The Effects of Allografts of Thymic Epithelial Reticular Cells on the Lymphoid Tissues of Neonatally Thymectomized Mice

By Esther Finch Hays

Neonatal thymectomy in the mouse results in a profound depletion of lymphoid tissue and a peripheral blood lymphopenia, as well as an impairment of the animal’s immunologic capacity. Restoration to normal lymphoid histology and immunologic function can be accomplished in several ways. Thymic grafts placed subcutaneously within the first seven days of life have proved effective. Massive inocula of thymus cells have been shown to restore these animals. However, reconstitution is more readily accomplished with inocula of viable spleen cells. Osoba and Miller have shown that an immunologic recovery can be accomplished when neonatally thymectomized mice are implanted at 7 days of age with diffusion chambers containing newborn and embryonic thymus. The histologic recovery in many of these animals was incomplete. The tissue in the chambers was shown by these authors to be composed of epithelial reticular cells only when the animals were sacrificed at 10 weeks of age. These studies were interpreted as evidence that a humoral factor liberated by these cells was responsible for endowing the host lymphoid cells with immunologic competence.

A lymphostimulatory effect of thymus epithelial reticular cells has been demonstrated in the work of Gregoire and Duchateau, who found that inoculation of these cells, or extracts prepared from them, produced hyperplastic alterations of the draining lymph nodes which were in their opinion significantly greater than those produced by administration of lymphatic tissue.

In the studies reported herein, a single neonatal AKR thymus was sealed in a diffusion chamber which was then implanted intraperitoneally in an adult mouse and removed after 8 days. This resulted in a remnant which had lost the cortical cells and which was composed principally of epithelial reticular cells. This remnant was removed from the chamber and placed subcutaneously into 4 to 6 day old neonatally thymectomized C3HHeB mice. Observations were made of the histology of the thymic grafts, and the
capability of these thymic remnants to reconstitute the lymphoid tissue and immune function of these animals was studied.

**Materials and Methods**

**Animals.** Mice from strains C3HeB/Fe, AKR and (AKR × T6) F1 hybrid were used. They were given water and Wayne mouse breeder pellets ad libitum throughout the experiments. Because of a high incidence of infant diarrhea in the C3HeB/Fe colony, all of the females of this strain were placed on drinking water with added tetracycline as soon as they were seen to be pregnant. All animals were maintained on this drinking water throughout the experiment.

**Thymectomy.** The animals were thymectomized within 24 hours of birth. Ether anesthesia was used and the thymic lobes were removed by gentle suction. The completeness of thymectomy was checked at the time of autopsy by gross and histologic examination of the mediastinal tissues.

**Diffusion Chambers.** In order to obtain thymic epithelial reticular cells in the absence of cortical cells, the following procedure was carried out: Diffusion chambers were constructed using lucite rings sealed on either side with Millipore membranes 0.45μm pore size. The two thymuses were removed aseptically from newborn AKR mice and placed in Hank’s balanced salt solution (BSS) with added penicillin and streptomycin (100 μg/ml). Single organs were placed in the chamber with several drops of Hank’s solution. The chambers were then sealed with acetone, moistened with Hank’s solution, and implanted intraperitoneally, two per animal, in adult C3HeB mice. They were removed after 8 days and placed in Hank’s BSS with 100 μg/ml of penicillin and streptomycin. The membranes were cut away from the rings and the tiny visible remnant was trimmed but left attached to the graft, and it was then implanted subcutaneously into the neonatally thymectomized C3HeB/Fe mice of 4 to 6 days of age. Neonatally thymectomized littermate control animals were grafted subcutaneously at the same age with pieces of Millipore membrane of comparable size. Fluothane anesthesia was used for the grafting procedures.

**Sacrifice.** The animals were sacrificed using ether at intervals after birth. The blood for hematocrit, white cell, and differential counts was taken from the still-living anesthetized mice by section of the axillary blood vessels. The gross appearance of the organs was noted and the spleen and kidneys were weighed. The liver, spleen, axillary lymph nodes, mesenteric lymph node, Peyer’s patch, and mediastinal tissue were removed and fixed in Bouin’s fluid. Hematoxalin and eosin sections cut at 6μ were prepared and examined. The bone marrow was obtained by pressing a 24-gauge hypodermic needle through the shaft of the femur and placing the marrow plug on a cover slip with one drop of serum. The marrow particle was smeared, air dried, and stained with Wright’s stain. Differential counts were performed on these preparations.

**Skin Grafts.** The technic of skin grafting was that of Billingham and Medawar.7 Grafts of adult A/J skin from like sexes were placed on the right posterior thorax of adult mice which had been neonatally thymectomized and grafted with thymus remnants. Intact adult animals of the same strain were used as controls.

**Leukemic Cell Grafts.** Cell suspensions were prepared in Hank’s BSS from spleen and lymph nodes of leukemic mice. The suspensions were made by teasing the organs with a forceps. The suspensions were inoculated intraperitoneally in 0.2 cc. volumes, each inoculum containing approximately 2 × 10⁶ viable cells.

**Cytology.** The thymuses of (AKR × T6) F1 hybrid mice were implanted in intraperitoneal diffusion chambers for one week and used for grafts in neonatally thymectomized C3H mice. The animals were sacrificed at intervals and the grafts as well as the spleens of these animals were studied for the presence of the T6 marker chromosome using the following procedure: The animals were given intraperitoneal inoculations of 0.02 ml. colchicine per gram of body weight. One hour later cell suspensions from the spleen and thymus graft were prepared and incubated in 0.97 M sodium citrate for 20 minutes at room temperature, centrifuged very gently, fixed repeatedly in freshly made Carnoy’s fluid for several
ALLOGRAFTS OF THYMIC EPITHELIAL RETICULAR CELLS

Fig. 1.—Typical appearance of an AKR newborn thymus after 8 days in a diffusion chamber. Note loss of cortical cells and a persistence of plump viable epithelial reticular cells.

minutes. The cells were finally spread by an air-drying procedure and stained with Giemsa stain. The metaphases were counted under the phase contrast microscope.

Results

Appearance of the Fragments at the Time of Grafting

Twenty-two groups of animals were grafted with diffusion chamber remnants of neonatal AKR thymus. A representative graft from each group was examined histologically. All 22 of them were found to contain plump viable epithelial reticular cells (Fig. 1). Fibroblasts were also seen, surrounding the epithelial reticular (E-R) cells of 8 remnants; foci of pyknotic nuclei were seen in 11, and such an area is illustrated in Figure 2. Necrotic central portions were seen in nine of the remnants, and viable medullary cells were found at their periphery. In two instances a few cells which appeared to be viable cortical lymphoid cells were seen. These remnants did not resemble the intact thymus in any way. The normal mouse thymus during the first week of life has a predominance of cortical lymphoid cells with many large immature lymphoid cells in the subcapsular zone of the organ. Several of these remnants when placed in culture in glass petri dishes with N-16 medium with 15 per cent fetal calf serum, attached to the glass and grew slowly, spreading out in a syncitium from the attached cells. The cells of these cultures were large with abundant cytoplasm and 1 to 2 nucleoli in the nucleus.

Observations of Grafted and Control Mice

The histologic observations made on the remnant-grafted animals are summarized in Figure 3. The grafted animals sacrificed during the third
Fig. 2.—AKR newborn thymus after 8 days in diffusion chamber showing pyknotic nuclei believed to represent degenerating cortical cells.

Fig. 3.—Chart summarizing the histologic observations of the thymectomized remnant-grafted mice. The right side of the figure presents the observations of lymphoid tissue exclusive of that in the bone marrow, and the left side of the figure presents the observations made on the thymus remnant grafts.

week of life showed grafts composed of epithelial reticular cells only. One graft resembled a normal thymus but there was no evidence of lymphoid reconstitution in the host. The histologic appearance of the host lymphoid organs was identical to that of the thymectomized controls, both showing depletion of lymphoid cells in the spleen, lymph nodes, and Peyer's patches.

At 4 weeks of age the grafted animals still showed depleted lymphoid tissues and the grafts were varied in appearance. Some showed epithelial reticular cells and foci of myelopoiesis and others showed lymphocytes
Fig. 4.—Histologic appearance of a graft of a 44 day old C3HeB mouse showing diffuse infiltration of lymphoid cells surrounding the plump medullary cells. The lymphoid tissue of this animal showed some repletion of lymphocytes.

scattered among the epithelial reticular cells. These lymphocytes were smaller with more dense nuclei than the typical lymphocyte. No erythroid cells could be seen, although admittedly the differentiation between these cells and small lymphocytes is difficult.

At 5 weeks, one grafted animal demonstrated partial lymphoid reconstitution of a lymph node. Lymphoid depletion was present in the other animals of this group. The grafts at 5 weeks showed epithelial reticular cells with scattered small lymphocytes with dense nuclei and foci of myelopoiesis were present in some.

The 6-week-old mice showed the first consistent evidence of lymphoid reconstitution in their nodes and spleens. The grafts showed epithelial reticular cells and lymphocytes, and two of them had the histologic appearance of a normal thymus with a predominance of actively mitotic thymic cortical cells. The appearance of a 6-week graft is illustrated in Figure 4. These cells were larger and the nucleus was less dense than that of the "lymphocytes" described in the grafts from animals at 4 to 5 weeks of age. The lymphoid depletion in spleen, lymph node, and Peyer's patch of the thymectomized controls was marked at 6 weeks.

There was evidence of lymphoid reconstitution in all animals of 8 to 12 weeks of age, and two of them were completely normal histologically. One graft was composed of medullary cells only and the host showed partial lymphoid reconstitution. Five of the grafts showed epithelial reticular cells and lymphocytes. Another graft resembled a normal thymus and the microscopic appearance of the host lymphatic tissue was normal.
Fig. 5.—Mean absolute lymphocyte counts of the peripheral blood of thymectomized remnant-grafted, thymectomized, and normal C3HeB mice.

After 12 weeks of age the grafted animals demonstrated normal lymphoid tissue. All of their grafts resembled a normal thymus. They were also of comparable weight and general health to intact animals of the same age. The few control animals that survived to this period were small, with poor hair growth. They were sacrificed when moribund at 12 weeks. Their lymphoid tissue was characterized by the striking depletion of lymphocytes previously described in neonatally thymectomized mice.2

The body weight of the grafted mice remained below normal until 8 to 12 weeks of age, whereas the thymectomized littermate controls never attained normal body weight.

The mean peripheral blood lymphocyte counts of the thymectomized and grafted mice are compared with those of normal mice in Figure 5. There was persistent lymphopenia in both grafted animals up to 8 weeks of age. The absolute lymphocyte count rose in the grafted mice after eight weeks but did not reach normal levels. Table 1 presents the data regarding lymphocyte counts in a more detailed form.

Observations of the bone marrow lymphocytes in these animals were of considerable interest and are summarized in Table 2. Normal C3H mice had a mean bone marrow lymphocyte percentage of 28 with a range of 18 to 42 per cent. These figures apply to animals from 1 to 8 weeks of age as well as to adults. At 2 to 3 weeks of age the bone marrow lymphocytes of six thymectomized mice were within the normal range, and those of three of five remnant-grafted mice of this age were also normal. At 3 to 5 weeks
Table 1.—Summary of Data Obtained on Peripheral Blood Lymphocytes in Thymectomized, Thymectomized and Fragment Grafted, and Normal C3HeB Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age in Weeks</th>
<th>Lymphocytes (Cells/mm.³) Mean-Range</th>
<th>Lymphocytes (%) Mean-Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymectomy</td>
<td>3-5</td>
<td>629 (133-1222)</td>
<td>38 (12-70)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>3-5</td>
<td>1149 (245-4175)</td>
<td>33 (10-48)</td>
</tr>
<tr>
<td>Normal</td>
<td>3-5</td>
<td>1484 (460-2052)</td>
<td>59 (44-72)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>6-8</td>
<td>705 (108-1938)</td>
<td>20 (4-40)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>6-8</td>
<td>872 (370-1976)</td>
<td>22 (4-38)</td>
</tr>
<tr>
<td>Normal</td>
<td>6-8</td>
<td>2568 (1252-5438)</td>
<td>58 (44-76)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>10-12</td>
<td>731 (112-1368)</td>
<td>24 (12-40)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>10-12</td>
<td>2763 (780-3050)</td>
<td>41 (12-78)</td>
</tr>
<tr>
<td>Normal</td>
<td>10-12</td>
<td>5119 (3876-7254)</td>
<td>73 (68-78)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>12+</td>
<td>535 (150-966)</td>
<td>26 (6-46)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>12+</td>
<td>2488 (1190-5016)</td>
<td>61 (34-78)</td>
</tr>
<tr>
<td>Normal</td>
<td>12+</td>
<td>5029 (4134-6204)</td>
<td>70 (62-78)</td>
</tr>
</tbody>
</table>

Table 2.—Bone Marrow Lymphocyte Percentages in Individual Thymectomized and Remnant Grafted Mice at Different Ages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age in Weeks</th>
<th>Percentage Lymphocytes</th>
<th>(Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymectomy</td>
<td>2-3</td>
<td>42, 40, 40, 34, 22, 21</td>
<td>(33)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>2-3</td>
<td>42, 31, 23, 12, 9</td>
<td>(23)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>3-5</td>
<td>20, 17, 16, 11, 11, 12</td>
<td>(13)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>3-5</td>
<td>39, 23, 22, 18, 13, 11, 9</td>
<td>(19)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>5-8</td>
<td>13, 12, 6, 5, 5, 3</td>
<td>(7)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>5-8</td>
<td>21, 18, 12, 11, 10, 8, 6, 6</td>
<td>(12)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>8-12</td>
<td>10, 6, 5, 4, 3</td>
<td>(6)</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>8-12</td>
<td>32, 22, 16, 14, 11</td>
<td>(19)</td>
</tr>
<tr>
<td>Thymectomy</td>
<td>12+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Thymectomy &amp; Graft</td>
<td>12+</td>
<td>34, 20, 26, 25, 22, 20, 20, 16</td>
<td>(24)</td>
</tr>
</tbody>
</table>

three of the thymectomized animals were within normal range and three below, and three of seven of the remnant-grafted animals had a marrow lymphopenia. At 5 to 8 weeks the bone marrow lymphocytes were low in six thymectomized and in six of eight grafted mice. At 8 to 12 weeks all the thymectomized mice had lymphocytes below the normal range while three of five grafted mice had normal lymphocyte percentages in their marrow. After 12 weeks of age, there were no surviving thymectomized mice but the eight grafted animals demonstrated normal lymphocyte values.

Response of the Grafted Mice to Allografts

To determine if these mice with thymic grafts had the ability to reject allografts in a normal manner, the following study was carried out. Skin from A/Jax (H-2*) mice was grafted to eight thymectomized remnant-
Table 3.—Cytology of Thymus Grafts and Spleens from Neonatally Thymectomized C3H Mice Grafted at 8 Days of Age with Diffusion Chamber Remnants of (AKR \times T6) F1 Thymus

<table>
<thead>
<tr>
<th>Week of Age</th>
<th>No. Mice Examined</th>
<th>Per Cent Metaphases with Donor Cell Characteristics in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Remnant Grafts</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>4,0</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>4, 0</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

*Each figure represents a cytological preparation of an individual animal. At least 40 metaphases were examined in each preparation. — no scorable dividing cells in preparation.

grafted C3HeB mice (H-2*). Eight normal C3HeB mice of the same age were also given A/Jax skin allografts. The animals were 3 to 6 months of age at the time of grafting. The median survival time of the grafts of normal animals was 13 days, with a range of 9 to 17 days; all of the thymectomized remnant-grafted animals rejected their grafts at 12 days.

In a second study, $2 \times 10^6$ lymphoma cells from a CF#1 mouse with a spontaneous disease were inoculated intraperitoneally into 2-month old mice from the following groups: neonatally thymectomized, neonatally thymectomized and grafted with thymic epithelial reticular cell remnants, and intact animals. There were two animals in each group. Four weeks later a lymphoma of CF#1 type developed in one of the neonatally thymectomized mice. The other animals remained healthy and were sacrificed without evidence of leukemia 4 months after the cell inoculation.

In a third study, $2 \times 10^6$ AKR spontaneous lymphoma cells were given to three normal-appearing, 12-week-old C3HeB mice which had been neonatally thymectomized and grafted with AKR thymus epithelial reticular cells during the first week of life, as well as to three intact mice of the same age and strain. These experimental animals all developed generalized lymphoma of AKR type 15 days after inoculation of cells. The animals at autopsy showed gross and microscopic involvement of the spleen, lymph nodes with lymphoma, absence of a thoracic thymus, and well developed thymus grafts which resembled a normal organ and were not infiltrated with lymphoma cells. The intact tumor cell-inoculated C3HeB mice did not develop lymphoma.

**Cytology of the Thymus Grafts**

The results of cytologic investigation of thymus grafts of neonatally thymectomized C3H mice bearing subcutaneous grafts of medullary remnants of (AKR \times T6) F1 hybrid thymus are shown in Table 3. All grafts were removed at a time when the lymphoid repopulation had been shown to be present. They contained a majority of dividing cells of host origin and only a few with the cytologic characteristics of those of the donor of the graft. There were very few donor cells seen in the spleens of these mice. Also when remnant allografts were given to neonatally thymectomized CBA/T6T6 mice, 99 per cent of the dividing cells populating the grafts at 5 weeks bore the markers and thus were of host origin.
The cortical population of the neonatal thymus as shown by these studies is extremely labile. These cells do not withstand the diffusion chamber incubation and disappear after one week. These results suggest that the thymus lymphocyte is a very short-lived cell, or a cell very sensitive to environmental changes. The marked changes produced in the thymus by radiation and cortisone—i.e., a loss of thymus lymphocytes—are compatible with the latter observation. The appearance of the thymus in diffusion chambers after one week in our studies and after 10 weeks in those of Osoba and Miller indicates that the epithelial reticular cells can survive under these conditions and have no capacity to regenerate thymus lymphocytes, and suggests that these cells, in the postnatal animal at least, are not the precursors of the thymus lymphocytes, but merely provide a site for the localization and a stimulus for the proliferation of these cells. The situation in the mouse embryo is somewhat different. Auerbach has shown that epithelial rudiments of embryonic mouse thymus can give rise to the lymphoid tissue. However, Ackerman's studies demonstrate the point that "undifferentiated" epithelial cells undergo two distinct lines of differentiation in the embryonic hamster thymus—i.e., into lymphocytes and into stellate reticular epithelial cells which form the organ parenchyma. The cells of the thymus remnants in the present study presumably are those of the latter group and do not have the capacity for lymphoid differentiation.

The experiments described in this report have also shown that the thymic medullary cell grafts have formed a basis for the reconstruction of a new thymus in the thymectomized host and that this is followed by a lymphoid and immunologic reconstitution of the host animal. Studies using a marker chromosome have demonstrated that cells reconstituting the thymus fragment graft are of host origin. Dukor and Miller have also shown that host cells repopulate the intact thymus graft in neonatally thymectomized mice. Speculation as to the source of these repopulating cells is of interest. Harris and Ford have shown, using chromosome marker technics, that when cells of the bone marrow are injected into lethally irradiated mice they are found in the thymus as well as the bone marrow and lymph nodes. These workers, as well as Gowans and Knight, have also pointed out that lymphoid cells from the nodes and thoracic duct do not enter the thymus in substantial numbers. These observations taken along with those reported herein—i.e., the presence of myelopoiesis in the regenerating thymus and the persistent lymphocytosis in the bone marrow of neonatally thymectomized mice up to 5 weeks of age—suggest that the bone marrow could be a source of the "lymphoid" cells seen to repopulate the medullary grafts in these lymphoid depleted animals. A fall in bone marrow lymphocytes at 5 to 7 weeks, in the grafted mice, a time when lymphoid reconstitution of the thymus graft is occurring, could reflect their utilization for this purpose. Their depletion in the bone marrows of thymectomized mice after 8 weeks suggests that the presence of thymus medullary cells is necessary for their continued proliferation. Support for the concept that these depleted marrows can respond...
to a thymic stimulus is obtained in the work of Cross et al. which demonstrated that bone marrow from 8-week-old mice which had been neonatally thymectomized was able to restore immunologic capacity in lethally irradiated nonthymectomized mice.

These studies provide no direct evidence for release of a humoral factor by the thymic medullary cells. They do suggest that these cells have an effect on lymphoid development of neonatally thymectomized mice that is directly related to them and not to any products of cortical cell breakdown. In the experiments of Osoba and Miller the intact thymus was placed in a Millipore chamber in the neonatally thymectomized host, and products from the degenerating cortical cells could have been released through the pores of the membrane.

Representative thymectomized remnant-grafted mice were found to have a normal capacity to reject skin and tumor allografts. This is evidence to indicate a return of their lymphatic system to functional normality, for it has been demonstrated that neonatally thymectomized, lymphoid-depleted mice lose their ability to reject allografts. The observation that the reconstituted remnant-grafted mice supported the growth of lymphoma cells from mice of the same strain as that of the graft indicates that these grafts were able to induce a state of immunologic tolerance to AKR lymphoma cells. This is of interest in view of the findings of Billingham and Silvers which demonstrated that small numbers of thymus cells were not effective producers of skin homograft tolerance. The results observed here may be attributed to proliferation of the grafted cells to supply the necessary amount of tolerance-conferring antigen, or to an instructional function of the AKR thymus medullary cells to the lymphoid cells entering and leaving the graft, conferring on them the capacity of tolerance for AKR cells. These data also provide indirect evidence for the persistence of AKR medullary cells in the thymus grafts, for a state of tolerance has been shown to be associated with cellular chimerism.

The thymus epithelial reticular cell has been shown by these studies to promote immunologic and lymphatic tissue normality in neonatally thymectomized hosts without any apparent lymphocytic contribution from the thymus grafts. These cells appear to exert an effect on lymphocytes either through the liberation of a humoral factor or by directly affecting lymphocytes passing through the thymus.

**Summary**

Neonatal thymus placed in a Millipore diffusion chamber for one week loses its cortical cells, while the epithelial reticular cells remain viable.

Grafts of these remnants in neonatally thymectomized mice are completely reconstituted from cells of the thymectomized host. Evidence is presented which suggests that the bone marrow may be a source of cells which reform the thymus cortex.

These remnant grafts result in lymphoid reconstitution of neonatally
thymectomized mice. The grafted animals also reject allografts of skin and lymphoma cells in a normal manner. However, these C3H mice, neonatally thymectomized and reconstituted with allografts of AKR thymic epithelial reticular cells, are tolerant to grafts of AKR lymphoma cells.

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**REFERENCES**


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