ROLE OF THE THYMUS IN PROPYLNITROSOUREA-INDUCED THYMIC LYMPHOMAGENESIS IN F344 RATS

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The role of the thymus in propylnitrosourea (PNU)-induced thymic lymphomagenesis was studied in F344 rats with genetically determined high susceptibility. The thymus was absolutely required for thymic lymphomagenesis, since thymectomy prior to or after PNU treatment abolished lymphomagenesis, whereas grafting of a normal neonatal thymus before PNU treatment restored it. Exposure to PNU for 42 days resulted in the appearance of potentially lymphomatous cells first in the thymus, and overt T-lymphomas subsequently appeared. Such cells seemed to be thymus-dependent, since intrathymic transfer of the thymus cells from 42-day PNU-treated rats induced T-lymphomas much more efficiently than intravenous transfer. Further, grafting of the thymus from 42-day PNU-treated rats into thymectomized rats resulted in T-lymphomas of donor origin without additional PNU treatment. Cells from the spleen or bone marrow from the same donors did not give rise to T-lymphomas irrespective of the route of cell transfer and sublethal irradiation of the recipients. Morphologically atypical cell foci were detected first on the 28th day in the thymus and were most pronounced during the 35th–42nd days. Therefore, the thymus is the essential organ in which the early events of PNU-induced rat T-lymphomagenesis take place.

Key words: Thymus — Rat leukemia — Thymic lymphoma — Chemical carcinogen — Propylnitrosourea

In thymic lymphomagenesis of the mouse, the requirement for the thymus has been well substantiated. Irrespective of the method of induction, lymphomagenesis is prevented by thymectomy and is restored by grafting of the thymus from an appropriate donor. The role of the thymus in lymphomagenesis, however, is still controversial. The thymus may provide a source of target cells susceptible to carcinogenic agents, a site of viral expression and replication or microenvironments supporting the growth and progression of early lymphoma cells. These functions are not necessarily mutually exclusive but may work in various combinations depending on the experimental model under observation.

In the rat, spontaneous T-lymphomas are extremely rare. Furthermore, most chemical carcinogens that induce high incidences of T-lymphomas in mice fail to induce similar leukemias in rats. However, this does not mean that rats are totally unsusceptible to T-lymphomas since they can be induced by neonatal injection of rat-adapted retroviruses. Ogiu et al. reported that Fischer 344 rats have high susceptibility to T-lymphoma induction by a chemical carcinogen, propylnitrosourea (PNU). Our recent study has shown that this susceptibility is dictated by two autosomal dominant genes Tls-1 and Tls-2. Tls-1 determines the type of disease, i.e., T-lymphoma vs erythroleukemia, and Tls-2 shortens the latent period. In an attempt to elucidate the mechanisms of action of these genes, we studied the role of the thymus in PNU-induced lymphomagenesis in Fischer 344 rats and the cellular events in the thymus during the early stage of lymphomagenesis. Chemically induced rat lymphomagenesis is advantageous since it is less likely to be complicated by activation of endogenous retroviruses.

Abbreviations: PNU, propylnitrosourea; Tls-1, thymic lymphoma susceptible-1; Tls-2, thymic lymphoma susceptible-2.

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Materials and Methods

Rats  Inbred male and female Fischer 344/CuCrj (F344) rats were purchased from Charles River Japan Inc., Atsugi, Kanagawa.

PNU Administration  PNU (Iwai Kagaku Co., Ltd., Tokyo) was dissolved in deionized water at a concentration of 400 ppm immediately before use. The rats were given PNU in drinking water ad libitum from 5 p.m. to 9 a.m., but no water was given thereafter. Administration of PNU started at the age of 40 days and continued for 90 days unless otherwise noted. After this period, the rats were given PNU-free water ad libitum. All the rats were killed when they became moribund or at the age of 12 months and full autopsy including histological examination was carried out. T-lymphoma was diagnosed on the basis of both involvement of the thymus and expression of Thy-1.1 antigen detected by a cytotoxicity test as described previously. To follow the early changes of lymphoid tissues induced by PNU, groups of 3-9 rats were killed at 3, 7, 10, 14 days, and thereafter weekly, after the start of PNU administration. The thymus, spleen and bone marrow were examined histologically.

Thymectomy, Thymus Grafting and Cell Transfer  Thymectomy was carried out at the age of 35 days unless otherwise noted. Some thymectomized female rats were grafted with a whole thymus from a newborn male rat under the left kidney capsule or with a PNU-exposed adult thymus. For cell transfer, pooled cells of 5-6 male donors were washed, suspended in medium 199 and injected either intrathymically or intravenously into age-matched normal female recipients. For intrathymic injection, the recipients were lightly anesthetized and the thymus was exposed by a small parasternal incision. In one experiment, sublethally irradiated female rats were used as recipients.

Irradiation  Female rats at the age of 50±2 days were exposed to a sublethal dose (700 or 900 rads as indicated later) of X-ray delivered from a Shimadzu Shin-Al 250 X-ray machine, operated under the following conditions; 250 kVp, 16 mA, with filters of 0.5 mm Cu plus 1 mm Al, HVL 1.2 mm Cu, average exposure rate 95-100 rads per min, and at a distance of 50 cm from the focus of the X-ray tube.

Chromosome Analysis  In order to determine the origin of lymphoma cells induced in chimeric rats, sex chromosome analysis was done. Chromosomal preparations were made from the thymus, spleen, and bone marrow by the modified air-drying methods described by Kurita et al. Approximately 80 metaphases were examined in each specimen. Sex-karyotype was readily determined since the X-chromosome of F344 rats was subtelocentric. The number of large subtelocentric chromosomes in female cells was 6, while that in male cells was 5.

Results

Requirement for Thymus in PNU-induced T-lymphomagenesis in Rats

As shown in Table I, oral administration of PNU in our protocol induced a high incidence of T-lymphomas in F344 rats (41/42). However, when the rats were thymectomized 5 days prior to the start of PNU, T-lymphomagenesis was totally abolished (0/22). When the thymectomized rats were grafted with a syngeneic neonatal thymus and subsequently given PNU, T-lymphomas involving the graft developed (14/16). However, thymus grafts carried out after PNU administration failed to restore this susceptibility. In this group, one of 12 leukemias

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of rats</th>
<th>All leukemias</th>
<th>Thymic lymphomas</th>
<th>Other leukemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNU a)</td>
<td>42</td>
<td>41 (98)%</td>
<td>41 (105)%</td>
<td>0 (—)%</td>
</tr>
<tr>
<td>Tx b) + PNU</td>
<td>22</td>
<td>7 (32)</td>
<td>0 (—)</td>
<td>7 (171)</td>
</tr>
<tr>
<td>Tx + Thymus grafting c) + PNU</td>
<td>16</td>
<td>14 (88)</td>
<td>12 (217)</td>
<td>2 (170)</td>
</tr>
<tr>
<td>Tx + PNU + Thymus grafting c')</td>
<td>36</td>
<td>12 (33)</td>
<td>1 (234)</td>
<td>11 (254)</td>
</tr>
</tbody>
</table>

a) PNU: PNU treatment for 90 days starting at the age of 40 days.
b) Tx: Female rats were thymectomized at the age of 35 days.
c) Thymus grafting: Normal 1-day-old male thymus was grafted at the age of 38 days (before PNU treatment).
c'): or at the age of 132 days (after PNU treatment) (c').
d) Numbers in parentheses indicate percentage of leukemias.
e) Numbers in parentheses indicate mean latent time (days) of leukemias.
f) Thy-1.1 positive lymphomas (grafted thymus enlarged).
g) Thy-1.1 positive lymphomas (grafted thymus not enlarged).
was found to bear Thy-1.1 but without involvement of the graft. These observations indicate that thymus is absolutely required for PNU-induced T-lymphomagenesis in rats and that exposure of the thymus to PNU may be essential.

Role of the Thymus  In our standard protocol, F344 rats were exposed to PNU for 90 days to ensure a high incidence and short latent period. However, as shown in Table II, exposure for 42 days was enough for lymphoma induction, although the average latent period of 187 days was much longer than that of 105 days observed with the standard protocol. Day 42 may well be a prelymphomatous stage, since there was no clinically evident T-lymphoma, but T-lymphomas developed without further exposure to PNU. To determine the initial site of appearance of early lymphomatous cells and to establish their properties, we further examined lymphoid tissues at this stage.

As shown in Table II, thymectomy carried out 3 days after termination of PNU administration markedly reduced the incidence of T-lymphomas. Grafting of the thymus from 42-day PNU-exposed rats into 20 thymectomized recipients produced 7 lymphomas all involving the graft and bearing Thy-1.1. These results may indicate that the thymus at this stage either contains transformed cells or undergoes certain modulations to support early transformants arising in the thymus or migrating from elsewhere. To test the first hypothesis, we injected normal syngeneic rats intrathymically or intravenously with lymphoid cells from PNU-exposed donor rats as summarized in Table III. With 35-day PNU-exposed donors, out of 10 rats injected intrathymically with thymocytes, two developed T-lymphomas of donor origin with longer latency, whereas none of 24 rats receiving either spleen or bone marrow cells developed T-

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**Table II. Development of Leukemias in Fischer 344 Rats Treated with PNU for a Period of 42 Days**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of rats</th>
<th>All leukemias</th>
<th>Thymic lymphomas</th>
<th>Other leukemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNU</td>
<td>18</td>
<td>15 (83)b</td>
<td>15 (187)c</td>
<td>0 (—)c</td>
</tr>
<tr>
<td>PNU + Txae</td>
<td>16</td>
<td>6 (38)</td>
<td>2d (268)</td>
<td>4 (231)</td>
</tr>
</tbody>
</table>

| a) Tx: Thymectomy was performed at the age of 85 days, namely 3 days after PNU administration had been completed.  
| b) Numbers in parentheses indicate percentage of leukemias.  
| c) Numbers in parentheses indicate mean latent time (days) of leukemias.  
| d) Thy-1.1 positive lymphomas. |

**Table III. Potentially Lymphomatous Cells Demonstrated by Transplantation Bioassay in the Thymus of 42-Day PNU-exposed Rats**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Exposure to PNU (days)</th>
<th>Route of transfera</th>
<th>T-lymphomas/No. of recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thymusb</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>it</td>
<td>1/6 (212)c</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>it</td>
<td>1/4 (183)</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>it</td>
<td>4/4 (140)</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>it</td>
<td>3/4 (167)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv</td>
<td>0/2</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>it</td>
<td>5/5 (107)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iv</td>
<td>1/5 (147)</td>
</tr>
<tr>
<td>6d</td>
<td>42</td>
<td>iv</td>
<td>2/12 (322)</td>
</tr>
</tbody>
</table>

| a) it, intrathymic; iv, intravenous.  
| b) Cell dose: 2-6 × 10⁶ thymus cells, 10-30 × 10⁶ spleen cells or 3-10 × 10⁶ bone marrow cells were injected.  
| c) Average latent time (days).  
| d) Hosts were irradiated before cell transfer: 700 R for recipients of thymus cells and 900 R for recipients of spleen and bone marrow cells. |
lymphoma (exp. 1 and exp. 2). In contrast, the intrathymic transfer of thymocytes from 42-day PNU-exposed donors effectively produced T-lymphomas (exp. 3, 4 and 5). However, intravenous transfer of cells from the same source was much less effective (exp. 4 and exp. 5). Transfer of spleen cells or bone marrow cells did not produce any leukemias, irrespective of the route of injection. In exp. 6, the recipients were sublethally irradiated and were injected intravenously with PNU-exposed donor cells. Two out of 12 recipients of thymocytes developed T-lymphomas with longer latency, but none of 28 rats receiving either spleen or bone marrow cells did. In this study, however, the effect of host conditioning was not studied systematically. Transplantation of fully developed lymphoma cells invariably killed the recipients within 3 weeks irrespective of route and host conditioning. These observations indicate that potentially lymphomatous cells appear first in the thymus and are somehow dependent on thymus for further progression. In these experiments, the second hypothesis that PNU modulates the thymus to support transformants migrating from elsewhere was not excluded. However, frequent development of lymphomas in normal recipients of the PNU-exposed thymocytes suggests that such modulation of the thymus, if any, may not be essential for lymphomagenesis.

**Histological Changes of the Thymus during Lymphomagenesis** In order to delineate early changes taking place in possible target organs, a sequential histological study was carried out. Continuous oral administration of PNU induced a two-phase change in the weight of thymus (Fig. 1). Immediately after the start of PNU, the relative thymus weight (thymus weight (mg)/body weight (g)) progressively decreased until the 35th–42nd day. Thereafter the thymus weight recovered gradually and the first histologically evident lymphoma was noted at the 77th day. The earliest change in the thymus, observed as early as 7 days after the start of PNU, was progressive depletion of lymphocytes, resulting in thinning of the cortex. After the 10th day, however, regeneration of the cortex occurred by repopulation of lymphocytes with various degrees of maturation. Foci of atypical cells (Fig. 2) were first observed in the deep cortex of thymus at the 28th day. Such foci had ill-defined margins and were composed of relatively high-density clusters of larger lymphocytes with cellular atypism. Thereafter, the number and size of foci gradually increased and they tended to fuse with each other. Emergence of atypical foci was most pronounced when the relative thymus weight was the smallest at 35–42 days after the start of PNU. At this stage, both bone marrow and spleen were highly hypoplastic and free from morphologically atypical cells. Sequential histological changes may reflect the behavior of potentially lymphomatous cells detected by the transplantation bioassay.

**Origin of the Lymphomas Induced in Thymectomized, Neonatal Thymus-grafted Rats** The origin of lymphoma cells in 14 female rats that had been thymectomized, grafted with neonatal male thymus, and treated with PNU (Table I) was determined by sex chromosome analysis. As shown in Fig. 3, out of 12 T-lymphomas, 5 that developed in the early stage were of donor origin (male type), 2 that developed...
in the late stage were of host origin (female type), and 5 that developed in the intermediate stage were of mixed origin. Two erythroblastic leukemias were of host origin. In this experiment, all leukemia cells recovered from either thymus, spleen, or bone marrow were of the same origin in each case.

**Other Types of Tumors and Leukemias**

As shown in Tables I and II, thymectomized rats developed several forms of leukemias other than T-lymphomas. They were 5 erythroblastic and 2 myelogenous leukemias in 22 thymectomized, PNU-treated rats; 2 erythroblastic leukemias in 16 thymectomized, thymus-grafted, PNU-treated rats; and 5 erythroblastic and 6 myelogenous leukemias in 36 thymectomized, PNU-treated, thymus-grafted rats. There were a few scattered cases of lymphomas with Thy-1.1 induced in thymectomized rats. In rats, unlike mice, Thy-1.1 is expressed in a broader range of hemolymphatic cells, so that their exact origin remains obscure.

Various non-hematopoietic neoplasms were also observed. Of 77 non-leukemic neoplasms that developed in 74 thymectomized female rats, 48 arose in the alimentary tract and 29 in other sites, including 9 hepatomas, 2 hemangioendotheliomas of the liver, 9 mammary tumors, 2 ovarian tumors, 6 ear duct carcinomas, and 1 nephroblastoma. They occurred with much longer latency (mean survival time, 282 days), and were frequently accompanied with leukemias.

**DISCUSSION**

F344 rats exhibit genetically determined susceptibility to thymic lymphomagenesis by PNU. The present study confirmed and extended the observations accumulated on both spontaneous and induced T-lym-
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in mice, i.e., that the thymus is the essential determinant of the type of leukemias. Thymectomy carried out either before or after PNU administration virtually abolished the occurrence of lymphomas with T-cell phenotypes, and grafting of neonatal thymus prior to PNU administration restored this susceptibility.

The origin of T-lymphoma cells has been controversial. In the mouse, several investigators showed that potentially lymphomatous cells first appear among bone marrow cells spontaneously in young AKR mice or after exposure to leukemogenic virus or X-rays. They postulated that the initial transformants occur among bone marrow prothymocytes and subsequently migrate to the thymus. Hays and Kato defined prelymphoma cells as cells giving rise to donor origin thymic lymphomas when injected into 400 R-irradiated recipients after a longer latent period. In the case of transplantation of fully developed lymphoma cells, lymphomas develop with much shorter latency, usually without involvement of the thymus, and conditioning of the recipients is not required. Although the present study does not exclude the possibility that initiation could occur externally, the thymus is the site at which potentially lymphomatous cells first appear as a result of exposure to PNU. Administration for as short a time as 42 days is enough for such cells to be induced. At this stage, focal proliferation of atypical lymphocytes becomes increasingly manifest in the regenerating thymus but evident lymphomas appear much later. Similar chronological changes in the thymus were reported by Ogiu et al. The potentially lymphomatous cells are localized in the thymus, and in the transplantation bioassay they exhibit special preference for the intrathymic route for further progression. In contrast to the prelymphoma cells defined in mice, conditioning of the host seems not to be essential for progression to frank lymphomas in the secondary hosts. However, it is still possible that transformants at earlier stages could be detected either in the thymus or elsewhere by careful manipulations of recipients. Therefore, we prefer the term early or potentially lymphomatous cells instead of prelymphoma cells to denote the transformed cells in the thymus of 42-day PNU-exposed rats.

Exposure of the thymus to PNU seems to be essential for lymphogenesis. Thymectomized rats exposed to PNU for 90 days and subsequently grafted with neonatal thymus rarely developed T-lymphomas. This seems inconsistent with the hypothesis that the transformants arise outside the thymus and migrate into the thymus in the later stage of lymphogenesis. In this study we did not explore the possibility that PNU, in addition to a direct transforming effect, may modify the thymic microenvironments somehow to promote lymphogenesis. However, the fact that the thymus cells from 42-day PNU-treated donors could produce T-lymphomas in the normal thymus of secondary hosts may argue against this possibility.

Thymectomized rats with a neonatal thymus graft developed lymphomas involving the graft on PNU administration. The origin of the lymphomas varied depending on the latent period. The lymphomas developing earlier were of donor origin, but later, lymphomas of host or mixed origin arose. Occurrence of lymphomas of donor origin indicates that the transforming events in these cases took place within the thymus. Our preliminary data show that a thymus graft is almost totally replaced by host cells as early as 21 days after grafting. It is conceivable the late-occurring lymphomas of host origin may arise from the host cells that have immigrated to the thymus. The heterogeneity of the origin of lymphomas observed here may well be related to the stage of repopulation of the grafts by the host cells and the timing of lymphomagenic action of PNU on intrathymic target cells.

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