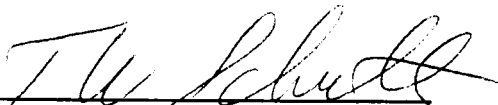
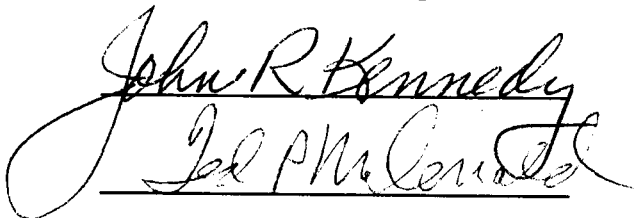


To the Graduate Council:

I am submitting herewith a thesis written by Ronnie Wayne Culberson entitled "Modification Of Splenic Megakaryocytopoiesis In Sublethally Irradiated Mice By A Thrombocytopoiesis Stimulating Factor". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.


T. W. Schultz, Major Professor

We have read this thesis
and recommend its acceptance:




Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate School

**MODIFICATION OF SPLENIC MEGAKARYOCYTOPOIESIS IN
SUBLETHALLY IRRADIATED MICE BY A THROMBOCYTOPOIESIS-
STIMULATING FACTOR**

A Thesis
Presented for the
Master of Science
Degree
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Ronnie Wayne Culberson

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ABSTRACT

The radioprotective effects of TSF (thrombocytopoiesis-stimulating factor or thrombopoietin) have been studied in irradiated mice in relation to bone marrow megakaryocytopoiesis, but not splenic megakaryocytopoiesis. The work reported here was undertaken to determine the effects of TSF on splenic megakaryocytopoiesis in mice exposed to 3.0 Gy of ^{137}Cs . Mice were given Step II TSF 1 hour before and 8 hours after whole-body irradiation for a total of 4 U of TSF per mouse. For controls, additional mice were injected with human serum albumin (HSA), normal rabbit serum (NRS), or rabbit anti-mouse platelet serum (RAMPS). Blood was collected from each mouse on days 10 and 14 after irradiation for measurement of platelet counts, $\%^{35}\text{S}$ incorporations into platelets, platelet sizes, packed cell volumes (PCV), RBC counts, and WBC counts. Spleens were removed and processed for measurement of megakaryocyte size and number. Severe thrombocytopenia and leukopenia were observed on days 10 and 14, but severe anemia was not present on either day. TSF caused significant increases in platelet count ($P < 0.005$), $\%^{35}\text{S}$ incorporation into platelets ($P < 0.025$) and WBC count ($P < 0.005$) on day 10 as compared to the values of mice treated with HSA. Similarly on day 10, RAMPS caused significant increases above NRS-treated mice in platelet count ($P < 0.005$), $\%^{35}\text{S}$ incorporation into platelets ($P < 0.0005$), WBC count ($P < 0.05$), platelet size ($P < 0.0005$) and PCV ($P < 0.025$). As compared to their irradiated controls, both TSF and RAMPS caused significant increases ($P < 0.0005$) on day 10 in total megakaryocyte mass (TMM) and megakaryocyte number, but neither significantly increased megakaryocyte diameter on day 10. Neither TSF nor RAMPS significantly increased the peripheral blood measurements, megakaryocyte numbers, or TMM on day 14 as compared to their irradiated controls. TSF significantly increased ($P < 0.05$) splenic

megakaryocyte diameter above HSA controls on day 14. The present studies showed that TSF caused significant increases above irradiated control mice in platelet count, ^{35}S incorporation into platelets, splenic megakaryocyte number, and TMM on day 10, and caused significant increases in splenic megakaryocyte diameter on day 14. These results show that TSF is an effective radioprotective agent and could be helpful in elevating platelet production in patients subjected to chemotherapy or radiation treatments.

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LIST OF ABBREVIATIONS

BFU-Mk, burst forming unit-megakaryocyte

CFU-Mk, colony forming unit-megakaryocyte

EDTA, ethylenediaminetetraacetic acid

HEK, human embryonic kidney

HSA, human serum albumin

IL-6, interleukin-6

Meg-CSF, megakaryocyte-colony stimulating factor

NRS, normal rabbit serum

PCV, packed cell volume

PRP, platelet-rich plasma

RAMPS, rabbit anti-mouse platelet serum

RBC, red blood cell

SACHe+, small acetylcholinesterase-positive

TMM, total megakaryocyte mass

TSF, thrombocytopoiesis-stimulating factor

VIP, Vacuum Infiltration Processor

WBC, white blood cell

1. INTRODUCTION

The effects of radiation on the thrombocytopoietic system of rodents has been well studied. It is known that radiation exposure leads to decreases in platelet count [1-9], platelet production [2,8], medullary megakaryocyte number [3-6,10], and increases in medullary megakaryocyte size [3,4]. In addition, studies using TSF (thrombocytopoiesis-stimulating factor or thrombopoietin) from human embryonic kidney (HEK) cell culture medium [11] or platelet specific antiserum [7-9,11] to induce acute, severe thrombocytopenia, have shown that TSF decreases the severity and duration of radiation-induced thrombocytopenia by enhancing platelet production in the irradiated mice. Both TSF and platelet specific antiserum are known to stimulate thrombocytopoiesis and megakaryocytopoiesis in normal mice [12]. Furthermore, the evaluation of megakaryocytes in the bone marrow of the irradiated mice treated with TSF showed that the enhanced platelet production was associated with dramatic increases in megakaryocyte size but marked decreases in megakaryocyte numbers [11]. However, the effects of TSF on splenic megakaryocytes of irradiated mice have not been investigated.

The purpose of the present investigation was to examine splenic megakaryocytopoiesis in sublethally-irradiated mice treated with TSF or rabbit anti-mouse platelet serum (RAMPS). Platelet production was measured by determining platelet counts, platelet sizes, and ^{35}S incorporation into platelets [13]. Splenic megakaryocytopoiesis was measured by evaluating splenic megakaryocyte size and numbers, and the total megakaryocyte mass (TMM) [14]. The present study shows that TSF caused an increase in platelet counts, enhanced ^{35}S incorporations into platelets, increased splenic megakaryocyte numbers and sizes, and elevated TMM in mice exposed to a sublethal dose of radiation. In addition to previous work [7-9,11],

these results further demonstrate that TSF will stimulate thrombocytopoiesis and megakaryocytopoiesis in irradiated mice. These findings indicate that TSF may be an effective radioprotective agent, which could be used to raise platelet counts in cancer patients undergoing radiation therapy.

2. BACKGROUND

Megakaryocytes

Megakaryocytes, the large polyploid cells responsible for the formation and release of blood platelets, represent only a small percentage of the cells in the mammalian bone marrow [15,16]. The ploidy of normal bone marrow megakaryocytes from most species has been shown to be 8N to 32N (i.e., 4 to 16 nuclei) [17-19] with the modal ploidy being 16N [19]. Jackson et al., however, have shown that the normal bone marrow megakaryocytes from mice of the C3H strain have a higher modal ploidy (i.e., 32N) than most other mouse strains, but have similar platelet counts, megakaryocyte sizes, and megakaryocyte numbers [19].

In contrast to the high ploidy of mouse bone marrow megakaryocytes, the ploidy of megakaryocytes from the mouse spleen was reported to be much lower. Davis et al. [20] have reported that the modal ploidy of splenic megakaryocytes in normal C57BL/6J mice was only 2N. However, Cullen and McDonald have shown that the diameters of splenic megakaryocytes from male C3H mice were not significantly different from bone marrow megakaryocytes [21]. If splenic megakaryocytes were lower in ploidy than bone marrow megakaryocytes, one would not expect splenic megakaryocytes to be similar in size to bone marrow megakaryocyte, since it has been shown that the diameter and ploidy of megakaryocytes are related [17,18]. Therefore, it is unlikely that the modal ploidy of murine splenic megakaryocytes is 2N.

Additionally, the large recognizable megakaryocytes do not undergo cellular division, and therefore, their maintenance depends on a continued influx of stem cells from the pluripotent stem cell compartment [22]. To date, the earliest identifiable

progenitor cells of the megakaryocytes have been suggested to be the burst forming unit-megakaryocyte (BFU-Mk) and the colony-forming unit megakaryocyte (CFU-Mk) [23]. Since BFU-Mk derived colonies take longer to appear and produce more megakaryocytes than do colonies derived from CFU-Mk, the BFU-Mk appears to be an earlier committed megakaryocyte progenitor than the CFU-Mk.

In addition to the BFU-Mk and CFU-Mk, workers have identified other cells in the murine bone marrow that appear to be transitional cells in the megakaryocyte lineage [24-26]. These cells are called small acetylcholinesterase-positive (SACHe+) cells (or immature megakaryocytes) and are believed to represent the stage of commitment where the precursor cell leaves the precursor pool to begin endomitosis (nuclear division without cytoplasmic division). It has been shown that these immature megakaryocytes will develop into large single mature megakaryocytes by 60 hours of growth on semisolid agar cultures in the presence of a thrombopoietic stimulating factor (TSF) [26,27], but are not responsive to a megakaryocyte-colony stimulating factor (Meg-CSF) [27,28]. Furthermore, the observation that Meg-CSF primarily promotes the proliferation of CFU-Mk progenitor cells and not their differentiation led to the suggestion that the *in vitro* regulation of megakaryocytopoiesis is controlled by two factors (i.e., Meg-CSF for CFU-Mk proliferation and TSF for megakaryocyte maturation) [22,26,28].

The maturation of the immature megakaryocytes has been proposed to occur in four stages [17,22]. As described by Williams and Levin [22], the Stage I megakaryocytes (6-24 μm) are the earliest megakaryocytes and have the highest nuclear/cytoplasmic ratio. Stage II megakaryocytes (14-30 μm) have a horseshoe-shaped nucleus, a demarcation membrane system, and a nuclear/cytoplasmic ratio still greater than 1:1. Stage III megakaryocytes (16-56 μm) have a large granular

eosinophilic cytoplasm and a developed demarcation membrane system. The Stage IV megakaryocytes (20-50 μm), responsible for platelet production [17,22], have tightly packed nuclei and an eosinophilic cytoplasm. After endomitosis, massive increases in the cytoplasm occur in which platelets eventually form. Levine et al. showed that 80% of the 4N-8N megakaryocytes were immature and 95% of the platelet-shedding megakaryocytes were 16N-32N [17]. Furthermore, maturation and polyploidization appeared to be linked; megakaryocyte size increased with maturation stage (I-IV) within each ploidy group (4N-32N) and megakaryocyte volume doubled with each ploidy doubling ($2 \times N$) within each maturation stage [17].

The mechanism of platelet production and release by megakaryocytes is not well understood. Two models have been suggested: the proplatelet model and the flow model [29]. In both models, the demarcation membrane is associated with cytoplasmic maturation and platelet release. According to the proplatelet model, the demarcation membrane encloses regions of the megakaryocyte cytoplasm that mature and form the platelets. Thereafter, the mature megakaryocyte fragments into proplatelets when platelets are needed. The proplatelets then exit the bone marrow through the marrow sinusoid, enter the circulation, and break down to release their platelets. The flow model suggests that the role of the demarcation membrane is not to "demarcate" platelets, but to provide the additional membrane needed for the formation of megakaryocytic pseudopods which extend into the bone marrow sinusoids for platelet release [29].

Thrombopoietin and Assays Used for Thrombopoietin

TSF is now known to be the major controlling factor of thrombocytopoiesis and megakaryocytopoiesis [12]. An important step toward the elucidation of TSF was the

development of a sensitive bioassay for thrombopoietin. McDonald found that mice are more sensitive to exogenous sources of thrombopoietin in a period of "rebound" thrombocytosis than normal mice [30]. When mice are given a single injection of a platelet specific antiserum, such as RAMPS, acute platelet depletion (thrombocytopenia) results within 4 hours, and, in response to the acute thrombocytopenia, thrombopoietin is released from the kidneys [31,32]. Thereafter, platelet counts quickly return to normal and then "overshoot" the normal values (thrombocytosis) before leveling off to normal levels. Because mice have an increased sensitivity to exogenous TSF given at the time of "rebound" thrombocytosis than normal mice, the immunothrombocythemmic mouse assay has been found to be a reliable assay for thrombopoietin [31].

In addition to platelet counts, the response to exogenous TSF has been measured via platelet size and ^{35}S incorporation into platelets [13,30,33,34]. The ^{35}S incorporation into platelets, however, has been shown to be the most sensitive indicator of increased platelet production [13]. Since ^{35}S is chiefly incorporated into new platelets, the ^{35}S incorporation assay detects the slightest increase of thrombocytopoietic activity. In addition, not only do new platelets incorporate ^{35}S but they also tend to be larger in size [13,34]. Although not as sensitive as platelet count or ^{35}S platelet incorporation, platelet size is frequently used as an indicator of stimulated thrombocytopoiesis. Additionally, megakaryocyte size and number [35,36], megakaryocyte DNA content [37], and measurement of SACH E^{+} cells [38,39] have been used as assays for the effects of thrombopoietin on megakaryocytopoiesis. TSF from HEK cell culture media has been shown to increase all seven parameters mentioned above to the same degree or near the same degree as

seen in RAMPS-treated animals [13,30,33-39]. However, studies show that the main effect of TSF is to increase the maturation of immature megakaryocytes [26,27,40].

Effects of Radiation on Thrombocytopoiesis and Megakaryocytopoiesis (Bone Marrow)

The effects of radiation on the thrombocytopoietic system of rodents has been well studied. It is known that radiation exposure initially leads to decreases in bone marrow megakaryocyte number [3-6,10], followed by decreases in platelet counts [1-9], platelet production [2,8], and increases in bone marrow megakaryocyte size [3,4]. Simpson [6] found that after rats were exposed to radiation there was first a suppression in megakaryoblasts, followed by a decrease in basophil megakaryocytes, then a reduction in granular and mature megakaryocytes, and finally a decrease in peripheral platelets. Odell et al. [5] reported similar results and suggested that radiation primarily damages the proliferating megakaryocyte progenitors. Therefore, megakaryocytes in the endomitotic phase of maturation are insensitive to radiation damage [4-7]. Moreover, immature megakaryocytes exposed to radiation may prematurely enter endomitosis and thus, continue to mature and release their platelets [5]. Megakaryocytes induced to enter prematurely the into endomitotic phase of growth at a higher ploidy level (4N), may be the reason for the megakaryocytic macrocytosis often seen in irradiated animals [4]. Therefore, radiation exposure reduces the megakaryocyte population by damaging the proliferating megakaryocytic progenitors [4-7].

Effects of Radiation on Thrombocytopoiesis and Megakaryocytopoiesis (Splenic)

Some of the first work using radiation to study splenic hematopoiesis was done by Jacobson and colleagues [1,41]. They found that if the spleens of mice given 6.0 Gy of whole-body irradiation were protected with lead boxes, there was a rapid transfer (within 24 to 48 hours) of erythropoiesis from the damaged bone marrow to the spleen with the mean erythrocyte, hemoglobin, and hematocrit values not being significantly different from controls. The mean platelet count in the unprotected mouse fell slowly to a low level of 15,000 per mm^3 on day 8 post irradiation and did not reach a normal value until day 18 post-irradiation. However, in mice with lead-protected spleens, the platelet count fell to a low level of only 230,000 per mm^3 on day 9 and reached a normal value by day 11 post-irradiation. It was found that the bone marrow and unprotected spleens became aplastic after whole body irradiation [1,41].

In irradiated mice with lead protection of the spleen, granulocytopoiesis and megakaryocytopoiesis were significantly increased as compared to irradiated mice without lead protection of the spleen. Furthermore, as erythropoiesis, granulopoiesis, and megakaryocytopoiesis in the spleen increased, splenic lymphatic tissue decreased to almost 75% less than controls [1]. Even when the whole-body radiation levels were 10.25 Gy, there was regeneration of the hematopoietic tissues of mice with protected spleens [41]. In contrast, there was not any apparent regeneration of hematopoietic tissues in unprotected mice even 10 days after 10.25 Gy of radiation. Although Cole et al. [42] were able to show that hematopoietic cells from the lead-protected spleens were responsible for the repopulation of hematopoietic tissues observed in the irradiated mice, it has also been observed that recovery begins earlier after whole-body irradiation in rats when one hind-leg was covered with a lead shield

[6]. In fact, the spleen did not appear to play any significant role in hematopoietic recovery of unprotected mice exposed to whole-body irradiation [2,3].

In addition to these studies, Ebbe and Phalen reported an apparent increase in megakaryocyte number in the spleens of CF₁ mice given 6.5 Gy of whole-body irradiation [4]. Megakaryocyte number was counted in 12 μ m thick sections taken from the center of the spleen. The number of splenic megakaryocytes was found to be elevated following recovery from irradiation in one experiment and 29 days after radiation-induced megakaryocytopenia in another experiment. In contrast, tibial megakaryocytes decreased 50-80% from day 29 through day 70 after radiation with platelet counts remaining normal or falling only 20% of normal [4]. The maintenance of normal or near normal platelet counts was found to be reflective of increased tibial megakaryocyte size of 130-150% of controls. The contribution to platelet production by the splenic megakaryocytes in the irradiated mouse was not clear. However, additional work using splenectomized mice showed that splenic megakaryocytes did not contribute significantly to platelet recovery in irradiated mice [2,3].

Splenic Hematopoiesis

Even though megakaryocytes are commonly found in the normal murine spleen [43-45], hematopoietically, the adult murine spleen is mostly erythropoietic [46-48]. Normally, the murine spleen is responsible for as much as 30% of erythropoiesis [47], but can produce as much as half of the total erythropoiesis in the bled mouse [48]. In contrast, the murine spleen does not normally contribute to thrombocytopoiesis, but can produce platelets in extreme cases of bone marrow suppression [2,20]. In fact, Davis et al. showed that mice whose bone marrow was destroyed by strontium-90 (⁹⁰Sr) survived 119 days with death occurring only in

cases where the spleen was removed [20]. In addition, the removal of the spleen has been shown to cause an increase in platelet production in rats [49-52] and in mice [2,53,54] instead of a decrease in platelet production. However, Bozzini et al. showed that splenectomy resulted in a 15% decrease in total red cell mass [46]. Since splenic megakaryocytes do not normally contribute significantly to platelet production, the purpose of their presence in the spleen is not completely known.

Radioprotection by TSF

It has been observed in previous studies that mice made thrombocytopenic by a platelet specific antiserum before exposure to sublethal doses of radiation have a greater recovery of platelet production than untreated irradiated controls [7-9]. It is now believed, however, that the endogenous thrombopoietin released in response to the immune-induced thrombocytopenia was responsible for the enhanced platelet production in the irradiated mice [11].

Using TSF from HEK cell culture medium, a well known reliable source of exogenous TSF [12], Carter was able to duplicate those results previously observed in irradiated mice treated with a platelet specific antiserum [11]. Therefore, irradiated mice treated with TSF, either exogenous or endogenously administered, have increased platelet counts [7-9,11], enhanced incorporation of [^{75}Se] Selenomethionine [8] or [^{35}S] Sodium sulfate [11] into platelets, elevated hematocrits [8,11], increased bone marrow megakaryocyte size [11], and decreased bone marrow megakaryocyte number [11]. The results showing the effects of TSF on WBC counts have been ambiguous; although different radiation dosage was used, Carter [11] found that TSF significantly increased WBC counts in irradiated mice, but Ebbe et al. [8] did not.

One explanation for the inverse relationship of megakaryocyte size and number in the mouse bone marrow observed by Carter [11] would be that the TSF mediated increase in megakaryocyte size led to the down regulation of megakaryocyte number via autoregulation as proposed by Ebbe and Phalen [4]. However, another explanation may be that the radiation-induced megakaryocytopenia stimulated the megakaryocyte size to dramatically increase, via the action of TSF, as a compensatory mechanism [11]. Whether or not TSF affects splenic megakaryocytopoiesis in a similar fashion as bone marrow megakaryocytopoiesis in irradiated mice has not been previously studied.

3. MATERIALS AND METHODS

Step II TSF Purification and Dose-Response Experiment

Step II TSF was purified from HEK cell culture medium using the procedure described by McDonald et al. [55]. Partial purification of crude TSF-rich medium by Sephadex G-75 column chromatography was followed by ethanol precipitation at 0-40% ethanol concentrations to achieve Step II TSF purification. To determine the activity of the Step II TSF, a dose-response experiment using the immunothrombocytopenic mouse assay was performed and analyzed by least squares regression analysis [55]. Additionally, Step II TSF was tested for the presence of IL-6 and endotoxin. Utilizing the ELISA assay, only 2500 units of IL-6 per unit of TSF were present; < 2.5 units of endotoxin per unit of TSF were detected by the E-toxate *Limulus* Amebocyte Lysate test (Sigma Chemical Company, St. Louis, MO). The small amount of IL-6 and endotoxin in Step II TSF did not warrant additional control experiments for this study [11].

Irradiation and Treatments

Eighty male C3H mice, between 6 to 8 weeks of age and weighing approximately 25 g, were sublethally irradiated with 3.0 Gy at an average rate of 0.43 Gys/min from a ^{137}Cs source (Siemens Inc., Des Plaines, IL) [11]. Fourteen mice were used as normal unirradiated controls for a total of 94 mice. The mice were randomly divided into two separate identical experiments consisting of 40 mice each. Each experiment was further divided into 4 test groups: TSF, human serum albumin (HSA), rabbit

anti-mouse platelet serum (RAMPS), and normal rabbit serum (NRS). Data from each experiment were pooled.

The mice were injected with Step II TSF or its carrier protein, HSA. TSF and HSA-treated mice were given one subcutaneous injection (s.c.) of TSF or HSA one hour before irradiation and another injection approximately eight hours after irradiation [11]. A total of 4 Units of TSF in 1.08 mg of protein was injected into each TSF-treated mouse. The amount of material in milligrams of protein that is required to increase the percent ^{35}S incorporation into platelets of immunothrombocytopenic assay mice by 50% above baseline is defined as a Unit (U) of TSF [55]. HSA-treated control mice were injected with 1.08 mg of HSA.

To evaluate the effectiveness of the Step II TSF in sublethally-irradiated mice, a comparison to an endogenous source of thrombopoietin was needed. Since it is known that thrombopoietin is endogenously released in thrombocytopenic animals [12], mice were given a single intraperitoneal injection (i.p.) of RAMPS to induce acute thrombocytopenia one hour prior to whole-body irradiation. Additional mice were given an i.p. injection of NRS for negative controls. A total of 0.1 ml of absorbed RAMPS [11] added to 0.4 ml of saline was injected into each RAMPS-treated mouse. NRS was diluted and injected at the same concentration as RAMPS.

Measurements of Peripheral Blood

To measure platelet production rates, mice were given a single i.p. injection (30 μCi) of $\text{Na}_2^{35}\text{SO}_4$ [30] on days 9 and 13 post irradiation. Twenty-four hours after radioisotope injection, on days 10 and 14, platelet counts, % ^{35}S incorporation, packed cell volumes (PCV), RBC counts, and WBC counts were measured. Both RBC and WBC counts were determined using an Electronic Coulter Counter, Model

Z_F with an orifice of 100 μ m (Coulter Electronic, Inc., Hialeah, FL). A 1:500 and 1:50,000 dilution of retroorbital sinus blood in isotonic buffered saline was used for WBC and RBC counting, respectively. For platelet counting, blood from the retroorbital sinus was initially diluted (1:100 dilution) with filtered 1% ammonium oxalate to lyse the red blood cells. The sample was loaded onto a hemacytometer chamber and platelets were counted using direct phase microscopy. To determine PCV, additional blood was drawn from the retroorbital sinus into heparinized capillary tubes, centrifuged for 5.5 minutes in a micro hematocrit centrifuge, and measured using a micro hematocrit reader. All mice were anesthetized with a single i.p. injection of 0.5 ml of heparin-sodium pentobarbitol solution [30].

Blood used to measure $\%^{35}\text{S}$ incorporation was collected via cardiac puncture into plastic syringes containing 1 ml of 1.0% ethylenediaminetetraacetic acid (EDTA). The blood was further diluted with additional EDTA solution to a total volume of 3 ml and centrifuged at 450 g for 4.5 minutes to obtain platelet-rich plasma (PRP). The PRP was centrifuged at 800 g for 15 minutes to form a platelet pellet. The platelets were washed in 1% ammonium oxalate and saline as previously described [30,33] and were re-suspended in 0.30 ml of isotonic buffered saline. A 1:2000 dilution of the platelet suspension was made and the number of platelets in suspension determined. A 100 μ l sample of the platelet suspension was added to scintillation vials containing 7 ml of scintillation fluid (a mixture of dioxane, naphthalene, phenyl-oxazolyl-phenyl-oxazolyl-phenyl or POPOP, and polyphenylene oxide or PPO). Radioactivity was then measured using a Beta Scintillation Counter (Packard Instrument Company, Downers Grove, IL). The $\%^{35}\text{S}$ incorporation was determined as described by McDonald [30].

For platelet sizing, a 20 μ l sample was taken from the PRP and was further diluted into 10 ml of isotonic buffered saline. The dilution was then used for platelet sizing [34]. An Electrozone/Celleoscope with a 48 μ m orifice (Particle Data, Inc., Elmhurst, IL) was used to determine the mean platelet size of each mouse.

Preparation of Splenic Tissue and Morphometry

The spleen from each mouse was removed and placed in 10% neutral-buffered formalin. To enhance fixation, the spleens were bisected sagittally and processed in a Vacuum Infiltration Processor (VIP; Miles Laboratory, Inc., Elkhart, IN). After being processed, the spleens were embedded in paraffin, sectioned on a microtome at a thickness of 5 μ m at 100 μ m intervals, and stained with hematoxylin and eosin. The quality of the splenic tissue was found to be best when processed as shown in Table 1.*

Megakaryocyte number and size were obtained from splenic tissue using stereological techniques described by Cullen and McDonald [36]. The areas of 50-200 megakaryocyte profiles were measured at a magnification of 1200X via video overlay onto the x-y digitizing tablet of the Micro-Tech 100 Morphometric and Densometric Image Analyzing System (Analytical Imaging Concepts, Irvine, CA).

By treating megakaryocytes as spheres [56], the area of each profile was used to calculate its diameter:

$$d = 2 \sqrt{(\text{area}/\pi)}$$

Upon the determination of the diameter of each megakaryocyte profile, the profiles were grouped according to size; there were 19 possible size classes. Once all

*All Tables and Figures may be found in the Appendix.

the megakaryocyte profiles were determined, a histogram was constructed from the profile frequency distribution. The number of megakaryocyte profiles too small to be identified and counted, called "lost caps" or optically lost profiles, was estimated using the method described by Cullen and McDonald [36]. The frequency distribution was then corrected to account for the optically lost profiles and used to calculate the mean diameter of section profiles ($\bar{d}$) which was then used to calculate the mean megakaryocyte diameter ($\bar{D}$):

$$\bar{D} = 4 \times \bar{d} / \pi$$

The number of section profiles per unit area (N_A) and $\bar{D}$ were used to calculate the number of spherical particles per unit volume (N_V) while taking into account the thickness (t) of the tissue section:

$$N_V = N_A / (\bar{D} + t)$$

The number of section profiles per unit area was estimated using a transparent square grid of equidistant lines superimposed on the video field [39]. The intersections of the lines (100/field) were considered as sampling points. The $\bar{D}$ and N_V values were corrected for 15% tissue shrinkage and used to calculate the total megakaryocyte mass (TMM) [14]:

$$\text{TMM } (\mu\text{m}^3/\text{mm}^3) = \frac{4}{3} \cdot \pi \cdot \left(\frac{\bar{D} (\mu\text{m})}{2} \right)^3 \cdot N_V$$

Normalizing Data and Statistical Analyses

The data for all treatment groups of each experiment were converted to percent of control by dividing them by the means of their respective controls (i.e., NRS/NRS, RAMPS/NRS, HSA/HSA, and TSF/HSA). The percentages were then multiplied by the pooled means (Experiments 1 and 2) of the irradiated controls to obtain a new

value. The purpose of this procedure was to reduce the error term by the amount of variation from experiment to experiment, and in doing so, the standard deviation and standard error of the irradiated controls (i.e., NRS and HSA) were lowered without changing their mean. Statistical differences between irradiated control mice and irradiated mice given RAMPS or TSF were determined by the Student's *t* test. The dose-response experiment utilizing Step II TSF was analyzed by least squares regression analysis.

4. RESULTS

Dose Response Experiment

The results of the dose-response experiment used to determine the specific activity of Step II TSF are depicted in Table 2. Response to TSF, as measured by percent ^{35}S platelet incorporation, increased as the dosage of TSF was increased. A significant linear correlation between TSF dosage and percent ^{35}S platelet incorporation was found ($r^2 = 0.90$, $P < 0.0005$). The specific activity of the Step II TSF preparation was determined to be 3.7 U of TSF per mg protein.

Effects of Radiation on Peripheral Blood Elements

Figure 1 shows the peripheral platelet counts of unirradiated mice and irradiated mice treated with NRS, RAMPS, HSA, or TSF. As compared to the unirradiated controls, exposure to a radiation dose of 3.0 Gy resulted in the suppression of the platelet counts in all treatment groups on days 10 and 14. Although decreases were found in TSF-treated mice on day 14 as compared to HSA controls, on day 10 both RAMPS and TSF significantly elevated ($P < 0.005$) platelet counts over the values of their irradiated controls (i.e., NRS and HSA, respectively). In support of these findings, additional data shown below demonstrates that RAMPS and TSF enhanced platelet production in the sublethally-irradiated mice.

Percent ^{35}S incorporation into platelets of the unirradiated control mice and the irradiated mice treated with NRS, RAMPS, HSA, or TSF are presented in Figure 2. The effects of radiation on platelet production as measured by the percent ^{35}S incorporations into platelets were similar to those seen in platelet counts, that is, platelet production in all treatment groups on days 10 and 14 was lower than the

platelet production of the unirradiated controls. When compared to the NRS-control mice on day 10, the percent ^{35}S platelet incorporation was significantly enhanced ($P < 0.0005$) in the RAMPS-treated mice. Likewise on day 10, platelet production in the TSF-treated mice was significantly elevated ($P < 0.025$) above the HSA-control mice. Even though a reduction was observed on day 14 in the RAMPS and TSF-treated mice as compared to their irradiated controls, these results are in agreement with Figure 1 showing that the platelet production in irradiated mice was elevated by treatment with RAMPS and TSF.

In addition to peripheral platelet counts and percent ^{35}S incorporation into platelets, platelet sizes were also used as a measurement of platelet production (Figure 3). All treatment groups on day 14 showed platelet sizes greater than the normal unirradiated mice: NRS (114%), RAMPS (115%), HSA (110%), and TSF (107%). In contrast, platelet sizes on day 10 were decreased in the irradiated mice compared to unirradiated controls except for the RAMPS-treated mice which had platelet sizes significantly larger ($P < 0.0005$) than the NRS-control mice.

Although the results above show that a severe thrombocytopenia was present on days 10 and 14 in mice after exposure to 3.0 Gy of radiation, the occurrence of anemia of equal severity was not observed. Figure 4 shows the packed cell volume (PCV) of unirradiated mice and irradiated mice treated with NRS, RAMPS, HSA, or TSF. In relation to the unirradiated mice, the PCV was only slightly suppressed in the irradiated mice on both days 10 and 14. Although the PCV of irradiated mice treated with TSF was not significantly increased above the HSA-control mice on either day 10 or day 14, the PCV of mice treated with RAMPS was found to be significantly higher ($P < 0.025$) than the NRS-treated mice on day 10. To further

investigate any possible effects of RAMPS and TSF on erythropoiesis in mice exposed to a radiation dose of 3.0 Gy, additional data were collected.

Figure 5 depicts the red blood cell (RBC) counts of the irradiated mice, as well as the unirradiated control mice. Similar to the PCV in Figure 4, the RBC counts on days 10 and 14 were only slightly decreased in mice treated with NRS, RAMPS, HSA, or TSF when compared to the unirradiated controls. Furthermore, neither RAMPS nor TSF caused significant increases above their irradiated controls in RBC counts on either day 10 or 14. This finding further suggests that TSF may not be effective at stimulating the erythropoietic system of mice exposed to 3.0 Gy.

Figure 6 illustrates the white blood cell (WBC) counts of the same mice used for the RBC counts in Figure 5. When compared to the unirradiated mice, it is clear that irradiation lowered the white blood cell (WBC) count regardless of the treatment. All treatment groups on both days 10 and 14 reflected about the same level of decrease in WBC counts (35 to 47% of unirradiated normals). Even though significant differences from their irradiated controls were not found in either the RAMPS or TSF-treated mice on day 14, RAMPS was found to increase ($P < 0.05$) WBC counts above the values of the NRS-treated mice on day 10. Similarly on day 10, TSF-treated mice had significantly higher WBC counts ($P < 0.005$) than the HSA-control mice.

Effects of Radiation on Megakaryocytes

Megakaryocyte numbers which were measured in the spleens of unirradiated control mice and in irradiated mice treated with NRS, RAMPS, HSA, or TSF are depicted in Figure 7. This figure shows that 3.0 Gy of radiation greatly reduced splenic megakaryocyte number in all treatment groups on days 10 and 14. Although the number of splenic megakaryocytes in unirradiated mice was determined to be

794 ± 53 (S.E.) megakaryocytes per mm^3 , the megakaryocyte numbers on day 10 of the NRS and HSA-treated irradiated mice were reduced to 8.5% and 7%, respectively, of the unirradiated values. However, as demonstrated in Figure 7, both RAMPS and TSF caused elevations in the number of splenic megakaryocytes on day 10 that were significantly higher ($P < 0.0005$) than their NRS and HSA control mice; these factors did not significantly elevate megakaryocyte number on day 14.

Figure 8 records the results of determining the size of the megakaryocytes in the spleens of unirradiated control mice and irradiated mice. As shown, there was not any significant difference in mean splenic megakaryocyte diameter among treatment groups on day 10, but the mean splenic megakaryocyte diameter in all treatment groups were higher than unirradiated mice on day 10: NRS (115%), RAMPS (110%), HSA (117%), and TSF (113%). Likewise, similar results were seen on day 14, except for that TSF caused a significantly increased mean splenic megakaryocyte diameter compared to the values of the HSA-treated control mice ($P < 0.05$).

Since Figures 7 and 8 illustrated that RAMPS and TSF increased splenic megakaryocyte number on day 10 after irradiation but not megakaryocyte size, the total megakaryocyte mass (TMM) was determined (Figure 9). Since mass is reflective of both splenic megakaryocyte size and number [16], TMM is a better indicator for the effects of irradiation and TSF on splenic megakaryocytes. As seen in Figure 9, sublethal whole-body irradiation reduced the TMM on both days 10 and 14 in all treatment groups to levels well below that of the normal unirradiated mice. While significant differences were not observed on day 14, both RAMPS and TSF significantly increased ($P < 0.0005$) the TMM on day 10 above the values of their NRS and HSA controls which were reduced to 13% and 11%, respectively, of the unirradiated controls.

5. DISCUSSION

In a previous study [11], administration of TSF to mice prior to whole-body irradiation was found to result in a significant increase in bone marrow megakaryocyte size but a marked decrease in their number. However, megakaryocyte size and number were not measured in the spleens of irradiated mice treated with TSF. Therefore, to determine the effects of TSF on splenic megakaryocytes, thrombocytopoiesis was stimulated in irradiated mice by injections of RAMPS or a Step II preparation of TSF from HEK cell culture medium. The results of these studies agree with previous work showing that mice which were exposed to irradiation and treated with RAMPS or TSF have elevated platelet counts [7-9,11] and enhanced radioisotope incorporation into platelets [8,11]. In addition, the present work showed that TSF caused increases in splenic megakaryocyte size and numbers, and splenic TMM of sublethally-irradiated mice.

In agreement with studies by McDonald et al. [55], the present work showed that Step II TSF significantly increased the ^{35}S incorporation into platelets of immunothrombocythemic assay mice (Table II). These data also demonstrated that the Step II preparation of TSF had a dose-dependent, stimulatory effect on thrombocytopoiesis as demonstrated by the production of a linear dose-response relationship between ^{35}S platelet incorporation and the amounts of Step II TSF injected into the assay mice. In addition to the ability of Step II TSF to stimulate thrombocytopoiesis, it also significantly increases the DNA content of bone marrow megakaryocytes in normal mice [37]. Therefore, purification of TSF from HEK cell culture medium by Sephadex G-75 column chromatography and ethanol precipitation yields a partially purified Step II TSF of high specific activity [55].

In the present study, it was found that RAMPS and the partially purified Step II preparation of TSF from HEK cell culture medium contributed significantly to the enhancement of platelet counts in sublethally-irradiated mice. These results agree with a previous study [11] using a Step I TSF showing that thrombopoietin was responsible for the elevated platelet counts seen in irradiated mice treated with a platelet specific antiserum [7-9]. Although the work of Carter [11] and the present study used a different source of TSF (Step I versus Step II) with a different specific activity, similar platelet counts on day 10 in both studies were observed in irradiated mice treated with TSF. In comparison, Carter used a Step I TSF purified from HEK cell culture medium by Sephadex G-75 column chromatography, whereas the Step II TSF used here was additionally purified by ethanol precipitation at 0-40% concentrations [55]. The additional purification step resulted in a TSF with a specific activity (3.7 U/mg protein) that was approximately 23 times more potent than the specific activity of Step I TSF (0.16 U/mg protein). Despite its greater potency, however, mice treated with the Step II TSF were not found to have significantly enhanced platelet counts on day 14 after 3.0 Gy of radiation as compared to the HSA-control mice. In contrast, Step I TSF has been shown to significantly elevate platelet counts above HSA-treated mice 14 days after whole body irradiation [11].

The prolonged stimulation of platelet production in irradiated mice by the Step I TSF may not have been a direct result of the TSF, but possibly an indirect effect of the contaminants present in the preparation; Step II TSF is a purer source of TSF than the Sephadex-purified TSF [55]. Nevertheless, if the contaminants in the exogenous TSF were the sole reason for the differences observed in the platelet counts on day 14 in the TSF-treated, irradiated mice in this work and in Carter's study, the results seen in the RAMPS-treated, irradiated mice on day 14 in both studies would be expected to

be similar, but they were not. Whereas Carter found that RAMPS significantly increased platelet counts above NRS-controls on day 14, the data reported here did not show any significant differences on day 14. Therefore, differences observed between these 2 studies may be more related to differences in the timing of treatments or differences in the calibration of the ^{137}Cs radiation unit. Nevertheless, additional measurements support the results discussed above showing that Step II TSF will enhance platelet production in sublethally-irradiated mice.

In addition to the platelet counts, ^{35}S incorporation into platelets, used as an index of platelet production, was also increased in both the RAMPS and TSF-treatment groups on day 10 as compared to their irradiated controls (Figure 2). These data support the findings of other workers showing that RAMPS [8,11] and TSF [11] will increase radioisotope incorporation into platelets of irradiated mice. Moreover, the results indicate that TSF is effective in protecting the murine thrombocytopoietic system from radiation damage. However, the results reported on day 14 demonstrating that ^{35}S incorporation into platelets was reduced, further support the present observations that platelet production was decreased in the RAMPS and TSF treatment groups on day 14. On the other hand, Carter found that platelet production in the control irradiated mice was significantly less than irradiated mice treated with RAMPS or TSF [11] indicating that the platelet production in the RAMPS and TSF-treated mice of the present study were suppressed. Since the plasma of irradiated mice has been shown to contain thrombopoietin [57], the rapid recovery seen here in the NRS and HSA-control groups may have been caused by the endogenous release of thrombopoietin. The endogenous release of thrombopoietin may have been suppressed in irradiated mice treated with RAMPS or TSF. Additionally, Ebbe and Phalen found that mice given 4.0 Gy of radiation had higher platelet counts at 16 days

after radiation exposure than mice given the same radiation dose plus an anti-platelet antibody [7]. As mentioned above, the contrasting results observed on day 14 of this work and Carter's study may not be attributed differences in the TSF used, but may be related to additional unknown factors.

Like the platelet counts and percent ^{35}S platelet incorporation for the RAMPS-treatment group on day 10, platelet sizes of the RAMPS treated mice were also significantly increased above the NRS-controls on day 10; platelet sizes of the TSF-treated mice were not significantly different from HSA controls on day 10 (Figure 3). These observations agree with previous studies showing that platelet sizes increase with the stimulation of thrombocytopoiesis [13,34]. However, if this was always true, the platelet sizes on day 10 in mice treated with TSF should be significantly increased above HSA-treated controls as indicated by the increased platelet counts (Figure 1) and increased platelet production (Figure 2) of the TSF-treated mice, but they were not. Instead, the platelet sizes were similar in both TSF and HSA treatment groups on day 10. Therefore, it appears that platelet size is not a very sensitive index for thrombopoietin-stimulated thrombocytopoiesis [34]. Although the results reported in Figures 1,2, and 3 show the suppressive effects of 3.0 Gy on murine thrombocytopoiesis, additional data indicate that the same dosage of radiation may only have a minor negative effect on erythropoiesis.

The results of the present work show that 3.0 Gy will only slightly suppress murine erythropoiesis as indicated by small decreases in the RBC count (Figure 5) and PCV (Figure 4) of NRS and HSA-treated mice. These data are in agreement with other studies showing that the murine thrombocytopoietic system may be more sensitive to sublethal doses of radiation than the erythropoietic system. Although Figure 5 illustrates that treatment with RAMPS or TSF did not significantly change

RBC counts on either day 10 or on day 14, irradiated mice treated with RAMPS were shown to have increased PCV on day 10 (Figure 4). In contrast, the study of Ebbe et al. [8] found that when mice were exposed to 6.5 Gy of radiation and given a single injection of platelet specific antiserum, the hematocrits, as well as platelet production, were significantly increased. Therefore, their results suggested that the production of platelets and red blood cells might be linked [8]. Although a lower dosage of radiation (3.0 Gy) was used, the present results do not indicate any connection between the erythropoiesis and thrombopoiesis. Moreover, Step II TSF does not appear to stimulate erythropoiesis in the irradiated mouse, but the data show that TSF will enhance leukocyte production.

In this investigation, radiation-induced leukopenia was alleviated by TSF treatment. As seen in Figure 6, both RAMPS and TSF were observed to significantly increase WBC counts on day 10 above the values of the NRS and HSA-treated controls, respectively. Whereas these results are in agreement with the study of Carter [11], they do not agree with the study of Ebbe et al. [8] who found that leukocyte counts in mice given 6.5 Gy of radiation were not significantly increased. Again, the contradiction may be related to the difference in radiation dose; the present investigation and Carter's study used a radiation dose of 3.0 Gy. Nevertheless, WBC production was significantly increased above NRS and HSA-treated controls in irradiated mice given either RAMPS to induce the endogenous release of TSF or the exogenous TSF from HEK cell culture medium, respectively (Figure 6). This present work demonstrates that thrombopoietin may be capable of stimulating WBC production in the irradiated mouse, possibly through an indirect mechanism.

In addition to the results showing that TSF and RAMPS caused an increased platelet count (Figure 1) and an enhanced platelet production (Figure 2) in irradiated

mice on day 10, both Step II TSF and RAMPS caused significant increases in splenic megakaryocyte number on day 10 (Figure 7) without significantly changing the splenic megakaryocyte size (Figure 8). Other workers have reported increases of splenic megakaryocyte number in sublethally irradiated mice [4] and in mice given a platelet specific antiserum [58], but splenic megakaryocytopoiesis in mice both irradiated and treated with RAMPS or TSF had not been examined before. Furthermore, the effects of TSF on splenic megakaryocytopoiesis in irradiated mice on day 10 reported here are in contrast to another study [11] which found that TSF decreased the number and increased the size of bone marrow megakaryocytes in irradiated mice on day 10. These present findings appear to indicate that bone marrow and splenic megakaryocytopoiesis are regulated differently in the sublethally-irradiated mouse. One explanation for the inverse relationship between size and number of bone marrow megakaryocytes in the irradiated mice has been autoregulation [4,12].

Autoregulation hypothesizes that megakaryocytes regulate themselves through changes in their number or size based on their existing current condition [4,12]. For example, autoregulation is commonly seen in mice in which the radiation-induced reduction of megakaryocyte numbers [3-6,10] is compensated by the enlargement of megakaryocyte sizes [3,4]. Although autoregulation of megakaryocytopoiesis has been observed in the spleens of hypoxic mice [21], autoregulation of splenic megakaryocytopoiesis was not apparent in this study. It is possible that such a mechanism of regulation does not exist in the spleens as it does in the bone marrow of irradiated mice. Since it has been shown that splenic megakaryocyte progenitors are a more rapidly-dividing population than that in the bone marrow [45] and since TSF from kidney cell culture medium has been shown to increase the number of SACHe+

cells [38,39], it is possible that the TSF-mediated recovery of splenic megakaryocyte number may have negated autoregulation. Furthermore, as seen in Figure 10, significant stimulation of splenic megakaryocytopoiesis by RAMPS or TSF was found on day 10 by significant increases in TMM as compared to NRS and HSA-irradiated controls. The existence of a rapidly-dividing population of megakaryocyte progenitor population may explain the enhanced recovery in megakaryocyte number and TMM of the NRS and HSA-treated mice on day 14. Nevertheless, it is possible that megakaryocytopoiesis in the bone marrow and the spleen may be under different controlling mechanisms [21,45].

Whether or not the TSF mediated increase of splenic megakaryocytopoiesis contributed significantly to the observed enhanced platelet recovery is not known. However, it was noted that in the RAMPS and TSF-treated mice the increased platelet counts and ^{35}S incorporations into platelets on day 10 appeared to be associated with increased splenic megakaryocytopoiesis on day 10, and additionally, the decreased platelet counts and ^{35}S platelet incorporations of the RAMPS and TSF treatment groups on day 14 also appeared to be correlated with a reduction in splenic megakaryocytopoiesis on day 14. Although previously reported studies using splenectomized-irradiated [2,3] and radiostrontium-treated [2,20] mice have indicated that the murine spleen does not normally contribute to platelet production, the mice in these studies were not treated with TSF. It would be interesting to study the effects of TSF in splenectomized-irradiated and radiostrontium-treated mice.

In summary, the present work indicates that TSF may be effectively used in patients undergoing chemotherapy or radiation treatments to enhance platelet recovery and shows for the first time that TSF from HEK cell culture medium can stimulate megakaryocytopoiesis in the spleens of irradiated mice. In addition, the present work

agrees with other studies [21,45] which indicate that the humoral regulation of bone marrow megakaryocytopoiesis may be different from splenic megakaryocytopoiesis.

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APPENDIX

Table 1. Tissue Processing of Spleens on the Vacuum Infiltration Processor (VIP).

Step	Reagents ^a	Purpose	Time (min.)	Temp. °C
1	10% formalin	Fixation	10	40
2	10% formalin	"	10	40
3	80% ethyl alcohol	Dehydration ^b	30	40
4	95% ethyl alcohol	"	30	40
5	95% ethyl alcohol	"	30	40
6	100% ethyl alcohol	"	30	40
7	100% ethyl alcohol	"	35	40
8	100% ethyl alcohol	"	35	40
9	xylene	Clearing	45	20
10	xylene	"	45	20
11	paraffin	Infiltration	45	60
12	paraffin	"	45	60

^a Fresh reagents were used for each step.

^b Graded dehydration was performed to prevent tissue shrinkage.

Table 2. Effect of Step II TSF on ^{35}S incorporation into platelets of immunothrombocytopenic assay mice.

Treatment	Number of mice	Dose (mg/mouse)	Response ^{a,b,c} (% control)
Saline	10	----	----
Step II TSF	4	0.054	109
Step II TSF	8	0.110	138.8 ^{***}
Step II TSF	4	0.220	133.5 ^{**}
Step II TSF	5	0.320	171.8 ^{*****}
Step II TSF	5	0.540	186.8 ^{*****}

^a Response was measured by % ^{35}S platelet incorporation into platelets of immunothrombocytopenic mice [30].

^b Significant differences between Saline controls and TSF treatments were:

$**P < 0.025$, $***P < 0.01$, $*****P < 0.0005$.

^c Coefficient of determination of data was: $r^2 = 0.90$.

Figure 1.

Platelet counts of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation and treated with normal rabbit serum (NRS), rabbit anti-mouse platelet serum (RAMPS), human serum albumin (HSA), or thrombocytopoiesis-stimulating factor (TSF). Each treatment consisted of 10 mice each except for the unirradiated control group in which 14 mice were used. Vertical bars represent the SE. Values were significantly different from control mice: ****P < 0.005.

PLATELET COUNT

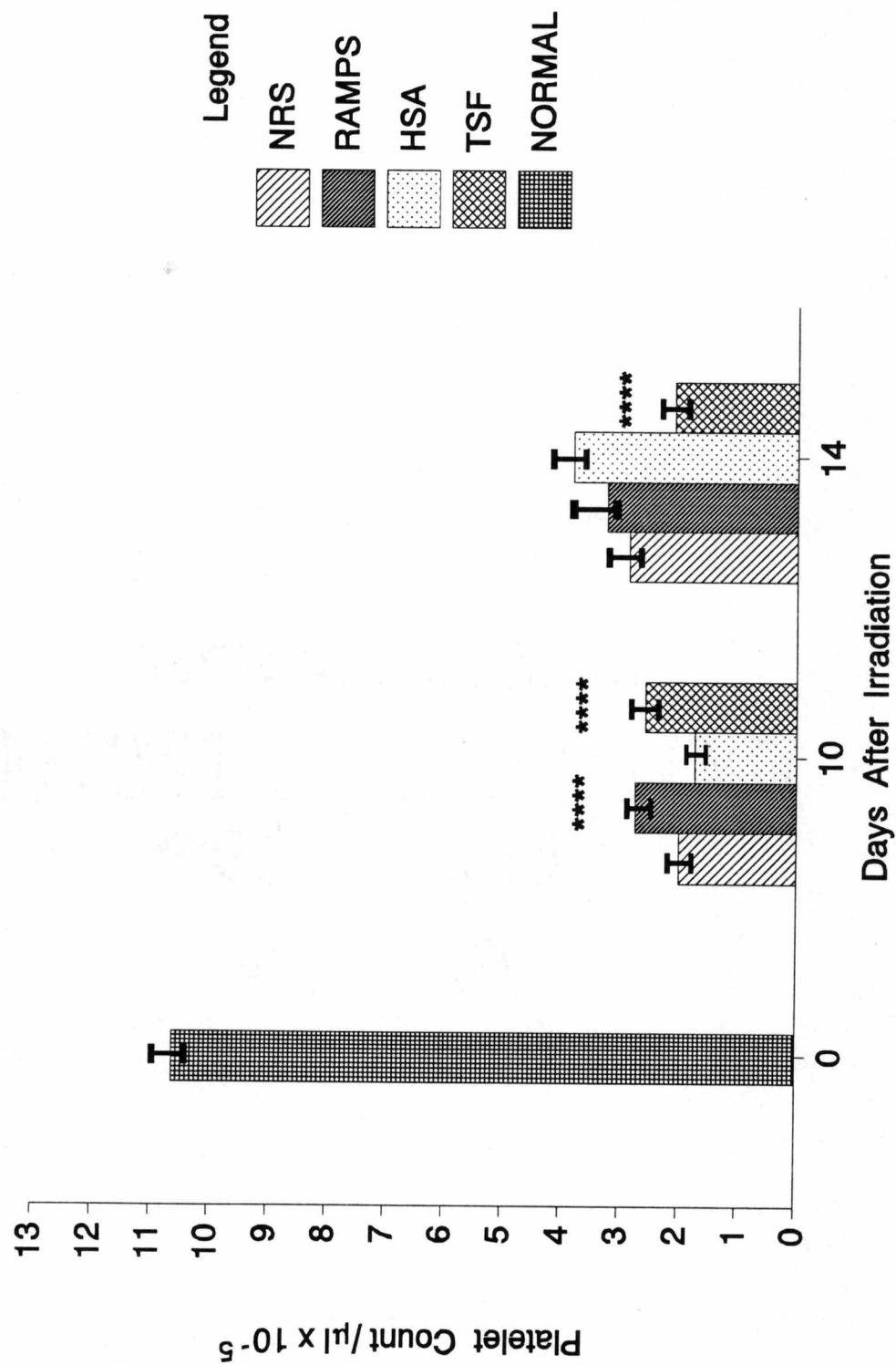


Figure 2.

^{35}S incorporation into platelets of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice each except for the TSF group on day 10 and the unirradiated control group in which were 9 and 14 mice, respectively. Vertical bars represent the SE. Values were significantly different from control mice: $**P < 0.025$, $****P < 0.005$, $*****P < 0.0005$.

PERCENT ^{35}S INCORPORATION

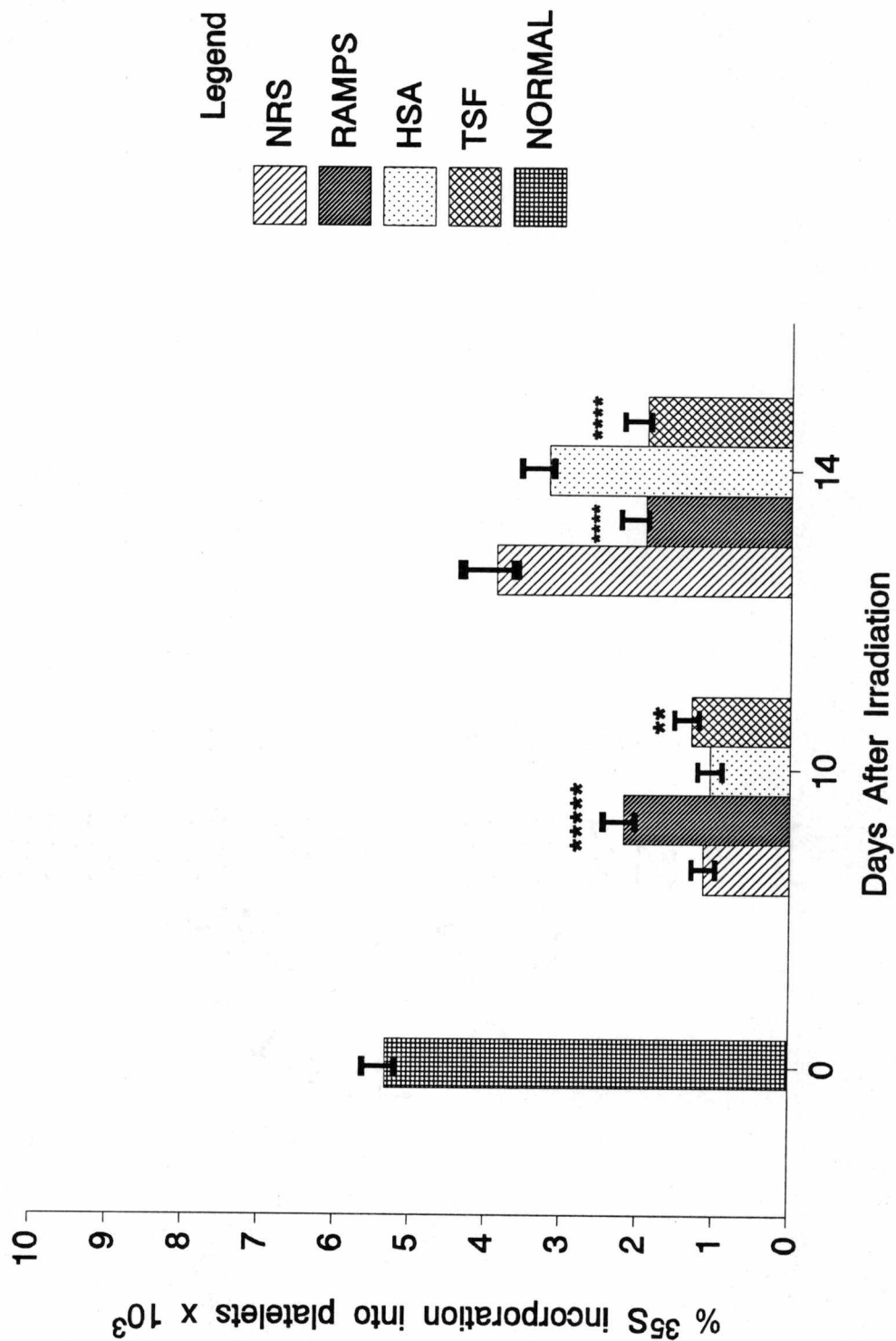


Figure 3.

Platelet sizes of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. The number of mice in each treatment group were: 5 mice each in the NRS and HSA treatment groups, 10 mice each in the RAMPS and TSF treatment groups, and 14 mice in the unirradiated control mice. Vertical bars represent the SE. Values were significantly different from control mice: *****P < 0.0005.

PLATELET SIZE

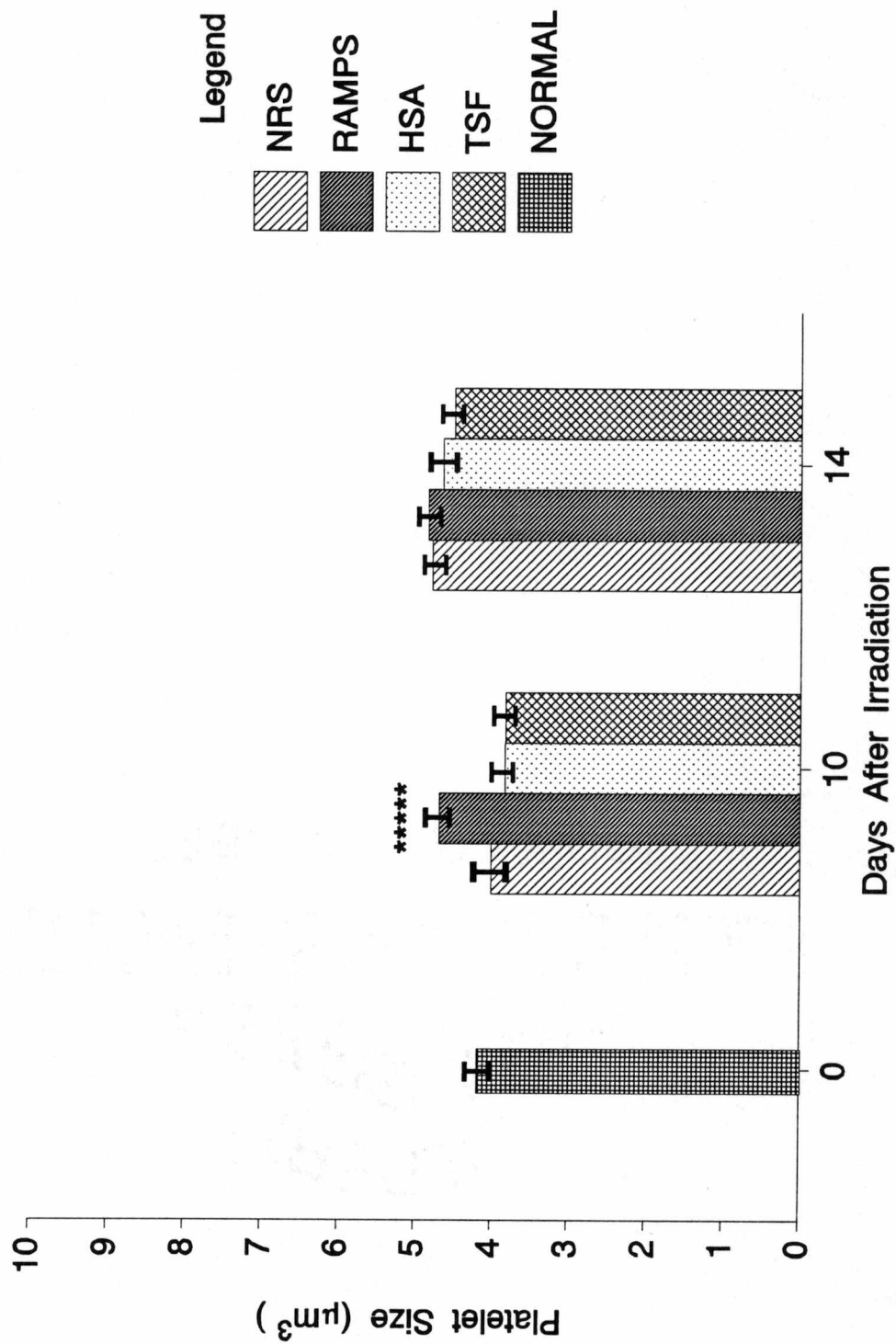


Figure 4.

Packed cell volumes (PCV) of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice each except for the unirradiated control group in which 14 mice were used. Vertical bars represent the SE. Values were significantly different from control mice: $**P < 0.025$.

PACKED CELL VOLUME

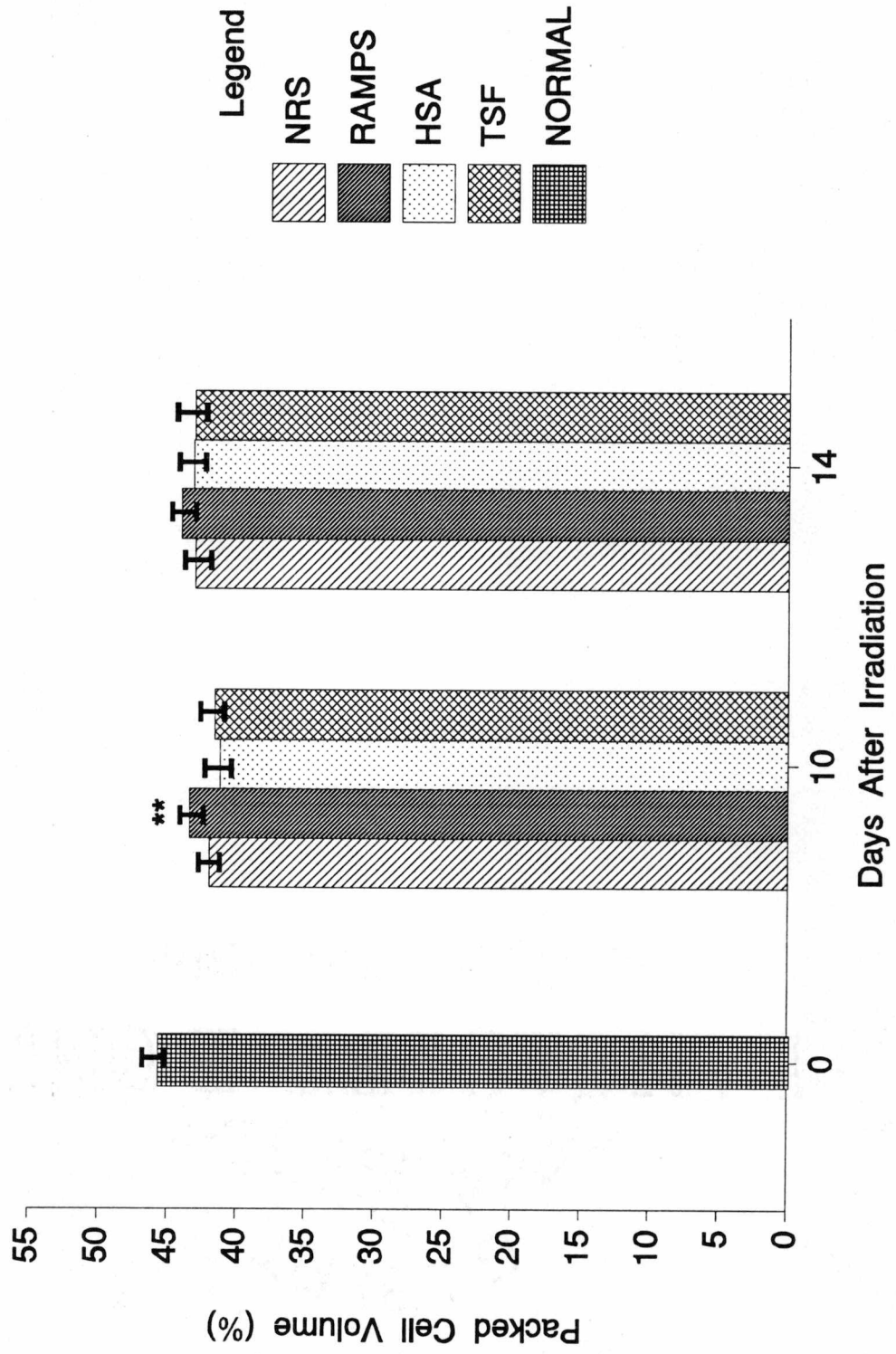


Figure 5.

Red blood cell (RBC) counts of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice each except for the unirradiated control group in which 14 mice were used. Vertical bars represent the SE. Values were not significantly different from control mice.

RED BLOOD CELL COUNT

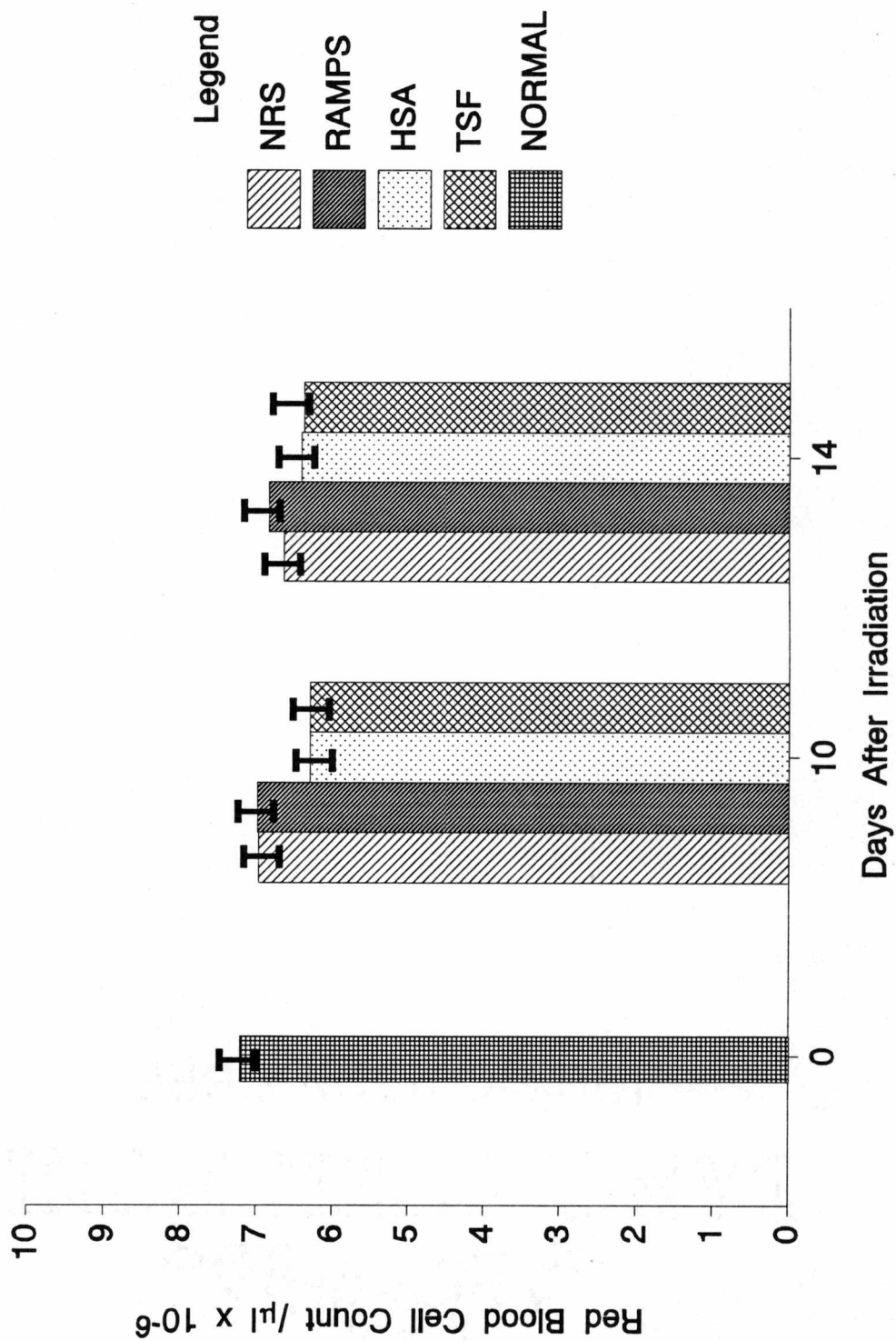


Figure 6.

White blood cell (WBC) counts of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice each, except for the NRS group on day 10 and the unirradiated control group in which were 9 and 14 mice each, respectively. Vertical bars represent the SE. Values were significantly different from control mice: * $P < 0.05$, **** $P < 0.005$.

WHITE BLOOD CELL COUNT

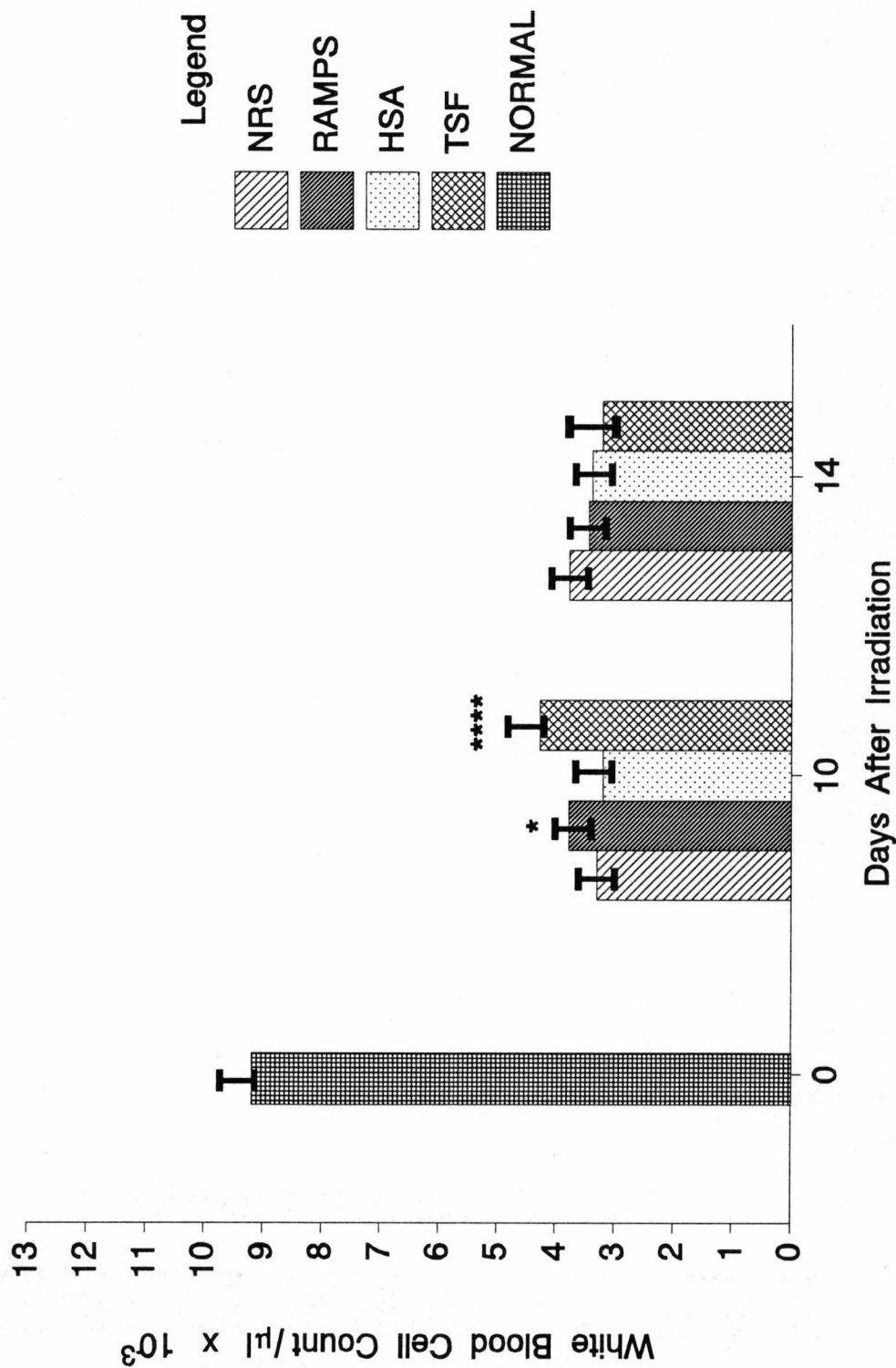


Figure 7.

Splenic megakaryocyte number of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice each except for the unirradiated control group in which were 14 mice. Vertical bars represent the SE. Values were significantly different from control mice: *****P < 0.0005.

MEGAKARYOCYTE NUMBER

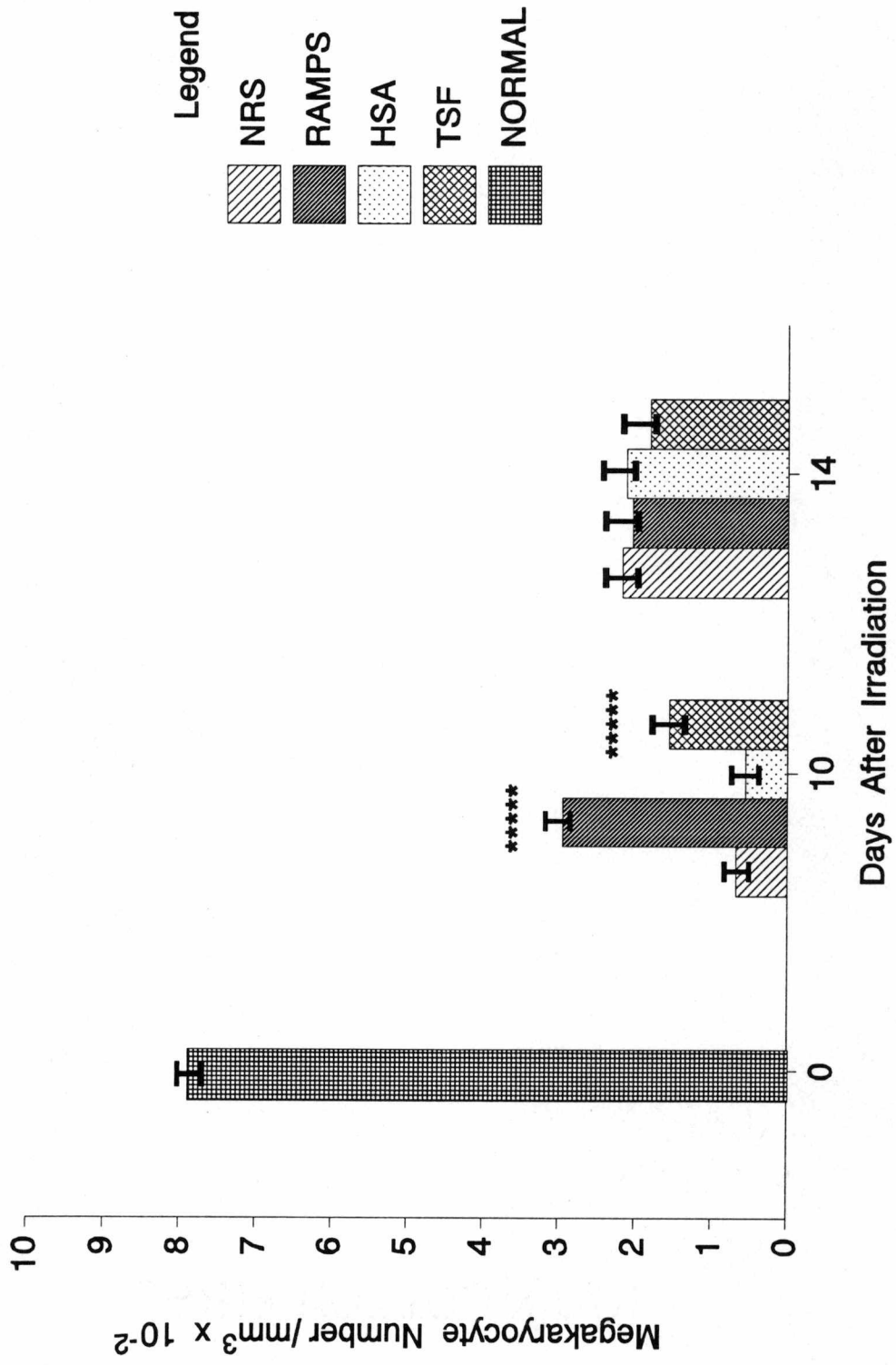


Figure 8.

Splenic megakaryocyte diameter of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice except for the unirradiated control group in which 14 mice were used. Vertical bars represent the SE. Values were significantly different from control mice: * $P < 0.05$.

MEGAKARYOCYTE DIAMETER

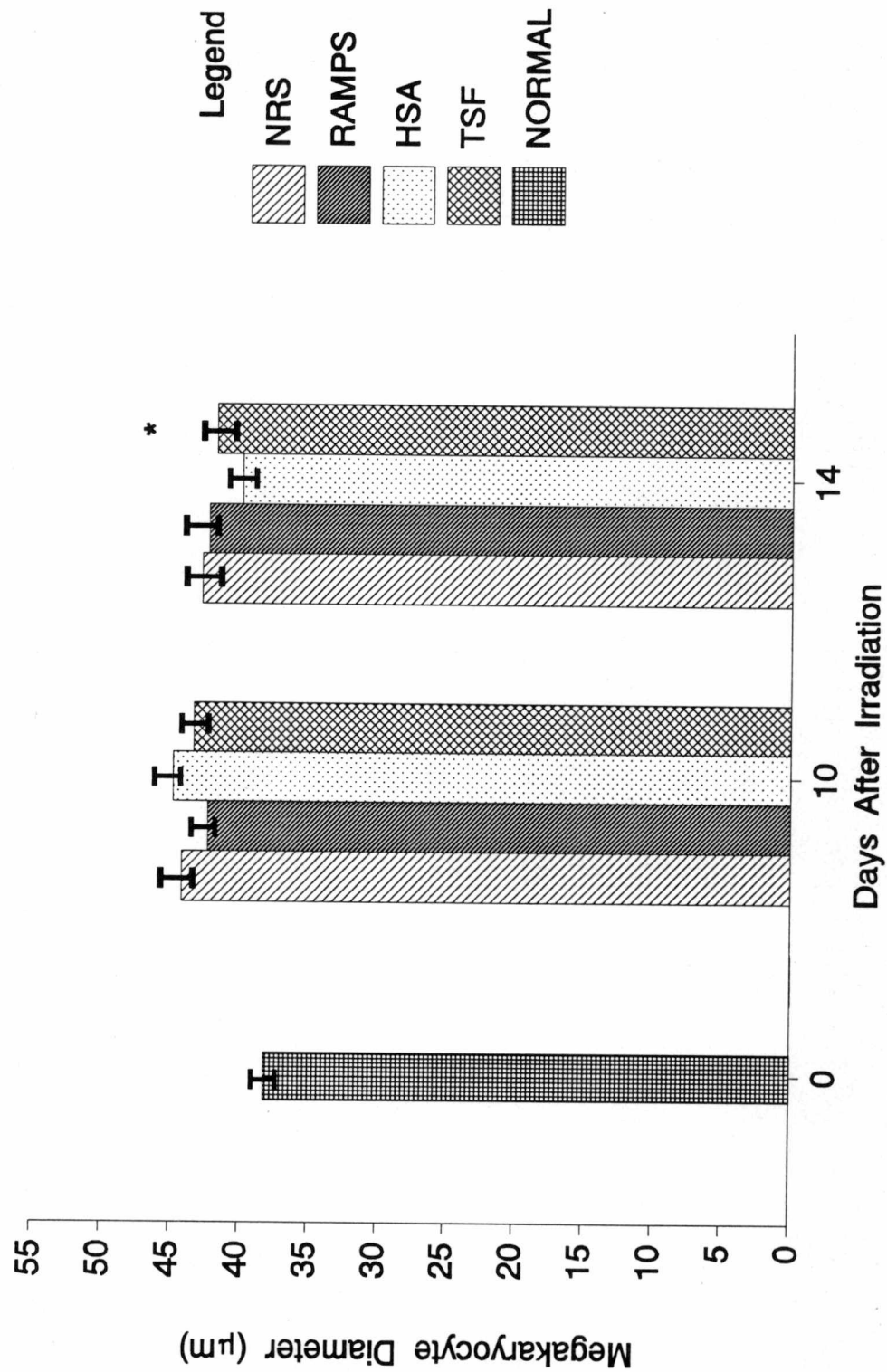
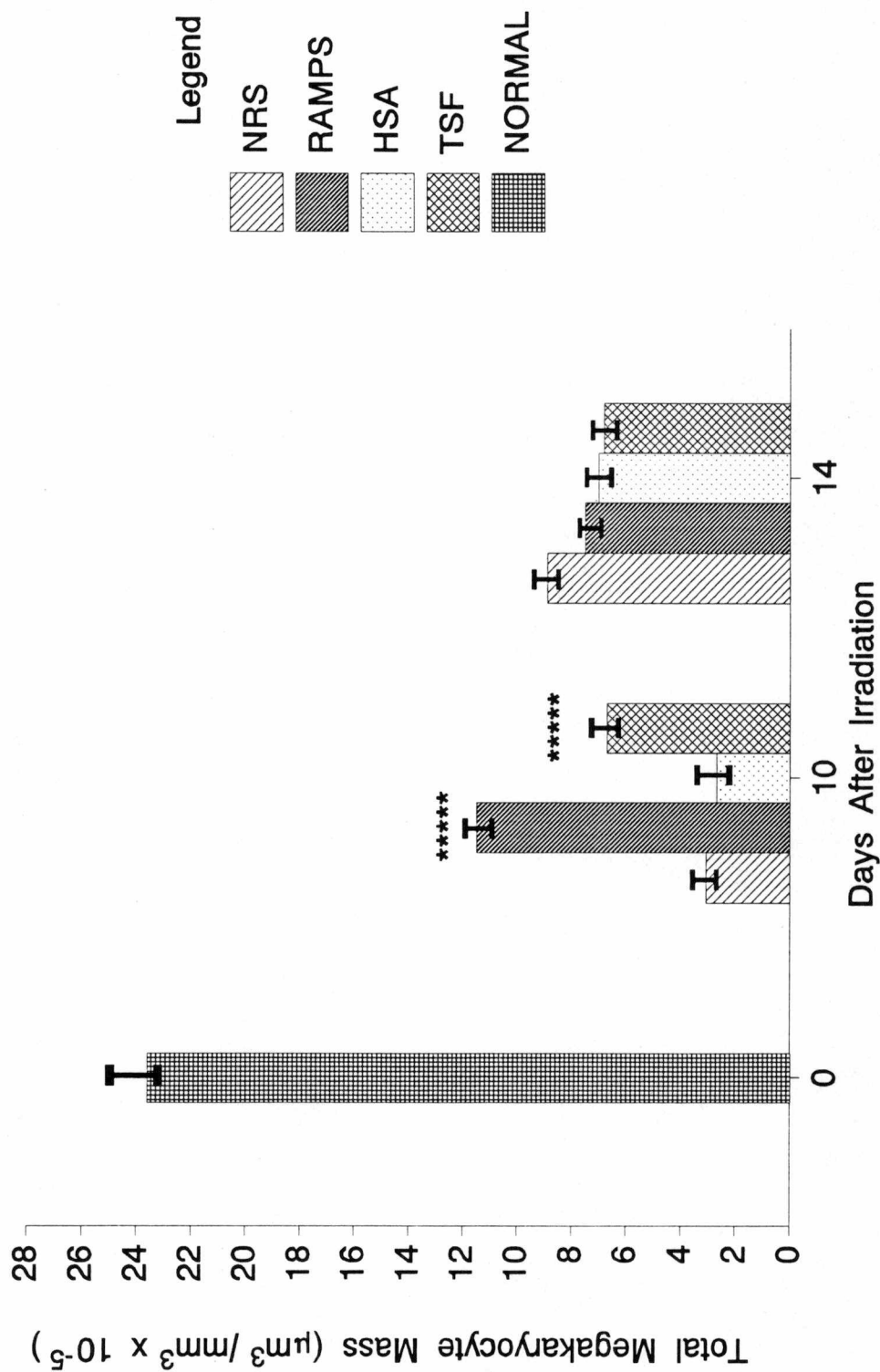


Figure 9.

Total megakaryocyte mass (TMM) of normal unirradiated mice and mice exposed to a sublethal dose (3.0 Gy) of whole-body irradiation treated with NRS, RAMPS, HSA, or TSF. Each treatment group consisted of 10 mice except for the unirradiated control group in which 14 mice were used. Vertical bars represent the SE. Values were significantly different from control mice: *****P < 0.0005.

TOTAL MEGAKARYOCYTE MASS



VITA

Ronnie W. Culberson was born on March 31, 1967 in Rome, Georgia. He attended local elementary and secondary schools, and graduated with honors from Model High School in 1985. The following August of that same year, he entered Shorter College, Rome, Georgia, where he graduated Cum Laude with a Bachelor of Arts degree in Biology in May, 1989. In January of 1990, he moved to Knoxville, Tennessee and began work toward a Master's degree in Comparative and Experimental Medicine. In May 1992, he became the second of eight children to receive his Master's degree.