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

Application of Methotrexate for Suppression of Immune Response

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Received January 31, 1966

1) The early administration of methotrexate (MTX) was effective for suppression of homograft response in mice lethally gamma-irradiated and transplanted with homologous bone marrow (HBM), but survival rate thus obtained was still less than that when isologous bone marrow (IBM) was transplanted.

2) The effect of MTX for suppression of homograft response in the mice lethally irradiated and transplanted with homologous spleen cells (HSC) was not satisfactory.

3) MTX for suppression of homograft response in the mice sublethally irradiated and treated with HBM or HSC was highly effective.

4) The possible mechansism of MTX for suppression of homograft response was discussed.

INTRODUCTION

As previously described[1], pre-irradiation of the donor could not satisfactorily prevent so-called homograft response or delayed death. Therefore, in order to inhibit the homograft response the use of immunologically immature fetal liver as a protective agent in place of adult HBM has been studied, but the result does not seem to be always good[2]. Drug treatment as a method of modifying the homograft response has been tried. Uphopff[3] reported that the use of folic acid antagonist, methotrexate (MTX), might spare mice that would normally succumb to a homograft response following lethal total body X-irradiation and HBM treatment in 1958 and, within a few years after-wards, 6-mercaptopurine[4], azathiopurine[5] and cyclophosphamide[6] were in use for this purpose. Recently, Ferrebee et al.[7] also reported that the early administration of MTX prevented the delayed foreign marrow reaction. The purpose of this experiment reported here is to ascertain the effect of MTX in the mice gamma-irradiated lethally or sublethally under high dose rate and treated with homologous bone marrow (HBM) or homologous spleen cells (HSC).

MATERIALS AND METHODS

Dd/s and Na2 strain mice were used as recipients and donors, respectively.

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They were supplied from the Kyoto University Animal Center. A Co 60 gamma-irradiation facility which belongs to the Institute for Chemical Research of Kyoto University was used in the present experiments. The conditions of irradiation were described previously[1]. Lethal irradiation in this study means approximately 900 r irradiation which is 100 % lethal to mice within 30 days. LD 100 dose of gamma-rays to mice can be given in less than half a minute. MTX (1.5 mg per kg body weight) usually injected intraperitoneally 4 times every other day, beginning 1 day after total body irradiation and HBM or HSC transplantation. This is the early administration of MTX, while the delayed administration of MTX means that injections of MTX begin at 8 days or 14 days after irradiation. Bone marrow suspension was obtained by the method described previously[1]. Spleen cell suspension was obtained by the following way ; removed donors' sleens were washed in cold Tyrode's solution, excluded surrounding tissues and capsules, scissored in the solution, pippeted more than ten times and the coarse particles settled down by gravity within half a minute. The remaining supernatant fluid was practically a single cell suspension. The number of the nucleated cells of bone marrow and spleen cell suspension inoculated per mouse was 10×10^6 to 15×10^6.

RESULTS

1) Survival rate

At 900 r, as summarized in Table 1, ten types of experiments were carried out. In the experiment 1, the early administration of MTX was used, i.e., MTX was given at 1, 3, 5 and 7 days to the irradiated and HBM transplanted mice. The survival rates were 78 % at 21 days and 73 % at 30 days. It should be noted that they were much better than those of 30 % at 21 days and 20 % at 30 days in the HBM transplantation alone in experiment 5. On the other hand,

Table 1. Suvival rate of lethally (900r) gamma-irrated mice treated with HBM (or HSC) and Methotrexate (MTX).

Exp.	infused cell type	MTX given on days	No. of mice	% Survival (at days)					
				7	14	21	30	60	90
1	HBM	1, 3, 5, 7	22	100	91	78	73	45	32
2	HBM	8, 10, 12, 14	33	100	82	64	52	21	18
3	HBM	14, 16, 18, 20	10	70	70	50	40	40	40
4	HBM	1; 3, 5, 7, 9, 11, 13, 15	18	100	50	28	17	5	5
5	HBM	None	10	60	50	30	20	10	10
6	HSC	1, 3, 5, 7	64	84	69	45	36	16	11
7	HSC	None	20	85	65	35	10	5	5
8	ISC	1, 3, 5, 7	8	100	88	88	88	88	88
9	ISC	None	8	100	100	100	88	88	88
10	None	None	20	40	0				

MTX was given 1.5 mg/kg/mouse

the 30 day survival rates of the mice treated with both HBM and the delayed administration of MTX were 52 % and 40 % in experiments 2 and 3, respectively. The mice treated with both HSC and the early administration of MTX showed 36 % survival at 30 days in experiment 6. This survival rate was a little better than that of 10 % at 30 days in the HSC transplantation alone in experiment 7. At 90 days, there was not so much difference in the survival rate between the HSC-MTX treated mice and the HBM or HSC alone treated mice, while in both the isologous spleen cells (ISC)-MTX treated mice and the ISC alone treated mice, the survival rates were 88 % at 90 days as seen in experiments 8 and 9.

At 700 r, as shown in Table 2, the survival rate of the only irradiated mice in experiment 7, was 92 % at 90 days. This was not so much different from that of the mice treated with ISC or IBM. When the irradiated mice were treated with HSC or HBM, they showed very reduced survival, i.e., 6 % or 33 %, respectively, at 90 days as seen in experiments 2 and 4. But the survival rate of the mice which were treated with HBM (or HSC) and MTX was 80 % or 69 %, at 90 days in experiments 1 and 3, respectively.

At 800 r, the only irradiated mice showed 44 % survival at 90 days, while most of the HSC treated mice died by 21 days.

Table 2. Survival rate of sublethally gamma-irradiated mice treated with HBM (or HSC) and Methotrexate (MTX).

Exp.	Dose of irrad.	infused cell type	use of MTX	No. of mice	% Survival (at days)					
					7	14	21	30	60	90
1	700r	HBM	Yes	10	100	100	100	80	80	80
2		HBM	None	20	100	50	40	40	33	33
3	700r	HSC	Yes	26	100	100	85	81	73	69
4		HSC	None	34	91	82	68	24	6	6
5	700r	IBM	None	10	100	100	100	90	90	90
6		ISC	None	20	100	100	100	95	95	95
7	700r	None	None	24	96	92	92	92	92	92
8		None	Yes	8	100	100	100	100	100	100
9	800r	HSC	Yes	18	67	61	55	55	55	55
10		HSC	None	18	89	39	17	11	11	11
11		None	None	16	94	81	50	44	44	44

MTX was given on days 1,3,5, and 7.

2) Body weight change

At 900 r, body weight changes in both the HSC treated mice and the HBM or HSC and MTX treated mice are shown in Fig. 1. The weight of the HBM-MTX treated mice reached almost the preirradiation level approximately at 20 days after total body irradiation, and then decreased again. The early administration of MTX could not completely prevent delayed body weight change. In the HSC-MTX treated mice, body weight began to decrease again at 20 days or so after irradiation and did not show any tendency of recovery, while in both

the ISC and the ISC-MTX treated mice, body weight changes were almost the same as each other, i.e., they initially decreased, but afterwards showed tendency of recovery and then reached the preirradiation level by 30 days and thereafter increased gradually.

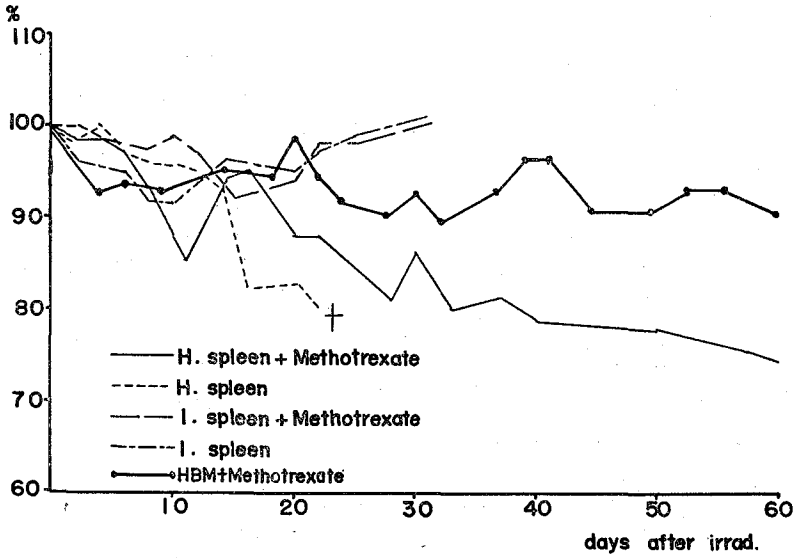


Fig. 1. Body weight changes (900r).

At 700 r, as shown in Fig. 2, body weight changes in both the HBM-MTX treated mice and the HSC-MTX treated ones were almost the same, i.e., they did not show any decreasing tendency. When the mice were treated with HBM or HSC alone, they showed initial decrease in body weight, but that of the former gradually increased and almost at 20 days reached the preirradiation level and that of the latter temporarily increased, followed by a tendency of

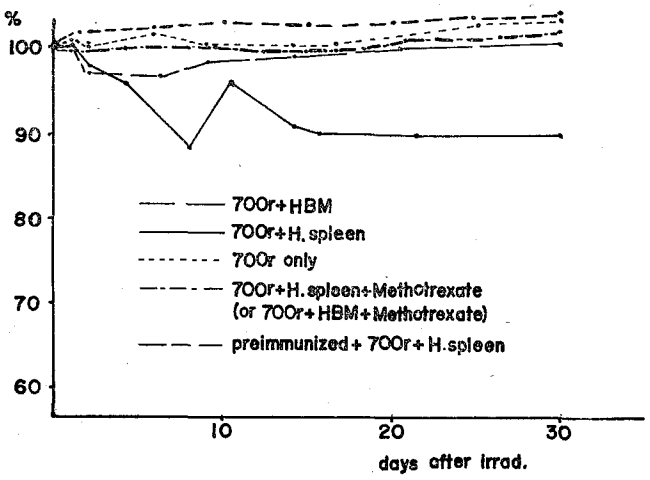


Fig. 2. Body weight changes (700r).

gradual decrease and never reached the preirradiation level by 30 days. When the recipients were preimmunized with donor tissue homogenate 7 days previously and transplanted with HSC after the irradiation, they did not show any decreasing tendency in body weight.

3) **Hematological findings**

At 900 r, as shown in Figs. 3, 4, 5 and 6, changes of erythrocyte count platelet count and hemoglobin content of the ISC-MTX treated mice were almost the same as those of the ISC alone treated mice, except that the former had a 2～3 day delayed beginning of recovery of leucocyte and reticulocyte count which subsequently increased quickly. In the HBM or HSC and MTX treated mice nearly the same tendency of retarded recovery of reticulocyte and leucocyte count was also observed. In general, the HSC treated mice seemed more anemic, even with the early administration of MTX.

At 700 r, as shown in Figs. 7, 8, 9 and 10, the IBM or ISC treated mice showed more rapid recovery of erythrocyte, reticulocyte and leucocute count and hemoglobin content than those of the untreated mice. In the HBM or HSC treated mice here were not marked changes of leucocyte and reticulocyte count as compared with those of the ISC treated mice, but the erythrocyte count and hemoglobin content were decreased at the delayed stage of post-irradiation days which were prevented by the early administration of MTX.

In the present study, as to peripheral lymphocyte count no marked changes

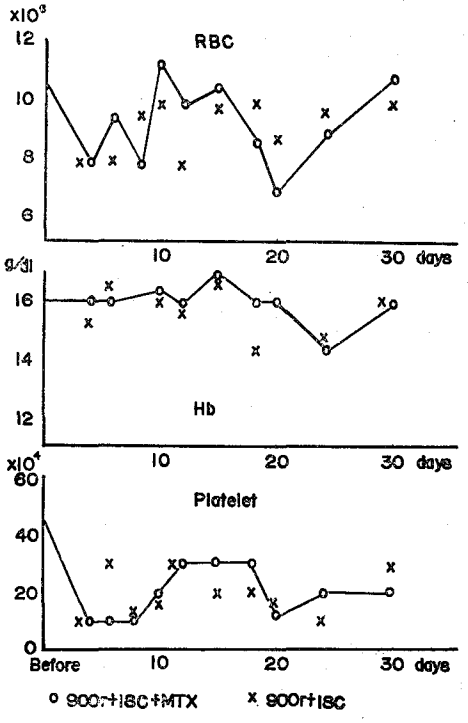


Fig. 3. Changes of RBC, Hb and platelet count (900r).

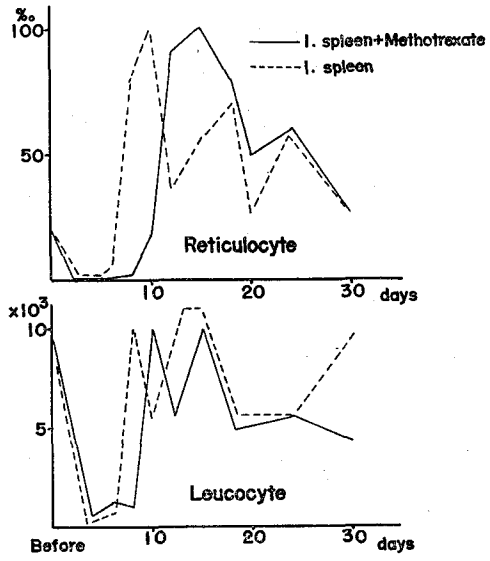


Fig. 4. Changes of leucocyte and reticulocyte count (900r).

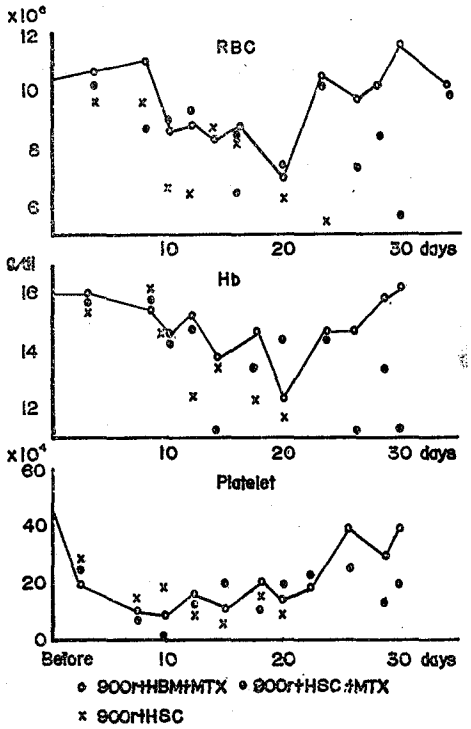


Fig. 5. Changes of RBC, Hb and platelet count (900r).

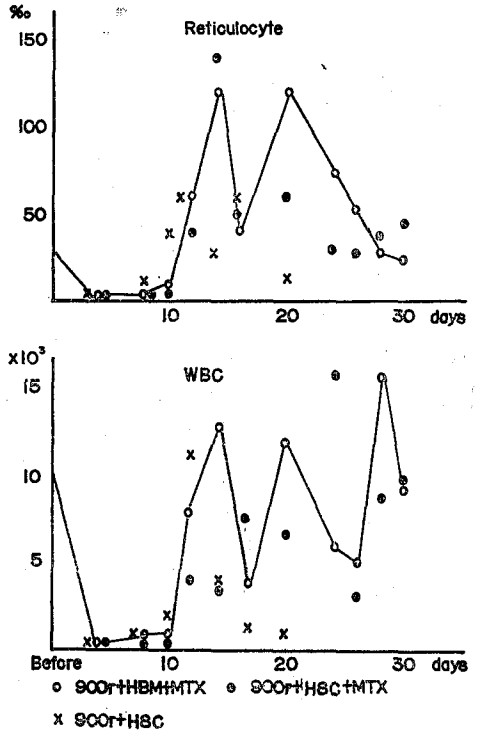


Fig. 6. Changes of leucocyte and reticulocyte count (900r).

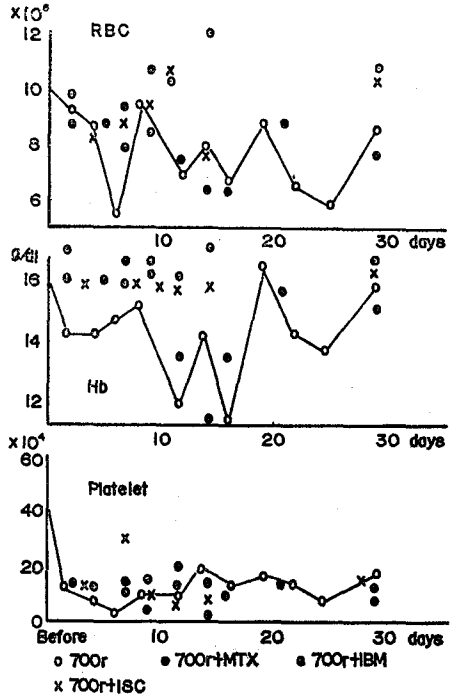


Fig. 7. Changes of RBC, Hb and platelet count (700r).

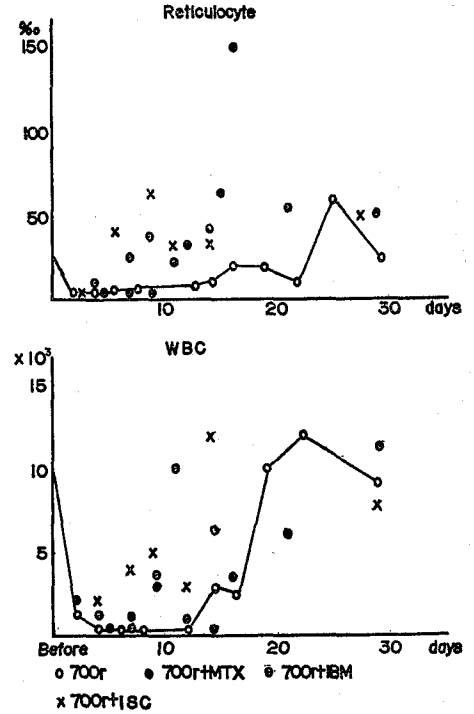


Fig. 8. Changes of leucocyte and reticulocyte count (700r).

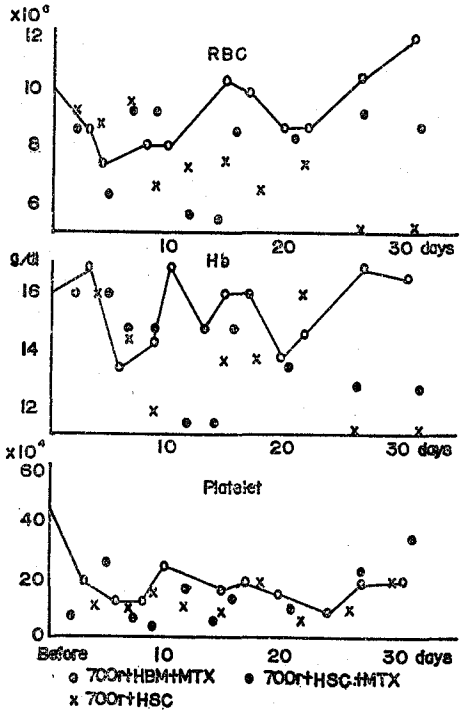


Fig. 9. Changes of RBC, Hb and platelet count (700r).

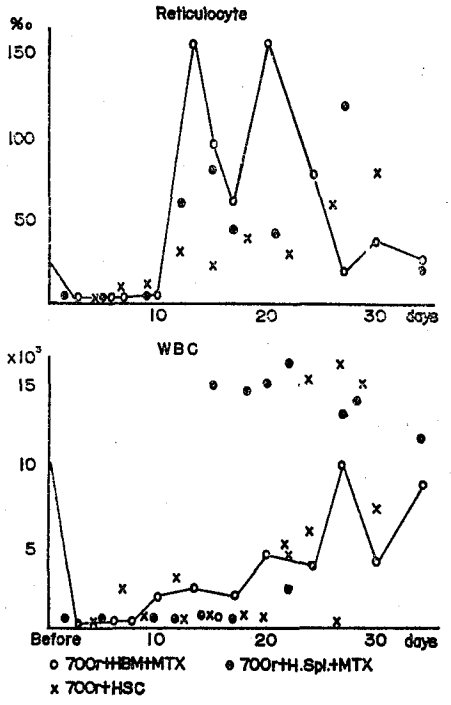


Fig. 10 Changes of leucocyte and reticulocyte count (700r).

were observed following the administration of MTX.

4) **Histological findings**

At 900 r, there were no marked qualitative differences of histological findings of hematopoietic tissues between the HBM or HSC and MTX treated mice and the controls which were treated with HBM alone, i.e., early regeneration of bone marrow occurred at 5~10 days after irrdiation and treatment, the red plup of spleen began to recover at about 9~10 days, and the white plup of spleen and the lymph nodes of some mice showed a few germinal center at the same days In some mice the regeneration of thymic cortex became marked by 8~10 days (Fig. 11). But some other mice which survived beyond 21 days had hypoplastic or aplastic bone marrow (Fig. 12~15), aplasia of lymphoid tissues including thymus (Fig. 16), emaciation, hemorrhage, diarrhea and ruffled fur, i.e., secondary bone marrow aplasia with wasting syndrome. Most of the survivors beyond 40 days showed almost complete recovery of the bone marrow and all lymphoid tissues.

At 700 r, histological findings of bone marrow and spleen of the HBM or HSC and MTX treated mice were not so much different from those of the HBM or HSC alone treated mice ; almost complete wasting by 4 days after irradiation followed by spotty regeneration of the bone marrow and early phase of the recovery of the red pulp of the spleen beginning at 4~5 days in the HBM or

HSC treated mice, while the recovery of the HBM or HSC and MTX treated mice began at 6~10 days. Even in the controls which were irradiated and untreated, the bone marrow and the red pulp of the spleen began to recover at 6~10 days. As to thymic recovery, there was a tedency of some retardation in the HBM or HSC alone treated mice except for some animals showing marked regeneration at 8~10 days, the majority of which belonged to the HBM treated mice. On the contrary, the irradiated and untreated mice showed comlete recovery of the thymus at 8~10 days. The HBM or HSC and MTX treated mice showed various manifestations of thymic recovery which were almost complete at 10~13 days in some mice and incomplete in others with aplasia of other lymphoid tissues during the period of 60 post-irradiation days. Thymic recovery of the mice survived for 90 days was almost complete.

DISCUSSION

The ability of the folic acid antagonist to suppress immune response was first shown by Malmgren *et al.*[8] who found that repeated injections of MTX inhibited antibody production in the mice that had been injected with sheep red cells. Subsequent works have confirmed the effect of this agent on production of humoral antibody[9~12]. It has also been shown to inhibit the induction of delayed hypersensitivity[13~16] and homograft response[17~19]. The effectiveness of MTX to suppress homograft response in bone marrow transplantation was shown by Uphoff in 1958[8], who reported that the use of MTX spared mice that would otherwise succumb to a homograft response following total body X-irradiation and HBM transplantation and that the secondary weight loss which might be caused by the homograft response was also suppressed by the administration of MTX every other day 9 times beginning at 14 days after the total body irradation. But other workers[7] reported that the survival rates of mice treated with HBM and delayed administrations of MTX were 90% at 10 days and 20% at 30 days, while early administrations of MTX prevented delayed death, i.e., the survival rate was 100% at 90 days. In the present study reported above the survival rates of the mice lethally irradiated and treated with HBM and MTX which was given at 1, 3, 5 and 7 days after irradiation were 73% at 30 days and 32% at 90 days. It should be noted that they were much better than those of 30% at 21 days and 20% at 30 days in the group treated with HBM alone. Body weight changes of the HBM-MTX treated mice reached almost preirradiation level approximately at 20 days after irradiation, and then gradually decreased again. The survival rate of them also decreased to 45% at 60 days. When the delayed administration of MTX beginning at 8 days or 14 days was used, the survival rates were 52% or 40% at 30 days, respectively. These results suggest that an immune response was suppressed by the early administration of MTX at least for about 30 days, though it occurred in some mice later. But more than half of the mice lethally irradiated and treated with both HSC and early administered MTX died by 30 days, and their body weight began to decrease again at about 20 days after irradiation and did not show any tendency of recovery, though the

survival rate was a little better than that of the group treated with HSC alone. It is well known that spleen cells can protect lethally irradiated mice, but it is also believed so far that there are more lymphoreticular cells in the spleen of mice than in the bone marrow. And it is generally accepted that the lymphoreticular cells as the cells closely associated with cellular antibody play a major role in homograft response[20], though some workers insist that homograft response depends upon serum antibody[21,22]. These facts suggest that the effect of MTX must diminish when the number of the transplanted immunologically comptent cells increases. Therefore, it may be thought that the effect of MTX for immune suppression of the HSC transplanted mice is less than that of MTX for suppression of the HBM transplanted mice. The survival rates and histological findings in ISC transplantation with and without MTX were almost the same as each other. Therefore, it goes without saying that this dose of MTX used has no marked toxicity in lethally gamma-irradiated mice, while Uphoff[3] suggested that MTX might be more toxic in lethally irradiated mice than in normal mice. As for the effect of the early administration of MTX on homograft response in the lethally irradiated and HBM transplanted mice, Ferrebee *et al.*[7] discussed that, if the reaction of immunologically competent cells on the marrow infusion could be restrained during a short interval, either these cells passed through their life-span and died without injuring the host, or they and their progeny acquired tolerance to their environment without first having injured it and themselves by reaction. In the present study, as above described, the immunologically competent cells also seem to react in the early stage of recovery after the irradiation and it is probable that the more the number of them is, the stronger the immune response occurs. The effect of MTX is attributable largely to its ability to bind folic acid reductase, an enzyme responsible for the conversion of folic to tetrahydrofolic acid. The latter is a coenzyme for many biochemical reactions, the most important of which is probably thymidine synthesis[24]. Werkheiser[25] suggests that the different susceptibility of various tissues to folic acid antagonists may be partly explained by differences in their permeability to the antagonist and the time during which they can resist folic acid reductase inhibition without sustaining irreversible damage. On entering the cell, the antagonist binds folic acid reductase and thus strongly inhibits thymidine synthesis and processes dependent on it, such as cell divission. Synthesis of proteins, including folic acid reductase, is only moderately affected and Werkheiser suggests that the cells recovers when sufficient folic acid reductase has been produced to enable thymidine synthesis, unless it has suffered irreversible damage in the meantime. Berenbaum *et al.*[26] stated that, during the early stage of the immune response, antigenically stimulated cells might be irreversibly damaged by only a few hour exposure to folic acid antagonists, i.e., antigenically stimulated cells may have the special susceptibility to MTX. Therefore, it should be considered that the immunologically competent cells infused to the irradiated but antigenically rich environment may also have the special susceptibility to MTX during the early stage of immune response. In the present study MTX was noted to be highly effective for the suppression against the development of the homograft response during 30

Fig. 11. Beginning marked recovery of thymic cortex; 10 days after 900r irradiation and HBM-MTX treatment. Bone marrow showed marked regeneration. Nucleated cell count of one femur was 1.2×10^6.

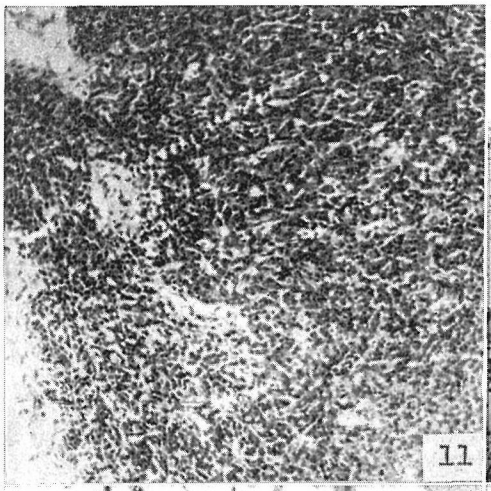
Fig. 12. Aplastic bone marrow; 36 days after 900r irradiation and HSC-MTX treatment. Hemorrhages were seen in the intestinal tract and mesenteric lymph node. Body weight was 24.3g before irradiation and decreased to 18.3g at the day of autopsy. All lymphoid tissues were wasted. Nucleated cell count of one femur was 1.0×10^6.

Fig. 13. Aplastic bone marrow; 27 days after 900r irradiation and HBM treatment. Hemorrhages were seen in the intestinal tract and mesenteric lymph node. Body weight was 22.0g before irradiation and decreased to 17.5g at the day of autopsy.

Fig. 14. Hypoplastic bone marrow; 31 days after irradiation and HBM-MTX treatment. Mesenteric lymph node showed a few germinal center. Thymus was almost wasted. Nucleated cell count of one femur was 2.2×10^6.

Fig. 15. Localized aplastic bone marrow; 28 days after irradiation and HSC-MTX treatment. All lymphoid tissues were almost wasted. Body weight was 22.3g before irradiation and decreased to 11.9g at the day of autopsy.

Fig. 16. Complete wasting of the thymus of the mouse described in Fig. 12.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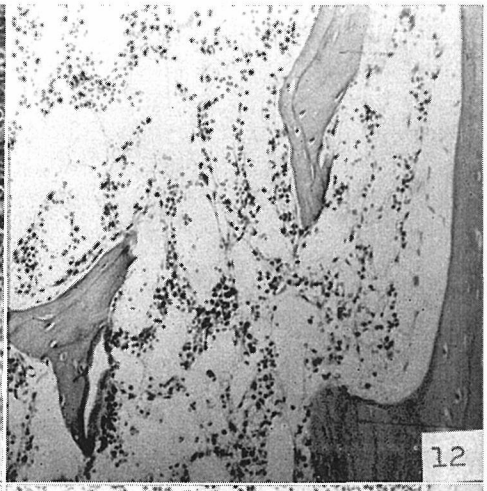
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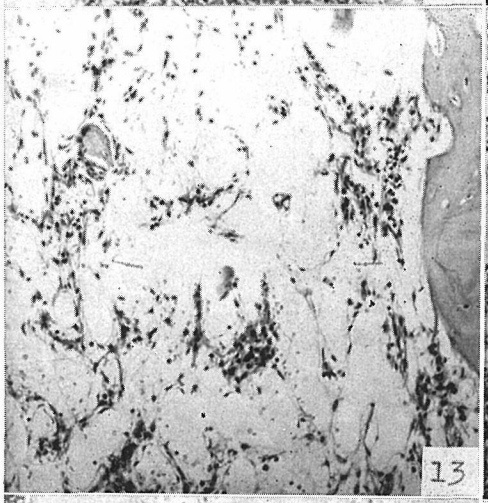
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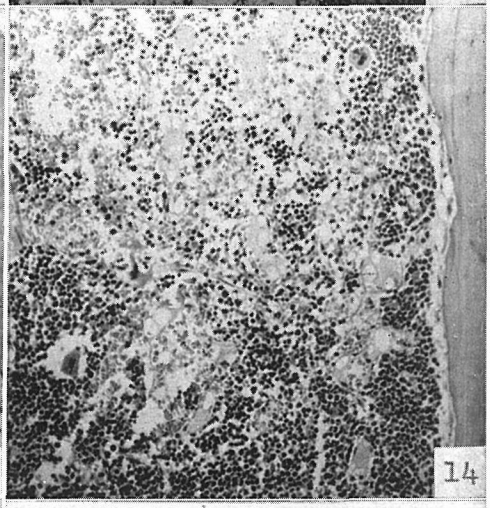
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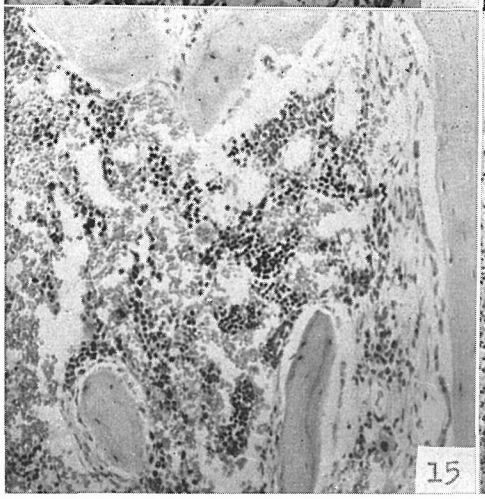
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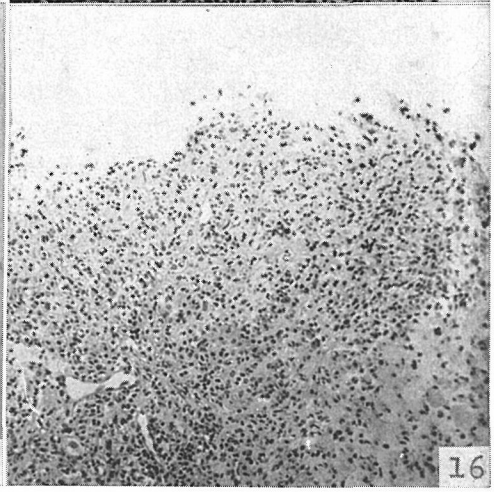
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days after irradiation and HBM transplantation when the early administration of MTX in the dose of demonstrating no toxicity to normal mice was used. But the survival rate thus obtained was still less than that after the IBM transplantation. In order to suppress only immune response without causing lethal side effect of the use of a large amount of MTX, Berenbaum *et al.* showed that the delayed administration of folinic acid reduced the toxicity of MTX for those tissues (presumably intestinal epithelium and bone marrow)[27], damage to which causes weight loss and death, but did not reduce the toxicity of MTX for cells participating in the early stage of the immune response. Therefore, it is considered that the combination of early MTX and delayed folinic acid administration may be more effective to prevent homograft response or delayed death, because much MTX can be given to the mice without causing serious side effects.

Recently the killing effect of infused homologous[28~30] or heterologous[31] hematopoietic cells for sublethally irradiated host mice was reported. According to Uphoff's report, the killing effect depended on the strain combinations used and the effect in certain combinations was modified by : delayed marrow inoculation, use of substrain C3HeB as the irradiated host, and preimmunization of the irradiated host. In the present study the lethal effect of Na2 HBM or HSC against dd/s host was also found ; the 30-day survival rates without transplantation were 92 % at 700 r and 44 % at 800 r, while they were 24 % and 40 % at 700 r with HSC and HBM, respectively, and 11 % at 800 r with HSC inoculation after irradiation. But MTX, when used after irradiation and HBM or HSC inoculation, altered the lethal effect ; the 30-day survival rates were about 80 % at 700 r and 55 % at 800 r. These data also indicate the MTX suppresses homograft response. But, at the sublethal dose, the HBM or HSC transplanted and MTX administered mice without early mortality are probably thought to show reversion to the host phenotype, because the recovery pattern of their lymphoid tissues, which used to show important changes in homograft response, was almost the same as that in the irradiated and untreated mice, though some mice of the former group showed a tendency of retarded recovery of lymphoid tissues. Furthermore, the peripheral blood pictures showed quicker recovery than that of the irradiated and untreated mice. Uphoff stated[30] that an experiment in which mice survived for 90 or more days after sublethal irradiation and HBM treatment provided further evidence for marrow rejection without lethality, i.e., these mice rejected tumor grafts of a strain specific neoplasm that grew only in the strain of the marrow donor : long-term protection might be a tiding-over phenomenon. The results obtained in the present study also suggest that, as Uphoff's theoretical implications, there may be not a simple graft rejection, but a tiding-over effect of the inoculated homologous cells with subseqent regression of th infused cells and reversion to the host phenotype. In general, it is thought that the early mortality of the sublethally irradiated and HBM or HSC treated mice is due to stronger and quicker recovery of host's immune state[31]. Furthermore, Trentin showed[32] that the early motality at sublethal dose of irradiation was observed in the parent mice infused with F_1 hybrid, not in the F_1 hybrid infused with parent marrow, i.e., this type of mortality resulted from

recovery of the host's ability to reject temporarily grafted marrow and there was usually secondary bone marrow aplasia. Schwartz *et al.*[28] also reported that some of the mice infused with parental spleen cells died of bone marrow aplasia : in other mice, however, the bone marrow was cellular and the immediate cause of death could not be given. In the present study, at 700 r, secondary bone marrow aplasia or hypoplasia in some mice, especially in the HSC alone treated mice, was also observed, but the cause of much lower 30-day survival of the HSC alone treated mice than that of the HBM alone treated mice did not seem to be ascribed only to secondary bone marrow aplasia. In order to clarify this difference between inoculations of HSC and HBM, further experiments are needed. As for the secondary bone marrow aplasia at the lethal dose of irradiation, de Vries *et al.*[38] noted that it was caused by host against graft reaction and chiefly occurred before 30 days after irradiation, and that so-called homologous disease or delayed foreign marrow reaction which took place after the 30 th post-irradiation day was of a complex nature ; impaired recovery of radiation-induced lesions, decreased resistance to bacterial infections due probably to generalized atrophy of the lymphoid tissues, and also an immunological reaction of the graft against host were postulated to be causal factors. In the present study secondary bone marrow aplasia with marked secondary weight loss, ruffled fur, diarrhea and hemorrhage was also found. These results incicate that even in the stage of secondary bone marrow aplasia, the cause of death should be considered to be of a complex nature.

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