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Kinetics of Neutrophils in Mice Exposed to Radiation and/or Granulocyte Colony-Stimulating Factor Treatment

A. L. Romero-Weaver, X. S. Wan, E. S. Diffenderfer, L. Lin, and A. R. Kennedy¹

Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Abstract

Astronauts have the potential to develop the hematopoietic syndrome as a result of exposure to radiation from a solar particle event (SPE) during exploration class missions. This syndrome is characterized by a reduction in the number of circulating blood cells (cytopenias). In the present study the effects of SPE-like proton and γ radiation on the kinetics of circulating neutrophils were evaluated during a one-month time period using mice as a model system. The results revealed that exposure to a 2 Gy dose of either SPE-like proton or γ radiation significantly decreased the number of circulating neutrophils, with two nadirs observed on day 4 and day 16 postirradiation. Low circulating neutrophil count (neutropenia) is particularly important because it can increase the risk of astronauts developing infections, which can compromise the success of the mission. Thus, two granulocyte colony-stimulating factors (G-CSFs), filgrastim and pegfilgrastim were evaluated as countermeasures for this endpoint. Both forms of G-CSF significantly increased neutrophil counts in irradiated mice, however, the effect of pegfilgrastim was more potent and lasted longer than filgrastim. Using the expression of CD11b, CD18 and the production of reactive oxygen species (ROS) as markers of neutrophil activation, it was determined that the neutrophils in the irradiated mice treated with pegfilgrastim were physiologically active. Thus, these results suggest that pegfilgrastim could be a potential countermeasure for the reduced number of circulating neutrophils in irradiated animals.

INTRODUCTION

Exploration class missions outside of low-Earth orbit are planned for the near future, and it is expected that astronauts will experience more adverse biological effects of space radiation in these missions than in previous missions. Some of the adverse biological effects may be caused by exposure to solar particle event (SPE) radiation, which is composed mainly of low-energy protons (1). It is estimated that astronauts will receive a deep tissue/organ dose of up to 2 Gy as a result of exposure to SPE radiation during extravehicular activities (EVAs), which can lead to the development of the acute radiation syndrome (ARS) (2). The hematopoietic syndrome, which is characterized by a reduction in the number of circulating blood cells, is one of the expected ARSs that astronauts could experience if exposed to SPE

radiation during EVAs. Using a mouse model system, we and others have reported that exposure to 2 Gy of low-energy protons (simulated SPE radiation) can cause significant decreases in circulating blood cells (3, 4). Of the circulating blood cells, neutrophils constitute the first line of immune defense, their reduction can increase the risk of infections for the crew and jeopardize the success of the space mission.

Neutrophil production is initiated in the bone marrow from hematopoietic stem cells (HSCs). These cells give rise to multipotent progenitors (MPPs), which can commit to specific blood lineages and further differentiate into different types of mature blood cells in highly controlled processes involving many regulators required for maintenance of hematopoietic homeostasis (5, 6). One of the critical factors in neutrophil differentiation is granulocyte colony stimulating factor (G-CSF) (7). Under normal conditions, the majority of mature neutrophils remain in the bone marrow and only about 2% of differentiated mature neutrophils are released into the blood stream, where they circulate for 10–24 h, and then they migrate into tissue to survey for signs of infection (8, 9). In response to infection or inflammation, host cells and invading pathogens secrete potent inflammatory mediators and neutrophil chemoattractants, which trigger the production of G-CSF by some host cells as well as adhesion molecule expression on the surface of vascular endothelial cells and neutrophils (10–12). G-CSF has been shown to induce the release of neutrophils from the bone marrow reserve into the blood circulation (7, 13). During infection or inflammation, the release of circulating neutrophils can increase by tenfold in a matter of hours (14). The adhesion molecules expressed on the surface of activated neutrophils, such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), bind to other adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, on the surface of activated vascular endothelial cells near the site of infection or inflammation (14–16). This mediates the exit of neutrophils from the circulation by transmigration across the vascular endothelium to the site of infection. The neutrophils ingest microorganisms by phagocytosis and kill them by a combination of cytotoxic components released from neutrophil granules and the production of reactive oxygen species (ROS) (9), by a process known as the respiratory burst (17).

Low neutrophil counts (neutropenia) are associated with increased risks for infections (18, 19). Different forms of recombinant G-CSFs (rG-CSF) are currently available for treatment of congenital neutropenia and neutropenia induced by chemotherapy or radiotherapy as well as for stimulation of neutrophil recovery after bone marrow transplantation. Two commercially available rG-CSFs are filgrastim (Neupogen®) and pegfilgrastim (Neulasta®), which is the pegylated form of filgrastim. Pegfilgrastim has a longer *in vivo* half-life; therefore, it requires less frequent administrations than filgrastim (20–22), which may make it more suitable for use in space exploration. The present study was conducted to assess the acute effects of SPE radiation on the kinetics of circulating neutrophils and to evaluate filgrastim and pegfilgrastim as countermeasures against radiation induced neutropenia using our mouse model system. The neutrophil counts measured in the irradiated animals without G-CSF treatment were used as baseline values for comparison to determine the effects of both countermeasures. Since neutrophil dysfunction is associated with increased risk for infections (23, 24), the activation of selective neutrophil functions

was determined by comparing the expression of specific neutrophil activation markers, CD11b and CD18, as well as ROS production capability. The expression levels of these markers and ROS production were determined in neutrophils isolated from irradiated mice treated with pegfilgrastim, and compared to the expression levels and ROS production in neutrophils isolated from unirradiated control mice treated with vehicle to determine whether the pegfilgrastim-stimulated neutrophils in irradiated mice maintain normal physiological activities.

MATERIALS AND METHODS

Mice

Female ICR (Imprinting Control Region) mice at 6 weeks of age were purchased from Taconic Farms Inc. (Hudson, NY). Animals were acclimated for 7 days in the University of Pennsylvania animal facility. Five animals were housed per cage and given *ad libitum* access to water and food pellets. The animal care and treatment procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Radiation

The mice were placed in custom designed Plexiglass chambers and exposed to total body doses of 0.5, 1 or 2 Gy, at a dose rate of about 0.5 Gy/min of either gamma or proton radiation. Gamma radiation was administered by a Cesium 137 Gammacell 40 irradiator (Nordion, Ottawa, ON, Canada) located at the University of Pennsylvania (Philadelphia, PA).

The proton beam was produced by the IBA cyclotron (IBA Particle Therapy, Jacksonville, FL) located in the Roberts Proton Therapy Center at the University of Pennsylvania. The 230 MeV proton beam extracted from the IBA cyclotron was reduced in energy with a graphite degrader and the phase space was restricted by divergence and momentum slits in the energy selection system to a nominal energy of 151 MeV or a range of 16 cm water equivalent thickness (WET).² The degraded beam was delivered in double-scattering mode with a uniform spread out Bragg peak (SOBP) modulation width of 5 cm using a horizontal beam with gantry angle 270°. A 23 cm × 17 cm opening in the tungsten multileaf collimator shaped the beam into a useable field size (>95% of maximum within the flat region) of 20.6 cm × 17 cm at the gantry isocenter. Eight mouse enclosures, with dimensions of 7.2 cm × 4.1 cm × 4.1 cm, were arranged in a 2 × 4 array forming a 14.2 × 16.4 cm target area. The center of the enclosure array was placed at the gantry isocenter with an additional 11 cm of Solid Water® Slab (Gammex Inc., Middleton, WI) placed directly in front of the array to degrade the proton beam energy further to approximately 74 MeV or a range of ~4.5 cm. Five centimeter of Solid Water® Slab was placed directly behind the enclosure array. The mouse enclosures were irradiated with a range of proton energies forming the uniformly modulated dose region of the SOBP. The dose-averaged linear energy transfer (LET) of the proton radiation is low (<10 keV/μm) within the mid-SOBP where the mice are located and rises to higher LET (>10 keV/μm) towards the downstream edge of the SOBP, which lies

²IBA Proton Therapy System Maintenance Manual for the Roberts Proton Therapy center.

beyond the mouse enclosures (25). For purposes of this study, this radiation is defined as “SPE-like protons” and the depth dose profile in water is shown in Fig. 1. Dosimetry verification was performed before the irradiations with a 2D ion chamber array (I²mRT MatriXX, IBA Dosimetry, Schwarzenbruck, Germany) placed at a depth of 13.3 cm. These irradiation conditions result in a homogeneous dose distribution of SPE-like proton irradiation in the mice.

Unirradiated control mice were also placed in the custom designed Plexiglass chambers and all mice (irradiated and unirradiated) were maintained in the plexiglass chambers for the same time period.

Filgrastim or Pegfilgrastim Treatment

Filgrastim and pegfilgrastim were kindly provided by Amgen, Inc. (Thousand Oaks, CA). The effects of filgrastim and pegfilgrastim were evaluated in a series of experiments, in which filgrastim and pegfilgrastim were injected subcutaneously (s.c.) in the back of the neck at the indicated doses in a total volume of 100 μ l per animal. Groups of irradiated and unirradiated animals were injected with 100 μ l of PBS and included in the experiments as control groups for comparison with the filgrastim- or pegfilgrastim-treated irradiated and unirradiated groups.

In the initial experiments performed to determine the duration of filgrastim and pegfilgrastim effects on neutrophils, mice were irradiated with 2 Gy of γ rays and injected with 100 μ l of PBS, filgrastim (300 μ g/kg) or pegfilgrastim (600 μ g/kg) on day 3 postirradiation, and the neutrophil count was monitored for up to 8 days postirradiation (5 days after filgrastim or pegfilgrastim administration). Unirradiated mice were treated with PBS as a control group. In addition, unirradiated mice treated with filgrastim or pegfilgrastim were also included in the experiment as controls to confirm that treatment with filgrastim and pegfilgrastim, at the doses administered, was capable of increasing neutrophil counts. Once it was established that filgrastim and pegfilgrastim treatment resulted in increased neutrophil counts at the doses administered in our mouse model, the unirradiated filgrastim or pegfilgrastim treated groups were not included in the remaining experiments. Instead, the neutrophil count data obtained in the initial experiment for the unirradiated mice treated with pegfilgrastim (Fig. 3) were used for comparison with the neutrophil count data obtained in the experiments shown in Figs. 4 and 5. The omission of a positive control group (i.e., a control group of unirradiated mice treated with filgrastim or pegfilgrastim) in subsequent experiments was to minimize animal and drug usage, and because the results of such a positive control group would not be useful in the major experimental aim, which was to determine whether filgrastim or pegfilgrastim could increase neutrophil counts in irradiated animals, compared to irradiated animals that did not receive filgrastim or pegfilgrastim treatment.

In the next experiment, performed to determine the most effective time of administration of pegfilgrastim, mice irradiated with 2 Gy of γ rays were treated with pegfilgrastim (600 μ g/kg) either immediately after irradiation, one day before irradiation or one day after irradiation. Neutrophil counts were determined on day 3 after irradiation. To determine the relationship between the pegfilgrastim dose and its effect on neutrophil counts, a subsequent

experiment was performed in which mice irradiated with 2 Gy of γ rays were treated with 150, 300 or 600 $\mu\text{g/kg}$ of pegfilgrastim immediately after the radiation exposure and neutrophil counts were determined on day 3 after irradiation. Mice with or without the radiation exposure were treated with PBS and included in each of these two experiments as control groups.

In experiments performed to determine whether multiple pegfilgrastim injections could completely prevent radiation-induced decreases in neutrophil counts, mice irradiated with 2 Gy of γ rays were treated with pegfilgrastim at doses of 300 $\mu\text{g/kg}$ immediately after irradiation, 150 $\mu\text{g/kg}$ on day 3 and day 9 after irradiation and 75 $\mu\text{g/kg}$ on day 14 after irradiation. Mice irradiated with 2 Gy of γ rays and injected with PBS at the same times were included in the experiment as controls. The neutrophil count was monitored for up to 22 days after irradiation.

To determine whether pegfilgrastim could increase neutrophil counts after SPE-like proton radiation in a manner similar to that observed for mice exposed to γ radiation, mice were exposed to 2 Gy of SPE-like proton radiation or 2 Gy of γ radiation and were treated with a dose of 300 $\mu\text{g/kg}$ of pegfilgrastim, administered immediately after irradiation and 150 $\mu\text{g/kg}$ administered on day 3 postirradiation. Neutrophil counts were obtained every other day after irradiation for up to 6 days.

For all experiments, mice were weighed individually to calculate the dose of filgrastim or pegfilgrastim; these agents were diluted in sterile PBS to 100 μl .

Blood Sample Collection and Processing

At the indicated times, irradiated and unirradiated mice with or without rG-CSF treatment were euthanized by carbon dioxide (CO_2) inhalation and approximately 1 ml of blood was collected from each animal by cardiac puncture. An aliquot of about 250 μl of blood was placed into lavender top blood BD microtainer® collection tubes containing ethylenediaminetetraacetic acid (EDTA, no. 365973; Becton Dickinson, Franklin Lakes, NJ) and sent to Antech Diagnostics (Lake Success, NY) for complete blood cell count analysis, which includes white blood cells, red blood cells, platelets, lymphocytes, monocytes, eosinophils and basophils in addition to neutrophils, which were the focus of this study. Another aliquot of about 750 μl blood was placed into green top blood BD microtainer® collection tubes containing lithium heparin (no. 365971; Becton Dickinson, Franklin Lakes, NJ) for flow cytometry analysis. The use of the Antech Diagnostic facility has been previously validated (26).

Neutrophil Counts in Mice after Irradiation with Gamma or SPE-like Proton Radiation

To determine and compare the kinetics of circulating neutrophils in mice irradiated with γ rays or SPE-like protons, mice were irradiated with γ rays or protons at total body doses of 0 (control), 0.5, 1 and 2 Gy. On day 0, 1, 2, 4, 6, 8, 10, 13, 16, 19, 23 and 30, blood was drawn from each animal. At each time point, there were 5 animals from each radiation dose group. This was consistent in both experiments with either γ rays or proton radiation. An unirradiated control group was included at each time point.

Flow Cytometry Analysis

Each blood sample was divided into 100 μ l aliquots and red blood cells were removed by the addition of 1 ml ammonium buffer (containing 155 mM ammonium chloride, 100 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.4). The ammonium buffer was neutralized by the addition of 1 ml Hanks' balanced salt solution (HBSS) buffer without phenol red (Mediatech, Manassas, VA). Then samples were centrifuged at 3,000 relative centrifugal forces (rcf) for 5 min and the pellets were resuspended in 100 μ l of Flow Cytometry Staining Buffer (eBioscience, San Diego, CA). The resuspended cells were either unstimulated (treated with PBS) or stimulated by the addition of 60 ng/ml (final concentration) of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Co., St Louis, MO) and incubated for 15 min at 37°C. After incubation, 10 μ l of CD11b-FITC (clone M1/70) or CD18-PE (Clone M18/2) antibodies (both from eBioscience Inc., San Diego, CA) or 4 μ g/ml (final concentration) of dihydrorhodamine 123 (DHR 123) (Cayman Chemical Co., Ann Arbor, MI) were added. Samples with DHR123 were incubated for 15 min at 37°C. Samples with CD11b or CD18 were incubated for 30 min at 4°C. At the end of the incubation, 1 ml of HBSS was added and samples were centrifuged at 3,000 rcf for 5 min. The pellets were resuspended in 500 μ l of flow cytometry staining buffer and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuestPro software (BD Biosciences). Cells were sorted by light scatter based on the relative cell size (forward-scatter) and granularity (side-scatter). A dot plot was generated with the forward-scatter (y-axis) and the side-scatter (x-axis) and a gate was set around the neutrophil population (Fig. 8). The neutrophil population was isolated for further functional analyses by applying the same gated region to all the dot plots that were generated from each sample from all groups, i.e., the unirradiated group treated with PBS, the unirradiated group treated with PMA, the irradiated group treated with PBS, and the irradiated group treated with pegfilgrastim. Next, the population of CD11b, CD18 or Rhodamine 123 positive cells was detected in the neutrophil gated population. A single parameter histogram was chosen to determine the percentage of neutrophils that have the fluorochrome-conjugated antibody bound to the surface. A marker was set to select the positively stained cells (with increased fluorescence intensity) in the PMA stimulated samples, using the unstimulated samples as the negative cells. The percentage of positively stained CD11b, CD18 or Rhodamine 123 neutrophils in the gated region were determined by the CellQuestPro software in a total of 10,000 gated cells. Lastly, a bar graph was constructed to show the mean of the calculated percentages from 3 experimental replicates with each sample representing the blood from one mouse and the results of analyses performed to determine whether there were statistically significant differences between treatment groups (Fig. 9).

RBE Data and Statistical Analyses

The neutrophil count in each animal at each time point after irradiation was divided by the mean neutrophil count of the control group at the same time point and expressed as fraction of control for further analysis. The neutrophil data for the animals at each time point after proton- and γ -radiation exposures were fitted using a simple exponential dose-response function: $y = e^{-\alpha D}$, where α is the slope parameter and D is the radiation dose. The ratio of the proton dose-response curve slope parameter to the γ -ray dose-response curve slope

parameter was calculated as the estimate of proton relative biological effectiveness (RBE). The 95% confidence interval (95% CI) was calculated for each dose response curve slope parameter and compared between the proton and γ radiation to determine the statistical significance of the RBE. An RBE of 1 was inferred if the dose-response curve slope parameters were not significantly different between the γ ray and proton radiation (i.e., the proton dose-response curve slope parameter was within the 95% CI of the γ -ray dose-response curve slope parameter). The dose-response curve fitting and 95% CI calculation were performed using Stata/IC 12.1 statistical software.

The means of neutrophil count expressed as fraction of control for different treatment groups and/or times were compared by one-way ANOVA followed by Tukey's test or by a t test using SigmaPlot 12.0 software.

RESULTS

Kinetics of Circulating Neutrophils in Mice Exposed to SPE-like Proton or Gamma Radiation

The neutrophil counts in mice exposed to 2 Gy of γ rays or 2 Gy of SPE-like protons were monitored for up to 30 days postirradiation (Fig. 2). Neutrophil counts decreased significantly with two nadirs observed on different days. The first nadir occurred 4 days postirradiation when neutrophil numbers decreased by approximately 65% and 70% in animals irradiated with 2 Gy of γ rays or 2 Gy of SPE-like protons, respectively. By day 10 postirradiation, neutrophil numbers recovered to values that were not significantly different from those of the controls. The neutrophil numbers decreased again subsequently and exhibited the second nadir by day 16 postirradiation when the neutrophil numbers decreased by approximately 80% and 75% in animals exposed to 2 Gy of γ rays or 2 Gy of SPE-like protons, respectively. The neutrophil numbers recovered again thereafter and by day 23 postirradiation, the neutrophil counts were not significantly different from those of the controls.

The neutrophil data from the animals irradiated with 0 (control), 0.5, 1 and 2 Gy of γ rays or protons were fitted with an exponential decay model ($y = e^{-\alpha D}$) to determine the dose-response relationship for each time point after irradiation. The slope parameter of the dose-response curves changed at the time of the first and second nadirs occurring on day 4 and day 16 (Table 1), respectively. Based on the ratio of the dose-response curve slope parameters between the SPE-like protons and γ rays, the proton RBE determined at day 1 after irradiation was estimated to be 0.5, which was significantly below 1.0, indicating that the protons were only about half as effective as γ rays in inducing the initial decrease in the number of circulating neutrophils after irradiation. In the subsequent 21 day period from day 2 to day 23 after irradiation, the proton RBE values fluctuated within a range of 0.8 to 1.4 and were not significantly different than 1.0. A meaningful proton RBE value could not be calculated for day 30 after irradiation because no significant dose-response relationship was observed after irradiation with either γ rays or SPE-like protons.

Evaluation of Countermeasures for Low Neutrophil Counts Caused by Radiation Exposure

Two forms of rG-CSF, filgrastim and pegfilgrastim, were evaluated as potential countermeasures for low neutrophil counts resulting from radiation exposure. Since the γ rays and SPE-like protons were not significantly different in their effectiveness at decreasing the neutrophil counts, except on day 1 after irradiation, γ radiation at a single dose of 2 Gy was used in the experiments performed to evaluate the effects of the countermeasures. The results indicated that within a day after treatment with 300 $\mu\text{g/kg}$ of filgrastim (day 4, Fig. 3A), the neutrophil counts increased by approximately threefold in the unirradiated control group treated with filgrastim (bold-solid line) compared to the unirradiated group treated with PBS (thin-straight line) and 3.2-fold in the irradiated mice treated with filgrastim (dashed line) compared to the irradiated group treated with PBS (dotted line). The stimulatory effect of filgrastim lasted for 2 days and was statistically significant. By day 3 after filgrastim administration, the neutrophil counts in the irradiated and unirradiated control animals treated with filgrastim returned to levels that were similar to those of the irradiated and unirradiated control mice treated with PBS (Fig. 3A). Treatment with 600 $\mu\text{g/kg}$ of pegfilgrastim (day 6, Fig. 3B) increased neutrophil counts by up to 15-fold in the unirradiated control group treated with pegfilgrastim (bold-solid line) compared to the unirradiated control group treated with PBS (thin-straight line) and by up to 7.3-fold in the irradiated group treated with pegfilgrastim (dashed line) compared to the irradiated group treated with PBS (dotted line). The stimulatory effect of pegfilgrastim lasted for 3 days and was statistically significant. By day 4 after pegfilgrastim administration, the neutrophil counts in the irradiated and unirradiated animals treated with pegfilgrastim returned to levels that were not significantly different than those in the irradiated and unirradiated animals treated with PBS (Fig. 3B). These results demonstrated the stimulatory effects of filgrastim and pegfilgrastim on circulating neutrophil counts in both the unirradiated mice and irradiated mice. Since the stimulatory effect of pegfilgrastim was stronger and lasted longer than that of filgrastim, with the maximum effect observed on day 3 after pegfilgrastim administration, the subsequent experiments were performed using pegfilgrastim and not filgrastim.

To determine the most effective time of pegfilgrastim administration relative to the radiation exposure, 3 separate groups of mice were treated with pegfilgrastim either one day prior to irradiation, immediately after irradiation exposure, or one day after irradiation and the neutrophil counts were determined on day 3 after pegfilgrastim administration. The results demonstrated that pegfilgrastim significantly increased the neutrophil counts in the irradiated mice regardless of whether it was administered immediately after irradiation or one day before or after irradiation (Fig. 4).

To determine whether a lower dose of pegfilgrastim would be effective in our experimental model, mice were irradiated and given 150, 300 and 600 $\mu\text{g/kg}$ doses of pegfilgrastim. The results showed that the stimulatory effects of pegfilgrastim were dose dependent and all three doses resulted in statistically significant increases in neutrophil counts in the irradiated mice treated with pegfilgrastim compared to the irradiated mice treated with PBS (Fig. 5). The neutrophil counts in the irradiated mice treated with 300 and 600 $\mu\text{g/kg}$ pegfilgrastim were higher than the neutrophil counts in the unirradiated mice treated with PBS.

In the next experiment, the effects of several pegfilgrastim administrations in irradiated animals were evaluated. We hypothesized that providing irradiated mice with a higher dose of pegfilgrastim before the first nadir in neutrophil counts, followed by lower pegfilgrastim doses thereafter, would maintain high numbers of neutrophils for an extended period after radiation exposure. We found that a treatment regimen, consisting of 300 µg/kg of pegfilgrastim administration immediately after radiation exposure, followed by 150 µg/kg of pegfilgrastim on days 3 and 11, and by 75 µg/kg of pegfilgrastim on day 14 after radiation exposure, significantly increased the neutrophil counts on day 2, 4, 6, 10, 12, 14 and 16 in the irradiated animals treated with pegfilgrastim, compared to the neutrophil counts in the irradiated animals treated with PBS (Fig. 6). The maximum increase in neutrophil counts was observed on day 4 after radiation exposure, which coincided with the time of the first nadir of neutrophil counts observed in the irradiated animals treated with PBS.

To test whether pegfilgrastim could increase neutrophils in mice irradiated with 2 Gy of SPE-like protons in a similar manner to that observed in mice exposed to 2 Gy of γ radiation, an experiment was performed in which mice were either unirradiated and treated with PBS or exposed to 2 Gy of γ rays or 2 Gy of SPE-like proton radiation and treated with either PBS or 300 µg/kg pegfilgrastim immediately after exposure, followed by administration of either PBS (unirradiated control mice) or 150 µg/kg pegfilgrastim (irradiated mice) on day 3 postirradiation. Neutrophil counts were determined on days 0, 2, 4 and 6 postirradiation and were expressed as fraction of control. The results indicate that pegfilgrastim increased neutrophil counts in 2 Gy of SPE-like proton irradiated mice and the increase was comparable to that observed in pegfilgrastim treated mice exposed to 2 Gy of γ radiation (Fig. 7).

Determination of Activation of Neutrophil Functions in Irradiated Mice Treated with Pegfilgrastim after PMA Stimulation

CD11b and CD18 are cell surface proteins that regulate neutrophil adhesion and migration, and ROS production has been shown to be involved in neutrophil phagocytosis. Neutrophil functional activity was determined by measuring the expression of the activation markers CD11b and CD18 and ROS production. ROS production was determined indirectly through the measurement of Rhodamine 123 fluorescence (27). Whole blood samples were treated with PBS or PMA to assess neutrophil activation. In flow cytometry measurements, neutrophils were gated by their characteristic forward-scatter and side-scatter distribution (Fig. 8). The expression of activation markers, CD11b and CD18, and Rhodamine 123 were determined in the neutrophil gated population in samples obtained 4 days after irradiation from irradiated mice treated with pegfilgrastim and compared to the normal distribution of the marker expression in unirradiated mice treated with PBS (for details refer to the Materials and Methods section). The results from the PMA stimulated samples indicated that the neutrophil gated population in irradiated mice treated with pegfilgrastim resulted in a larger percentage of cells expressing CD11b or CD18 (Fig. 9A and B, respectively), compared to the PMA stimulated samples isolated from the unirradiated control mice treated with PBS. The expression of both markers was increased, in a statistically significant manner. These data suggest that pegfilgrastim-stimulated neutrophils in irradiated mice are functionally active and moreover, have a higher percentage of cells expressing the activation

markers, CD11b and CD18, compared to the normal circulating neutrophils in naïve mice (unirradiated mice treated with PBS). However, ROS production, represented by the expression of Rhodamine 123, was not significantly different between the samples taken from irradiated animals treated with pegfilgrastim and samples taken from unirradiated control mice treated with PBS (Fig. 9C), indicating that both neutrophil populations (pegfilgrastim-stimulated or not) were comparable in their ability to produce ROS upon PMA stimulation. Taken together, these studies suggest that the neutrophils isolated from irradiated mice treated with pegfilgrastim are functionally active.

DISCUSSION

Kinetics of Mouse Neutrophils in Response to SPE-like Proton and Gamma Radiation

Exposure of mice to 2 Gy of SPE-like proton and 2 Gy of γ radiation resulted in a statistically significant decrease in the number of circulating neutrophils, which occurred with two nadirs observed on different days in the irradiated mice. The declining phase of the neutrophil counts lasted for 4 days in the first drop (from day 1 to day 4 postirradiation) and 6 days in the second drop (from day 10 to day 16 postirradiation). Each declining phase was followed by a recovery phase, which took approximately 6–7 days to complete. At the nadir points, which occurred on day 4 and day 16 after irradiation, the circulating neutrophil counts were below 500 neutrophils/ μ l, which is considered clinically critical (18). Assuming there would be a similar response in astronauts exposed to an internal tissue/organ dose of 2 Gy of SPE radiation, it might be expected that astronauts would experience a significant reduction in circulating neutrophils, which could threaten their health and mission success due to increased risks of developing infections. Therefore, countermeasures for SPE radiation induced neutropenia are needed.

Statistically significant decreases in neutrophils were observed as early as one day after irradiation in the present study (Figs. 2 and 6). Significant decreases in neutrophils have also been observed to occur at later times, since they are known to be more radiation resistant than lymphocytes (28). The two neutrophil nadirs were clearly detected in these studies in which neutrophil counts were determined at several closely spaced times after irradiation. Previously reported studies have suggested similar results. For example, Gridley *et al.* (4) reported decreases in granulocyte counts at 4 and 21 days after a single dose of 2 Gy proton or γ radiation. Some studies performed in animals and astronauts, however have shown an increased number of neutrophils after space flight (29–31). This neutrophilia has been associated with several factors, such as stress and exposure to a microgravity environment (32), but none of these previous studies in animals or astronauts included exposure to significant doses of SPE radiation. The neutropenia observed in our studies was the sole result of exposure to photon or SPE-like proton radiation. To better assess the risk of astronauts for a significant reduction in circulating neutrophils resulting from exposure to SPE radiation in space missions, it would be necessary to determine the combined effect of SPE and microgravity exposure on neutrophil numbers and functions.

Evaluation of Countermeasures for Low Neutrophil Counts Caused by Radiation Exposure

In the present study, the stimulatory effect of pegfilgrastim on circulating neutrophils lasted for 3 days, which was longer than the effect of filgrastim. In the irradiated animals, the effect of pegfilgrastim appeared to be independent of the time of pegfilgrastim administration relative to the radiation exposure, which is consistent with observations by other investigators who reported that the effect of pegfilgrastim was independent of the time of its administration relative to time of treatment with other agents that decrease neutrophil counts, such as cyclophosphamide or other chemotherapeutic agent (33, 34). We observed that pegfilgrastim treatment is effective when administered either one day before radiation exposure, immediately after radiation exposure or even one day after radiation exposure. The magnitude of the increase in the neutrophil count was smaller in the irradiated mice treated with pegfilgrastim than in the unirradiated mice treated with pegfilgrastim, indicating that radiation exposure compromises the bone marrow in such a way that stimulation by pegfilgrastim augments neutrophil production but not to the same degree as in an animal whose bone marrow has not been compromised.

In these studies, the effect of pegfilgrastim on neutrophils was dose-dependent in the range of 150 to 600 $\mu\text{g/kg}$. In human studies, some dose-escalation studies did not show a dose dependency (35, 36), whereas other studies demonstrated a clear dose dependency of the pegfilgrastim induced neutrophil recovery (37, 38). It is conceivable that the dose response for pegfilgrastim induced neutrophil recovery may have a plateau, and the results of the present study indicate that the plateau concentration of pegfilgrastim in the mouse model is probably higher than 600 $\mu\text{g/kg}$.

The results reported here also indicated that a regimen of 4 pegfilgrastim injections is sufficient to maintain the neutrophil counts in irradiated mice at an acceptable level over a 22 day experimental period after irradiation. Although pegfilgrastim administration was equally effective, when administered one day before, one day after or immediately after irradiation, the first injection was given immediately after radiation exposure in these experiments. As SPEs are very difficult to predict, it is expected that pegfilgrastim treatment would begin soon after SPE radiation exposure in space.

The majority of the experiments reported here were performed using 2 Gy of γ radiation since the effects of 2 Gy of SPE-like proton and 2 Gy of γ radiation on neutrophil counts were similar, with the RBE not significantly different from 1 (Table 1 and Fig. 2), and the γ -ray source was more easily accessible and cost effective for experimental use. It was demonstrated that pegfilgrastim was as effective in its ability to increase neutrophil counts in mice exposed to 2 Gy of SPE-like proton radiation as it was in mice exposed to 2 Gy of γ radiation (Fig. 7). It should be noted that the gamma and SPE-like proton radiation may have different effects on some other biological endpoints; thus, the similarity in the effects of these two types of radiation on neutrophil counts can not necessarily be extrapolated to other biological endpoints.

The results of the neutrophil activity measurements indicated that neutrophils isolated from irradiated mice treated with pegfilgrastim expressed activation markers CD11b and CD18, as well as Rhodamine123 upon PMA stimulation, in a similar fashion to that observed for

neutrophils isolated from unirradiated mice treated with PBS. In fact, we observed a higher percentage of neutrophils expressing CD11b and CD18 in the pegfilgrastim treated irradiated mice, compared to the unirradiated control mice and treated with PBS. Since the exact number of events or neutrophil number, were gated in both groups of mice (irradiated with pegfilgrastim treatment and unirradiated with PBS treatment), we conclude that PMA is capable of inducing the expression of CD11b and CD18 in a higher percentage of neutrophils in the pegfilgrastim treated irradiated animals. Expression of integrins CD11b and CD18 are essential for migration of activated neutrophils. Although Rhodamine 123 expression was not significantly different after PMA stimulation in both animal groups, the expression of this marker was comparable between both groups, suggesting that comparable numbers of neutrophils are able to produce ROS in both the unirradiated mice treated with PBS and the irradiated mice treated with pegfilgrastim. These data confirm that the circulating neutrophil population in irradiated animals treated with pegfilgrastim is indeed physiologically active.

There are a limited number of studies on the effects of pegfilgrastim on neutrophil activity in either animals or patients. The majority of the studies have been performed with the non-pegylated forms of rG-CSF and the results have been conflicting. Some authors have reported enhancement of neutrophil functions in terms of phagocytosis, superoxide anion generation, expression of CD11b/CD18 and bacterial killing (39–42), while others report reduced chemotaxis and altered expression of surface markers (43–45). In general, treatment with the non-pegylated forms of rG-CSF has been shown to result in a modest decrease in the incidence of febrile neutropenia, with no significant impact on overall survival (46). Few studies have been conducted with pegfilgrastim to assess neutrophil function. In a comparison study of three forms of rG-CSF, Ribeiro *et al.* (47) have shown that treatment with filgrastim caused a significant decrease in neutrophil chemotaxis and expression of CD11b, whereas treatment with pegfilgrastim or lenograstim (a glycosylated, non-pegylated rG-CSF) resulted in a small, but not significant, decrease in the expression of CD11b, with no significant reduction in chemotaxis. A slight reduction in neutrophil ROS production was observed after treatment with all three forms of rG-CSF (47). Two other studies suggested that there could be changes in neutrophil chemotaxis with pegfilgrastim treatment, as evidenced by the observed abnormalities in actin polymerization in breast cancer patients undergoing chemotherapy and treated with pegfilgrastim (48, 49). However, it is not clear whether this effect was due to chemotherapy or to pegfilgrastim treatment, since decreased neutrophil chemotaxis, which is associated with defects in actin polymerization (48), has also been reported with chemotherapy alone (50). In cancer patients undergoing chemotherapy, significantly fewer episodes of febrile neutropenia and neutropenia-related hospitalizations have been reported after pegfilgrastim treatment than after filgrastim treatment (51–54), suggesting that pegfilgrastim is a better choice for the treatment of neutropenia. The results described in the present study indicate that pegfilgrastim has the potential to be an effective countermeasure for neutropenia expected after SPE radiation exposure. Further studies are needed to determine whether pegfilgrastim treatment can also prevent or reduce the number of infections in mice exposed to SPE-like proton radiation. Also, it is necessary to evaluate other agents that have effects similar to those observed for

pegfilgrastim but can be administered by routes other than injections, which may be safer and easier to provide in a space setting.

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FIG. 1.
The depth-dose profile of the SPE-like protons in water.

FIG. 2.

Effect of 2 Gy of SPE-like proton or 2 Gy of γ radiation as a function of time on circulating neutrophil counts in mice. Neutrophil numbers from irradiated mice were normalized with neutrophil numbers from corresponding controls from each day and the results are expressed as fraction of control. Results are from a representative experiment from two independent experiments that were performed with similar results (* $P < 0.05$ for a comparison of samples from mice exposed to 2 Gy of γ rays or 2 Gy of SPE-like protons with corresponding samples from unirradiated control mice, by one-way ANOVA followed by Tukey's test, $n = 5$ mice for each treatment group at each time point).

FIG. 3.

Duration of filgrastim (panel A) or pegfilgrastim (panel B) effects on neutrophils from control mice or mice exposed to 2 Gy of γ radiation. Unirradiated and irradiated mice were treated with PBS, filgrastim (300 $\mu\text{g/kg}$) or pegfilgrastim (600 $\mu\text{g/kg}$) on day 3 postirradiation, and then neutrophil counts were determined for up to 8 days postirradiation. Results are from a representative experiment from two independent experiments that were performed with similar results ($*P < 0.05$ for the comparison of samples from irradiated mice treated with filgrastim or pegfilgrastim with corresponding samples from irradiated mice treated with PBS, $^{\#}P < 0.05$ for a comparison of samples from unirradiated control mice treated with filgrastim or pegfilgrastim with corresponding samples from unirradiated control mice treated with PBS, and $^{\forall}P < 0.05$ for a comparison of samples from irradiated mice treated with PBS to unirradiated control mice treated with PBS, by a t test. Bars represent standard deviation, $n = 5$ mice for each treatment group at each time point).

FIG. 4.

Effect of pegfilgrastim administered at different times relative to a 2 Gy of γ radiation exposure in mice. Mice were irradiated with 2 Gy of γ rays, and injected, s.c., with either PBS or pegfilgrastim (600 μ g/kg) at the times indicated. Unirradiated mice treated with PBS were included as controls. Blood samples were collected on day 3 after the pegfilgrastim administration for analysis. Results are from a single experiment (* $P < 0.05$ for a comparison of samples from irradiated mice treated with pegfilgrastim to corresponding samples from irradiated mice treated with PBS, # $P < 0.05$ for a comparison of samples from unirradiated control mice treated with pegfilgrastim to corresponding samples from unirradiated control mice treated with PBS, and ‡ $P < 0.05$ for a comparison of samples from irradiated mice treated with PBS to corresponding samples from unirradiated control mice treated with PBS, by a t test. Bars represent standard deviation, $n = 5$ mice for each treatment group). Results of unirradiated control mice treated with pegfilgrastim (600 μ g/kg) at 3 days after pegfilgrastim administration from Fig. 3 have been incorporated into this figure for comparison with the other treatment groups.

FIG. 5.

Relationship between the pegfilgrastim dose and neutrophil counts in irradiated mice. Mice were irradiated with 2 Gy of γ rays and then injected with pegfilgrastim at the doses indicated immediately after radiation exposure. Blood samples were collected 3 days after the pegfilgrastim administration for analysis. Results are from a single experiment [$*P < 0.05$ for a comparison of samples from irradiated mice treated with pegfilgrastim (at doses of 600, 300 and 150 $\mu\text{g/kg}$) to corresponding samples from irradiated mice treated with PBS, $\#P < 0.05$ for a comparison of unirradiated control mice treated with pegfilgrastim compared to corresponding samples from unirradiated control mice treated with PBS, $\text{¥}P < 0.05$ for a comparison of samples from irradiated mice treated with PBS with corresponding samples from unirradiated control mice treated with PBS and $\text{£}P < 0.05$ for a comparison of samples from irradiated mice treated with pegfilgrastim (at doses of 600, 300 and 150 $\mu\text{g/kg}$) to corresponding samples from unirradiated control mice treated with PBS, by a t test. Bars represent the standard deviation, $n = 5$ mice for each treatment group]. Results of unirradiated control mice treated with pegfilgrastim (600 $\mu\text{g/kg}$) at 3 days after pegfilgrastim administration from Fig. 3 have been incorporated into this figure for comparison with the other treatment groups.

FIG. 6.

Effect of multiple injections of pegfilgrastim on neutrophil counts in irradiated animals. Mice were irradiated with 2 Gy of γ rays and injected with PBS or pegfilgrastim at the times and doses indicated. Blood samples were collected at the times indicated for analysis. Results are from a representative experiment from two independent experiments that were performed with similar results. (* $P < 0.05$ for a comparison of results from samples taken from irradiated mice with pegfilgrastim treatment to the results from corresponding samples from irradiated mice treated with PBS, by a t test. Bars represent standard deviation, $n = 8$ mice for each treatment at each time point.)

FIG. 7.

Effect of pegfilgrastim administration on neutrophil counts in mice irradiated with 2 Gy of SPE-like proton radiation compared to the effect on neutrophil counts in mice exposed to 2 Gy of γ radiation. Mice were exposed to either 2 Gy of γ rays or 2 Gy of SPE-like proton radiation and treated with either PBS or pegfilgrastim at the indicated time and doses. Neutrophil counts were determined at the indicated times and expressed as fraction of control. (* P < 0.05 for a comparison of results from samples from γ -irradiated mice treated with pegfilgrastim with corresponding samples from γ -irradiated mice treated with PBS, # P < 0.05 for a comparison of results from samples from SPE-like proton irradiated mice treated with pegfilgrastim with corresponding samples from proton-irradiated mice treated with PBS, by a t test. Bars represent standard deviation, n = 5 mice for each treatment group at each time point.)

whole blood from unirradiated control mouse

FIG. 8.

Identification of the neutrophil population. Whole blood samples were analyzed by light scatter. The neutrophil subpopulation was selected based on its forward- and side-scatter distribution in the dot plot. The same exact rectangular gate, which selected for the neutrophil subpopulation, was applied to each of the dot blots for further functional analyses.

FIG. 9.

Determination of neutrophil activation after PMA stimulation. Blood samples collected from unirradiated mice or mice irradiated with 2 Gy of γ rays were stimulated by PMA treatment. First, whole blood samples were gated for the neutrophil population (shown in Fig. 8; for details see Materials and Methods section). From the gated population in each sample, the percentage of cells expressing CD11b (panel A), CD18 (panel B) or Rhodamine 123 (panel C) after PMA stimulation in both the unirradiated control group treated with PBS (black bar) and the irradiated group treated with pegfilgrastim (gray bar) were determined. The bar graphs represent the average percentage of cells expressing each surface marker from 3 experimental replicates [$\pm$ standard deviation (SD)]. The results shown represent one of two independent experiments performed with similar results (* $P < 0.05$, for a comparison between PMA stimulated samples isolated from unirradiated mice treated with PBS and PMA stimulated samples from irradiated mice treated with pegfilgrastim, by a t test.).

TABLE 1

Dose-Response Curve Slopes and Proton RBE Estimates for Neutrophils^a

Time (days)	Slope of the dose-response curves for γ rays			Slope of the dose-response curves for protons				RBE	Significantly different from 1.0?
	Fitted value	95% CI		Fitted value	95% CI				
		Lower limit	Upper limit		Lower limit	Upper limit			
1	0.594	0.375	0.813	0.319	0.135	0.503	0.5	yes	
2	0.242	0.132	0.351	0.278	0.107	0.450	1.2	no	
4	0.860	0.582	1.138	0.703	0.555	0.850	0.8	no	
6	0.278	0.168	0.388	0.331	0.216	0.446	1.2	no	
8	0.258	0.143	0.372	0.271	0.163	0.379	1.1	no	
10	0.134	-0.017	0.284	0.191	0.032	0.349	1.4	no	
13	0.435	0.311	0.559	0.495	0.351	0.640	1.1	no	
16	0.899	0.681	1.117	0.806	0.564	1.047	0.9	no	
19	0.549	0.233	0.864	0.430	0.178	0.682	0.8	no	
23	0.123	0.014	0.231	0.116	0.017	0.215	0.9	no	
30	-0.108	-0.229	0.013	0.048	-0.084	0.181	NM ^b	no	

^aThe dose-response curves were established by fitting the neutrophil count data (expressed as fraction of control) using an exponential decay model: $y = e^{-\alpha D}$, where α is the slope parameter and D is the radiation dose (Gy).

^bNot meaningful due to the insignificant dose response on day 30.