 Gy irradiation for 30 days. Panel B: Dots indicate individual platelet counts for days 3, 7, 17 and 22. #No radiation vs. 6.11 Gy; *6.11 Gy vs. 6.11 Gy + PLAG 250 mg/kg. #/*$P < 0.05$, ##/**$P < 0.01$, ###/***$P < 0.005$.

DISCUSSION

Since the Fukushima nuclear power plant accident in 2011, public awareness of the threat of radiation has greatly increased, and has prompted the formation of contingency plans for the prophylaxis and treatment of ARS (*1, 32, 33*). Many government agencies involved in national security and public health, including the U.S. FDA, have been searching for suitable radiation countermeasures for the treatment of H-ARS and GI-ARS for several decades (5–7). However, to date, only a limited number of medical options are available, including G-CSF and PEGylated G-CSF, as mitigators of H-ARS (*8–11*). Therefore, the development of safe and effective radiation countermeasures is a priority project for the government.

Here we report that gamma-ray TBI at a lethal dose ($LD_{70/30}$, 6.11 Gy) has a mortality rate of approximately 70%, and results in substantial reduction of body weight and H-ARS symptoms, including neutropenia, thrombocytopenia and anemia in BALB/c mice. Administration of PLAG 50 mg/

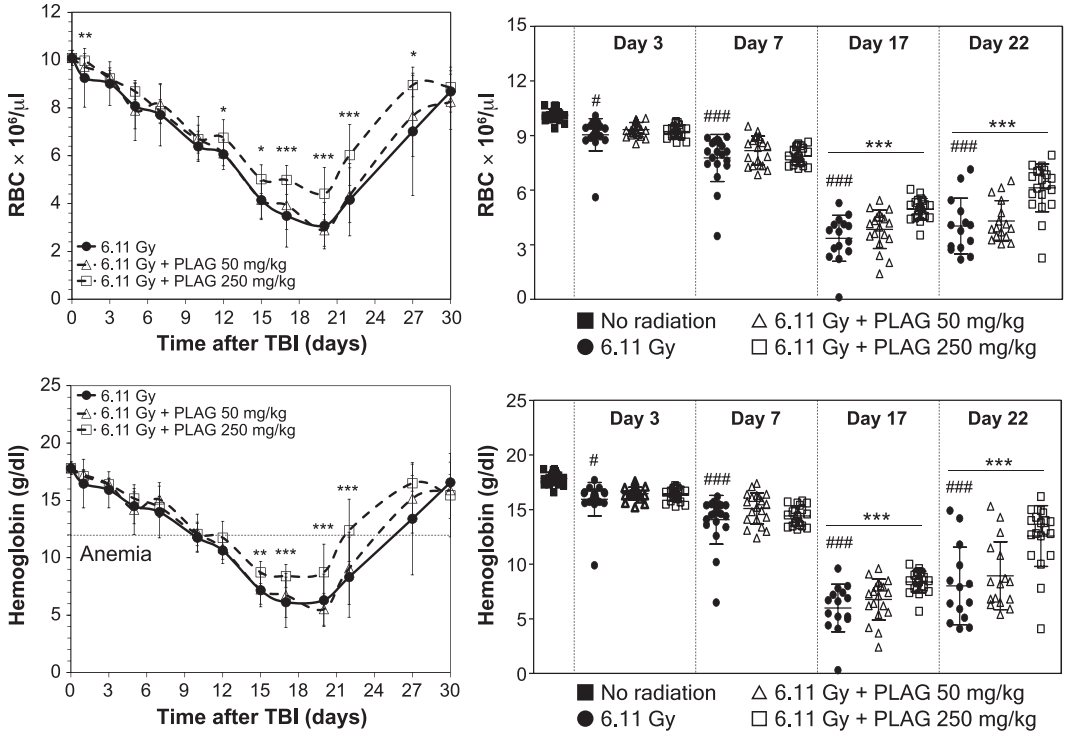

FIG. 5. PLAG mitigated the depletion of RBC counts and hemoglobin levels in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 20 per group, 10 males and 10 females) received the $LD_{70/30}$ dose (6.11 Gy) of gamma rays and were administered 50 or 250 mg/kg of PLAG once a day starting the day after irradiation. Panels A and C: Effect of PLAG administration on the kinetics of RBC counts and hemoglobin levels after 6.11 Gy irradiation for 30 days. Panels B and D: Dots indicate individual platelet counts or hemoglobin levels for days 3, 7, 17 and 22. #No radiation vs. 6.11 Gy; *6.11 Gy vs. 6.11 Gy + PLAG 250 mg/kg. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.005$.

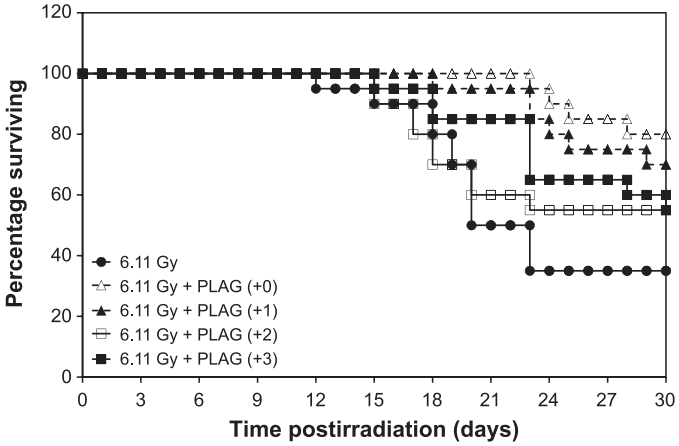

FIG. 6. Time effect of PLAG administration on survivability and body weight loss in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 20 per group, 10 males and 10 females) received an $LD_{70/30}$ dose (6.11 Gy) of gamma rays and were administered 250 mg/kg of PLAG once a day starting the same day (+0), as well as 24 h (+1 day), 48 h (+2 days) or 72 h (+3 days) postirradiation. Survival was monitored for 30 days. $^{*}P < 0.001$, 6.11 Gy + PLAG (+0 day) vs. 6.11 Gy; $^{*}P < 0.001$, 6.11 Gy + PLAG (+1 day) vs. 6.11 Gy; $^{*}P = 0.008$, 6.11 Gy + PLAG (+2 days) vs. 6.11 Gy; $^{*}P = 0.0012$, 6.11 Gy + PLAG (+3 days) vs. 6.11 Gy (log-rank test).

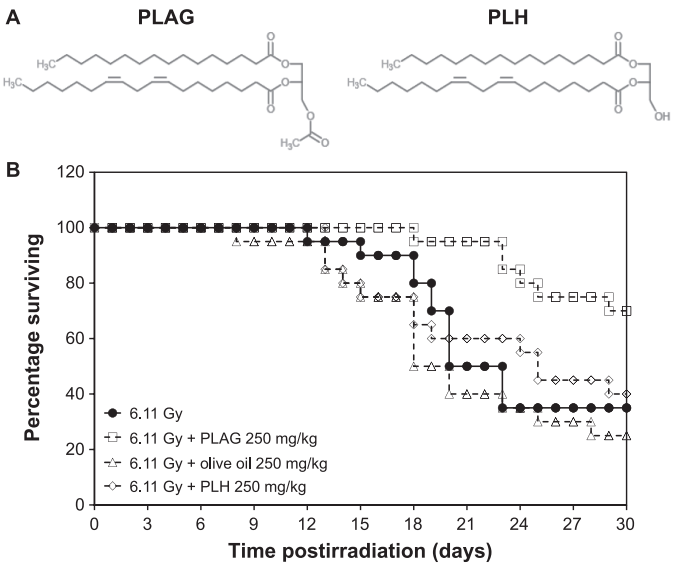

FIG. 7. Effects of administration of PLAG, olive oil and PLH on survivability in mice receiving an $LD_{70/30}$ dose of gamma rays. Mice (n = 20 per group, 10 males and 10 females) received the $LD_{70/30}$ dose (6.11 Gy) of gamma rays and were administered 250 mg/kg of PLAG, olive oil and PLH once a day starting the day after irradiation and continuing until the final day of observation. Panel A: Chemical structures of PLAG and PLH. Panel B: Survival was monitored for 30 days. $^{*}P < 0.001$, 6.11 Gy + PLAG 250 mg/kg vs. 6.11 Gy; $^{*}P = 0.432$, 6.11 Gy + olive oil 250 mg/kg vs. 6.11 Gy; $^{*}P = 0.069$, 6.11 Gy + PLH 250 mg/kg vs. 6.11 Gy (log-rank test).

TABLE 7
Time Effect of Administration of PLAG 250mg/kg on Survivability and Average Life Duration of Irradiated Mice

	No. of mice that survived/total	Survivability (%)	Survival time of decedents (days)		Log-rank test *P**
			Mean ± SEM	Median	
Control	7/20	35	19.2 ± 0.9	20	
PLAG (+0 day)	16/20	80	25.0 ± 1.1	24.5	<0.001
PLAG (+1 day)	14/20	70	23.7 ± 1.4	23.5	<0.001
PLAG (+2 days)	11/20	55	18.1 ± 0.8	18	0.008
PLAG (+3 days)	11/20	55	22.3 ± 1.6	23	0.0012

kg and 250 mg/kg resulted in 60% and 85% survival, respectively, after irradiation, and significantly mitigated radiation-induced severe weight loss. We extended the 15-day observation beyond the end of PLAG administration to ensure the durability of the mitigating effect on survival enhancement. During 15 days of the extended period, the survivability of all groups did not change (data not shown). Moreover, PLAG 250 mg/kg significantly alleviated radiation-induced neutropenia, thrombocytopenia and anemia by increasing the counts of neutrophils, platelets and RBCs, respectively, in peripheral blood.

As reported elsewhere, it has been shown that PLAG is an effective therapeutic candidate in different inflammatory-associated diseases, such as rheumatoid arthritis, atopic dermatitis, asthma and hepatitis (*20, 34–36*). In addition, the therapeutic efficacy of PLAG has been demonstrated in chemotherapy and radiation-induced oral mucositis in hamster and mouse models, through the effective attenuation of neutrophil infiltration into cheek pouches and tongues and a decrease in inflammatory cytokines (*37*). PLAG is currently undergoing a phase II clinical trial for chemotherapy and radiation-induced oral mucositis. We believe that the efficacy of PLAG in modulating an excessive inflammatory response would also contribute to the improved biological indices in ARS.

Ionizing radiation releases very high energy, which damages molecular components in cells of various tissues (*4*). DNA is the most susceptible target of radiation, and its damage results in chromosomal aberrations, mutations and eventually cellular death (*38, 39*). Radiation-induced cellular death by mitotic death and necrosis is associated with the inflammatory response, resulting in hyperinflammatory and hypercytokinemia-like syndromes (*40, 41*). We observed that PLAG effectively decreased the radiation-induced release of damage-associated molecular patterns (DAMPs) in the bloodstream that induce systemic inflammation, such as high motility group box 1 and s100A9 (data not shown). We believe that the putative mechanism of PLAG in ARS is the accelerated resolution of inflammation by effectively eliminating radiation-induced inflammatory factors.

For the study of the hematopoietic syndrome, most researchers have used, in addition to canine models and nonhuman primates, inbred mouse strains, including BALB/c, C3H/HeN, B6D2F1/J and C57BL/6. Of these strains, BALB/c is the most sensitive to radiation, and C57BL/6 is the most resistant (*18*). Because radiation directly affects single- and double-stranded DNA breaks and/or impairment of DNA-damage repair machinery (*42*), the relatively high sensitivity of BALB/c mice to radiation can be attributed to defects in double-stranded DNA repair (*43*). In this study, BALB/c mice were used to evaluate the effect of PLAG, since it was believed that this strain, due to its inherent genetic defects, would more apparently exhibit a radiation-induced systemic inflammatory response by releasing DAMPs. However, it is necessary to assess the efficacy of this drug in more than one mouse strain because of strain variables with respect to radiation response.

The cells in the bone marrow and gastrointestinal (GI) tract are the most sensitive to radiation damage, because these cells are highly proliferative and have a low sensitivity threshold to radiation, caused by fast cellular turnover (*1, 44, 45*). Along with hematopoietic injury, GI-ARS is a serious injury that causes various clinical manifestations, from those occurring in the mouth to those in the colon and rectum. Patients with GI-ARS suffer not only from malnutrition due to reduced digestive capacity, but also from excessive inflammation and bacterial overgrowth after radiation exposure (*46, 47*). The co-appearance of GI damage with hematopoietic injury increases the mortality rate even at low radiation doses (*48, 49*). Therefore, it is

TABLE 8
Effect of administration of PLAG, Olive Oil, PLH 250 mg/kg on Survivability and Average Life Duration of Irradiated Mice

	No. of mice that survived/total	Survivability (%)	Survival time of decedents (days)		Log-rank test *P**
			Mean ± SEM	Median	
Control	7/20	35	19.2 ± 0.9	20	
PLAG 250mg/kg	14/20	70	23.7 ± 1.4	23.5	<0.001
Olive oil 250mg/kg	5/20	25	17.9 ± 1.5	18	0.432
PLH 250mg/kg	8/20	40	18.8 ± 1.6	18	0.069

necessary to investigate whether PLAG also attenuates the signs and symptoms of GI injury after radiation exposure.

In conclusion, the administration of PLAG significantly improved gamma-ray-induced mortality and body weight loss in BALB/c mice. Moreover, it effectively attenuated radiation-associated hematopoietic injuries, including neutropenia, thrombocytopenia and anemia. Together, these findings demonstrate that PLAG has therapeutic potential as a radiation countermeasure for the improvement of survivability and treatment of the hematopoietic subsyndrome of ARS.

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