Bone Marrow-Thymus Interactions during Thymic Lymphomagenesis Induced by Fractionated Radiation Exposure in B10 Mice: Analysis Using Bone Marrow Transplantation between Thy 1 Congenic Mice

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Bone marrow transplantation (BMT) experiments were conducted using B10.Thy 1 congenic mice to explore the nature of bone marrow-thymus interactions during thymic lymphomagenesis induced by fractionated whole-body X-irradiation (FX). BMT from normal Thy 1 congenic donors into FX-treated recipients one day after FX-treatment resulted in the suppression of tumor development; the suppression being exponentially proportional to the increasing number of bone marrow cells injected. The suppression of tumor development by BMT was shown to be due to prevention of the appearance of prelymphoma cells. BMT from FX-treated donors, which are deficient in pre T cells, into lethally (9 Gy) irradiated Thy 1 congenic recipients resulted in the development of high incidence of thymic lymphomas most of which (~76%) were host-derived, whereas no lymphomas were recovered from the recipients of normal bone marrow. These results suggest that intrathymic T cell precursors which initially repopulate the depleted thymus are prone to undergo preneoplastic changes in the absence of recruitment of more primitive T cell precursors (pre T cells) from the bone marrow but they undergo normal differentiation when large number of pre T cells are available. It was concluded that primary cause of the FX-induced thymic lymphomagenesis was a shortage in supply of pre T cells from the bone marrow to the depleted thymus, which caused differentiation arrest of the progeny of regenerating intrathymic T cell precursors, followed by development of prelymphoma cells that eventually evolve into autonomous lymphoma cells within the thymus.

INTRODUCTION

During the early 1950's Kaplan reported that treatment of C57BL mice with fractionated whole-body X-irradiation (FX) beginning with the age of 33±3 days resulted in the development of high incidence of thymic lymphomas. He and his collaborators then found that the development of thymic lymphomas induced by FX-treatment could be prevented when bone marrow or spleens were shielded by lead during irradiation or when normal bone marrow cells were infused intravenously shortly after FX-treatment. They also found that the development of thymic lymphomas could be prevented by thymectomy (Tx) after FX-treatment and that subcutaneous grafting of unirradiated neonatal thymuses into FX-treated
Tx mice resulted in the development of thymic lymphomas most of which were donor-derived\(^3\)-\(^5\), implying that radiogenic lymphomas were induced by 'indirect' mechanism. They then showed that cell-free extracts from such radiation-induced lymphomas, inoculated into syngeneic newborn mice, elicited identical tumors\(^6\). Thus, in 1964, Kaplan summarized his view on the possible mechanism of FX-induced thymic lymphomagenesis as follows\(^7\).

Radiation produces three simultaneous effects, all of which are essential to the induction process: 1) injury to the normal sites of storage of the latent virus with concomitant release of virus; 2) injury to the thymus, followed by regeneration; 3) injury to the bone marrow, which in turn interferes with the regeneration of irradiated thymus, producing a maturation arrest in which large numbers of highly immature lymphoid cells are made available for a sustained period for the oncogenic expression of the virus.

Since then numerous attempts have been made to unravel the mechanism of radiation lymphomagenesis with emphasis on the identification of the virus or the virus-like agent and its possible role in radiation lymphomagenesis\(^8\)-\(^23\). However, the direct proof that radiation actually induces virus or virus-like agent that causes transformation of regenerating immature lymphoid cells has not been obtained yet even with the use of modern molecular techniques\(^20,22\)-\(^23\). Thus, the question of the mechanism of radiation lymphomagenesis still remains unsettled. During the past decade, we have conducted a series of transplantation experiments by using B10.Thy 1 congenic strains, in which donor and/or host had been variously treated or untreated depending on the purpose of each experiment. We then analyzed the fate of the donor- as well as the host-type cells by the use of Thy 1 alloantigen markers\(^12\)-\(^16\).

In the present study, we conducted bone marrow transplantation (BMT) experiments in order to explore the nature of bone marrow-thymus interactions during thymic lymphomagenesis induced by FX-treatment. The results indicated that the most important cellular events that led to the development of thymic lymphomas were the thymic atrophy and the depletion of pre T cells in the bone marrow. They also showed that cellular changes that were most critical for the appearance of prelymphoma cells\(^14\)-\(^16\) occurred within two weeks after FX-treatment. Evidence was also obtained which strongly suggested that intrathymic T cell precursors, which initially repopulated the depleted thymuses, were prone to undergo preneoplastic changes in the absence of recruitment of more primitive T cell precursors (pre T cells) from the bone marrow but they apparently underwent normal differentiation when sufficiently large number of pre T cells were supplied by BMT from normal donors. We will discuss a possibility that introduction of 'infectious' virus or virus-like agent is not necessary to explain the mechanism of 'indirect' induction of radiogenic lymphomas described above.

**MATERIALS AND METHODS**

**Mice**

Male B10/Sn (Thy 1.2, H-2b) and B10.NRH-Thy 1\(^a\) (Thy 1.1, H-2b) mice were used in the present study. They were designated as B10.Thy 1.2 and B10.Thy 1.1 mice, respectively. In
one experiment, (B10.Thy 1.1 × B10.Thy 1.2)F1 or B10.Thy 1.1/1.2 mice were also used. They were bred from our own colonies at the Animal Production Facility of this institute and were maintained within a microbiologically clean animal facility.

**Fractionated whole-body X-irradiation (FX)**

FX was performed with a Shinai-III X-ray generator (Shimadzu Seisakusho Ltd., Kyoto), which is located within the animal facility, at dose-rate of 0.6 Gy/min (200 KVP, 20 mA, 0.5 mm Al + 0.5 mm Cu filters; half-value layer, 1.24 mm Cu; target-skin distance, 52 cm). Mice were whole body irradiated at 8-day intervals with four doses of 1.61 Gy each beginning with the age of 33 ± 3 days.

**Bone marrow transplantation (BMT)**

Two kinds of BMT were performed. First, bone marrow cells from normal 10–16 week old B10.Thy 1.1 donor mice were injected intravenously into FX-treated B10.Thy 1.2 mice 1, 10 or 30 days after exposure to the last dose of FX-treatment. The number of bone marrow cells injected and the time of BMT after FX-treatment varied depending on the purpose of experiment and are described in the RESULTS section. Second, bone marrow cells from FX-treated B10.Thy 1.1 donor mice (28–30 days after the last dose of FX-treatment) were injected into lethally (9 Gy) irradiated B10.Thy 1.2 recipients within a few hours after X-irradiation. In the first experiment of this series, bone marrow cells from two femuri of each FX-treated mouse were injected individually into one each recipient mouse. But, in later experiments, 2×10⁷ bone marrow cells pooled from a group of 10–20 FX-treated mice were injected instead.

**Intrathymic (i.t.) injection**

Method of i.t. injection has been described in detail elsewhere⁴⁻⁶.

**Thy 1 typing of tumors and regenerating thymus cells**

When bone marrow reconstituted mice became moribound, they were sacrificed and tumor cells were typed with respect to Thy 1 markers by using immunofluorescence technique. Relative proportion of host- and donor-type cells within the regenerating thymuses of the recipients of bone marrow from Thy 1 congeneric donors was also examined similarly. Briefly, cell suspensions prepared from lymphoid tumors as well as from thymuses of the recipients of bone marrow were typed for Thy 1 markers with the use of immunofluorescence microscopy employing biotin-conjugated monoclonal anti-Thy 1.1 or anti-Thy 1.2 antibodies combined with FITC-conjugated avidin (Meiji Nyugyo Ltd., Tokyo) as described previously⁴⁻⁶⁻⁻²⁴.

**Florescence microscopy**

Olympus fluorescence-phase contrast microscope, Model BHS-RFK, equipped with x100 fluorescence-phase contrast objective and x10 ocular lenses, was used to score total as well as fluorescence positive cells smeared on the slide glass. Total cells in a randomly selected field were counted first using phase contrast microscopy without UV light. Fluorescence
positive cells were then scored in the same field by using fluorescence microscopy. More than 30 randomly selected fields were scored to obtain more than 500 cells for each slide.

RESULTS

Suppression of Lymphoma Development in the FX-treated B10 Mice by BMT from Normal Thy 1 Congenic Donors

In the first experiment of this series, we injected varying numbers ($2 \times 10^4$ – $8 \times 10^7$) of bone marrow cells from normal B10.Thy 1.1 donors into FX-treated B10.Thy 1.2 mice one day after the last dose of FX-treatment. We then examined the tumor incidence/mortality and performed Thy 1 typing of the developed tumors. The results, shown in Fig. 1, indicate that the incidence of thymic lymphomas decreased exponentially as the number of bone marrow cells injected was increased. The maximum suppression was observed at the highest bone marrow cell dose employed, i.e., $8 \times 10^7$ cells. Result of the Thy 1 typing of the developed tumors in this series indicated that 100% (34/34) were host-derived. This observation indicates that thymus cells derived from the normal bone marrow never get

![Graph](image-url)
transformed by the putative virus-like agent that has been claimed to be released from the
irradiated hosts and cause transformation of the regenerating immature thymus cells\(^6\)\(^-\)\(^9\).

We then analyzed the kinetics of the repopulation of donor- and host-type cells within
the thymuses of the FX-treated mice that received varying numbers of bone marrow cells
from normal Thy 1 congenic donors. The results indicated that the larger was the number
of bone marrow cells injected the earlier was the time of the repopulation of donor-type
cells within the host thymuses. Thus, for example, more than 90% of the cells of the host
thymus were replaced with donor-type cells before 2 weeks after BMT in mice which
received \(8 \times 10^7\) cells, whereas the same level of replacement was observed after 3 ~ 4
weeks following BMT in the recipients of \(2 \times 10^5\) cells. As it was shown previously that
thymic prelymphoma cells, which are defined as preneoplastic cells of the T cell lineage
that require thymic microenvironment to further evolve into fully autonomous neoplastic
cells, or thymic lymphoma cells, appeared within 1–2 weeks after FX-treatment\(^1\(^4\)\(^-\)\(^6\),
these results suggested to us that the time of the repopulation of the donor-derived cells in
relation to the time of the appearance of prelymphoma cells was critical for the suppression
of the lymphoma development in the bone marrow reconstituted mice.

**Effect of Delaying the Time of BMT on the Suppression of Lymphoma Development in
FX-treated Mice**

In the following experiment, we examined the effect of delaying the time of BMT for
10 or 30 days on the development of thymic lymphomas in the recipients. In this experiment,
the number of bone marrow cells injected was \(8 \times 10^7\), which was the highest cell dose

![Fig. 2](image_url)
employed in our study. The results, shown in Fig. 2, indicate that suppression of the lymphoma development was greatly reduced when BMT was delayed for 10 days. When BMT was delayed for 30 days, suppression of the lymphoma development was not observed.

Analysis of the kinetics of the repopulation of the donor-type cells within the thymuses of the recipients indicated that when BMT was delayed for 10 days, repopulation of the donor-type cells was also delayed for about 10 days and more than 90% replacement of the thymus with donor-type cells took place between 3 and 4 weeks (Fig. 3).

Thus, when these two sets of data are combined, we note that for effective suppression of lymphoma development in FX-treated mice by BMT the repopulation of donor-type cells within the thymuses of the recipients must occur before the appearance of prelymphoma cells and that repopulation of donor-type cells after the appearance of prelymphoma cells is not effective to suppress the development of lymphomas.

Prevention of the Development of Thymic Prelymphoma Cells within the Thymuses of FX-treated Mice Reconstituted with Bone Marrow from Normal Donors

We then examined whether transplantation of bone marrow cells from normal donors into FX-treated mice one day after FX-treatment would indeed prevent the appearance of thymic prelymphoma cells within the thymuses of the FX-treated recipients. Thus, $8 \times 10^7$ bone marrow cells from normal B10.Thy 1.1 donor mice were injected into FX-treated

Fig. 3. Time course of the replacement of the recipient thymuses with donor-type thymocytes following BMT at day 10 as compared to that observed after BMT at day 1. Solid symbols, host-type cells; Open symbols, donor-type cells. Open columns, incidence of mice which developed prelymphoma cells within the thymuses of FX-treated mice not treated with BMT.\(^{(3)}\)
B10.Thy 1.2 mice one day after the last dose of FX-treatment. One month later, $5 \times 10^6$ thymus cells recovered from these recipient mice were injected individually into thymuses of the sublethally (3.78 Gy) irradiated B10.Thy 1.1/1.2 mice (secondary recipients) to assess the development of prelymphoma cells within thymuses of the primary recipients. The results showed that incidence of thymic lymphomas in the secondary recipients was only 13% (2/15) as compared to 64% expected from the earlier results reported for FX-treated mice that were not injected with normal bone marrow cells$^{14}$. The results of Thy 1 typing of the two thymic lymphomas recovered in this experiment showed that they were Thy 1.2 homozygous, i.e., they were of the primary recipient origin. Thus, these results are consistent with an interpretation that development of prelymphoma cells was indeed suppressed when normal T cell precursors entered thymuses of the FX-treated mice and repopulated there before the appearance of prelymphoma cells.

Development of Host-type Lymphomas in Lethally Irradiated Mice Reconstituted with Bone Marrow from FX-treated Donors

In the following experiments, we asked a question regarding the role of the bone marrow of the FX-treated mice on the development of thymic lymphomas. For this purpose, we injected bone marrow cells from two femuri of individual FX-treated B10.Thy 1.1 donors into lethally irradiated B10.Thy 1.2 mice. Control group received $2 \times 10^6$ bone marrow cells from normal B10.Thy 1.1 donors. We then examined the lymphoma incidence and performed Thy 1 typing of the developed lymphomas. The results are shown in Fig. 4. They show clearly that high incidence of thymic lymphomas was observed in the recipients
of bone marrow from FX-treated donors, whereas no lymphomas were recovered from the recipients of normal bone marrow before 400 days after BMT.

The result of the Thy 1 typing of lymphomas recovered from these mice showed that as high as 78% were Thy 1.2 positive, indicating that they were host-derived. It is important to note that recipients of bone marrow from FX-treated donors developed high incidence of thymic lymphomas most of which were host-derived, whereas no lymphomas were recovered in the recipients of normal bone marrow. These results indicate that the role of the bone marrow of the FX-treated mice in the development of thymic lymphomas is not to provide preneoplastic (or prelymphoma) cells but rather to create a condition that causes the regenerating thymus cells to undergo preneoplastic changes by as yet undefined mechanism.

We then compared regeneration of the thymuses of the lethally irradiated B10.Thy 1.2 mice which received bone marrow from FX-treated or normal B10.Thy 1.1 donors. The results, shown in Fig. 5, indicated that repopulation of the donor-derived cells within the thymuses of the recipients of bone marrow from FX-treated mice was greatly reduced as compared to that seen in the recipients of normal bone marrow, indicating a severe

![Fig. 5](image-url)
deficiency of the thymus-repopulating potential of the bone marrow from FX-treated mice. This observation is consistent with our earlier observation that thymus-repopulating potential of the bone marrow was greatly reduced in the FX-treated mice\textsuperscript{14}. Our preliminary estimate of the frequency of pre T cells in the bone marrow of FX-treated mice was as low as 2\% of that seen in the normal bone marrow\textsuperscript{14}. This would explain why thymuses of the FX-treated mice as well as those of the lethally irradiated mice reconstituted with bone marrow from FX-treated donors were poorly repopulated with bone marrow-derived cells.

To summarize the results presented above, it is clear that radioresistant T cell precursors which initially repopulate the thymuses of the lethally irradiated mice\textsuperscript{24} undergo normal differentiation when mice are reconstituted with normal bone marrow, whereas the same population of cells undergo preneoplastic changes when mice are protected with bone marrow from FX-treated donors that contain very few T cell precursors.

**DISCUSSION**

The results presented here provide some information that have important implications for our understanding of the mechanism of FX-induced thymic lymphomagenesis in mice.

First, in confirmation and extension of the earlier results of Kaplan\textsuperscript{2} and others\textsuperscript{17–18}, we found that injection of bone marrow cells from normal donors into FX-treated mice one day after the last dose of FX-treatment resulted in the suppression of the development of thymic lymphomas. The suppression of the tumor development by BMT was exponentially proportional to the number of bone marrow cells injected; the larger was the number of bone marrow cells injected, the more significant suppression of tumor development was observed.

Second, we showed that there was a high correlation between the time of repopulation of donor-type cells within the recipient thymuses and magnitude of the suppression of tumor development: the earlier was the repopulation of donor-type cells commenced, the more significant suppression of tumor development was observed. It appeared that extensive repopulation of donor-type cells, e.g., >90\% replacement, within the thymuses of the FX-treated mice must take place within two weeks after FX-treatment in order to effectively suppress the development of lymphomas. As it is known that prelymphoma cells normally begin to appear between 1–2 weeks following FX-treatment\textsuperscript{14–16}, these results suggest that suppression of lymphoma development by BMT may be caused by preventing the appearance of prelymphoma cells during early intervals after FX-treatment.

Third, it was shown that transplantation of large number (8x10\textsuperscript{7}) of bone marrow cells into FX-treated mice one day after the last dose of FX-treatment resulted in the suppression of the appearance of prelymphoma cells within the thymuses of the FX-treated mice.

Fourth, we found that transplantation of bone marrow cells from FX-treated donors into lethally irradiated Thy 1 congenic recipients resulted in the development of high incidence of thymic lymphomas most of which were host-derived, whereas no lymphomas were
recovered from the recipients of normal bone marrow cells. We then found that repopulation of thymuses of the lethally irradiated recipients was severely depressed when bone marrow cells were derived from FX-treated donors, suggesting strongly that bone marrow of the FX-treated mice is deficient in T cell precursors that are abundantly present in the normal bone marrow. Evidence to support this notion has been reported previously\textsuperscript{14}. Thus, when these results are combined, it appears that intrathymic radioresistant T cell precursors, which initially repopulate the thymuses of lethally irradiated mice\textsuperscript{24}, are prone to undergo preneoplastic changes if more primitive T cell precursors, or pre T cells, are not recruited from the bone marrow.

Taken together, the cellular events that lead to the development of thymic lymphomas following FX-treatment of the susceptible strain of mice may be summarized as follows (Fig. 6).

FX-treatment causes atrophy of the thymus as well as depletion of pre T cells in the bone marrow. Intrathymic T cell precursors which survived the exposure to FX-treatment begin to regenerate first in the absence of recruitment of pre T cells from the bone marrow. Under this condition, the regenerating immature T cells undergo differentiation arrest. Thus, it appears that massive recruitment of pre T cells from the bone marrow is essential
for the regenerating cells to proceed further along their differentiation pathways. On the other hand, when sufficiently large number of pre T cells are supplied by BMT from normal donors into FX-treated mice one day after the last dose of FX-treatment, normal growth and differentiation of initially regenerating immature lymphoid cells is restored. In other words, it appears that when thymic atrophy is induced under conditions of reduced supply of pre T cells from the bone marrow, a differentiation arrest will occur among regenerating immature lymphoid cells. Some of these regenerating cells will then undergo preneoplastic change, or transformation, due to a disregulation of the growth control which is probably brought about by imbalance of the levels of various growth factors and/or inhibitors that are produced by thymic epithelial cells, dendritic cells as well as macrophages and aberrant expression of receptors for these factors among regenerating thymus cells. We consider that transformation must occur as an epigenetic process rather than as a result of mutation(s) because it occurs within the thymuses of all FX-treated mice during short period after FX-treatment during which mitotic rate is extremely low.

The above-mentioned interpretation would explain why dose-response relationship for induction of thymic lymphomas in C57BL and its related strains by radiation show a ‘threshold’ around 2 Gy, which implies that extensive cell destruction within the thymus as well as in the bone marrow is a prerequisite for the development of thymic lymphomas after irradiation. We may further note that this interpretation would also explain the ‘indirect’ induction of radiogenic lymphomas described by Kaplan and others without assuming induction of ‘infectious’ virus or virus-like agent. The rationale for this notion is the following. It is well known that when normal newborn thymuses are grafted into histocompatible normal recipients most thymus cells present at the time of the grafting undergo necrosis and residual intrathymic T cell precursors regenerate first and are then replaced with host-derived cells later. Thus, when normal thymuses are grafted subcutaneously into FX-treated recipients, which are deficient in pre T cells in the bone marrow, the regenerating immature thymus cells must undergo differentiation arrest because pre T cells are not recruited from the bone marrow. Once thymic atrophy is induced in the environment in which pre T cells are not available, the subsequent events that occur within the grafted thymus must be the same as those discussed above for FX-treated mice. Thus, in conclusion, the primary cause of the FX-induced thymic lymphomagenesis is the shortage in supply of pre T cells from the bone marrow to the depleted thymus, which causes differentiation arrest of the progeny of regenerating intrathymic T cell precursors, followed by development of prelymphoma cells that eventually evolve into autonomous lymphoma cells under the influence of thymic microenvironment.

Another important notion suggested from the results reported here is that once prelymphoma cells are generated within the regenerating thymuses of the FX-treated mice it is not possible to prevent them to evolve into neoplastic cells even if T cell precursors derived from the normal bone marrow repopulate the thymuses in which prelymphoma cells have developed. This notion implies that suppression of lymphoma development may be achieved only by preventing the appearance of prelymphoma cells by supplying pre T cells from the normal bone marrow during very early stage of regeneration.
It is obvious that our next step is to dissect molecular mechanism of the postulated discordance of the growth and differentiation of regenerating T cells that leads to the manifestation of preneoplastic character and eventually to the development of fully autonomous lymphoma cells.

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