Modification of Hematopoietic Cell Transplantation in Mice Irradiated with Gamma Rays under High Dose Rate. (I)

Combined Use of AET and Bone Marrow Treatment

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1) The AET protected mice supralethally irradiated with gamma rays under high dose rate were treated with isologous bone marrow (IBM) or homologous bone marrow (HBM). The combined use of AET and IBM was effective for saving the mice exposed to supralethal irradiation, but most of the AET protected and HBM treated ones died after the 21th post-irradiation day.

2) The AET protected and gamma-irradiated donor HBM could not prevent delayed death.

INTRODUCTION

It is generally accepted that there are three types of acute radiation death of mammals[1] ; they are characterized by hematopoietic failure, intestinal injuries and central nervous system disorders. Bone marrow treatment which was first observed by Lorenz et al.[2,3] is available for hematopoietic failure, but less effective for intestinal injuries and central nervous system disorders of supralethally total body irradiated animals[4]. On the other hand, several chemical radioprotective agents are also known to protect the mammals from acute radiation death. AET (S-2-isothiuronium dihydrobromide)[5~8] is one of the most effective ones protecting animals, especially mice, from acute radiation death. AET has been shown to protect intestines more than hematopoietic organs[7]. Therfore, mice pretreated with AET and exposed to total body irradiation of otherwise supralethal dose may survive acute radiation death when they receive inoculation of bone marrow. Furthermore, it was suggested that AET protected predominantly non-antibody forming cells[8]. Therefore, recipient mice pretreated with AET and exposed to supralethal dose of radiation may easily take homologous bone marrow which is inoculated after irradiation, because prolonged complete wasting of lymphoid tissues, in which immunologically competent cells are probably formed[9,10], of recipients is observed and reduced number of immunologically competent cells ameliorates homograft response. On the other hand, when bone marrow of AET pretreated and irradiated homologous donor mice is inoculated to recipient mice, they may not succumb to so-called secondary disease after total

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body irradiation. Cudkowicz reported[11] that preirradiation of donor homologous bone marrow could prevent homologous disease as well as AET pretreated and X-irradiated donor marrow did.

In most of the experiments on bone marrow treatment, X rays were used to irradiate animals, but only a few[12,13] have used gamma-rays under high dose rate. A powerful Co^{60} gamma-irradiation facility has been furnished at our university. Therefore, the purpose of this experiment is to ascertain whether the combined use of AET and bone marrow treatment of supralethally gamma-irradiated mice is effective, and whether AET pretreated and gamma-irradiated HBM (homologous bone marrow) can prevent the recipient mice from secondary disease after total body gamma-irradiation under high dose rate.

MATERIALS AND METHODS

1) Mouse: Dd/s strain mice supplied from the Kyoto University Animal Center were used as recipients and isologous bone marrow (IBM) donors. Na2 strain mice were used as homologous bone marrow (HBM) donors. They were two to two and a half months old weighing 20-23 g. The sexes of the donors and recipients were identical. Irradiated mice were kept in wooden boxes measuring 16×12×30 cm, 8 to 10 in a box, were fed wheat, dried fish and vegetable and were given tap water ad libitum.

2) Preparation of cell suspension: The donor mice were sacrificed by cervical dislocation 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. Coarse particles settled down by gravity within half a minute.

3) Irradiation: A Co^{60} gamma-irradiation facility which belongs to the Institute for Chemical Research of Kyoto University was used in the present experiments. The facility has been described in detail[14,15]. The intensity of gamma rays in June 1962 was 139 kr/h, namely, 38.7 r/sec. which was quite high. LD 100/30 day dose of 900r was obtained in 27 sec. in October 1962, and in 32 sec. in October 1964.

4) AET: AET was supplied from the Takeda Pharmaceutical Industry, Ltd., Osaka, Japan. The mice were intraperitoneally given AET (6.0-10.0 mg/mouse) in physiological saline 15-20 minutes before irradiation. The bone marrow donor mice given AET were exposed to 100r, 450r or 900r 4 hours, 6 days or 8 days before sacrifice. In some instances, the donors without AET were exposed to 100r, 450r or 900r 4 hours, 6 days or 8 days before sacrifice.

RESULTS

I) Combined Use of AET and Bone Marrow Treatment for Irradiation Injuries

A) Survival rate. The survival rates and 30-day mortalities in 13 experi-

Table 1. Survival rate of AET protected, gamma-irradiated mice treated with IBM* (or HBM)*.

Exp.	Treatment	Number of mice	% Survival at days					
			7	14	21	30	60	90
1	450r	28	100	100	100	100	93	93
2	AET-900r	30	100	100	86	86	86	86
3	900r-IBM	28	100	100	100	88	88	88
4	AET-900r-IBM	8	87	87	87	87	75	75
5	900r	20	40	0				
6	AET-1300r	18	55	55	22	22	11	11
7	AET-1300-HBM	14	78	43	14	0		
8	AET-1500r	17	77	65	23	6	6	6
9	AET-1500r-IBM	15	73	73	66	60	60	60
10	AET-1500r-HBM	12	100	100	100	58	8	8
11	AET-1800r	14	14	0				
12	AET-1800r-IBM	15	27	6	0			
13	AET-2000r	15	0					

* Inoculated cell count is 10×10^6.

ments are shown in Table 1 and Fig. 1, respectively. At 900r, there were no 30-day survivals among the untreated mice, while the treated mice showed a significant percentage of survivals. At 1500r, survival rate of the AET alone treated mice was 6% at 30 days, while those of the AET-IBM and AET-HBM treated mice were 60% and 58% at 30 days, respectively. However, delayed death was observed only in the AET-HBM treated mice, namely, survival rate at 90 days was 8%. At 1800r or 2000r, all of the mice treated in any way died by 21 days. They showed heavy bloody diarrhea and weight loss.

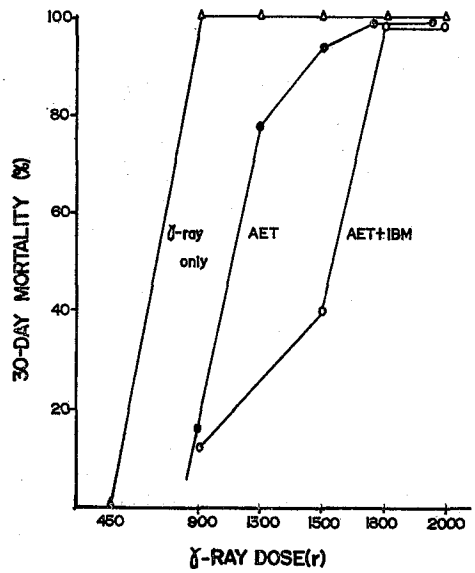


Fig. 1. Mortality of gamma-irradiated, AET protected mice treated with IBM.

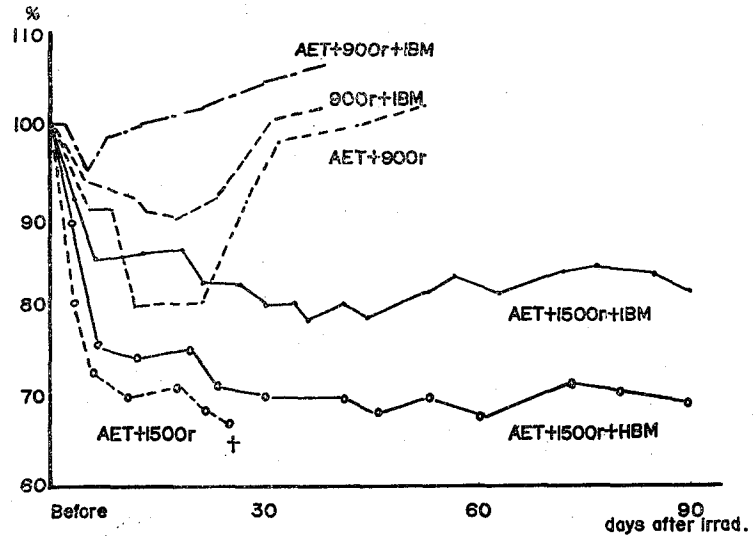


Fig. 2. Body weight changes.

B) **Body weight changes.** Body weight changes are shown in Fig. 2. At 900r, body weight of the AET treated mice reached almost preirradiation level by 30 days. In the AET-IBM treated mice body weight showed only a slight decrease during the period of initial 10 days and then increased rapidly. At 1500r, in most of the treated mice the weight loss stopped at 6 to 10 days, staying at 80-85% of preirradiation level in the AET-IBM treated mice and 70-75% in the

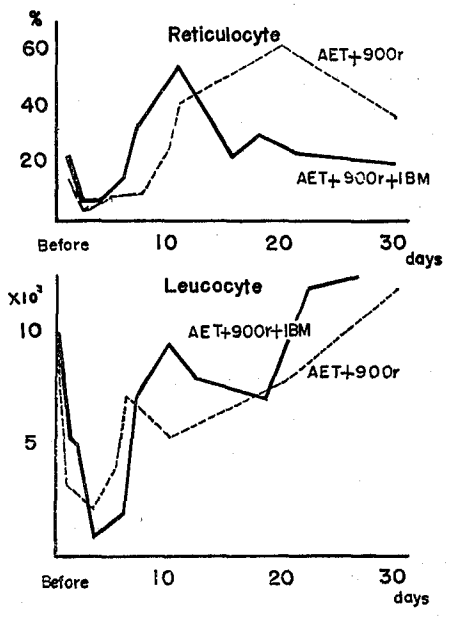


Fig. 3. Changes of reticulocyte and leucocyte count (900r).

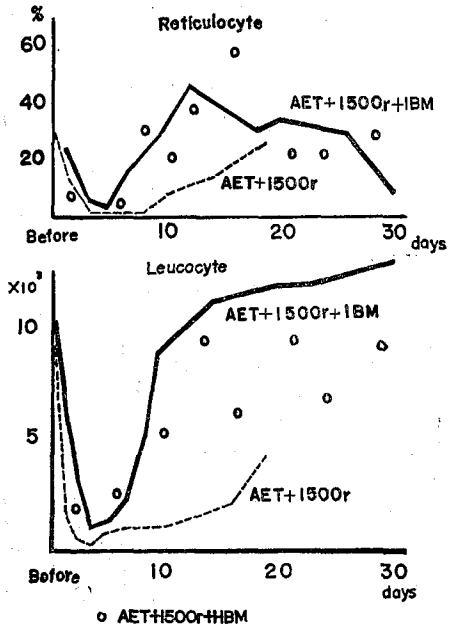


Fig. 4. Changes of reticulocyte and leucocyte count (1500r).

AET-HBM treated mice during the period of 90 days. At 1800-2000r, all of the treated mice died by 21 days, showing rapid weight loss.

C) **Hematological findings.** 1) Leucocyte count. At 900r, as shown in Fig. 3, peripheral blood leucocyte count in both the AET treated and the AET-IBM treated mice showed a rapid decrease, 2000 to 3000 at 2 days, 1000 at 3 days. In most of the irradiated and untreated mice there was no increase in leucocyte count up to the time of their death. In the AET or AET-IBM treated mice the leucocyte count began to increase at 5 or 6 days, the count being approximately 3000 to 5000 at 6 days and 5000 to 9000 at 10 days. Thereafter, the count of the AET-IBM treated mice increased to the normal level somewhat quicker than that of the AET treated mice and both groups reached the normal level by 30 days. At 1500r, as shown in Fig. 4, changes of leucocyte count in the AET-IBM treated mice were essentially the same as those in them at 900r and in the AET-HBM treated mice. In the AET treated mice at 1500r, the leucocyte count showed a rapid decrease, 0 to 500 at 4 days, followed by a slight increase, but never reached 5000 by 21 days and most of the AET treated mice died by 30 days.

2) Erythrocyte count, reticulocyte count, platelet count and hemoglobin content. At 900r, the change of the erythrocyte count following irradiation was less than in the leucocyte count. As shown in Figs. 3 and 5, in both the AET-IBM treated and the AET treated mice changes of erythrocyte count, platelet count and hemoglobin content were almost the same. They showed a slight decrease for initial 10 to 20 days, followed by a gradual increase to reach the normal level by 30 days. As for the reticulocyte count there was a definite difference between

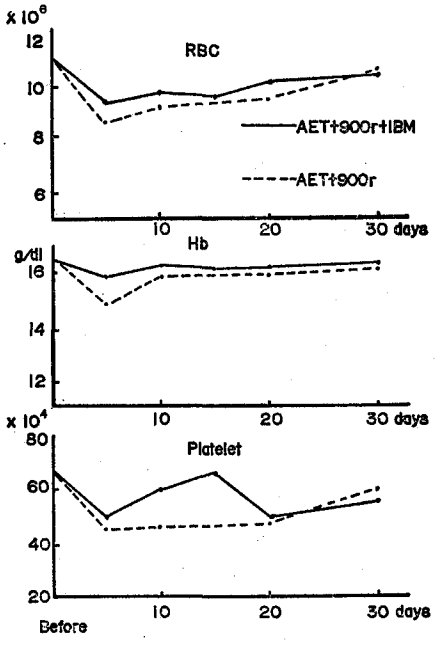


Fig. 5. Changes of RBC, Hb and platelet count (900r).

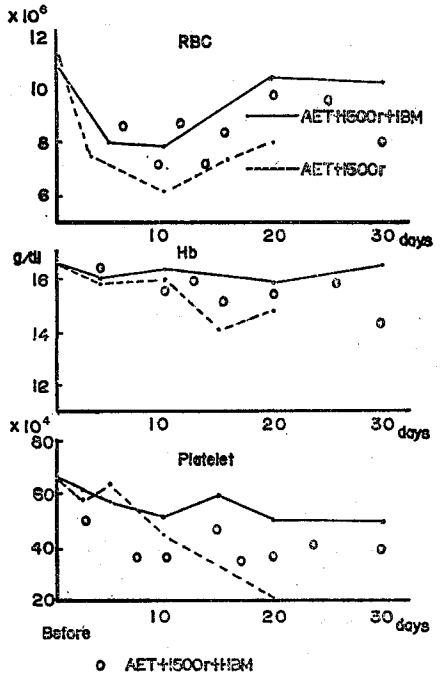


Fig. 6. Changes of RBC, Hb and platelet count (1500r).

the AET treated mice and the AET-IBM treated ones. The count in the former began to increase at 8 or 10 days, reaching its maximum at 20 days, while it in the latter began to increase at 5 to 7 days, reaching its maximum at 10 days. At 1500r, as shown in Fig. 4 and 6, there was a apparent difference between the AET treated and the AET-IBM treated mice. Erythrocyte and platelet count and hemoglobin content in the AET-IBM treated mice returned to normal by 30 days. In the AET treated mice the erythrocyte count and hemoglobin content decreased rapidly or gradually followed by a slight increase, but nerver reached normal by by 30 days and most of the mice died by 30 days.

D) Histological findings. 1) Bone marrow. At 900r, in both the AET treated and the AET-IBM treated mice only mature granulocytes, megakaryocytes and mononuclear cells were seen in the bone marrow cavity 24 hours after irradiation and the nucleated cell count of one femur was 3.0×10^6. These cells decreased in number to almost 1.4×10^6 at 3 days, but not to none. At 4 days many foci of young regenerating cells appeared in most of the AET-IBM treated mice and scattered groups of young cells appeared in most of the AET treated mice. Nucleated cell count of one femur was 6.5×10^6 in the former followed by return to the normal level by 8 days, and 3.0×10^6 in the latter followed by return to the normal level by 10 days. Autopsy on the AET treated mice that died between 14 and 21 days revealed considerably regenerated bone marrow and some mice showed pneumonia of the lung and intestinal injuries, namely, destructed intestinal epithelial cells. At 1500r, in both the AET-IBM (or-HBM) treated mice, and the AET treated mice, the bone marrow showed incomplete wasting even at 3 days, i.e., nucleated cell count of one femur was $0.8\text{-}1.0\times10^6$. And the early regeneration of bone marrow in the former occurred at 4-5 days, while it in the latter occurred at 8-9 days. The former that died by 21 days showed a marked degeneration of the intestinal epithelium, but most of the bone marrow recovered to the normal level by 10-14 days. The complete recovery of bone marrow in the latter was observed at 24 days. At 1800r, all of the AET treated mice that died by 14 days died of intestinal injuries and showed no recovery of the hematopoietic systems. But a few mice of the AET-IBM treated ones that died between 11 and 21 days showed almost complete bone marrow recovery.

2) Spleen. At 900r, in both the AET-treated and the AET-IBM treated mice lymphocytes in the white pulp of the spleen rapidly decreased in number after irradiation, but disappearance from the red pulp of the cells of the erythrocytic and myelocytic series was incomplete at 2 days, and a small number of lymphocytes always remained in the white pulp. The marked regeneration of the cells of the erythrocytic and myelocytic series in the red pulp usually began at 6 to 7 days and became nearly complete at 10 days in the AET-IBM treated mice. The white pulp of the AET-IBM treated mice did not recover earlier than that of the AET alone treated mice. At 1500r, in both the AET treated mice and the AET-IBM (or-HBM) treated mice, the disappearance from the red pulp of the cells was not complete at 3-4 days. A marked regeneration of the red pulp in the latter occurred at 5-8 days, but that of the former did at 9-10 days. The white pulps in both groups were still nearly wasted at 30 days.

3) Lymphnode. At 900r, the lymphnodes had wasted almost completely by 24 hours after irradiation, leaving a small number of lymphocytes. In both groups these wasted conditions continued for about three weeks. In the AET-IBM treated mice the number of lymphocytes in the lymphnodes became almost normal by 30 days, but in the AET treated mice it was still subnormal by 30 days. At 1500r, in both groups the lymphnodes had wasted almost completely by 24 hours after irradiation and these conditions continued for 30 days.

4) Thymus. At 900r, complete wasting of the thymus was followed by a fairly good recovery at 5 to 9 days in some of the mice in both groups. At 1500r, complete wasting of the thymus was followed by recovery of the cortex at 7 to 9 days in some of the mice in both groups. But the thymic recovery of the AET-HBM treated mice was not observed by 30 days.

5) Intestine. At 900r, slightly destructed villi and scattered vacuoles were seen in both groups but a marked degeneration could not be found. At 1500r, profound loss of the intestinal epithelium was found in some of the mice in all groups. At 1800r and 2000r, almost complete loss of the intestinal epithelium was found in most of both groups.

II) Effect of Treatment of AET Treated and Preirradiated Donor Marrow on Lethally Whole Body Irradiated Mice

A) Survival rate. The survival rates in 16 experiments are shown in Table 2. Both the 100r irradiated and the 6 days previously 100r irradiated HBM could not save well 900r irradiated host mice from early death, while the 6 days pre-

Table 2. Survival rate of lethally gamma-irradiated mice treated with IBM (or HBM) from the mice given AET and varying doses of gamma rays.

Exp.	Donor			Recipient		% Survival (at days)					
	Type	Treatment	BM suspension at days after irrad.	Treatment	Number of mice	7	14	21	30	60	90
1	HBM	100r	0	900r	12	63	18	18	18	9	9
2	HBM	100r	6	900r	12	66	16	16	16	16	16
3	IBM	100r	6	900r	12	66	66	66	66	66	66
4	IBM	AET-100r	0	900r	12	100	100	100	83	83	83
5	HBM	AET-100r	0	900r	18	72	28	28	17	11	6
6	IBM	AET-100r	6	900r	8	100	100	100	100	100	100
7	HBM	AET-100r	6	900r	28	86	57	45	39	25	21
8	IBM	AET-450r	0	900r	12	66	50	50	50	50	50
9	HBM	AET-450r	0	900r	20	80	20	20	20	5	5
10	IBM	AET-450r	8	900r	8	100	100	100	100	87	75
11	HBM	AET-450r	8	900r	7	14	0				
12	HBM	450r	0	900r	11	65	18	18	18	9	9
13	HBM	None	None	900r	20	60	30	20	20	10	10
14	IBM	AET-900r	8	900r	7	14	14	14	14	14	14
15	IBM	AET-100r	6	AET-1500r	8	75	75	75	75	75	63
16	HBM	AET-100r	6	AET-1500r	14	93	78	57	50	50	36

viously 100r irradiated IBM could save well the mice from early death, i.e., 21-day survival was 66%. Both the AET protected, 100r preirradiated IBM and the AET protected, 6 days previously 100r irradiated HBM could save early death fairly well, giving slightly increased survival rates. They were 100% and 45% at 21 days in the former and the latter group, respectively, but were 83% and 21% at 90 days, respectively. Both the AET protected and 100r irradiated HBM and the AET protected and 8 days previously 450r irradiated HBM could not save well the mice from early death. The AET protected and 1500r irradiated mice transplanted with the AET protected and 6 days previously 100r irradiated HBM showed a fairly good survival rate, i.e., 57% at 21 days, but the similarly treated host mice infused with AET protected and 6 days previously 100r irradiated IBM showed a good survival, i.e., 75% at 21 days and 63% at 90 days.

B) **Body weight.** There were no differences in the body weight between the mice treated with ordinary bone marrow and the one infused with pretreated bone marrow.

C) **Hematological findings.** There were not so much differences between the mice treated with ordinary bone marrow and the ones infused with pretreated bone marrow. As shown in Fig. 7, effectiveness of the AET protected and 6 days previously 100r irradiated IBM is stronger than that of the HBM treated in the same way.

D) **Histological findings.** There were not so much differences between the mice treated with ordinary bone marrow and the ones infused with pretreated bone marrow.

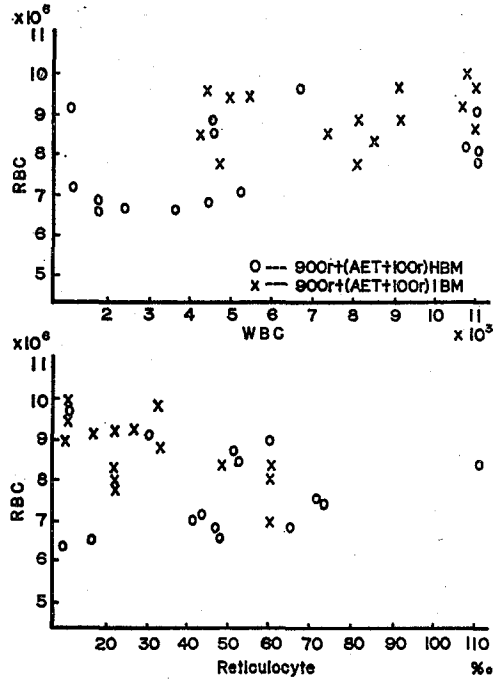


Fig. 7. RBC, WBC and Reticulocyte count between 14 and 30 days after irradiation.

DISCUSSION

It was first observed by Jacobson *et al.*[16,17] that shielding of the spleen of mice protected them from acute radiation death. Thereafter, Lorenz *et al.*[2,3] demonstrated that the mice exposed to lethal total body irradiation were protected by inoculation of bone marrow cells. Bone marrow treatment is effective for radiation injury by a lethal dose, namely, for hematopoietic failure, and can not save most of the mice that have received supra-lethal dose of total body irradiation in which intestinal damage plays a major role. Makinodan *et al.*[18] reported that percent survival of the mice exposed to 1300r and treated with IBM was lower than that of the ones exposed to 900r lethal irradiation and treated with IBM and we also observed[19] that all of the mice exposed to 1300r-1500r and treated with IBM died by 21 days due mainly to intestinal damage, though some mice showed good hematopoietic recovery at early post-irradiation days. On the other hand, a chemical radioprotective compound AET is known to protect both hematopoietic organs and intestinal tract. Doherty *et al.*[7] reported that the protective effect of AET was stronger on the intestines than on the hematopoietic system. Then, the combined use of AET and bone marrow treatment might save most of the recipient mice which should die due to intestinal damage after total body irradiation of otherwise supralethal dose. One of the purposes of these experiments reported here was to clarify the effect of the combined use of AET and bone marrow treatment under varying doses of total body irradiation. The survival rate of the AET treated mice exposed to 1500r total body irradiation was 6% at 30 days after irradiation, but apparently it was raised to 60% at 30 days if the mice were treated with IBM, i.e., the combined use of AET and IBM could save most of the mice exposed to 1500r from radiation death, though the survivors at 90 days after irradiation still had reduced body weights which were approximately 80-90% of preirradiation level. When irradiation dose was increased to 1800r-2000r, irradiated mice died of acute intestinal damage, even with the combined treatment of AET and IBM, showing heavy diarrhea and pancytopenia. AET protected and supralethally irradiated mice treated with HBM showed a somewhat different clinical course, i.e., all of the AET-HBM treated mice at 1300r died by 30 days, showing weight loss and diarrhea. This indicates that 1300r was not a supralethal dose, but sublethal to the AET protected mice, and then HBM inoculation gave lethal effect[20~23], which was often observed in ordinary HBM or HSC (homologous spleen cells) transplantation to sublethally irradiated mice, to the recipient mice. Most of the AET-HBM treated mice at 1500r survived for 30 days, but later they died by 60 days, showing still weight loss and diarrhea. Although most of the AET-IBM treated mice survived beyond 90 days, their body weight still did not reach the preirradiation level by 90 days. In the long term observations of IBM treated mice, a few workers[13,24] reported that the mice exposed to lethal irradiation and saved with bone marrow treatment had somewhat different manifestations from the unirradiated control mice, which included retarded increase of body weight, occurrence of some malignant neoplasms and nephrosclerosis. Therefore, it is probable that the survivors exposed to su-

Explanation of Plates

Plate 1. Small intestine ; 3 days after 1500r irradiation. Marked degeneration of intestinal epithelium.

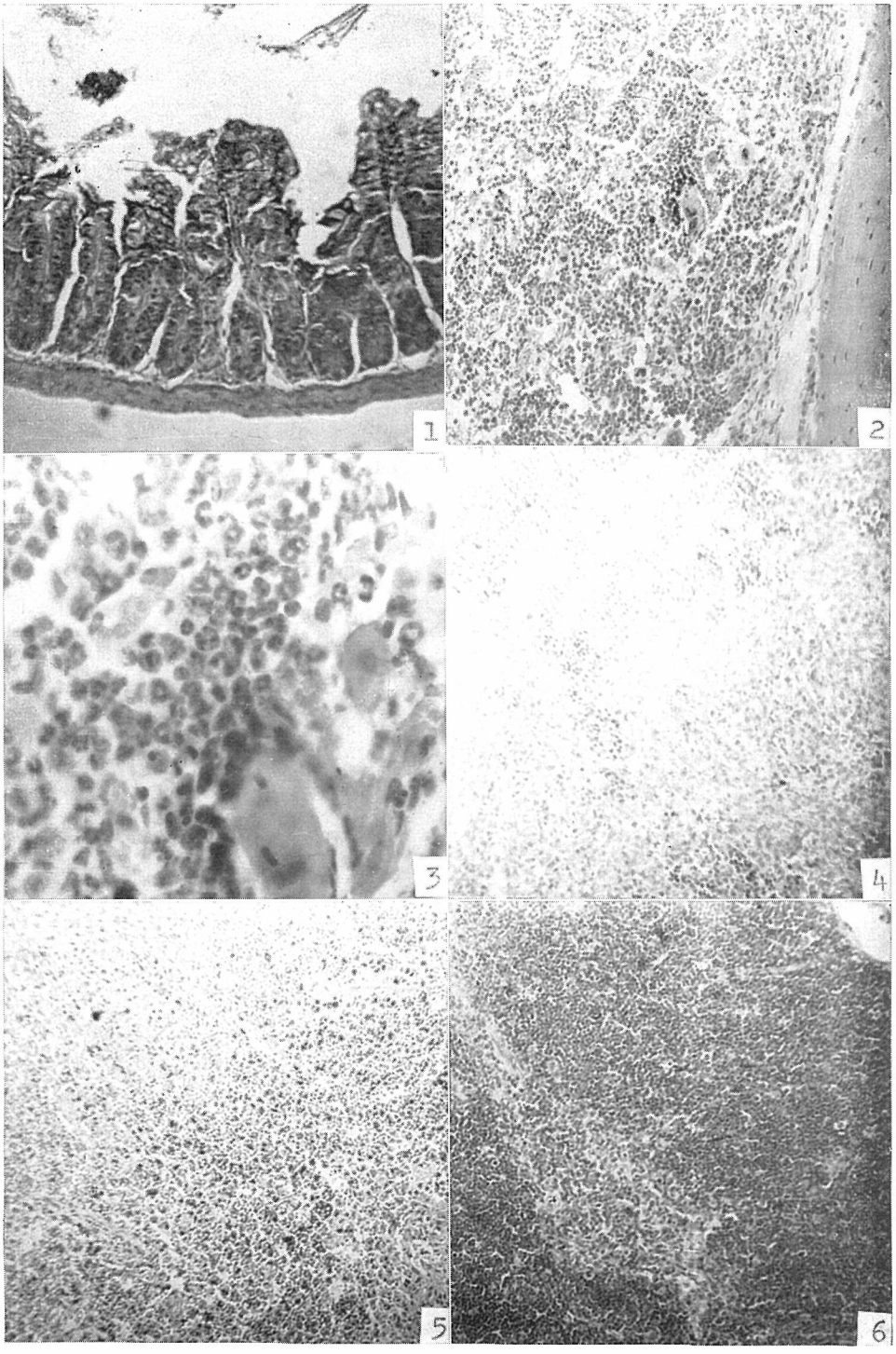
Plate 2. Bone marrow ; 4 days after 1500r irradiation in the mouse given AET and HBM. The bone marrow cavity is filled with neutrophile leucocytes and the spotty early regeneration is observed.

Plate 3. Bone marrow ; Higher magnification of Fig. 2. Many neutrophile leucocytes and spotty young cells.

Plate 4. Spleen ; 4 days after 1500r irradiation in the mouse given AET and HBM. Complete wasting of the white pulp, but sparse myelocytic and erythrocytic cells in the red pulp.

Plate 5. Mesenteric lymphnode ; 4 days after 1500r irradiation in the mouse given AET and HBM. Almost complete wasting.

Plate 6. Thymus ; 6 days after 100r irradiation and AET protection. Almost complete recovery. Nucleated cell count of one femur is 12×10^6 in this mouse.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pralethal irradiation and treated with AET-IBM (or-HBM) beyond 90 days suffered from chronic radiation injuries, after they were saved from acute radiation death. Histological studies on the AET pretreated mice at 4 days after 1500r irradiation showed that the bone marrow was not completely acellular and the white pulp of the spleen was wasted while the red pulp of the spleen was not completely wasted. Futhermore, damage to the lymph nodes was considerable. These findings also suggest that AET may protect predominantly non-antibody forming cells. It is suggested, at least, that the AET pretreated host exposed to 1500r irradiation could easily take HBM without early mortality and would not succumb to secondary bone marrow aplasia which was thought to be a main cause of early mortality[24,25] caused by host against graft reaction. On the other hand, even though the AET treated host exposed to supralethal irradiation could take inoculation of HBM easily, the repopulated immunologically competent cells from the donor mice in the host might react, according to the graft against host theory, against the host during the delayed stages after irradiation and transplantation of HBM. Therefore, in order to suppress so-called secondary disease, the donor's bone marrow must be pretreated. Cudkowicz reported[11] that pretreatment with Xrays on the donor mice prevented delayed death. The data reported here have so far been different from his results. In this present experiment, a dose as small as 100r or 450r gamma-rayirradiated HBM could not prevent even early death effectively. It has been observed by many investigators that D37 value is about 70r-115r[26~30]. Therefore, there might still be a small number of immunologically competent cells in the bone marrow when a radiation dose of 100r was given to the donor mice, and they might react against host antigens, even if pretreated HBM could prevent early death. Only the AET protected, 6 days previously 100r irradiated donor marrow showed 57% survival at 14 days and 45% at 21 days, but the recipient mice also succumbed to delayed death after the 30th post-irradiation day. Therefore, such pretreated HBM had the proliferative ability, but failed to prevent so-called homologous disease. Argyris reported[31] that the 400r irradiated HSC reduced the proliferative capacity of CBA spleen cells by about 95-96% and the immunologic activity by about 98-99%. In his studies a quantitative estimate was made of the decrease in proliferative capacity and immunologic activity of spleen cells exposed to 400r of total body X-irradiation. The decreased proliferative capacity of irradiated CBA spleen cells was inferred from a decreased ability to induce homograft tolerance in newborn C3H mice as compared to the ability of various doses of non-irradiated CBA spleen cells to induce homograft tolerance. The decreased immunologic activity of CBA spleen cells was estimated from a decreased capacity to induce a graft against host reaction in newborn C57BL/6 mice as compared to the capacity of standard doses of non-irradiated CBA cells. Makinodan[28] found an approximate 98% decrease in antibody producing capacity in terms of agglutinin production after 400r total body X-irradiation. Celada and Carter[29] reported that at lower doses of X-irradiation *in vitro* the homograft rejecting mechanism of mouse spleen cells is more radio-resistant than the agglutinin producing capacity. On the other hand, Goodman and Bender[32] reported that the radiosensitivity of immunologically com-

petent cells was similar to that of hematopoietic cells and 400r irradiated HBM could not prevent secondary disease in the strain combination of 1C3F1 and LAF1 or B6D2F1. In the present study the 450r gamma-irradiated HBM could not prevent even early death in the strain combination of Na2 and Dd/s and only the AET protected, 6 days previously 100r irradiated HBM could prevent early death, though they failed to prevent delayed death. Furthermore, the AET-IBM treated mice exposed to supralethal irradiation survived for 90 days, while most of the AET-HBM treated mice exposed to 1500r irradiation died after the 30th post-irradiation day.

To summarize, it has been thought that AET protects non-antibody forming cells predominantly, but its ability was found to be very weak, and the combined use of AET and bone marrow treatment in supralethally gamma-irradiated mice improved survival rates to a certain extent, whereas the AET pretreated and gamma-irradiated HBM could not prevent the recipient mice from delayed death after total body gamma-irradiation under high dose rate.

In order to prevent so-called secondary disease compeletely, further fundamental problems must be solved.

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