AFRRI SCIENTIFIC REPORT

Effects of endotoxin on survival of hypertransfused mice

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Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFRRI SR83-2	2. GOVT ACCESSION NO. A131 114	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) EFFECTS OF ENDOTOXIN ON SURVIVAL OF HYPERTRANSFUSED MICE		5. TYPE OF REPORT & PERIOD COVERED 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) R. M. Vigneulle and S. J. Baum		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Armed Forces Radiobiology Research Institute (AFRRI) Defense Nuclear Agency Bethesda, Maryland 20814		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NWED QAXM MJ 00040
11. CONTROLLING OFFICE NAME AND ADDRESS Director Defense Nuclear Agency (DNA) Washington, DC 20305		12. REPORT DATE February 1983 13. NUMBER OF PAGES 19
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Published in Experimental Hematology 10: Suppl. 12, 249-262, 1982.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The survival of hypertransfused B6CBF$_1$ female mice exposed to 8.5 and 9.0 gray of cobalt-60 gamma rays and immediately given 10 ug of endotoxin i.p. was significantly increased compared to either irradiated mice which were given endotoxin or to hypertransfused and normal mice that had been comparably irradiated and were given saline i.p. Animals which received the combined treatment (hypertransfusion, irradiation plus endotoxin) had increased survival compared to the other treatment groups at day 30 or at day 40 under controlled environmental conditions, but not when the recovery

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. ABSTRACT (continued)

occurred under less controlled environmental conditions. Hypertransfused mice have greatly expanded pools of uncommitted progenitor and myeloid precursor cells which apparently are unstimulated. After irradiation, when these pools were stimulated by endotoxin, granulocytopoiesis was enhanced which resulted in an increase in animal survival.

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Reprinted from EXPERIMENTAL HEMATOLOGY
Vol. 10, Supplement No. 12, 1982

EFFECTS OF ENDOTOXIN ON SURVIVAL OF HYPERTRANSFUSED MICE

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Animal survival following an exposure to a midlethal radiation dose usually depends on recovery of hemopoiesis. It is generally agreed that bone marrow failure following irradiation results in the perturbation of granulocytopoiesis. The animal is at great risk for the development of infections and consequently diminished capacity for survival because of a reduction in functional granulocytes during this first week or so after irradiation (1).

Administration of endotoxin as a single injection either before or immediately after irradiation increases survival in mice (2-8), rats (9), and canines (10-12). These studies indicated that the survival-promoting effect tended to diminish with increasing time following mid lethal irradiation and at 24 hours after midlethal irradiation endotoxin had little beneficial effect. The improvement in survival from endotoxin is a result of an increase in granulopoiesis in the postirradiated animal. Both before and after irradiation endotoxin accelerates myeloid recovery in mice (12-14) and canine (11,15). The time of injection markedly influences the pattern of change in the leukocyte counts. The most pronounced changes occur when the endotoxin is geven up to 24 hours before and immediately after irradiation. Besides endotoxin, substances such as leukogenerol (16) and 19S globin fraction of serum (17.18) have been reported to accelerate myeloid recovery postirradiation.

Since endotoxin stimulates granulocytopoiesis, this was believed to underlie its ability to protect against postirradiation infections. The production of CSF in response to endotoxin might be the humoral regulator of granulopoiesis as seen in endotoxin pretreated irradiated mice. Therefore this was a property of endotoxin that was carefully studied (19-21) as well as the cells of the monocyte-macrophage series which appear to be responsible for protection against infection (22). These studies demonstrated that the stimulation of granulocyte recovery by endotoxin seems to be mediated by its effect upon the sites of production and/or release of such factors rather than directly upon the granulocyte precursors.

Endotoxin injection elevates the number of cells forming granulocyte colonies in vitro (23) as well as the concentration of factors in serum of mice (24) and rats (25) which stimulate growth of granulo-

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Supported by Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00040. The views presented in this paper are those of the authors. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

cytic colonies.

Erythroid suppression prior to irradiation reduces the demand for erythropoiesis in hypertransfused mice which may leave more stem cells available for differentiation into nonerythroid lines (26-29). Moreover, other studies have demonstrated a decreased erythroid capacity by the reduction in CFU-E (30) and BFU-E (31) in hypertransfused mice and CFU-E but not BFU-E in the exhypoxic mice (32).

Suppression of erythropoiesis per se is not entirely responsible for the improved recovery of the myeloid system postirradiation (33,34). A single administration of endotoxin shifts erythropoiesis from the bone marrow to the spleen in erythroid suppressed mice. Endotoxin inhibited erythropoiesis in the bone marrow and stimulated it in the spleen (35-37). The decrease of erythropoiesis from the bone marrow by endotoxin may be associated with an increased mobilization into the blood of hematopoietic colony-forming cells (38). However, when mice are sublethally irradiated and hypertransfused, an increase in granulopoiesis was demonstrated with endotoxin (28,29) or other stimulatory factors (40).

If a reduced erythropoiesis exists during and after lethal irradiation and is followed by a stimulation for granulopoiesis, then granulopoiesis could be increased at a time when the postirradiated mouse is at great risk for infection for lack of functional neutrophils. The present investigation is concerned with the possibility that progenitors of the granulocytic series are further enhanced under these circumstances by competing for the early multipotent progenitors. We have approached this question by evaluating whether a single administration of endotoxin leads to changes in the granulocyte compartment indicative of improved myeloid capacity as measured by survival following a midlethal dose of cobalt-60 gamma rays to hypertransfused mice in which the demand for erythropoiesis is suppressed.

MATERIALS AND METHODS

ANIMAL STRAINS AND HUSBANDRY. Male and female B6CBF$_1$ (C57Bl6 x CBA)F$_1$ hybrid mice (Cumberland Farms, Clinton, TN) 2-3 months of age, were used. The mice were housed in plastic shoe box cages, five experimental mice per cage, Wayne Lab-Blox and hyperchlorinated tap water were supplied ad libitum. The mice in the first three replicate experiments were kept under the following controlled environmental conditions in the animal facility. The ambient room temperature was maintained at 74°C with 45% relative humidity and an alternate 12-hour light/dark cycle during the observation period. During this time, the mice were handled only when the cages were changed, twice weekly. Animal survival was observed on a daily basis and all dead animals were removed. The mice in the remaining three replicates reported here were housed in different quarters under less controlled environmental conditions during a transition period into a new animal facility. The data has been divided and treated separately to reflect the difference in environmental conditins. Some of the data has been

pooled using valid statistical limits and inferences made on these means.

HYPERTRANSFUSION. Both male and female mice were exsanguinated under ether anesthesia and the blood collected via the carotid artery into isoton (azide free) with a trace quantity of heparin and the erythrocytes were packed by centrifugation. The erythrocytes were washed in greater than equivalent volumes of ice cold isoton and packed by centrifugation. After the third blood cell washing procedure, the hematocrit of the packed erythrocytes was measured and, if necessary, adjusted to 65% with ice cold buffered saline. Groups of female mice were injected intraperitoneally with 1 ml of packed erythrocytes on 2 consecutive days. We observed that hematocrits of mice rendered plethoric by this procedure were 60% or greater for periods of 7-12 days following hypertransfusion. Erythroid suppression was confirmed 2-3 days following hypertransfusion by the disappearance of reticulocytes from the peripheral blood.

EXPERIMENTAL DESIGN. The female mice were divided into four treatment groups: I) irradiated mice given saline i.p.; II) irradiated mice given 10 ug endotoxin i.p. (Lipopolysaccharide Salmonella typhimurium, lot 1, List Biologicals, CA); III) plethoric irradiated mice given saline i.p.; and IV) plethoric irradiated mice given 10 ug endotoxin i.p. Either saline or endotoxin was given i.p. immediately following irradiation. Groups of plethoric mice and normal mice were bilaterally irradiated with 8.5, 9.0, 10.0 or 10.5 gray of cobalt-60 gamma rays in the AFRRI facility at a dose rate of 0.4 gray per minute. Each treatment group for each radiation dose used had at least five animals. The experiment was replicated three times under controlled environmental conditions and three times under less controlled environmental conditions. The data sets are presented separately since, although the hypertransfusion, radiation exposure, and endotoxin preparations were equal, the difference in the environmental factors after treatment resulted in noticeably different survival in the combined treatment groups at each radiation dose. Since each data set is composed of three experiments under similar environmental conditions, the data within each treatment group was pooled and comparisons were made between these groups of pooled data. The complete experiment had a total of 40 mice for each of the four treatment groups at doses of 8.5, 9.0, and 9.5 gray. The total mice in the 10.0 and 10.5 gray dose groups were, respectively, 15 and 5, for each of the four treatment groups. The survival data are plotted as a function of days postirradiation through day 40, the end of our observation period. The log-rank test of Savage (41) was used to assess statistical significance for the survival data.

HEMATOLOGY AND HEMATOPOIETIC CELL STUDIES. Some additional experiments were designed to evaluate the effect of 9.0 gray of cobalt-60 gamma rays on the hematopoietic cell precursors and peripheral blood hematology of the treatment groups during their early observation period. This was performed in order to assess the effect of the respective treatments in the plethoric mouse model. The data from

these studies will be used to demonstrate the effectiveness of treatment on hematopoiesis. These findings will be correlated with those from mortality studies.

PERIPHERAL BLOOD HEMATOLOGY. Peripheral blood samples were obtained by bleeding the mouse from the retroorbital venous plexus using a heparinized hematocrit tube. Hematocrit, RBC and WBC were determined by conventional methods. RBC and WBC were counted using a Coulter Counter Model ZBI. RBC were lysed from the WBC counting sample using zapoglobin. Hematocrits were determined from the first capillary tube of blood taken and centrifuged in an Adams Autocrit II.

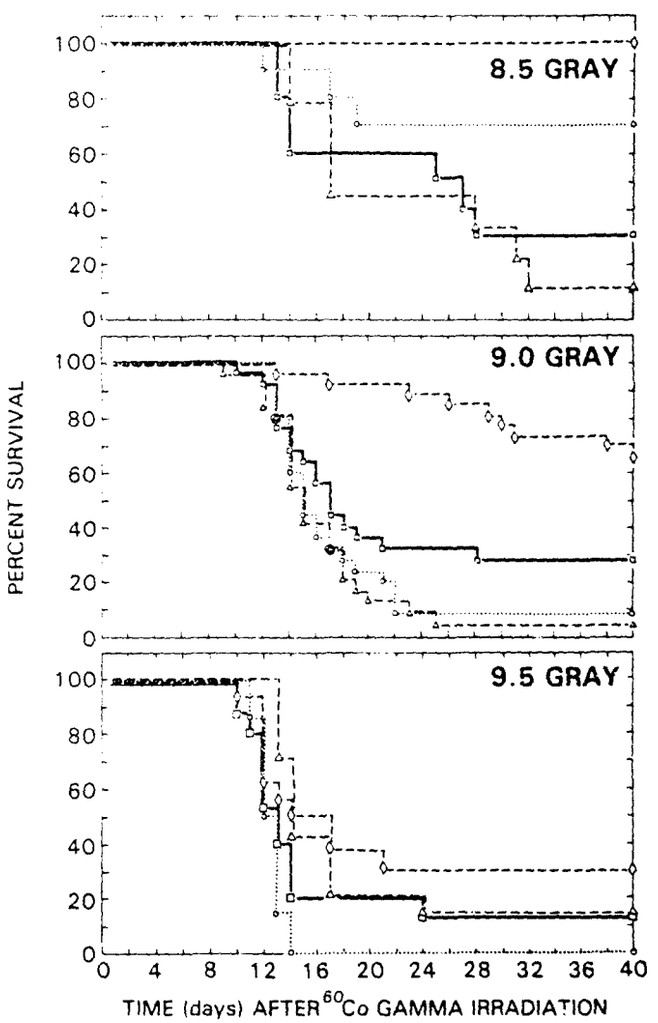
RESULTS

Figure 1 shows survival is radiation dose-dependent in the range 8.5-9.5 gray. The survival for the hypertransfused mice irradiated and given endotoxin, the combined treatment group, is significantly greater than that found for the other treatment groups at 8.5 and 9.0 gray. Survival of the combined treatment group is statistically significant from those groups of pletnoric mice which were irradiated and given saline, and from those groups of irradiated mice given saline, and from those groups of irradiated mice given endotoxin ($0.01 > P > 0.001$). At the highest radiation dose shown (9.5 gray), the magnitude of improvement in survival for the combined treatment group is less compared to irradiated mice given saline than observed during the 40 days after either 8.5 or 9.0 gray. No increase in survival was found for the combined treatment group at 10.0 and 10.5 gray (data not shown). These data show that 10 ug of lipopolysaccharide endotoxin administered i.p. immediately after irradiation in nonhypertransfused mice was less effective than when given to the hypertransfused mice. At 9.0 gray this effect was reduced. At 40 days after 9.0 gray, 66% survival was found in the combined treatment group as compared to 27% survival in the endotoxin treatment group and less than 10% survival in the other treatment groups.

Figure 2 shows the ratio of survival for each of the treatment groups to the survival of the 9.0 gray irradiated group plus saline at 30 and 40 days after irradiation. The bar graph of the ratios clearly illustrate the effectiveness of the combined treatment in increasing survival at this radiation dose at 30 days (9.6), the usual terminus of the mortality associated with acute hematopoietic failure and at a later time, 40 days (8.2). The combined treatment group was 2.9 and 2.5 times as effective in improving survival as compared to the endotoxin treatment group at day 30 and 40, respectively. Moreover, no increase in survival is apparent for hypertransfused mice which were irradiated and received a saline injection i.p. in lieu of endotoxin.

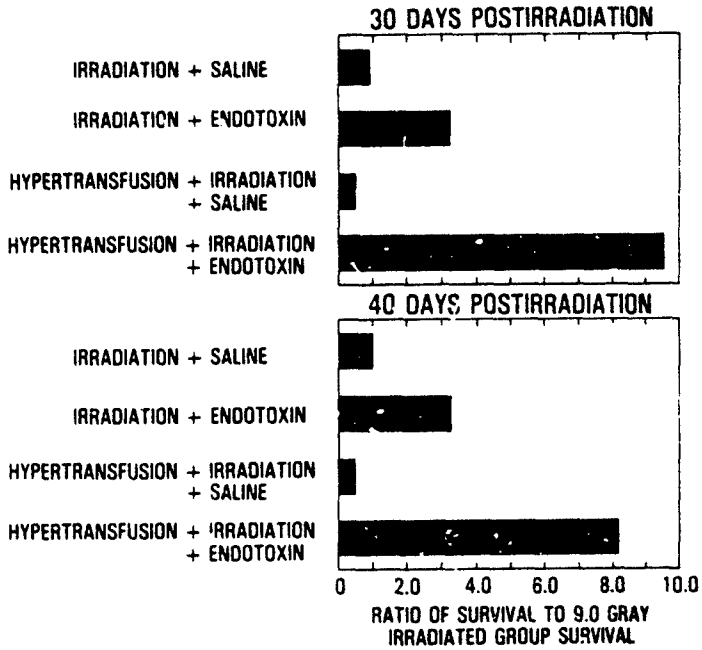
The environmental conditions under which the animals are kept during the observations period following irradiation can result in large differences in survival, including the combined treatment group. This is the case as seen for the second data set, replicates 4-6 (Fig 3). The survival curve for the combined treatment group shows an increase

FIGURE 1



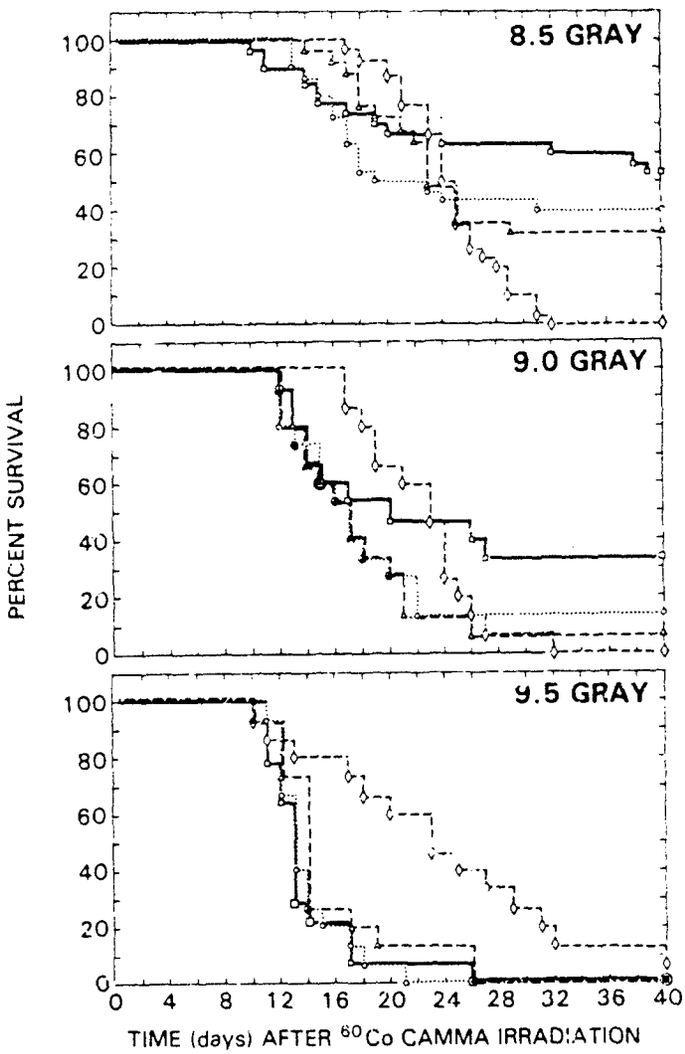
Acute survival curves of mice for data set 1, replicates 1-3, for the following 4 treatments: Irradiation plus saline, open circles; Irradiation plus 10 ug endotoxin, open squares; Hypertransfused, irradiated plus saline, open triangles; Hypertransfused, irradiated plus 10 ug endotoxin (combined treatment), open diamonds.

FIGURE 2



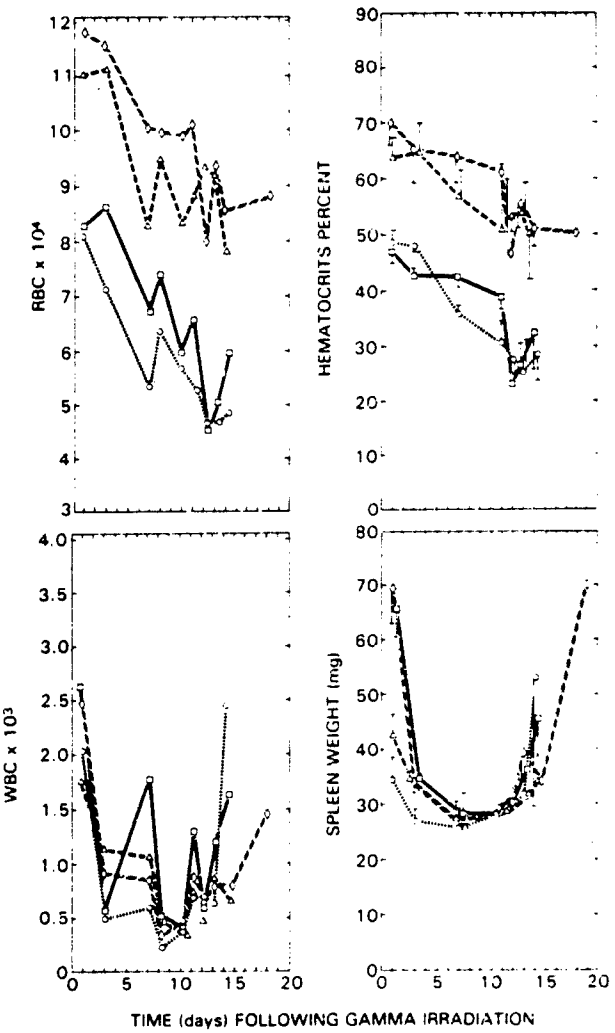
Histogram of ratio of survival of each treatment group to the 9.0 Gray irradiation group survival at day 30 and day 40 after irradiation.

FIGURE 3



Acute survival curves for mice for data set 2, replicates 4-6, shows survival for mice kept under different environment conditions than survival curves presented in Fig 1. The 4 treatments and symbols are comparable to that described in Fig 1. The combined treatment group symbole is the open diamond.

FIGURE 4



RBC, Hematocrit, WBC, and Spleen Weights for groups of mice which received each of the 4 treatments described in Fig 1 as a function of time (days) following irradiation. The combined treatment group symbol is the open diamond.

in animal survival time through day 22 as compared to the other treatment groups. However, under the apparently adverse environmental conditions existing during the recovery period, recovery is not complete since little or no increase in survival is demonstrated for the combined treatment group after day 22. These data indicate that poor environmental conditions subjected mice to increased chances for infection and decreased the capability for recovery of these animals even when receiving the combined treatment. These data suggest that the health of the animals (presumably infection) during these replicate experiments was clearly altered relative to those which are reported in Fig 1. Gross contamination of either the packed red blood cell preparation or the endotoxin and saline solutions was ruled out through routine broth culture and microbial screening. Some of the post-mortum data collected from a small sample of dead animals which, when found were not decomposed, did not indicate any unusual findings regarding the cause of death of the combined treatment group as compared to the other treatment groups that was not attributed to radiation induced lesions. No antibiotics were administered to any of these groups of mice before or after the irradiation.

Figure 4 shows data for RBC, WBC, hematocrits, and spleen weights for groups of mice treated according to the same protocol and sacrificed at 1, 3, 7, 9, 10, 11, 12, 13, 14, and 16 days following 9.0 gray of irradiation. The RBC and hematocrits are significantly elevated initially in the hypertransfused mice and following irradiation remain above the level observed in irradiated mice or irradiated mice given endotoxin. The number of erythrocytes in normal and hypertransfused groups of mice which were given endotoxin immediately after irradiation is greater than comparable groups given saline, respectively. WBC and spleen weight values decrease until their individual nadir is reached. These groups of normal mice or hypertransfused mice which received endotoxin demonstrated a smaller decline in WBC and spleen weight at day 1 as compared to the groups of normal and hypertransfused mice which were irradiated and received saline. Recovery of WBC and spleen weight values begins concomitantly although these parameters do not reflect the effectiveness of the combined treatment group as depicted by survival.

DISCUSSION

It is clearly established that endotoxin of various kinds and dosages given immediately after irradiation increases survival in mice (4,12). The data we have reported here are in agreement with those earlier studies. In addition, our new observation shows that survival can be further increased when endotoxin is administered to hypetransfused mice. The magnitude of the increase in survival is gamma radiation dose dependent for the combined treatment group.

Plethora induced in animals has been shown to increase hematopoietic progenitor cells (CFU-S, GM-CFC, BFU-E) in both bone marrow and spleen, although the erythroid committed cell, the CFU-E and ^{59}Fe incorporation were markedly reduced in the bone marrow and were

moderately reduced in the spleen (30,31). Plethora induced in mice prior to these midlethal doses of gamma radiation seems to have increased the number of uncommitted cells in the hematopoietic stem cell pool. This suggests that a larger concentration of uncommitted progenitor and/or precursor cells are available for differentiation into nonerythroid cell lines due to the reduced demand for erythropoiesis. Although the expansion of the uncommitted progenitor pool made additional precursor cells available for differentiation and amplification into the granulocytic series, data of the present study demonstrates that this can only be achieved by further indirect stimulation of endotoxin for these cells.

Hypertransfusion is altering some regulatory aspect of the hematopoietic cell compartment possibly including the microenvironment in order that granulocytopoiesis in hypertransfused mice could recover more rapidly from sublethal radiation induced bone marrow depression (27,34). The survival results agree with the suggestion that suppressed erythropoiesis seems not to be entirely responsible for the improved recovery of the myeloid system after irradiation (15,33,34). Although mice were rendered plethoric before midlethal irradiation in order that they could begin with an expanded pool of myeloblasts, promyelocytes, and myelocytes (34), endotoxin appears to be essential to promote survival in this investigation either as a stimulus for proliferation of myeloid cell compartment and/or as an inhibitor of erythroid cell compartment in the bone marrow.

Additional stress of adverse environmental conditions for the second data set shows the limited capacity of the recovery of the hematopoietic compartment to resist secondary infections during the acute survival interval following these midlethal doses. This suggests that although the combined treatment may be potentially beneficial to animal survival, other measures including antibiotics would be necessary to support experimental animals subjected to poor environmental conditions during the first few weeks postirradiation.

SUMMARY

The survival of hypetransfused $B6CBF_1$ female mice exposed to 9.5 and 9.0 gray of cobalt-60 gamma rays and immediately given 10 ug of endotoxin i.p. was significantly increased compared to either irradiated mice which were given endotoxin or to hypertransfused and normal mice that had been comparably irradiated and were given saline i.p. Animals which received the combined treatment (hypertransfusion, irradiation plus endotoxin) had increased survival compared to the other treatment groups at day 30 or at day 40 under controlled environmental conditions, but not when the recovery occurred under less controlled environmental conditions. Hypertransfused mice have greatly expanded pools of uncommitted progenitor and myeloid precursor cells which apparently are unstimulated. After irradiation, when these pools were stimulated by endotoxin, granulocytopoiesis was enhanced which resulted in an increase in animal survival.

ACKNOWLEDGEMENT

We wish to thank Mr. Richard T. Brandenburg for excellent technical assistance, Dr. Thomas J. MacVittie for valuable discussion and Miss Terrie Hunt and Mrs. Marianne Owens for typing the manuscript.

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