ABSTRACT

Intrathymic (i.t.) as well as i.p. injection of thymus cells from B10.Thy-1.1 mice manifesting overt thymic lymphomas, 4 months after split-dose irradiation, into B10.Thy-1.2 recipient mice resulted in the development of donor-type T-cell lymphomas, indicating that they contained "autonomous" lymphoma cells. In contrast, injection of thymus cells from apparently nonleukemic mice 1 month after split-dose irradiation resulted in the development of donor-type tumors only when they were injected i.t., suggesting that thymus cells from these mice contained "preneoplastic" cells that will eventually develop into thymic lymphomas under the influence of thymic microenvironment. These "thymus-dependent" neoplastic cells were termed "thymic prelymphoma cells." With the use of i.t. injection assay, it was shown that these thymic prelymphoma cells were detected in 26.1% (6 of 23) of the test donor thymuses when examined at 14 days and in more than 63% (15 of 24 and 14 of 22) when examined at 21 and 31 days after irradiation. To examine the possibility that thymic prelymphoma cells might appear first in the bone marrow before they become detectable within the thymuses of the split-dose-irradiated mice, bone marrow cells from B10.Thy-1.1 donors recovered at 8, 14, 21, and 33 days after split-dose irradiation were also injected i.t. into B10.Thy-1.2-recipient mice. The results indicated that none of these recipients developed donor-type T-cell lymphomas, suggesting that bone marrow is not the first site of the appearance of thymic prelymphoma cells.

INTRODUCTION

Fractionated, whole-body X-irradiation (four exposures of 1.61 Gy at 8-day intervals) of some mouse strains such as B10, B10.BR, or C57BL/Ka leads to the development of thymic lymphomas, with onset after about 3 months and a final incidence of more than 95% (1–3). Most of these tumors bear T-lymphocyte-specific surface antigens such as Thy-1, Lyt-1, and Lyt-2 (4). These radiation-induced thymic lymphomas appear to develop through a complex cellular processes involving interaction of the progenies of i.t.3 radiodensitive T-cell precursors and the bone marrow-derived T-cell precursors as well as the thymic epithelial cells and macrophages that constitute the thymic microenvironment (2, 3, 5, 6). To understand the mechanism of radiation-induced thymic lymphomagenesis, it is important to ascertain the site and the time at which prelymphoma cells, having the potential to develop into overt thymic lymphomas, will first appear after exposure to the split doses of radiation.

Several cell transfer assay systems have been used in the past to detect the prelymphoma/preleukemic cells that develop in the split-dose-irradiated mice (5, 7–9). They vary in methodological details and the conclusions drawn from different laboratories are not necessarily consistent with each other (5, 9, 10). Using a parent → F1, combination, Haran-Ghersa et al. (7, 8, 10) reported that the initial transformation event induced by X-irradiation occurred among prothymocytes present in the bone marrow and that these "preleukemic" cells required thymic microenvironment to further develop into overt T-cell leukemia (10). On the other hand, Boniver et al. (5) showed that preneoplastic cells, which had not yet acquired autonomous proliferation potential, were present in the thymus but not in the bone marrow when examined at 30 to 60 days after irradiation (5). Unfortunately, however, no attempts were made in both of these studies to determine whether the lymphomas studied in these experiments had expressed Thy-1 marker which is characteristic for T-cells. Thus, it was not clear from the published results whether these investigators were dealing with precursors of the same type of tumors or not. It is possible that different kinds of preleukemic cells that are committed to nonthymic lymphomas or leukemias may develop in the bone marrow or elsewhere of the lymphohemopoietic tissues when mice were exposed to radiations (11).

Thus, we consider that it is very important to define the cell lineage(s) or the type of leukemia/lymphomas with cell-type-specific markers when one talks about preleukemic cells in relation to their initial transformation and subsequent progression. In the present study, we used the term "thymic prelymphoma cells" to describe preneoplastic cells that will eventually develop into thymic lymphomas that express Thy-1 markers on their cell surfaces. We first examined the thymus dependency of growth potential of thymic prelymphoma cells and then studied the site and the time at which they first appear during radiation-induced thymic leukemogenesis. Results are presented here.

MATERIALS AND METHODS

Mice. Female B10f/Sn (Thy-1.2, H-2b) (hereafter called B10.Thy-1.2) and B10.NRH-Thy-1.1 (Thy-1.1, H-2d) (hereafter called B10.Thy-1.1) mice were used in the present study. They were bred from our own colonies at the Animal Production Facility of this institute (3) and were maintained within a microbiologically "clean" conventional animal facility (12).

Experimental Protocol. B10.Thy-1.1 mice were exposed to fractionated doses of X-rays. At varying periods after irradiation, cell suspensions (in Eagle's minimal essential medium supplemented with 5% fetal calf serum) prepared from the thymus (5 × 10^6) or the bone marrow (1 × 10^6) of these mice were injected i.t. into 6–9-week-old B10.Thy-1.2 mice which had been irradiated with 3.79 Gy immediately before injection. Injection was carried out from one individual donor to one individual recipient (Tables 1 and 2) unless otherwise stated (Figs. 1 and 2). Recipient mice were sacrificed when they appeared moribund or developed clinical evidence of disseminated lymphomas. Each lymphoma was tested for its genetic origin by means of cytotoxicity assay using anti-Thy-1.1 (T-11-D7, kindly supplied by Dr. H. Ishikawa, Keio University, Tokyo, Japan) or anti-Thy-1.2 antibodies (Olac, Ltd., Bicester, England) and rabbit complement (Low-Tox-M; Cedarian Laboratories, Ltd., Toronto, Ontario, Canada) as described previously (3).

Split-Dose X-Irradiation. Whole-body X-irradiation was performed with a Shinai-III X-ray generator (Shimadzu Seisakusho, Ltd., Kyoto, Japan) at 0.57 Gy/min irradiation parameters, 200 kVp, 20 mA, with filters of 0.5 mm Cu and 0.5 mm Al; half-value layer, 1.24 mm Cu;
RADIATION-INDUCED THYMIC PRELYMPHOMA CELLS

Thymic Prelymphoma Cells in the Thymus. Varying numbers of thymus cells taken from split-dose-irradiated B10.Thy-1.1 mice, 4 mo after irradiation and manifesting overt thymic lymphomas, were injected i.t. into 3.79-Gy-irradiated B10.Thy-1.2 mice, or i.p. into nonirradiated or thymectomized and 3.79-Gy-irradiated B10.Thy-1.2 recipient mice, and the incidence of donor-derived T-cell lymphomas was determined. The results, shown in Fig. 1A, indicate that the incidence of recipient mice that developed donor-type lymphomas was clearly dependent on the number of thymus cells injected and the environment to which they were injected. Thus, the injection via the i.t. route was 100 times more effective than the i.p. injection for supporting the growth of transplanted cells. However, when the number of thymus cells injected exceeded 10^7, 100% of the recipient mice developed donor-type lymphomas regardless of the route of their injection. These results indicate that the thymuses at 4 mo after split-dose irradiation contained lymphoma cells that proliferate autonomously anywhere in the syngeneic hosts, although their preference for thymic microenvironment was clearly indicated.

In contrast, when thymus cells taken from B10.Thy-1.1 donors 1 mo after split-dose irradiation, at which time none of the test donor mice manifested macroscopic evidence of lymphomas, were injected i.t. into 3.79-Gy-irradiated recipients or i.p. into nonirradiated or 3.79-Gy-irradiated thymectomized recipients, the donor-type tumors were recovered only when the thymus cells were injected i.t. (Fig. 1B). It may be noted that i.p. injection of as many as 8 x 10^7 thymus cells failed to show evidence of tumor development (Fig. 1B). These results indicate that the thymus at 1 mo after split-dose irradiation contains preneoplastic cells that will eventually develop into thymic lymphomas under the influence of thymic microenvironment.

Fig. 1. Incidence of thymic lymphomas that developed following i.t. and i.p. injection of thymus cells from split-dose-irradiated B10.Thy-1.1 mice at 1 and 4 mo after split-dose irradiation into B10.Thy-1.2 recipient mice. (A) Various numbers of thymus cells from female B10.Thy-1.1 mice 4 mo after irradiation were inoculated i.t. into 3.79-Gy-irradiated B10.Thy-1.2 mice (C) or injected i.p. into intact normal B10.Thy-1.2 mice (M) or thymectomized, 3.79-Gy-irradiated B10.Thy-1.2 mice (O). Three to 6 recipient mice (6-9 weeks old) were used for each point. (B) Various numbers of thymus cells from female B10.Thy-1.1 mice 1 mo after split-dose irradiation were inoculated i.t. into 3.79-Gy-irradiated B10.Thy-1.2 mice (C) or injected i.p. into intact normal B10.Thy-1.2 mice (M) or thymectomized, 3.79-Gy-irradiated B10.Thy-1.2 mice (O). Three to 6 female recipient mice (6-9 weeks old) were used for each point.

Table 1 Analysis of T-cell lymphomas developed after i.t. injection of thymus cells from split-dose-irradiated B10.Thy-1.1 mice

<table>
<thead>
<tr>
<th>Time (days) after split-dose irradiation</th>
<th>Thymic lymphoma-bearing mice</th>
<th>Donor type (Thy-1.1)</th>
<th>Host type (Thy-1.2)</th>
<th>Thy-1-negative lymphoma</th>
<th>No. of surviving mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0/16 (0.0%)</td>
<td>3/16 (18.8%)</td>
<td>240 d</td>
<td>1/16</td>
<td>12/16 (75.0%)</td>
</tr>
<tr>
<td>14</td>
<td>6/23 (26.1)</td>
<td>5/23 (21.7)</td>
<td>153 d</td>
<td>0/23</td>
<td>12/23 (52.2)</td>
</tr>
<tr>
<td>21</td>
<td>15/24 (62.5)</td>
<td>2/24 (8.3)</td>
<td>6/24</td>
<td>1/24</td>
<td>3/22 (13.6)</td>
</tr>
<tr>
<td>31</td>
<td>14/22 (63.6)</td>
<td>4/22 (18.2)</td>
<td>203 d</td>
<td>1/22</td>
<td></td>
</tr>
</tbody>
</table>

* Six- to 9-week-old female B10.Thy-1.2 mice were exposed to 3.79 Gy of X-rays, and 5 x 10^6 thymus cells, collected from split-dose-irradiated (1.61 Gy in 4 doses) B10.Thy-1.1 donors, were injected i.t. within 3 h after exposure to X-rays (transfer from one donor to one recipient).

† Number of T-cell lymphoma-bearing mice/total number of mice tested.

‡ Numbers in parentheses, percentage.

§ Numbers in parentheses, average latent period in days (d).

We define these thymus-dependent preneoplastic cells as "thymic prelymphoma cells" and distinguish them from a more general term, "preleukemic cells," (5, 9, 10) which may include preneoplastic cells of any lymphohemopoietic cell lineages and, therefore, can be quite confusing when one comes to discuss their nature and the first site(s) of their appearance.

Development of Thymic Prelymphoma Cells in the Thymus with Time after Irradiation. Thymus cells (5 x 10^7) recovered from B10.Thy-1.1 mice at day 8, 14, 21, and 31 after split-dose irradiation were injected i.t. into 3.79-Gy-irradiated B10.Thy-1.2 mice from one donor to one recipient and the genetic origin of T-cell lymphomas that developed in the thymuses of the recipient mice was determined. The results, shown in Table 1, indicate that: (a) thymic prelymphoma cells were not detected...
in the thymus at day 8 after irradiation; (b) they were first detected at day 14 after irradiation (6 of 23, or 26.1%); (c) the incidence of mice that contained thymic prelymphoma cells in their thymuses increased thereafter and it reached as high as 63% when examined at 21 and 31 days after irradiation. It may be added that about 10–20% of the recipient mice developed T-cell lymphomas which were most probably the primary tumors induced in the recipients by whole-body exposure to a single dose of 3.79 Gy. More recent results show that preirradiation of the recipient mice is not essential for the development of thymocyte precursors (pre-T-cells) present in the bone marrow of the split-dose-irradiated mice before they became detectable within the thymus, as suggested by the work of Haran-Ghera (7). Before we test this question, we thought it necessary to establish that the thymus could support the proliferation of thymocyte precursors (pre-T-cells) present in the bone marrow when they were injected directly into the thymus of 3.79-Gy-irradiated mice. Thus, in the following experiment, varying numbers of bone marrow cells from normal B10.Thy-1.1 mice were injected i.t. or i.v. into 3.79-Gy-irradiated B10.Thy-1.2 mice. One month later, the percentage of the donor- as well as the host-type T-cells within the thymus was analyzed by flow cytometry using fluorescence-labeled anti-Thy-1.1 or anti-Thy-1.2 antibodies. The results showed that when 10^4–10^5 of bone marrow cells were injected, the number of thymocytes developed from donor bone marrow cells after their i.t. injection was about 10 times higher than that observed after i.v. injection (Fig. 2). The i.t. transfer assay to detect thymocyte precursors in various lymphohemopoietic organs has also been reported by others (13–15), although the detail of the host treatment conditions used by these authors were slightly different from that used in the present study.

We then examined whether the thymic prelymphoma cells could be detected in the bone marrow of the split-dose-irradiated mice, by using our i.t. transplantation assay. Bone marrow cells (1 × 10^7) recovered from split-dose-irradiated B10.Thy-1.1 mice at days 8, 14, 21, and 33 after irradiation were injected directly into the thymus of 3.79-Gy-irradiated B10.Thy-1.2 mice from one donor to one recipient. The results indicated that no thymic lymphomas which manifested donor-type Thy-1 markers were recovered from the recipient mice (Table 2).

The results shown in Fig. 2 also indicate that the lowest number of donor bone marrow cells that resulted in the generation of donor-type T-cells in the recipient thymus was about 10^3 for cells from nonirradiated mice and about 10^5 for cells from split-dose-irradiated mice. These results show that the number and/or the efficiency to produce progeny of thymocyte precursors present in the bone marrow of split-dose-irradiated mice was greatly reduced as compared to that present in the normal bone marrow.

**DISCUSSION**

The primary purpose of the experiments reported in this paper was to determine the site and the time at which preneoplastic cells that will later develop into thymic lymphomas will first appear following exposure to split-dose irradiation. We first showed, by using i.t. transplantation assay of thymus cells between B10.Thy-1 congenic donor-host combinations, that thymus cells of the split-dose-irradiated B10.Thy-1.1 mice 1 mo after irradiation contained preneoplastic cells that require thymic microenvironment to evolve into overt T-cell lymphomas. In contrast, the "autonomous" lymphoma cells that were recovered from the thymus 4 mo after split-dose irradiation were shown to proliferate independently from the thymus (Fig. 1). We adopted the term "thymic prelymphoma cells," rather than "preleukemic cells," to describe preneoplastic cells of T-cell lineage because the latter term can include any preneoplastic cells of lymphohemopoietic lineages and therefore can cause confusion when one discusses the initial site(s) and time of their appearance which may vary considerably depending on the different cell lineages.

We also showed that these thymic prelymphoma cells first appeared in the thymuses after day 8 and prior to day 14 following split-dose irradiation and that they were present in the thymuses of more than 63% of mice when examined at days 21 and 31 after irradiation. More recent results indicate that when thymus cells from split-dose-irradiated mice at 2 mo after irradiation were injected i.t. or i.p. into recipients, 83.3% (10 of 12) of the i.t.-injected recipient and 16.6% (2 of 12) of the i.p.-injected recipient mice developed donor-type T-cell lymphomas, with 16.6% (2 of 12) having developed no tumors in both injection sites. These results indicate that some mice (16.6%) at 2 mo after split-dose irradiation had already developed thymic lymphomas in their thymuses but the same proportion of mice had not yet developed preneoplastic and/or neoplastic cells.

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**Table 2 Incidence and genetic origin of T-cell lymphomas developed after i.t. injection of bone marrow cells from split-dose-irradiated B10.Thy-1.1 mice**

<table>
<thead>
<tr>
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<td>1/20</td>
<td>3/20</td>
<td>16/20</td>
</tr>
<tr>
<td>33</td>
<td>0/18</td>
<td>0/18</td>
<td>3/18</td>
<td>15/18</td>
</tr>
</tbody>
</table>

* Six- to 9-week-old B10.Thy-1.2 mice were exposed to 3.79 Gy of X-rays and 10^6 bone marrow cells, collected from split-dose-irradiated (1.61 Gy in 4 doses) B10.Thy-1.1 donors, were injected i.t. within 3 h after exposure to X-rays. About one-half of male and female mice were used in each group (transfer from one donor to one same sex recipient).

* Number of T-cell lymphoma-bearing mice/total number of mice tested.

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M. Muto and T. Sado, unpublished results.

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![Fig. 2. Dose-response analysis of thymocyte precursors in the bone marrow by i.t. injection. Varying numbers of bone marrow cells from normal B10.Thy-1.1 mice (○) or from split-dose-irradiated mice 1 mo after irradiation (●) were injected i.t. into 3.79-Gy-irradiated B10.Thy-1.2 mice. To compare the relative sensitivities of assays, various numbers of bone marrow cells (□) from normal B10.Thy-1.1 mice were injected i.v. into 3.79-Gy-irradiated B10.Thy-1.2 mice. One mo later, the percentage of donor-type T-cells in the thymus was analyzed with flow cytometry. Each point represents the mean; bars, SE. Three to 8 female recipient mice (7–10 weeks old) were used for each point.](cancerres.aacrjournals.org)
We then asked whether or not the thymic prelymphoma cells might appear first in the bone marrow before they became detectable within the thymus of the split-dose-irradiated mice. Since it has been established that T-cell precursors present in the bone marrow can proliferate efficiently when they were injected i.t. into 3.79-Gy-irradiated recipients, we reasoned that i.t. injection of bone marrow cells from split-dose-irradiated mice should result in the development of donor-type T-cell lymphomas in the recipient thymuses if they contained "thymic prelymphoma cells" in the bone marrow. The results indicated that none of the recipients of the bone marrow cells from the split-dose-irradiated mice had developed donor-type T-cell lymphomas during the observation period of 13–14 mo when the test bone marrow cells were collected between day 8 and day 33 after irradiation (Table 2).

Thus, we conclude that the thymic prelymphoma cells will first develop within the thymus prior to day 14 after split-dose irradiation and that these thymic prelymphoma cells require thymic microenvironment to further develop into overt T-cell lymphomas. It may be added, however, that failure to detect donor type T-cell lymphomas in the recipients of the thymus cells from donors at day 8 after split-dose irradiation (Table 1) could be due to a possibility that the proportion of prelymphoma cells was too small to be detected by injection of $5 \times 10^4$ thymus cells. This same comment may also apply to the experiments in which $1 \times 10^7$ bone marrow cells from split-dose-irradiated mice were injected i.t. to examine the presence of thymic prelymphoma cells (Table 2). The experiments to test these possibilities by increasing the number of test cells injected i.t. are now in progress.

Finally, we wish to comment on the earlier results on the site(s) and the time at which the first prelymphoma/preleukemic cells will develop after split-dose irradiation. Haranghera et al. (7) reported that lymphomas could be detected, after a long latent periods, in the recipients of i.v.-injected bone marrow from donors that had been treated with split-dose irradiation and concluded that the test bone marrow contained preleukemic cells. Thus, our conclusion raises an apparent conflict with that reported by these authors if they were talking about thymic lymphomas. However, it is not clear from the published data whether they were dealing only with thymic lymphomas or not. More recently, Mori and Takamori (16) reported that when bone marrow cells from male NFS mice 30 days after split-dose irradiation were injected i.v. into 400-rad-irradiated syngeneic females, most of the lymphomas developed from the donor bone marrow cells were nonthymic lymphoblastic leukemias. This observation suggests that the bone marrow of some mice 1 mo after split-dose irradiation contains "preleukemic" cells that are committed to nonthymic lymphomas. Thus, all these observations point to a need to distinguish between preleukemic/prelymphoma cells for thymic and non-thymic lymphomas, because it is possible that the site and the time of the appearance of prelymphoma cells for these two different cell lineages as well as their subsequent growth requirements may not be the same.

We recently obtained convincing evidence that bone marrow cells form split-dose-irradiated B10.Thy-1.1 mice are highly competent in generating thymic lymphomas when they were injected i.v. into lethally irradiated B10.Thy-1.2 mice but most, if not all, of the lymphomas were originating from the recipients, pointing to a indirect action of split-dose-irradiated bone marrow on the generation of thymic lymphomas. Details of the experiments will soon be published.3

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Development of Prelymphoma Cells Committed to Thymic Lymphomas during Radiation-induced Thymic Lymphomagenesis in B10 Mice

Masahiro Muto, Eiko Kubo and Toshihiko Sado


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