Direct Action of a Leukemogenic Virus on the Thymus

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Summary

When a leukemogenic virus (radiation leukemia virus, RLV), extracted from radiogenic lymphomas of strain C57BL mice, was directly injected either into 1 lobe of the thymus in situ in newborn C57BL mice, or into intrarenal C57BL thymic grafts in thymectomized, irradiated C57BL or F1 hybrid hosts, a high incidence of lymphoid tumors developed after an unusually short latent period. No such tumors resulted when RLV was injected into intrarenal grafts of spleen or lymph node in similarly prepared hosts. In the neonatally inoculated animals, tumors originated invariably in the injected lobe, indicating that there is a direct neoplastic interaction between RLV and target cells in the thymus. Histologic examination and transplantation assays revealed that "transformed" cells with neoplastic potentialities are present in the thymus grafts as early as 1 week after virus inoculation. Virus injection into graft-holding hybrid hosts elicited a high proportion of tumors that exhibited the transplantation behavior characteristic of donor cell derivation. Data on other parameters, such as the route of virus injection, the interval between graft placement and virus injection, and the requirement for systemic or partial-body irradiation are also presented.

Materials and Methods

MICE. The mice used were of the inbred strains C57BL/Ka and AKR and F1 hybrids of C57BL/Ka × AKR, or BLK; AKR × C57BL/Ka, or KBL; and C57BL/Ka × BALB/c, or BLA, constitution. They were housed in plastic cages at constant room temperature and maintained on Purina Laboratory Chow and water. All dead mice were autopsied routinely; unless lymphoid tumor could be diagnosed unequivocally, the relevant tissues were examined histologically.

X-IRRADIATION. The mice were thymectomized when 33 ± 3 days old and 1 week later started on a course of 4 weekly whole-body X-ray exposures of 168 r each. The physical factors of irradiation were 250 kv, 1.0 mm Al + 0.25 mm Cu filter, 83 r/min, and 65 cm distance.

THYMUS GRAFTS. Strain C57BL and KBL hybrid mice were thymectomized, irradiated, and implanted with a C57BL thymus graft under the renal capsule; 8 days later, they received

EXPERIMENT 1: INOCULATION OF RLV INTO THE THYMUS IN SITU. The thymus of C57BL/Ka mice, aged 24 hr or less, was exposed by the technic described by Miller for neonatal thymectomy (10). A very small volume, less than 0.01 ml, of RLV was injected into the right thymic lobe, using a 30-gauge needle; it was impossible to avoid some leakage of the inoculated material. The opening in the chest wall was sutured, and the babies were caged with BALB/c foster mothers (to avoid cannibalism by their own mothers) until old enough to wean.

EXPERIMENT 2: INOCULATION OF RLV INTO INTRARENAL THYMUS GRAFTS. Strain C57BL and KBL hybrid mice were thymectomized, irradiated, and implanted with a C57BL thymus graft under the renal capsule; 8 days later, they received

Results

EXPERIMENT 1: INOCULATION OF RLV INTO THE THYMUS IN SITU. After neonatal intrathymic inoculation of RLV, the injected mice were sacrificed at 10-day intervals, and their thymus glands were removed for histologic examination. The earliest changes were noted at about 20 days; they consisted of small foci of large, pale immature lymphoid cells with abundant mitoses, in the cortical area of the injected lobe (Fig. 1). These microtumors increased gradually in size and invasiveness; between 30 and 40 days, the entire lobe became involved, and invasion of the uninjected lobe was seen in a few instances (Fig. 2). By 50 days, about 1/3 of the uninjected lobes had been invaded (Table 1). Control mice which had received an inoculation of saline instead of RLV were negative at all time intervals studied.

EXPERIMENT 2: INOCULATION OF RLV INTO INTRARENAL THYMUS GRAFTS. Strain C57BL and KBL hybrid mice were thymectomized, irradiated, and implanted with a C57BL thymus graft under the renal capsule; 8 days later, they received

1 This investigation has been supported by Research Grant CA 03352 from the National Cancer Institute, USPHS. Preliminary reports of this work were presented at the annual meetings of the American Association for Cancer Research in Chicago, Illinois, April, 1964, and Philadelphia, Pennsylvania, April, 1965.

2 Eleanor Roosevelt Foundation Fellow, 1963-1964, on leave from the Department of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel.
an injection of RLV directly into the graft. Control groups were similarly treated, except for the omission of irradiation or RLV injection. Some of the tumors arising in KBL hosts were transplanted intrarrenally to KBL and C57BL mice to determine whether their cells were of host or donor origin.

The results are summarized in Table 2. When RLV inoculation was omitted, lymphoid tumor incidence was only about 20%, and the average latent period was about 300 days, similar to the findings of Law et al. (4). Direct injection of RLV into the thymic grafts yielded tumors in 87% of KBL and 83% of C57BL hosts, with an average latent period of only 91-93 days. The same RLV preparation, checked for potency by i.p. inoculation into neonatal C57BL hosts, produced lymphomas in only 41%. Of particular interest were the results of the transplantation assay. Of 12 RLV-induced tumors tested, 9 were of donor (C57BL) genotype, whereas 7 tested tumors arising in the absence of RLV inoculation were all of host (KBL) origin.

Omitting irradiation reduced the frequency of lymphoid tumors in C57BL mice by nearly half and lengthened the median latent period greatly; in KBL mice, the diminution of response was even more striking (Table 2). Irradiation of C57BL mice with the thighs shielded (protecting the bone marrow) gave tumor yields as good as those obtained with whole-body irradiation. This result is in accord with our earlier observations concerning synergism between RLV and irradiation (6).

TABLE 1

<table>
<thead>
<tr>
<th>INTERVAL TO SACRIFICE (days)</th>
<th>NO. OF MICE WITH MICROTUMORS</th>
<th>TOTAL NO. OF MICE WITH LYMPHOMAS/NET NO. OF MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in 1 lobe</td>
<td>in both lobes</td>
</tr>
<tr>
<td>1-10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11-20</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>21-30</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>31-40</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>41-50</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>51-60</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

The interposition of a 30-day interval between irradiation and thymus grafting did not significantly modify the tumor response (Table 3). Similarly, varying the interval between thymus grafting and direct RLV injection from 0 to 20 days did not affect tumor incidence and only minimally prolonged the latent period. It may be noted that, in the latter groups, thymectomy of the C57BL host mice was omitted, without significantly altering tumor response. That such tumors originated from the intrarenal grafts rather than the thymus in situ is indicated both by their short latent period and by the fact that they consistently replaced the grafts, whereas the thymus in situ was seldom histologically involved.

To determine whether grafts of other lymphoid organs could substitute for the thymus, spleens and mesenteric lymph nodes were removed from C57BL mice aged 1-2 days and grafted intrarrenally into thymectomized, irradiated adult isogenic hosts; simultaneous controls received thymus grafts from the same donors. All were inoculated 8 days later with RLV. The lymph nodes did not grow well, and only a scar was seen when the graft site on the kidney was inspected several weeks later. The spleens and thymus grew. The only animals which became leukemic, however, were those with thymus implants; all of the 13 mice in this group developed tumors, with an average latent period of 63 days, whereas no lymphomas developed in mice with either node or spleen grafts.

**EXPERIMENT 3: ROUTE OF RLV INOCULATION.** C57BL mice were thymectomized, irradiated, and then implanted intrarrenally with a newborn C57BL thymus. They were then divided into 3 groups: in the 1st group, approximately 0.01-0.02 ml (the maximal retained volume) of RLV was injected directly into the thymic graft; in the 2nd, 0.2 ml of RLV was injected i.v.; and in the 3rd, 0.2 ml of RLV was injected i.p.

Injecting RLV i.v. instead of directly into the thymus graft made no significant difference in tumor incidence or latent period (Table 4). After i.p. injection, tumor incidence ultimately attained comparable levels, but required a much greater latent period. It must be noted, however, that the amounts of virus injected were not the same; perhaps if corresponding serial dilutions of RLV were injected via these 3 routes, the respective...
end-point dilutions might afford more valid and sensitive comparative indices.

**Experiment 4: Rate of Neoplastic Transformation in RLV-Injected Intrarenal Thymus Grafts.** C57BL mice were thymectomized, irradiated, and received an isologous intrarenal thymus graft. Nine days later, RLV was inoculated directly into the graft. At weekly intervals thereafter, groups of mice were biopsied. Approximately 1–4 of each graft was excised, the remainder being left undisturbed and subsequently observed for tumor development. A portion of the biopsy was fixed for histologic examination; the rest was reimplanted, as a bioassay for transplantability, under the kidney capsule of an untreated adult C57BL host.

Microtumors could be recognized histologically in biopsies taken as early as 1 week after virus inoculation; they gradually increased in number, extent, and invasiveness (Figs. 3, 4). The re-implanted thymic tissue grew well in the secondary hosts, and even biopsies taken only 1 week after virus inoculation became neoplastic. There was a good general correlation between these responses and tumor evolution in the original intrarenal graft (Table 5). It is concluded that potentially neoplastic cells apparently develop in the graft in as little as 1 week after inoculation of RLV. The possibility of virus being present in the graft and being carried over into the new host with the bioassay cannot be denied. However, the minute quantities of virus which might be transferred in this way would not, in our experience, induce lymphomas in adult untreated hosts with the frequency observed here. This point is now under further investigation;

**TABLE 3**

**Interval Between Irradiation and Grafting, and Between Grafting and RLV Inoculation, and Lymphoid Tumor Response in C57BL Mice**

<table>
<thead>
<tr>
<th>Treatment of Host</th>
<th>Thymectomy</th>
<th>Irradiation</th>
<th>Interval to thymus graft (days)</th>
<th>Interval to RLV injection (days)</th>
<th>No. Lymphomas/No. mice</th>
<th>Lymphoma incidence (%)</th>
<th>Av. latent period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ +</td>
<td>0</td>
<td>7-9</td>
<td>29/35</td>
<td>83</td>
<td>91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>30</td>
<td>7-9</td>
<td>22/26</td>
<td>85</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>0</td>
<td>0</td>
<td>16/16</td>
<td>83</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>0</td>
<td>7-9</td>
<td>14/15</td>
<td>93</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>0</td>
<td>20</td>
<td>15/15</td>
<td>100</td>
<td>115</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 4**

**Route of RLV Inoculation and Lymphoid Tumor Development in Intrarenal Thymic Grafts**

<table>
<thead>
<tr>
<th>RLV injection</th>
<th>No. Lymphomas/No. mice</th>
<th>Lymphoma incidence (%)</th>
<th>Av. latent period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.r.*</td>
<td>0.01–0.02</td>
<td>29/36</td>
<td>81</td>
</tr>
<tr>
<td>i.v.</td>
<td>0.2</td>
<td>24/27</td>
<td>89</td>
</tr>
<tr>
<td>i.p.*</td>
<td>0.2</td>
<td>19/28</td>
<td>68</td>
</tr>
</tbody>
</table>

* i.r., inoculation directly into intrarenal C57BL thymus graft.  
* All recipients were thymectomized, irradiated C57BL mice.

Hybrid animals are being used as recipients of the C57BL graft biopsies, and tumors arising in these recipients will be transplantation tested to determine whether they are of donor (grafted potential tumor cell) or host (contaminating virus) origin.

**Discussion**

These experiments indicate that RLV elicits its tumorigenic response by a local and direct interaction with cells in the thymus. It is apparent, from both histologic and transplantation assay evidence, that "transformed" cells with neoplastic potencies are present in the inoculated thymus as early as 1 week after virus injection. The rapid initiation of the neoplastic process may account for the apparent preponderance of donor-over host-type tumors observed; the transformed donor thymocytes, by retaining their reproductive potential, may prevent the entry and proliferation in the graft of host cells. This problem is currently under more detailed investigation.

The striking increase in tumor incidence and reduction in latent period which followed the direct injection of RLV into the thymus in situ or intrarenally supports the view that RLV from endogenous sources or injected i.p. is not efficiently delivered to the thymus. Perhaps significant amounts of virus are lost in the glomerular filtrate before reaching the intrarenal thymic grafts, which are supplied by the distal (efferent) arterioles of the tandem renal circulation. In any case, direct intrathymic injection appears to be the procedure of choice for the testing of tissue and plasma extracts for possible leukemogenic (viral) activity. Injection i.v. is almost as effective as direct inoculation of intrarenal thymic grafts, perhaps because the bolus of injected material is less efficiently cleared of virus at the glomerulus.

The special susceptibility of thymocytes to virus action is brought out by the inability of another lymphoid organ, the spleen, to support the development of lymphoid tumors under the same experimental conditions. The co-leukemogenic action of X-irradiation in virus-injected, thymus-grafted animals is quite evident in these experiments. However, radiation effects mediated by bone marrow injury are apparently not involved, since thigh shielding does not alter the response. The same interaction between irradiation and RLV-induced tumor incidence was observed (6) when virus was inoculated i.p. in nonthymectomized C57BL hosts. The possibility that irradiation augments susceptibility to RLV by transiently depressing immunologic responsiveness is now under investigation.
Acknowledgments

The authors wish to thank Miss Irene Sun and Mrs. Judith Hueter for technical assistance.

References

Fig. 1. C57BL thymus 5 weeks after direct neonatal inoculation with RLV. Note cortical microtumor (lower right) with immature cells and abundant mitoses. H & E, × 170.

Fig. 2. C57BL thymus 7 weeks after direct inoculation with RLV. The un.injected (right) lobe is normal, whereas the injected lobe is replaced by an invasive lymphoma. H & E, × 170.

Fig. 3. Intrarenal C57BL thymus graft, 4 weeks after direct inoculation with RLV. The normal cortical-medullary boundary is largely obliterated, and invasion through the capsule has already occurred near the tip of the graft. H & E, × 40.

Fig. 4. Higher magnification of same graft as in Fig. 3, to show infiltration by immature tumor cells with abundant mitoses. H & E, × 400.
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