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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
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Bethesda, Maryland

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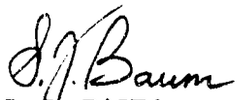

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FOREWORD
(Nontechnical summary)

The work reported herein was performed to test the hypothesis that rats could be made permanently anemic by repeated mixed gamma-neutron irradiations, and that once the maintenance of normal circulatory red blood cell concentration was lost, the administration of exogenous erythropoietin could not restore the production of red cells to normal levels.

Erythropoietin is a hormone which normally regulates the formation of red cells in the hematopoietic tissues of the body. Normally anemia, or other conditions which cause the oxygen tension of the body to decrease, results in the production of erythropoietin in the kidney (and perhaps elsewhere), and the appearance of the hormone in the circulation causes erythropoiesis (red cell production) to be stimulated in the bone marrow. Failure of radiation-induced anemia to respond to administered erythropoietin was tested to determine whether the achieved anemic steady-state condition was refractory to erythropoietin at high doses of erythropoietin.

Rats irradiated either with 9 periodic doses of 150 rads each of mixed gamma-neutron radiations, or with 300 rads each on 4 occasions, developed an anemia which was observed to be permanent over a period of 7 to 8 weeks. Following a 5-day series of erythropoietin injections (5 units/day) and a second regime of erythropoietin injections 38 days later (25 units/day for 5 days), the anemic state of the rats did not change.

The refractoriness of the circulating red blood cell production of the anemic rats was also corroborated by failure of tracer radioiron uptake to increase following erythropoietin injections. The reappearance of tracer radioactive iron in newly formed red cells is a measure of cellular production.

ABSTRACT

Experiments were performed to test the hypothesis that rats could be made permanently anemic by repeated mixed gamma-neutron irradiations, and that once the maintenance of normal circulatory red cell concentration was lost, the administration of exogenous erythropoietin could not restore the production of red cells to normal levels.

Rats were exposed to 9 periodic doses of 150 rads mixed gamma-neutron radiation or to 4 periodic doses of 300 rads. Hematocrits and erythrocyte counts obtained for 100 days or more after the final radiation exposure showed a significant reduction in erythrocyte production. This permanent anemia was not ameliorated by the treatment with 5 daily doses of either 5 units or 25 units of erythropoietin.

These findings appear to strengthen the hypothesis that the permanent anemia is caused by a reduced capability for cellular proliferation due to accumulation of residual injury in stem cells.

I. INTRODUCTION

Until fairly recently, it was not unreasonable to propose the hypothesis that a cellular renewal system, such as erythropoiesis, would completely repair injury sustained from exposure to ionizing radiation. This was supported by observations[10] that rats exposed to 50 rads daily over a period of 150 days showed no appreciable change in hemoglobin level or red cell count. Furthermore, other reports indicated that rats subjected continuously to ^{137}Cs gamma rays at a dose rate up to 84 rads per day, or to daily protracted doses up to 60 rads were able to maintain normal hemoglobin values for 40 days after the initiation of radiation exposure.[11,12] However, the hemoglobin concentrations declined beyond the 40th day until the animals died 10 to 15 days later. Furthermore, in a subsequent study[5] one of these authors observed that while continuous radiation (45 rads/day) did not decrease erythropoiesis in rats beyond the 5th exposure week, the repopulating ability of their bone marrow was severely decreased.

Evidence for postirradiation residual injury came from a different set of experiments.[1] Rats received 300 R x rays four times at 3-month intervals. At the beginning of each repeated exposure erythropoietic recovery was retarded, implying a reduction of total cellular space. However, there was no decrease in erythrocyte volume and hematocrit 89 days after the end of the first three exposures. A significant decrease in erythrocyte volume and hematocrit was observed 89 days after the 4th exposure. The author postulated that accumulated residual injury to the erythropoietic system had decreased its functional capacity for normal cellular production.

The radiation-induced residual injury could have been caused by faulty intracellular recovery of stem cells resulting in imperfect precursor proliferation.[4] There also could have been a physical reduction in stem cell numbers due to anatomical changes of the internal environment.[6] Finally, the possibility existed that insufficient quantities of erythropoietin were produced which would decrease stimulation for stem-cell release.

In order to test the latter hypothesis, a two-step approach was designed for the present experiment. First, rats were to be exposed at intervals to one of two sublethal doses of mixed gamma-neutron radiation. Upon the termination of periodic radiation exposures, the animals were to be tested for several weeks for their capability to produce new erythrocytes. Should a permanent anemia exist, the rats would be challenged with exogenous erythropoietin in an attempt to increase erythrocytosis.

II. METHODS

For this experiment 60 adult male Sprague-Dawley rats (Simonsen Laboratories, Inc., White Bear Lake, Minnesota) were used. The rats were 140 $\pm$ 3 days old at the beginning of the study and their weight ranged from 284 to 324 g. They were maintained individually in clean wire cages and were allowed free access to food and water.

Twenty rats were scheduled to be exposed to 150 rads of gamma-neutron radiation approximately once every 2 weeks, another 20 to 300 rads once every 4 weeks, and the remaining 20 were utilized as unirradiated controls. After 5 periodic

exposures to 150 rads and 2 to 300 rads, the reactor utilized for the radiation source became inoperative and was closed for repairs. Erythrocyte counts and hematocrit values obtained from each animal approximately every week indicated a return to normal values 25 days after the last radiation exposure; therefore, when the radiation source became available again, exposures were continued.

Eventually the 150-rad exposure group received 9 radiation doses (total 1350 rads) while the 300-rad group was subjected to 4 (total 1200 rads). When hematocrits and erythrocyte values measured with a Coulter counter indicated that the rats were anemic, half of the previously irradiated animals in each group received 5 units of Erythropoietin A, Step 1 daily for 5 days via the tail vein. This procedure commenced on day 167 after the first exposure for the 150-rad group and on day 153 for the 300-rad group. On the 209th day, the 150-rad group received another 5-day treatment regime of 25 units of erythropoietin per day and a similar procedure was followed for the 300-rad group beginning on the 195th day.

Six days before, and on the fifth day of the first erythropoietin treatment regime ^{59}Fe incorporation was measured in all rats in order to assess erythropoiesis. The rats were injected intravenously with a sodium citrate-buffered $FeCl_3$ solution containing 0.5 μCi of ^{59}Fe in 0.025 μg of total iron for the first test and 2.0 μCi of ^{59}Fe in 0.1 μg of iron for the second. Three days after the injection of the tracer radioactive iron, blood was withdrawn from each rat via the jugular vein and the radioactivity of 50 microliters of this blood was counted in an automatic dual-channel, well-type, gamma scintillation detector using a 3 x 3 thallium activated sodium iodide crystal. The method described by Sterling and Gray[13] using ^{51}Cr labeled

erythrocytes for blood volume measurements was utilized to determine total ^{59}Fe activity in the blood.

The AFRRI-TRIGA reactor described in a previous report[7] was the source for the mixed gamma-neutron radiation. The rats were placed in a radiation field with an isodose surface 292 cm from the reactor center line. The midline tissue dose rate was 20 rads per minute. The ratio of gamma to neutron kermas was 1.5. The procedures for dosimetry were published earlier.[4]

The statistical ranking test devised by White[14] and the "t" test were utilized to test the significance of hematocrit and erythrocyte count decrease, at the time when permanent anemia was indicated, between unirradiated controls and periodically irradiated animals.

III. RESULTS

Hematocrits and red cell concentrations were obtained approximately weekly in order to test for the anemic state of the rats, but not necessarily to determine erythrocyte injury and recovery conditions. Nevertheless, the responses observed in animals subjected periodically to 150 or 300 rads of mixed gamma-neutron irradiation (Figures 1 and 2) generally show the injury sustained after each exposure as well as the recovery. In the 150-rad group (Figure 1) subsequent exposures occurred at a time when erythropoiesis was accelerated in response to previous cellular injury which in turn would modify newly incurred injury. Animals exposed repeatedly to 300 rads (Figure 2) with 4-week intervals show the typical reduction in cellular production with low values approximately 2 weeks after irradiation. Both groups demonstrate a macrocytic anemia in response to the radiation damage.

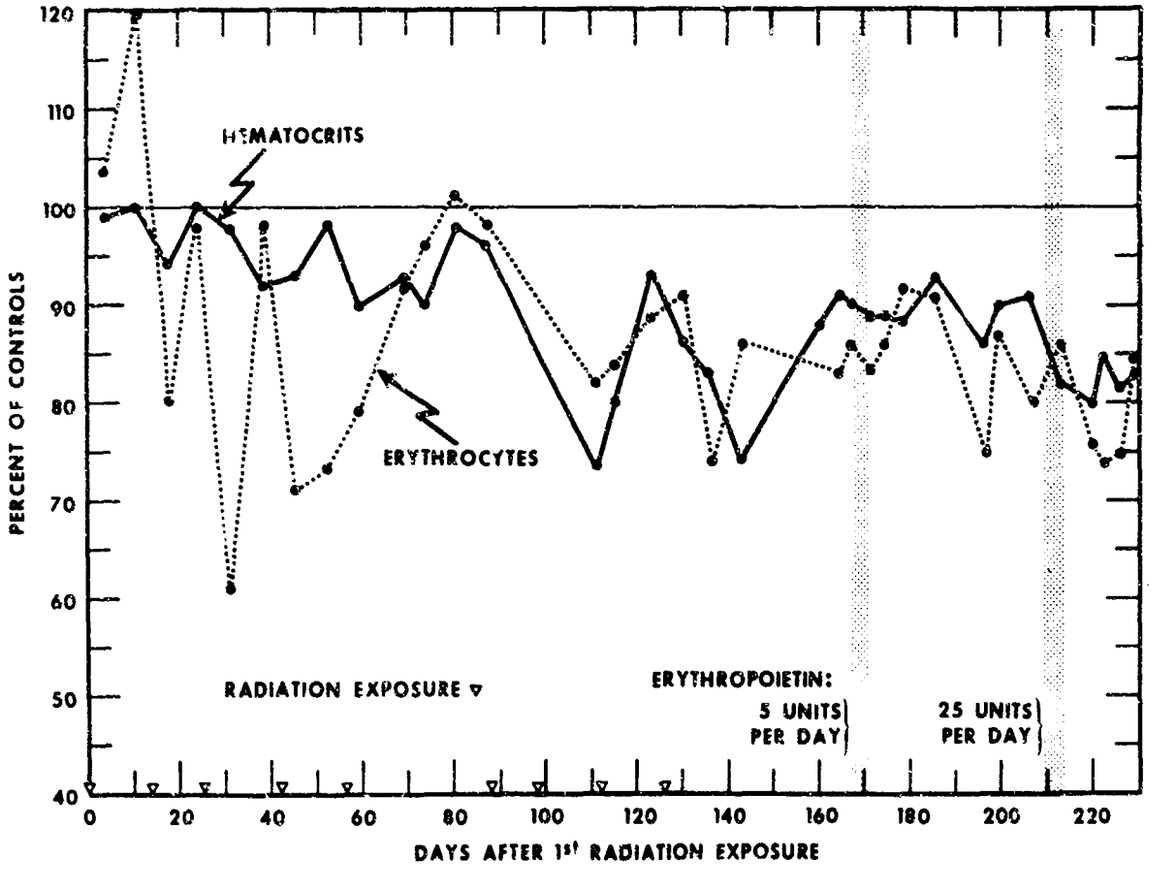

Figure 1. Percent changes in hematocrits and erythrocyte concentration of rats exposed periodically to 150 rads of mixed gamma-neutron radiation

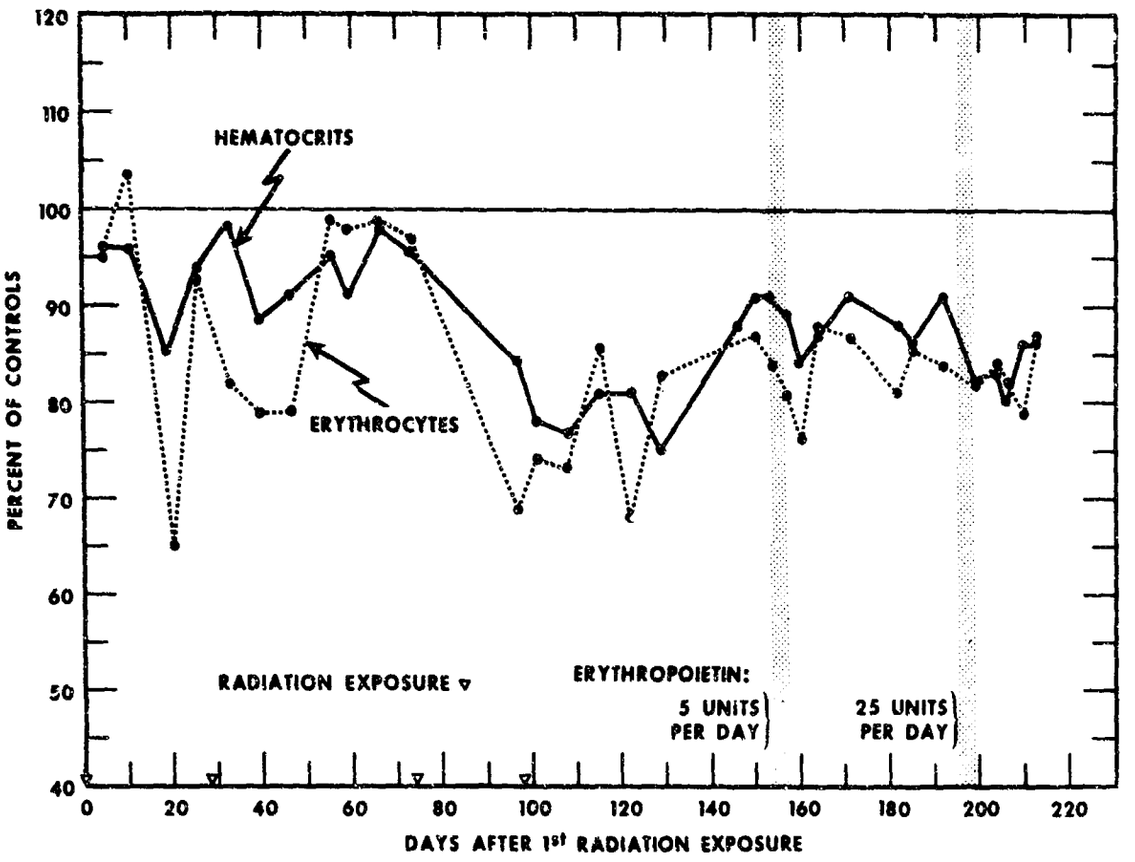

Figure 2. Percent changes in hematocrits and erythrocyte concentration of rats exposed periodically to 300 rads of mixed gamma-neutron radiation

The last radiation exposure for the 150-rad group occurred 126 days after the first, while that for the 300-rad group was on the 98th day. Since 6 animals of the first group and 10 of the second died at that time, it was decided to stop periodic irradiation. Erythrocyte production was measured for the next 7 to 8 weeks when it became clear that the animals in both exposure groups were indeed permanently anemic (Table I). As may be seen, the mean hematocrit in the exposure groups was significantly reduced ($p \leq 0.001$) from 47.1 percent to 40.0 and 40.7 percent, respectively.

Table I. Hematocrits of Rats just prior to the First Erythropoietin Administration

Radiation dose (rads)	Hematocrits					
	Number of rats	Mean and S.D. for all rats	p*	Number of rats	Mean and S.D. for selected group†	p*
150 (x9)	14	40.0 ± 2.6	0.001	7	38.1 ± 1.8	0.001
300 (x4)	10	40.7 ± 3.1	0.001	5	37.8 ± 1.1	0.001
0	20	47.1 ± 3.7				

* For comparison with controls.
† Rats with hematocrit below 40.

It was decided to utilize only rats with hematocrit below 40 for the erythropoietin test (Table I). Figures 1 and 2 clearly indicate that the administration of 5 units of erythropoietin per day for 5 days did not stimulate increased erythropoiesis. This is substantiated by the ^{59}Fe uptake which after the erythropoietin administration did not differ from values obtained before treatment (Table II).

Table II. Radioiron Incorporation in Radiation-Induced Anemic Rats Prior to and on the 5th Day After Erythropoietin Administration

Radiation dose (rads)	^{59}Fe Incorporation (percent of injected dose)	
	Before treatment*	After treatment*
150 (x9)	61.7 ± 5.1†	58.0 ± 5.8
300 (x4)	61.6 ± 5.1	62.9 ± 5.2
0	69.1 ± 3.2	67.1 ± 3.9

* 5 units Erythropoietin A, Step 1 daily for 5 days
† Standard Deviation

In order to be certain that adequate quantities of erythropoietin were used during the first treatment regime, a second regime was initiated 38 days later utilizing a fivefold increase in concentration of the hormone (25 units per day for 5 days). As may be seen from Figures 1 and 2 the anemic state of the rats was not changed. At the time of the final measurement, which was 100 days or more after the last radiation exposure, the hematocrits and red cell concentrations were significantly reduced by 15 percent from control values.

IV. DISCUSSION

The data obtained in the present study show that rats exposed to at least 5 periodic doses of 150 rads of mixed gamma-neutron radiation or to at least 2 periodic doses of 300 rads appear capable of recovery, with a return of peripheral erythrocytic values to preirradiation levels. However, after additional periodic exposures, the maintenance of normal circulatory cellular concentrations is lost and a permanent

anemia ensues. The return of a circulatory erythropoietic steady state should not be equated with complete recovery of the erythropoietic system since there is enough evidence to indicate that normal erythrocyte turnover is accomplished with the participation of only a small fraction of the total number of stem cells. This is substantiated in a report by Blackett[5] who observed no fall in red cell production even though the hematopoietic system was under considerable stress and the stem cell population was severely depleted. Furthermore, Baum[1] observed residual injury to the erythropoietic system after each of three repeated 300 R x-ray exposures. However, red cell volume and hematocrits were normal 89 days after each irradiation.

On the other hand, the present study demonstrates that radiation exposures beyond the numbers listed above result in a permanent decrease in circulatory red cells. This substantiates the findings reported earlier[1] that a fourth repeated exposure to 300 R x rays would cause anemia in rats. It was suggested that permanent anemia occurs only when the capacity of the erythrocyte precursors to proliferate cells is reduced below a certain limit. In a later paper[3] it was demonstrated that this residual injury occurs probably in the stem cell population. The results of the present experiment which demonstrate peripheral recovery after several replicated radiation exposures with a sudden significant reduction in cellular turnover after a specific number, strengthen the hypothesis which assumes accumulation of residual injury with replicated radiation doses.[4] When the accumulated residual injury reduces the total functional erythrocytic precursor cellular number to a level where a normal steady state can no longer be supported, a new and lower level is maintained.

The primary objective of this study was to determine if the anemia was caused by diminished titers of endogenous erythropoietin. Since it was reported previously[2] that the administration of 6 units of erythropoietin induces normal erythropoiesis in polycythemic rats, it was felt that 25 units would increase cellular release if the anemia was caused by its decreased endogenous production. However, no increase in erythropoiesis was noticed in terms of hematocrits, red cell numbers and in ^{59}Fe incorporation.

In order to be certain that the hormone was administered in sufficient quantities to stimulate measurable increases in erythropoiesis, a second treatment regime utilizing a total of 125 units over 5 days was undertaken. This fivefold increase in erythropoietin injection failed to ameliorate the anemic condition of the rats. Although reports in the literature[8,9] indicate that continous administration of erythropoietin for several weeks increases red cell production in normal rats or mice, the administration of 25 units per day for 5 days did not stimulate increased erythrocyte proliferation in the normal unirradiated animals in this study.

The findings of this experiment do not implicate diminished endogenous erythropoietin production as the cause for the permanent reduction in red cell production in rats exposed repeatedly to mixed gamma-neutron radiation. With the elimination of this possibility, the hypothesis proposed earlier[1] that the residual injury was caused by a reduced capability, either functional or anatomical, for cellular production is strengthened.

V. CONCLUSION

Rats exposed to periodic doses of either 150 or 300 rads of gamma-neutron radiation developed permanent anemia. This permanent anemia was not ameliorated by treatment with 5 daily doses of either 5 units or 25 units of erythropoietin. These findings appear to strengthen the hypothesis that the permanent anemia is caused by a reduced capability for cellular proliferation due to accumulation of residual injury in stem cells.

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13 ABSTRACT

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