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Author(s)	Yamagishi, Morihisa
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# Bone Marrow Treatment of Mice Lethally Irradiated with Gamma-Rays under High Dose Rate (I)

## Effect of Isologous Bone Marrow

Morihisa YAMAGISHI\*

First Division, Department of Internal Medicine, Faculty  
of Medicine, Kyoto University

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Mice were irradiated lethally with gamma-rays under high dose rate and were treated with isologous bone marrow. The irradiated mice responded well to the bone marrow treatment as evidenced by the quick improvement in the peripheral blood picture, body weight and histological findings of the hematopoietic organs, as well as by the reduced mortality rate.

### INTRODUCTION

Two organ systems have been well recognized as playing important roles in acute radiation death; the gastrointestinal system and hematopoietic system.

In the former, a massive dose of ionizing radiation causes the mucous membrane of the gastrointestinal tract to become abraded and ulcerated, followed by the invasion of bacteria and finally sepsis<sup>20)</sup>. This bacterial invasion through the intestinal wall is the main cause of death during the first week after lethal irradiation. In this disorder antibiotics such as streptomycin have been known to reduce mortality considerably.

In the latter, anemia, leucopenia and thrombopenia due to the devastation of the hematopoietic organs lead to the inability to resist invading organisms and to a bleeding tendency. This is the main cause of death during the second week. No effective therapy of the bone marrow injury had been found until Jacobson *et al.*<sup>15, 17-19)</sup> observed for the first time that shielding of the hematopoietic organs during irradiation caused early recovery of the peripheral blood picture and a lower mortality rate. It was found later that the injection of a homogenate or suspension of hematopoietic organs (spleen<sup>21)</sup>, bone marrow<sup>22)</sup>) could lessen the mortality. This kind of treatment has been confirmed as effective not only in mice and rats but also in guinea pigs<sup>23)</sup>, hamsters<sup>24)</sup>, rabbits<sup>25, 26)</sup>, dogs<sup>1)</sup>, and monkeys<sup>9)</sup>. Also in humans bone marrow therapy has been tried for the treatment of leukemia and other hematological diseases<sup>26)</sup>.

Gamma-rays have been known to be, with minor differences, similar to X-rays in biological effects, except that RBE is slightly lower in the former than in the latter. And in a few instances the radiation effect diminishes with very high dose rate<sup>22)</sup>, but there is little information about very intense in-

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stantaneous exposures such as atomic explosions.

In most of the experiments on bone marrow treatment, X-rays were used to irradiate animals, but only a few have used gamma-rays<sup>21)</sup>, and essentially none under high dose rate. One may be exposed to gamma-rays under high dose rate in an atomic explosion or in accidents at atomic energy plants. A powerful  $\text{Co}^{60}$  gamma-irradiation facility was furnished recently at our university and the following study was performed.

### MATERIALS AND METHODS

Inbred dd/s strain mice, male and female, were used as bone marrow donors and recipients. The mice were supplied from The Kyoto University Inbred Strain Animal Center. The donor mice were sacrificed by a blow on the head and bilateral femurs and tibias were removed aseptically. Both ends of the bones were cut, a small needle (size 1/5, used usually for tuberculin tests) was inserted into one end, cold sterile Tyrode's solution was flushed through the bone marrow cavity and bone marrow cells in it were washed out into a small tube. Many kinds of solutions have been used to suspend bone marrow cells by different workers: 0.8% NaCl plus 0.2% KCl<sup>37)</sup>, chilled M/15 phosphate buffer<sup>11)</sup>, buffered saline<sup>23)</sup>, salt-sucrose medium containing ATP and dextrose<sup>5)</sup>, physiological saline solution<sup>2)</sup>, and Tyrode's solution<sup>12,20,31)</sup>. Tyrode's solution was used in the present study because of its easy availability and of its theoretically good property of preserving living cells. The volume of Tyrode's solution used to suspend the bone marrow cells from four long bones was 1.2 cc. The total number of nucleated marrow cells thus obtained was  $4 \times 10^6$  to  $12 \times 10^6$  in 1.0 cc. of the solution in the early study, increased later to  $10 \times 10^6$  to  $20 \times 10^6$  by flushing the bone marrow cavity twice. Coarse particles settled out by gravity within half a minute. The remaining supernatant fluid was practically a single-cell suspension, but a small number of clumps consisting of several cells were still observed.

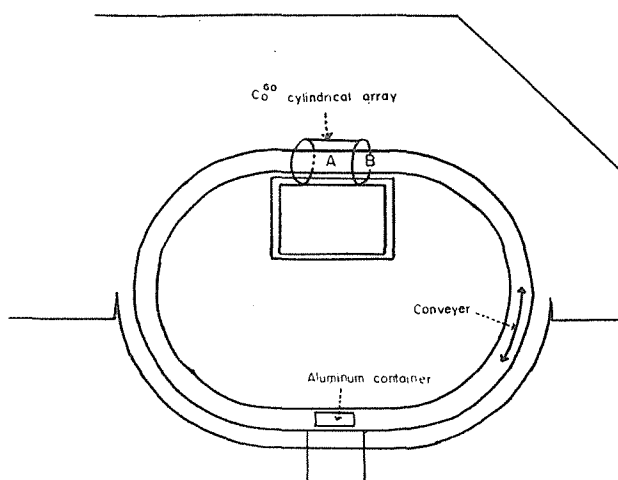


Fig. 1.  $\text{Co}^{60}$   $\gamma$ -ray irradiation facility.

A  $\text{Co}^{60}$  gamma-irradiation facility which belongs to the Institute for Chemical Research of Kyoto University was used in the present experiment (Fig. 1). This facility is described in detail in the first and second papers of this issue<sup>39,40</sup>. The intensity of gamma-rays in April, 1958 was  $2.34 \times 10^6$  r/hr and  $2.02 \times 10^4$  r/hr, at the center of the  $\text{Co}^{60}$  cylindrical array (A in Fig. 1) and at the place immediately outside of the array (B in Fig. 1), respectively. Three to four mice were placed in a  $10 \times 10 \times 10$  cm. paper box, which was then placed at the center of an aluminum container. The container with the box was moved in and out of the place of irradiation (A or B in Fig. 1) as quickly as possible in order for the mice not to be exposed to an extra dose of gamma-rays before and after receiving a certain dose of irradiation. Mice placed at A can be expected to receive equal doses of irradiation because gamma-rays come from practically all directions. Those placed at B, however, cannot be expected to do so, because gamma-rays come from one side and the distance from the cylindrical array to each animal in the box varies a little.

Irradiated mice were kept in wooden boxes measuring  $16 \times 21 \times 30$  cm, 6 to 9 in a box, were fed wheat, dried fish (every other day) and vegetables (twice a week) and were given tap water ad libitum.

## RESULT

### 1) Survival Rate

The survival rates in four experiments are shown in Table 1. There were no thirty day survivals among the controls, while the treated groups showed a significant percentage of survivals which varied from experiment to experiment. Female mice responded to the bone marrow treatment somewhat better than male mice. Most of the mice which survived for 30 days lived for the next 60 days or longer. After 90 days, two mice died at 99 and 106 in exp. #1, two at 125 and 130 days in exp. #2, and one at 163 days in exp. #3. Due to a considerable degree of post-mortem autolysis the cause of death in these

Table 1. Survival rate of gamma-irradiated mice treated with isologous bone marrow.

Experiment No.	Sex	Dose rate (r/min)	Dose (r)	No. of nucl. cells injected $\times 10^6$	Survival No. of living/No. of irradiated					% Survival		
					at 7 days	14	30	60	90	30 day	90	
1	♂	305	900	2.8~5.7	T	10/16	6/15	4/15	3/15	3/15	27	20
					C	7/9	2/9	0/9			0	
2	♀	3630	910	4.5~7.5	T	7/8	5/8	5/8	4/7	4/7	63	56
					C	3/4	0/4				0	
3	♀	3500	880	5.6	T	12/13	9/11	9/11	8/11	7/11	82	64
					C	4/5	0/5				0	
4	♂	3430	860	9.4	T	9/10	8/10	4/10	4/10	4/10	40	40
					C	7/8	1/8	0/8			0	

T : Treated

C : Control All of 75 control mice irradiated with more than 850r at A died within 21 days

long-lived mice was not clarified.

2) Body Weight Changes

Body weight changes are shown in Fig. 2.

a) Female. Both the control and treated mice showed a marked continuous weight loss for the first several days following gamma-irradiation. In the controls the weight loss continued until their death up to two weeks after irradiation. In the treated mice the weight loss stopped at 4 to 6 days and gradually increased thereafter. The increase was very gradual and never reached the preirradiation level, staying at 85 to 90% during the period of 60 days. Mice in exp. #3 were followed up to 90 days at which time the weight had recovered to 95%.

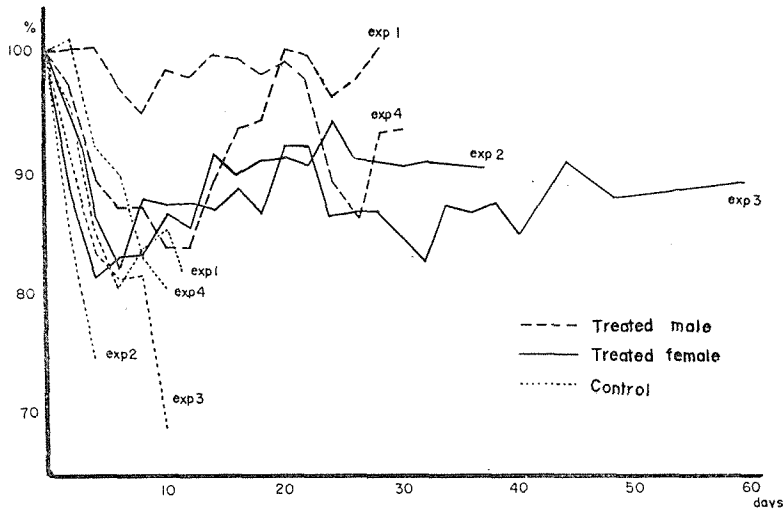


Fig. 2. Body weight changes in gamma-irradiated mice treated with isologous bone marrow.

b) Males. The weight change in the male controls was essentially the same as in the females except that there was a temporary increase in exp. #1. The treated mice in exp. #1 had a slower rate of weight loss than the females, followed by a rapid recovery to the preirradiation level by the fourth week. In exp. #4 there was only a slight weight loss with gradual recovery to the pre-irradiation level by the third week. There was, however, a period of abrupt decrease in the fourth week corresponding to the increased mortality.

3) Hematological Findings

a) Leucocyte count. The leucocyte count in both controls and treated mice showed a temporary increase within 24 hours after irradiation, followed by a rapid decrease, 2000 to 3000 at 2 days, 1000 at 3 days and less than 500 at 4 days. In most of the controls there was no increase in leucocyte count up to the time of their death except for a few which showed a slight recovery. In the treated mice the leucocyte count began to increase at 6 or 7 days, the count being approximately 1000 at 7 day and 5000 to 6000 at 10 days. Then it

gradually or quickly approached normal levels. The count was still somewhat below normal at 20 days (Fig. 4(c)), but had returned essentially to the normal level by 30 days.

**b) Erythrocyte count, reticulocyte count and hemoglobin content.** The change in the erythrocyte count following irradiation was less than in the leucocyte count. However, the effect of bone marrow treatment on the erythrocyte count can be seen in Fig. 3(a). At five days there was no apparent difference in erythrocyte count between the control mice and treated ones. At 9 days there was a definite difference between them, however. The difference was seen also in the hemoglobin content (Fig. 3(b)). These differences in both RBC and hemoglobin content were statistically significant with 95% probability. As for the reticulocyte count there was not yet much change 24 hours after irradiation, but it decreased to zero by 3 days. The reticulocyte count in the controls continued to be low, being at most 1 to 2 per thousand. The count in the treated mice began to increase at 5 or 6 days, reaching its maximum at 7 to 10 days when the count was much higher than the pre-irradiation level (Fig. 4(a)). The platelet count (by Rees-Ecker's method) was lowest around at 7 days being  $4 \times 10^4$  to  $6 \times 10^4$  in both controls and treated mice, followed by a gradual increase in the latter (Fig. 4(b)).

**d) Differential count.** In both the controls and treated mice, a relative increase in the number of granulocytes was observed for 24 hours after irradiation, 80 to 90% of the leucocyte count. This relative granulocytosis continued

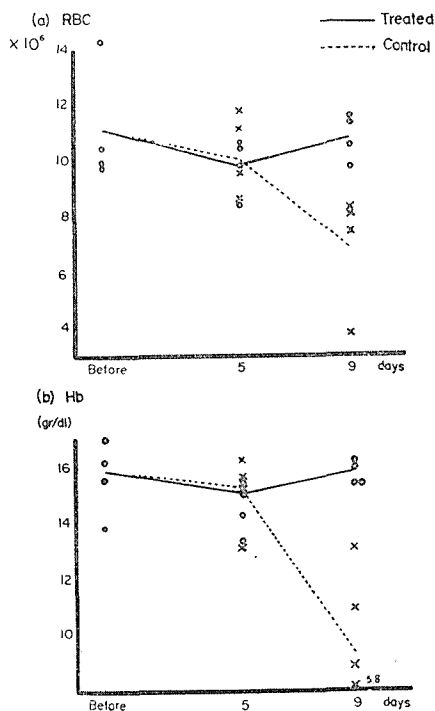


Fig. 3. RBC and Hb in gamma-irradiated female mice treated with isologous bone marrow.

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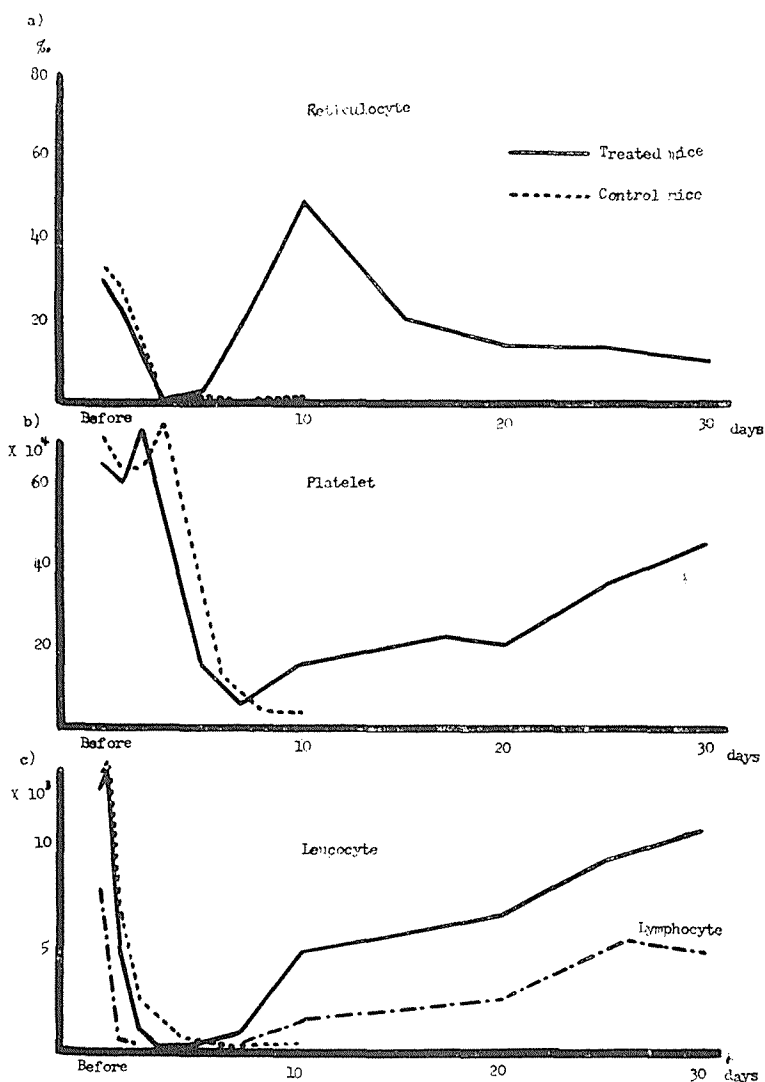


Fig. 4. Reticulocyte, platelet and leucocyte count in gamma-irradiated mice treated with isologous bone marrow.

to the next day with a few exceptions in which degenerated cells and nuclear debris were most of the leucocytes. At three days and thereafter, during the period of extreme leucopenia, only a small number of seemingly intact granulocytes and lymphocytes were seen among the degenerated leucocytes and nuclear debris. During the period of recovery in the treated mice both granulocytes and lymphocytes increased, the former being slightly predominant.

4) Histological Findings

a) **Bone marrow.** In both controls and treated mice, only mature granulocytes, megakaryocytes and monocytes were seen scattered in the bone marrow cavity 24 hours after irradiation. These cells rapidly decreased in number to almost none at 3 days (Fig. 5). However, a very small number of

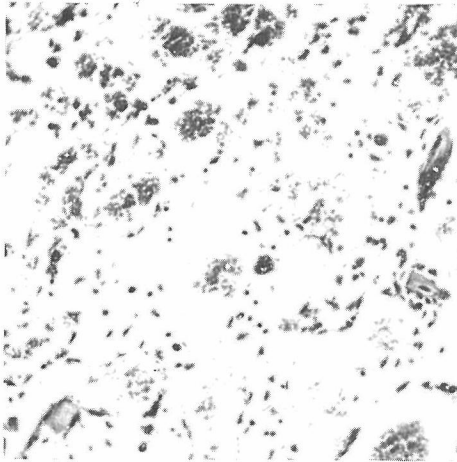


Fig. 5. Bone marrow ; 3 days after gamma-irradiation and isologous bone marrow treatment. The bone marrow is completely wasted. H-E stain  $\times 200$

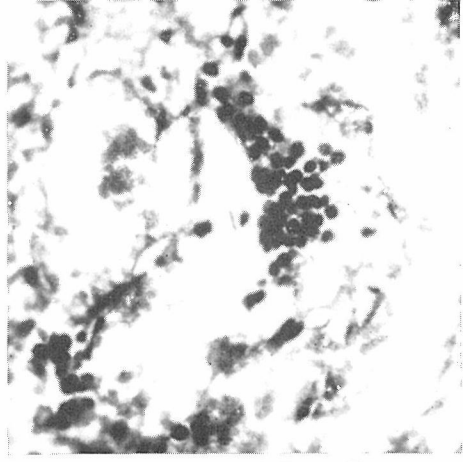


Fig. 6. Bone marrow ; 4 days after gamma-irradiation and isologous bone marrow treatment. A group of young bone marrow cells are seen to have appeared from the wasted bone marrow. H-E stain  $\times 400$

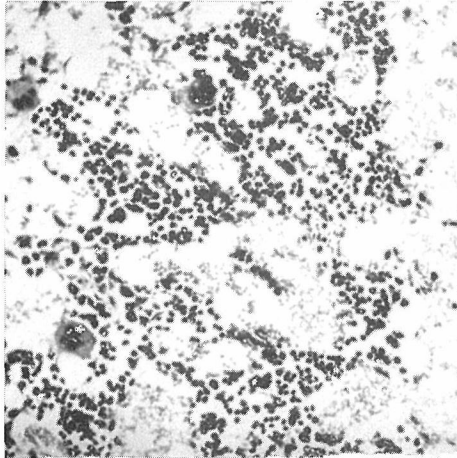


Fig. 7. Bone marrow ; 5 days after gamma-irradiation and isologous bone marrow treatment. Many foci of spotty regeneration. H-E stain  $\times 200$

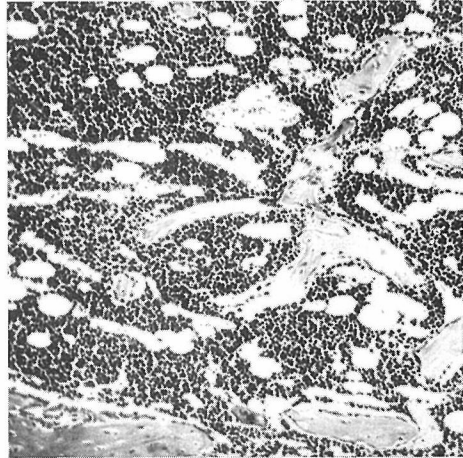


Fig. 8. Bone marrow ; 8 days after irradiation and isologous bone marrow treatment. The cellularity is normal except for scattered areas of fatty change. H-E stain  $\times 100$

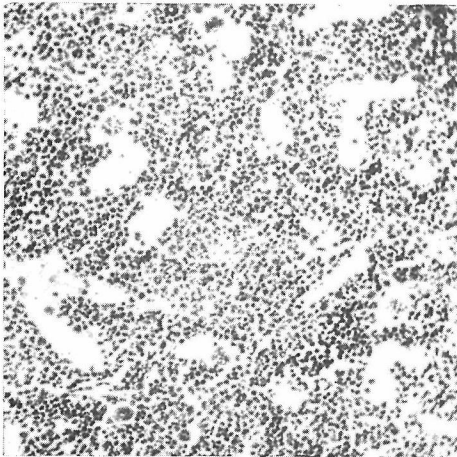


Fig. 9. Bone marrow ; 36 days after irradiation and isologous bone marrow treatment. The cellularity is normal except for the dilatation of sinuses. H-E stain  $\times 200$



Fig. 10. Spleen ; 4 days after irradiation. Completely wasted but a small number of lymphocytes are seen in the white pulp. H-E stain  $\times 200$



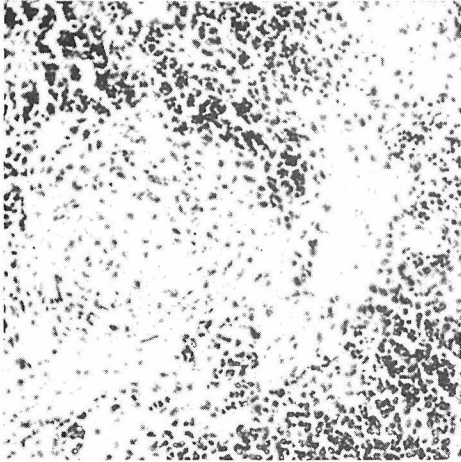


Fig. 11. Spleen; 7 days after irradiation and isologous bone marrow treatment. Marked regeneration of myelocytic and erythrocytic cells in the red pulp, but complete wasting of the white pulp. H-E stain  $\times 200$

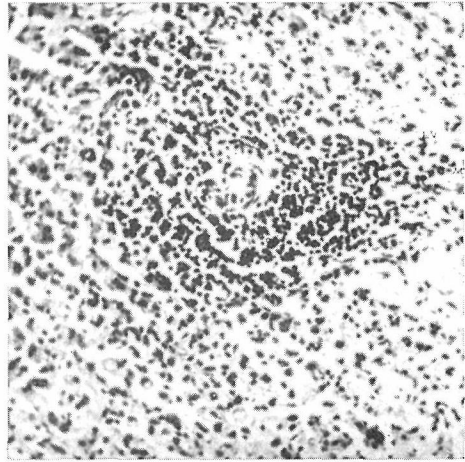


Fig. 12. Spleen; 14 days after irradiation. Although there is a moderate increase in number of lymphocytes in the white pulp, the red pulp is still wasted. H-E stain  $\times 200$

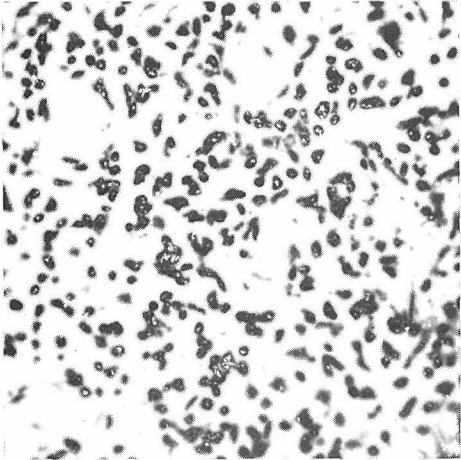


Fig. 13. Inguinal lymphnode; 14 days after irradiation and treatment with isologous bone marrow. Small number of scattered lymphocytes. H-E stain  $\times 400$

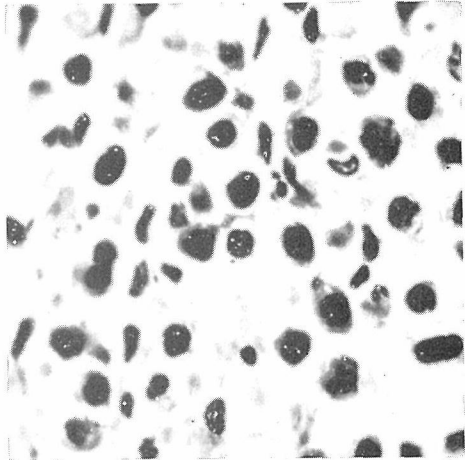


Fig. 14. Mesenteric lymphnode; 30 days after irradiation and isologous bone marrow treatment. The lymphnode is filled with plasma cells. H-E stain  $\times 400$



Fig. 15. Lung; Sacrificed immediately after injection of  $10^7$  bone marrow cells. A mass of injected bone marrow cells obstructing the lumen of the lung capillaries. H-E stain  $\times 200$

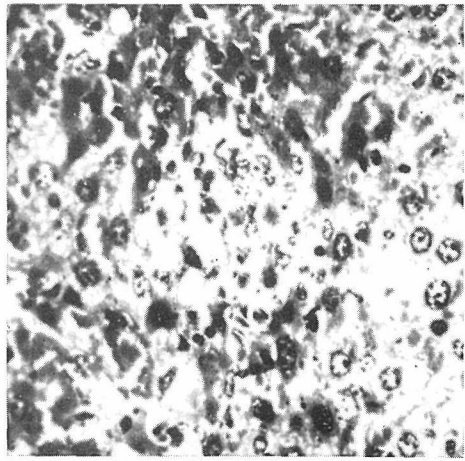


Fig. 16. liver; 20 days after irradiation and injection of isologous bone marrow. Focal necrosis without inflammatory reaction. H-E stain  $\times 400$

granulocytes were still observed even at this period. They did not appear to have regenerated because few young cells were seen among them. At 4 days scattered groups of regenerating young cells appeared in a few of the treated mice (Fig. 6). On the next day, in many treated mice the wasted bone marrow was studded with foci of young blood forming cells (Fig. 7). In the bone marrow of the mice which were in good condition at the time of sacrifice, two thirds to three fourths of the marrow cavity was filled with newly regenerated marrow cells at 7 days and almost normal cellularity except that there were scattered areas of fatty change and dilated venous sinuses were seen at 8 days (Fig. 8) as well as essentially normal cellularity at 11 days. Autopsy on the treated mice that died during the 30 day observation period revealed retarded recovery in most, if not all, some with no evidence of regeneration of hematopoietic cells even at 13 days. But the treated mice that died between 20 and 30 days had considerably regenerated bone marrow.

The bone marrow of the control mice had a slower rate of recovery than did that of the treated mice. No control mice, dying or sacrificed, showed evidence of bone marrow recovery before 8 days. The control mice in all experiments except for exp. #1 in which the mice might not have received uniform doses of irradiation died with bone marrow wasted or only partially recovered.

**b) Spleen.** In the control mice lymphocytes in the white pulp of the spleen rapidly decreased in number after irradiation, followed by the disappearance from the red pulp of the cells of the erythrocytic and myelocytic series leading to complete wasting of both the red and white pulp at 2 days. However, a small number of lymphocytes always remained in the white pulp (Fig. 10). The regeneration of the cells of the erythrocytic and myelocytic series in the red pulp did not usually begin before 10 to 12 days, except for a few mice showing early recovery at 8 days. All control mice except for those in exp. #1 showed little evidence of the recovery of white pulp at the time of their death.

The treated mice also showed initial wasting of the spleen as did the control mice, followed by a quick recovery of the red pulp in the sacrificed mice, beginning at 5 days and becoming nearly complete at 10 days (Fig. 11). Autopsy on the treated mice that had died revealed a somewhat retarded tendency for the red pulp to recover, but the recovery began at 10 days in most of them. The white pulp of the treated mice did not recover earlier than that of the controls. More than three or four weeks were needed before the white pulp resumed its pre-irradiation picture.

From the observations described above, it was noted that the red pulp of the treated mice recovered at least three or four days earlier than that of the controls.

**c) Lymphnode.** The lymphnodes had wasted almost completely by 24 hours after irradiation, leaving a small number of lymphocytes. This wasted condition continued for about three weeks (Fig. 13). After that an increase in number of lymphocytes was observed to begin. However, even at this period

some lymphnodes, particularly mesenteric, still showed little evidence of recovery and were full of plasma cells, macrophages and reticular cells (Fig. 14). Since all control mice died by two weeks after irradiation, it was not known which group, control or treated, showed earlier recovery.

d) **Other organs.** Complete wasting of the thymus was followed by a fairly good recovery at 7 to 10 days in some of the mice in both groups. Focal necrosis and degeneration without inflammatory reaction were seen in the liver of some mice in the treated group (Fig. 16). Since not all the livers were examined, it cannot be stated that these changes did not take place in the control mice. The adrenals, kidneys, and heart showed no significant change with H-E stain. Glomerulo-sclerosis was not observed in mice living longer than three months after irradiation. Aggregations of injected cells obstructing the lumina of the alveolar capillaries were observed immediately after injection of bone marrow cells (Fig. 15), followed by the quick disappearance of most of them within 24 hours. However, a few aggregations stayed in the capillaries for more than a week, showing disintegration of the cells.

#### DISCUSSION

It was shown in the present study that bone marrow treatment was effective in high dose rate gamma-irradiation as in ordinary X-irradiation. The 30 day survival rate in the reported experiments of 770 to 950 r X-irradiation and isologous bone marrow treatment was 76%<sup>23)</sup>, 45 to 100%<sup>37)</sup>, 40 to 100%<sup>5)</sup>, 94.3 to 100%<sup>8)</sup> and 68 to 100%<sup>22)</sup>. There were differences in the dose of X-rays and isologous bone marrow given. Urso *et al.*<sup>38)</sup> observed that the more bone marrow was injected, the quicker was the recovery of the cellularity of the bone marrow, and that the minimum number of the nucleated bone marrow cells to give 100% survival following 900 r X-irradiation was  $64.4 \times 10^6$ , and that  $12.8 \times 10^6$  was insufficient to give 100% survival (male 100% and female 90%). However, doses between  $64.4 \times 10^6$  and  $12.8 \times 10^6$  were not tested. When the dose was reduced to  $0.97 \times 10^6$ , the survival rate decreased to 75% in males and 53% in females. Lorenz *et al.*<sup>22)</sup> obtained 78 to 100% survival after 900 r X-irradiation and 1.5 mg. of isologous bone marrow treatment. From Cole *et al.*'s estimation,<sup>5)</sup> 1.5 mg. of bone marrow corresponds approximately to  $17 \times 10^6$ . Thus the result of Lorenz *et al.*'s experiment essentially agrees with that of Urso *et al.* From these studies 30 day survival rates of 50 to 90% can be expected when lethally irradiated mice are given  $1 \times 10^6$  to  $10 \times 10^6$  nucleated cells of isologous bone marrow. In the present study using gamma-rays under high dose rate the result was similar in the females to the expected survival rate. The survival rate in the males was low in the present study, however. Those mice surviving for a period of 30 days were in good condition for the subsequent 60 days, giving one the impression that they recovered almost completely from lethal gamma-irradiation injury. However, Hollcroft *et al.*<sup>14)</sup> observed that the life span of irradiated mice decreased irrespective of spleen shielding, the decrease being 19.7% of the natural life span after 900 r X-irradiation. At

least a part of the cause of the decreases of life span in irradiated animals protected by spleen shielding was an increase in the incidence of tumors in organs other than those of the lymphatic system and of glomerulo-sclerosis. Thus spleen shielding did not have a dose-reducing effect in terms of life span and the same situation is presumably present in bone marrow treatment. One half or more of the mice which survived for the initial 90 day period died within the next 6 months.

The initial decrease of body weight after irradiation is well known to be due mainly to anorexia<sup>28)</sup>. The rate of recovery of body weight after irradiation seemed to be related to the dose of irradiation and amount of bone marrow given. Hollcroft *et al.*<sup>14)</sup> have shown the smaller the dose of X-rays is, the longer is the period of subsequent weight increase. Urso *et al.*<sup>38)</sup> observed that with massive doses of bone marrow after irradiation the initial weight loss in mice was less than with ordinary dose of bone marrow, and that even the mice thus treated did not resume their pre-irradiation weight within 40 days. The reason for the slight degree of initial weight loss in exp. #4 could be due to a relatively small dose of irradiation and to a relatively large dose of injected bone marrow. However, the rather quick recovery of weight to the pre-irradiation level and its subsequent decrease seen in this group differ from the results of other workers, and this bizarre weight change is difficult to explain.

Three major mechanisms are considered to induce anemia in irradiated animals; the absence of erythropoiesis, active phagocytosis of erythrocytes, and increased excretion of bile pigments<sup>16)</sup>. When animals receive a massive dose of irradiation, diarrhea due to gastrointestinal damage leads to hemoconcentration within a few days after irradiation. However, anemia in irradiated animals at 9 days is unlikely to be modified by hemoconcentration. The fact that at 9 days anemia did not develop in the treated animals, which showed complete wasting of the bone marrow though transiently, is worthy of note. The recovery of erythropoiesis was early enough to offset the effects of anemia-inducing factors.

The life-saving mechanism of bone marrow treatment has been well known as promoting early recovery of hematopoietic organs, as seen also in the present study. Two major theories have been advanced to explain the mechanism of early recovery of hematopoietic organs. One is the so-called "humoral theory"; a certain subcellular substance or substances accelerate hematopoiesis. The other is "cellular theory"; the cells injected into irradiated animals seed in the hematopoietic organs and repopulate them. The humoral theory prevailed in the early days. However, many experimental data supporting the cellular theory have subsequently been reported. Lindsley *et al.*<sup>20)</sup> found that when rats were X-irradiated with a dose of LD50 and subsequently given homologous bone marrow carrying an immunologic marker, erythrocytes carrying the label could be detected for as long as 147 days and contributed as much as 80% of the peripheral erythrocytes. Other evidences of transplantation of homologous bone marrow cells into irradiated animals were shown by

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Merwin *et al.*<sup>25)</sup>, Ford *et al.*<sup>10)</sup>, and Porter<sup>29)</sup>. It had been known for some years that a certain number of mice fatally irradiated and given rat bone marrow survive<sup>1)</sup>, which had been thought to provide additional evidence in favor of the humoral theory because the idea of transplantation of heterologous rat cells into mice had been too fantastic to believe. However, Makinodan<sup>24)</sup> and Smith *et al.*<sup>33)</sup> immunologically, Nowell *et al.*<sup>27)</sup> histochemically, and Cole *et al.*<sup>5)</sup> using marrow transfer technique showed that rat bone marrow cells could seed and repopulate the marrow cavity of lethally irradiated mice. From these evidences the cellular theory has become the prevailing one. The humoral theory has not, however, faded away yet, because the mechanism of spleen shielding has not been explained adequately by the cellular theory and there are experiments in which lethally irradiated animals survived following the injection of heterologous bone marrow but repopulation by donor cells cannot be proved<sup>36)</sup>. In explaining the mechanism of isologous bone marrow treatment there is nothing against the cellular theory, but a humoral factor (or factors) may play a role. Hirsch *et al.*<sup>13)</sup> observed that isologous bone marrow was consistently effective in promoting early recovery of thymic weight, whereas homologous and heterologous marrows were not. It is not known, however, whether this difference is due to a humoral factor or to the immunological reaction involved in the homologous and heterologous bone marrow treatment.

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