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Single and Combination Cytokine Therapies for Treatment of Radiation-induced Hematopoietic Injury: Effects of *c-kit* Ligand and Interleukin-3

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Abstract

Following radiation exposures, severe hematopoietic depression can result from injury to hematopoietic stem and progenitor cell populations. In recent years, a variety of recombinant cytokines have been demonstrated to possess hematopoietic activity. While some cytokines are lineage restricted in their activity, others such as *c-kit* ligand and interleukin-3 (IL-3) appear to be capable of affecting early multilineage hematopoietic cell populations. Using a $B_6D_2F_1$ murine model of severe ^{60}Co radiation-induced hematopoietic hypoplasia, we have evaluated the ability of *c-kit* ligand (recombinant murine mast cell growth factor; rmMGF) and rmIL-3 to accelerate hematopoietic regeneration when administered either alone or in combination following radiation exposure. Hematopoietic regeneration was based on spleen and bone marrow spleen colony forming unit ($CFU\text{-}s_{12}$) and granulocyte-macrophage progenitor cell (GM-CFC) recoveries. MGF alone, administered subcutaneously (s.c) on days 1-17 postirradiation at 100 µg/kg/day or 200 µg/kg/day, accelerated bone marrow and splenic GM-CFC as well as splenic CFU-s recoveries in a direct dose-dependent manner. IL-3 alone (100 µg/kg/day, s.c. on days 1-17)

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also accelerated splenic GM-CFC and CFU-s recoveries. When these cytokines were co-administered (100 μg/kg/day each, s.c. on days 1–17), GM-CFC and CFU-s recoveries greater than those produced by either cytokine alone were observed. These studies illustrate a potential role for combined MGF and IL-3 in the treatment of radiation-induced hematopoietic injury.

Introduction

One of the most recent cytokines implicated in hematopoietic regulation is c-*kit* ligand, also known as mast cell growth factor (MGF), steel factor (SLF), and stem cell factor (SCF) (1–3). The c-*kit* ligand has been ascribed numerous hematopoietic and nonhematopoietic effects, although it was initially identified and purified based on its ability to stimulate mast cell growth (2–5). Multiple studies have focused on the *in vitro* effects of this factor, demonstrating that alone it has limited hematopoietic activity, but when combined with other hematopoietic cytokines, it synergizes to increase both the number and size of colonies generated from hematopoietic progenitors (3–11), and in some instances, to increase the replating potential of primitive progenitors (12). Furthermore, in combination with such factors, c-*kit* ligand also synergistically enhances the *in vitro* expansion of hematopoietic progenitors grown in liquid cultures (13–15). These effects are thought to result not only from the ability of c-*kit* ligand to potentiate progenitor cell proliferation but also from its ability to enhance progenitor cell survival (14,16).

IL-3, also known as multi-CSF, has previously been shown to enhance hematopoietic regeneration in irradiated animals based on recovery of peripheral blood white cells and platelets (17). Because *in vitro* studies have demonstrated synergistic hematopoietic stimulation produced by c-*kit* ligand combined with IL-3, we evaluated whether co-administration of these cytokines *in vivo* would synergize to further accelerate hematopoietic regeneration following radiation-induced hematopoietic hypoplasia.

Materials and methods

Cytokines

Recombinant murine c-*kit* ligand (MGF), was provided by Immunex (Seattle, WA). Recombinant murine IL-3 was provided by Behringwerke AG (Marburg, Germany). Cytokines were expressed in yeast and purified to homogeneity as previously described (10,18). Endotoxin contamination of cytokines was below the limit of detection using the Limulus amebocyte lysate assay. MGF was administered subcutaneously (s.c.) in a 0.1 ml volume at the dose of 100 or 200 μg/kg; IL-3 was administered s.c. at the dose of 100 μg/kg. In combination studies, mice received each cytokine at a separate injection site. All injections

were initiated 1 day following irradiation and continued daily for 17 days. Control mice were injected with an equal volume of sterile saline.

MICE

$B_6D_2F_1$ female mice (~20 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in an AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited facility in Micro-Isolator cages on hardwood-chip contact bedding and were provided commercial rodent chow and acidified water (Ph 2.5) *ad libitum*. Animal rooms were equipped with full-spectrum light from 6 a.m. to 6 p.m. and were maintained at 70°F +/- 2°F with 50% +/- 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were tested for *Pseudomonas* and quarantined until test results were obtained. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute Animal Care and Use Committee prior to performance.

IRRADIATION

The ^{60}Co source at the Armed Forces Radiobiology Research Institute was used to administer bilateral total-body gamma radiation. Mice were placed in ventilated Plexiglas containers and irradiated with 7.75 Gy at a dose rate of 0.4 Gy/min. Dosimetry was performed using ionization chambers (19) with calibration factors traceable to the National Institute of Standards and Technology. The tissue-to-air ratio was determined to be 0.96. Dose variation within the exposure field was < 3%.

CELL SUSPENSIONS

Cell suspensions for each assay represented tissues from three normal, irradiated, or irradiated and cytokine-treated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A medium (Flow Labs, McLean, VA) containing 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT). Spleens were pressed through a stainless steel mesh screen, and the cells were washed from the screen with 6 ml medium. The number of nucleated cells in the suspensions was determined by Coulter counter. Femurs and spleens were removed from mice euthanized by cervical dislocation.

SPLEEN COLONY FORMING UNIT ASSAY

Exogenous spleen colony forming units (CFU-s) were evaluated by the method of Till and McCulloch (20). Recipient mice were exposed to 9 Gy of total-body radiation to reduce endogenous hematopoietic stem cells. Bone marrow or

spleen cells were intravenously (i.v.) injected into the irradiated recipients 3-5 h later. Twelve days after transplantation, the recipients were euthanized by cervical dislocation, and their spleens were removed. The spleens were fixed in Bouin's solution, and grossly visible spleen colonies were counted. Each treatment group consisted of five mice.

GRANULOCYTE-MACROPHAGE COLONY-FORMING CELL ASSAY

Hematopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a double-layer agar granulocyte-macrophage colony forming cell (GM-CFC) assay in which mouse endotoxin serum (5% v/v) was added to feeder layers as a source of colony stimulating factors (21). Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 5% CO_2. Triplicate plates were cultured for each cell suspension.

STATISTICS

Results of replicate experiments were pooled and are represented as the mean +/- standard error (SE) of pooled data. Statistical differences were determined by Behrens-Fisher t-test analysis. Significance level was set at $p < 0.05$.

Results

The ability to accelerate hematopoietic regeneration in a murine model of severe radiation-induced hematopoietic hypoplasia was used to evaluate the potential of MGF and IL-3 to induce hematopoietic progenitor cell expansion *in vivo*. In preliminary studies, it was determined that a sublethal 7.75 Gy ^{60}Co radiation exposure induced severe hematopoietic hypoplasia from which recovery (especially in the spleen) became evident between days 14 and 17 post-irradiation (Figure 7.1). Based on these preliminary studies, subsequent studies evaluating the ability of cytokines to accelerate hematopoietic recovery focused on evaluation of bone marrow and splenic CFU-s and GM-CFC recoveries on days 14 and 17 post-irradiation.

The effects of MGF on CFU-s and GM-CFC recoveries in sublethally irradiated mice are illustrated in Figure 7.2 and Figure 7.3, respectively. At either the 100 µg/kg/day or 200 µg/kg/day dose, MGF alone accelerated bone marrow and splenic GM-CFC recovery. The 200 µg/kg/day MGF dose also accelerated splenic CFU-s recovery; however, no effect on bone marrow CFU-s recovery was observed at either MGF dose. Following administration of IL-3 alone, both splenic CFU-s (Figure 7.4B) and GM-CFC (Figure 7.5B) numbers were increased compared to saline-treated mice by day 17 postirradiation. No

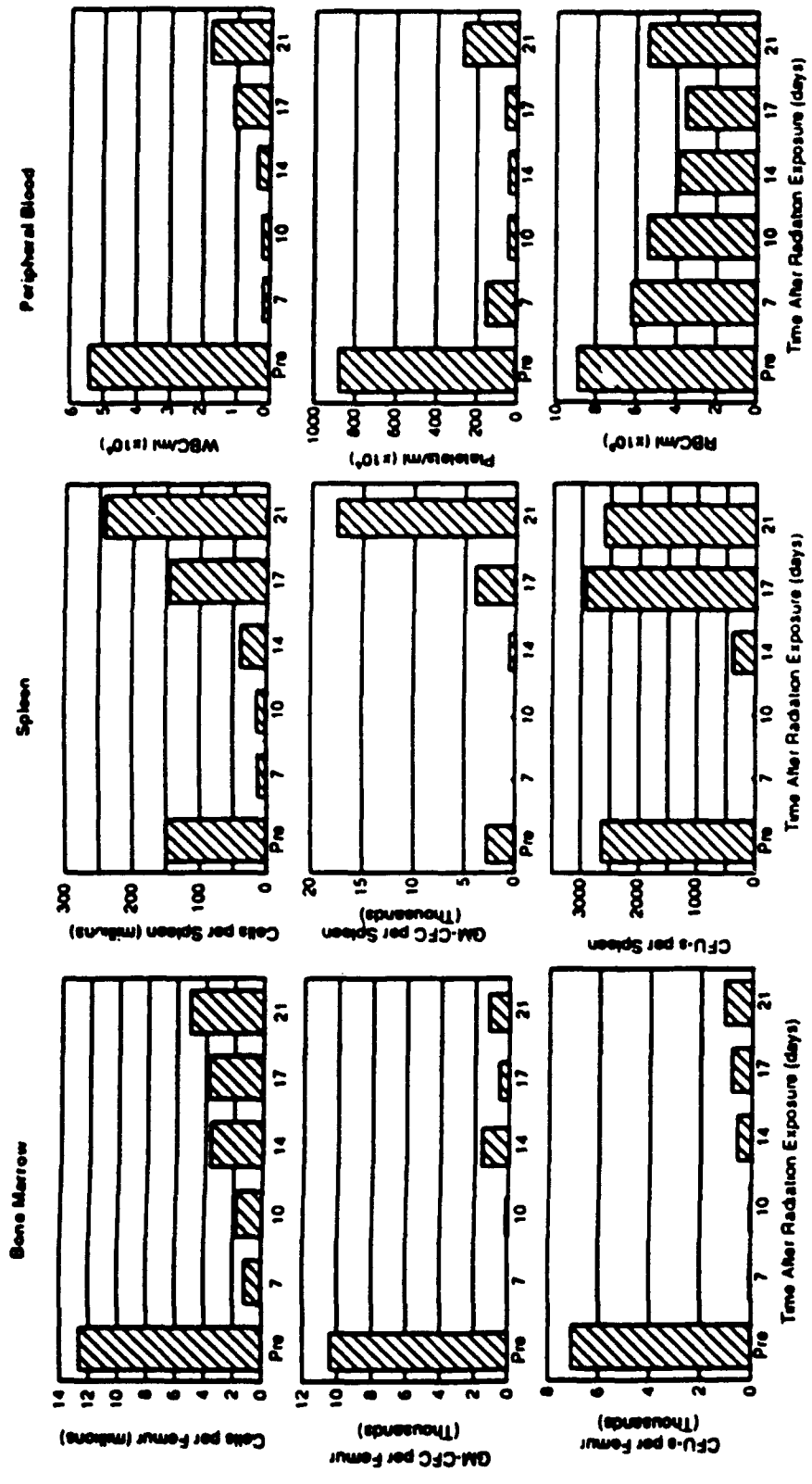
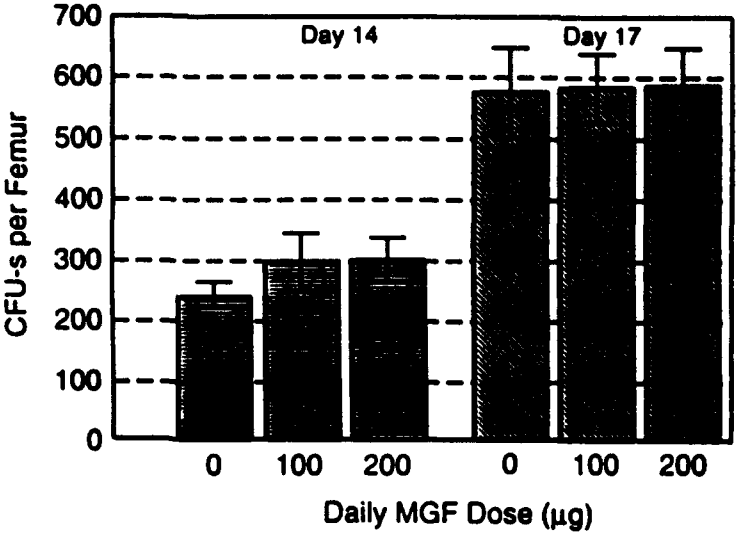
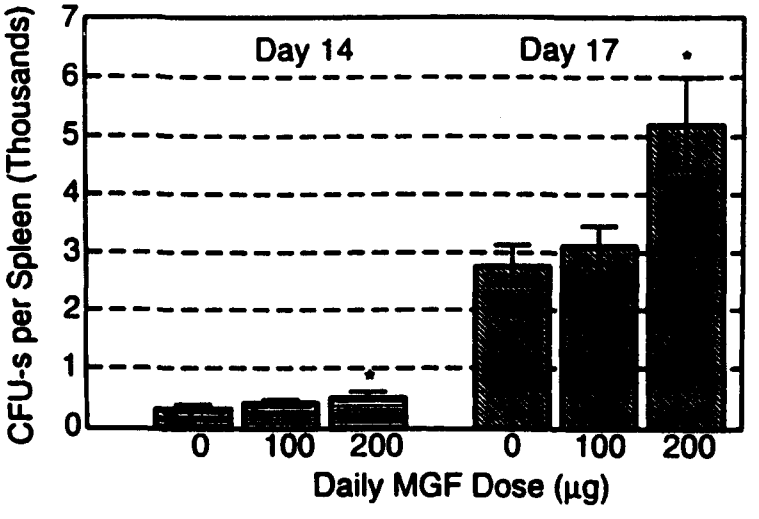


Figure 7.1 Hematopoietic suppression and recovery following a sublethal 7.75 Gy ^{60}Co radiation exposure in $B_6D_2F_1$ mice.

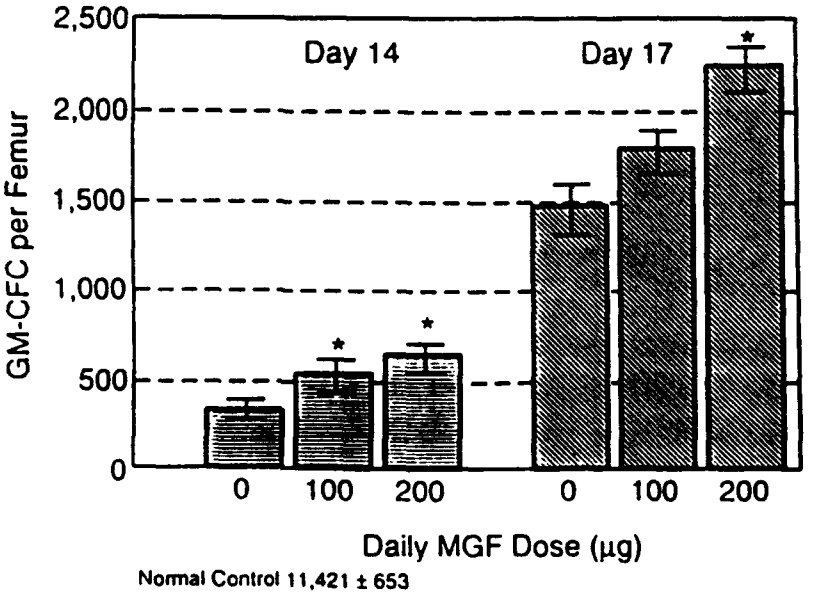


Normal Control 7,223 ± 150



Normal Control 2,722 ± 100

Figure 7.2 Effects of MGF administration (100 or 200 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) CFU-s recovery on days 14 and 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean +/- SE; * $p < 0.05$, with respect to saline controls.



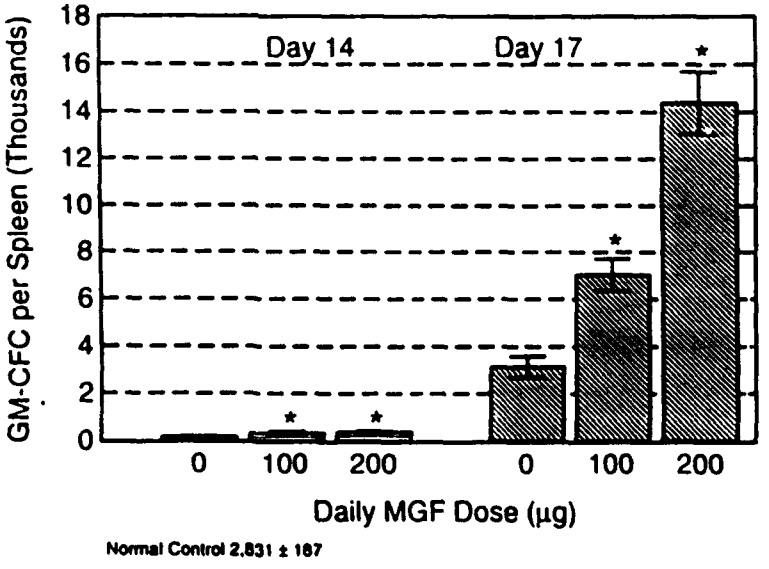
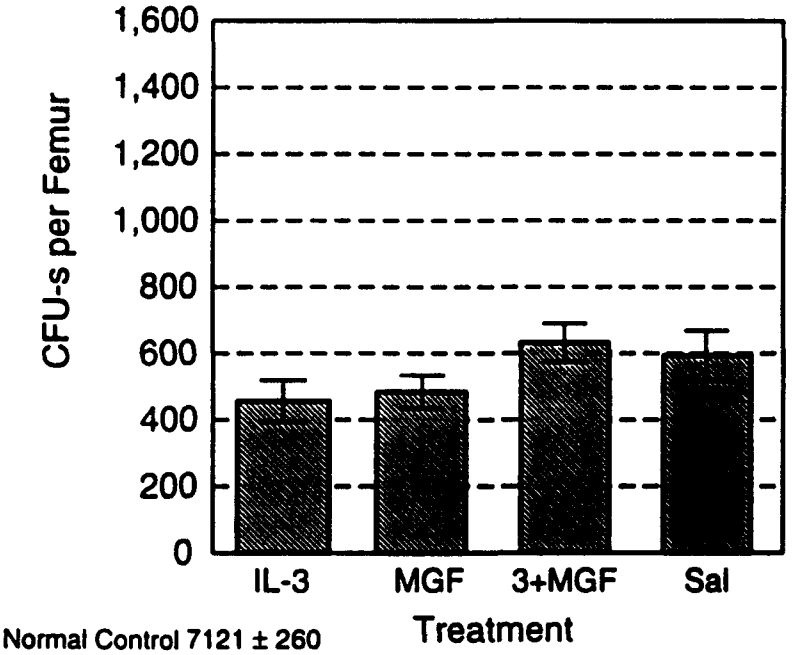


Figure 7.3 Effects of MGF administration (100 or 200 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) GM-CFC recovery on days 14 and 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean +/- SE; * $p < 0.05$, with respect to saline controls.



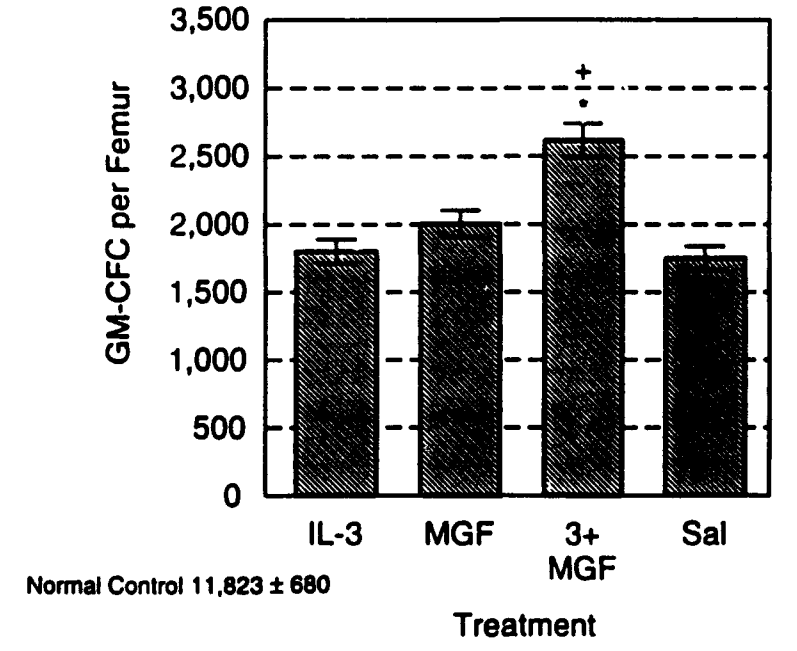
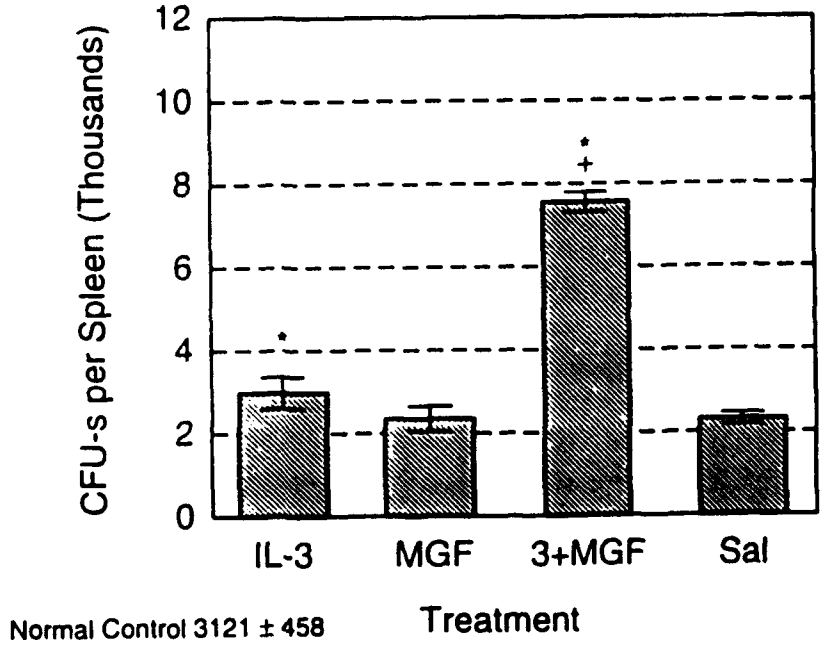


Figure 7.4 Effects of IL-3, MGF, and MGF plus IL-3 (each 100 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) CFU-s recovery on day 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean +/- SE; * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to IL-3 values.



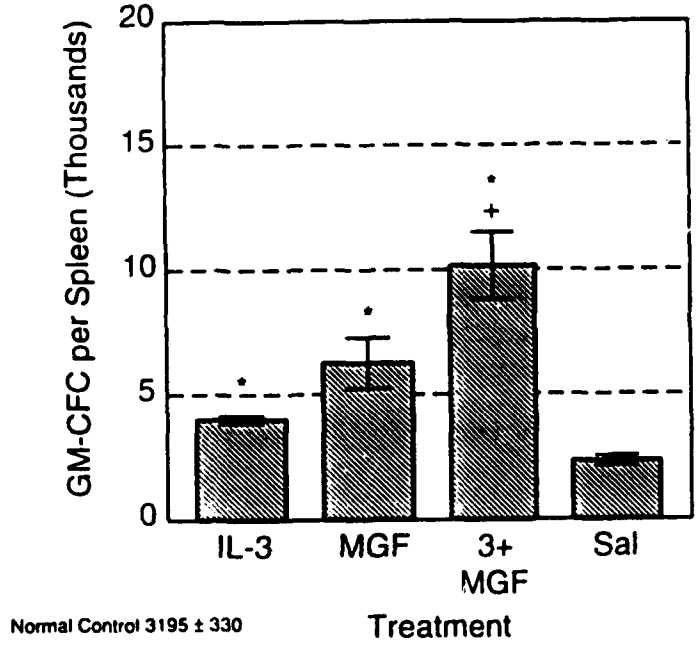


Figure 7.5 Effects of IL-3, MGF, and MGF plus IL-3 (each 100 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) GM-CFC recovery on day 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean +/- SE; * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to MGF values.

IL-3 effects, however, were observed on bone marrow recovery. When MGF was administered to sublethally irradiated mice in combination with IL-3, CFU-s (Figure 7.4) and GM-CFC (Figure 7.5) recoveries greater than those induced by MGF alone or IL-3 alone were observed, with splenic effects being more dramatic than bone marrow effects.

Discussion

Sustained hematopoietic recovery following chemotherapy or radiation exposure requires surviving pluripotent stem cells to self-renew as well as to differentiate into multipotent and committed progenitors capable of giving rise to functional mature cells. In recent years, administration of single hematopoietic growth factors, including G-CSF, GM-CSF, MGF, and IL-6, has been shown to stimulate hematopoietic regeneration following radiation- or chemotherapy-induced myelosuppression. In addition, some cytokine combinations, such as GM-CSF plus IL-3, have proven to surpass the effectiveness of single agents. Because c-*kit* ligand, *in vitro*, has been shown to synergize with IL-3 in stimulating progenitor cell proliferation and expansion, we hypothesized that administration of MGF in combination with this cytokine *in vivo* may further improve hematopoietic regeneration beyond that obtained with only MGF or only IL-3.

Our studies demonstrate that in irradiated mice:

1. MGF alone can accelerate hematopoietic regeneration.
2. IL-3 alone can also accelerate hematopoietic regeneration, and
3. when MGF and IL-3 are co-administered, hematopoietic recovery greater than that produced by either single cytokine can be obtained.

It remains to be determined whether the apparent lack of effect of MGF, IL-3, or the combination of these cytokines on bone marrow CFU-s regeneration concomitant with significant splenic CFU-s regeneration may be due to cytokine mediated bone marrow CFU-s mobilization. In spite of this, these studies suggest a potential role for combined MGF plus IL-3 in the treatment of hematopoietic hypoplasia.

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