EFFECTS OF SHORT-TERM EPITHELIAL RETICULAR CELL AND WHOLE ORGAN THYMUS GRAFTS IN NEONATALLY THYMECTOMIZED MICE*

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Neonatal thymectomy in mice results in a characteristic depletion of the lymphoid tissues and an impaired immunologic capacity (1–4). Animals so treated are subject to the development of a wasting disease characterized by diarrhea, weight loss, and early death (3). Thymus grafts are capable of normalizing a neonatally thymectomized host (1, 2). Evidence is also available to show that neonatal thymus enclosed in a cell-impermeable chamber in neonatally thymectomized mice can restore to some extent the characteristic defects of these animals (5). These experiments are interpreted as demonstrating a humoral factor liberated by the surviving epithelial reticular cells of the thymus, which is responsible for endowing lymphoid cells with immunologic competence. Previous experiments from this laboratory show that isografts and allografts of thymus remnants composed of epithelial reticular cells and placed subcutaneously in 4- to 6-day old neonatally thymectomized mice are reconstituted to the morphologic appearance of thymus (6). The grafted animals reject allografts of skin and lymphoma cells in a normal manner. Complete microscopic normality of the lymphoid tissues occurs when the animals are 8–12 wk of age.

The experiments comprising this study were designed to determine if subcutaneous and kidney capsular thymus epithelial reticular cell grafts placed for short periods of time in neonatally thymectomized mice could prevent wasting disease, restore the microscopic appearance of the lymphoid tissues, and return the animal's agglutinin response to sheep erythrocytes to normal. We studied both the immediate and prolonged effects of such grafts and compared these observations with those found in animals grafted with intact thymus for similar periods of time. Since these grafts were devoid of lymphoid cells at the time of implantation and in most instances were populated with these cells during the 2nd wk after grafting, we felt that their use in short-term studies might provide additional information regarding a specific activity

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of thymus epithelial reticular cells in the immunologic and histologic reconstitution of neonatally thymectomized mice.

Materials and Methods

Mice.—Mice used in these experiments were from inbred AKR, C3H/HeJ, and CBA/H-T6T6 strains which are maintained in this laboratory. They were given water and a pellet diet *ad libitum* throughout the experiments. Females giving birth to litters used for thymectomy were given water with added tetracycline as soon as they were seen to be pregnant and were maintained on this water until the thymectomized litters were weaned.

Grafts.—All of the thymuses used for grafting were from animals less than 48 hr of age.

The epithelial reticular cell remnants were prepared by a method described previously (6). Microscopic examination of representative remnants prior to grafting revealed them to be composed almost exclusively of epithelial reticular cells. When an intact organ was used, a single thymus was removed from a newborn animal and placed in Hanks' solution with 100 units per ml of penicillin and streptomycin. The organ was then grafted under the kidney capsule. Whenever possible, grafts between like sexes were carried out.

Thymectomy.—The animals were thymectomized within the first 24 hr of birth. Using halothane anesthesia, we removed the thymic lobes by gentle suction. To insure completeness of thymectomy, all animals when sacrificed had a block of superior mediastinal tissue removed for section after a close gross examination of the area. Animals in which thymus tissue was found were excluded from the experiments.

Removal of Grafts.—The subcutaneous grafts were removed by dissection. Identification was facilitated by their attachment to the Millipore membrane. The kidney capsule grafts were removed by nephrectomy, a method assuring a complete removal of all thymus tissue without trauma to the graft. Halothane anesthesia was used for the subcutaneous grafts; and a combination of halothane and sodium pentobarbital was used for the nephrectomy.

Sacrifice.—Animals were sacrificed at intervals after the grafts were removed. Blood for white cell counts, differential counts, and agglutinins to sheep red blood cells (SRBC), was taken from the living anesthetized animal by section of the axillary blood vessels. Bone marrow was studied by removing cells from the cut end of the femur with a small artist's brush dipped in serum and streaking them on a slide. Marrow cellularity was determined by making sections of marrow tissue removed from the opposite femur with a 24 gauge needle. The tissues were fixed in Bouin's fluid, and hematoxin- and eosin-stained serial sections cut at 5 μ were prepared. All of the marrows showed dense cellularity without fat or fibrous tissue. The blood and marrow films were stained with Wright's stain and differential counts of 100 blood and 200 marrow cells performed.

Grading and Staging of Tissues and Grafts.—For each animal, we graded the microscopic appearance of the spleen, mesenteric lymph node, axillary lymph node, and Peyer's patch, as follows:

A. *Depleted:* diminished numbers of lymphocytes around the periarteriolar areas of the splenic follicles, decrease in size of follicles with small or poorly defined germinal centers; depletion of lymphocytes in the intermediate zone of the lymph node; aggregates of cortical small lymphocytes uniformly present, germinal centers in these aggregates absent or reduced in size; some reduction in over-all numbers of cortical lymphocytes; loss of lymphocytes in perivascular areas, but germinal centers usually present, in Peyer's patch; decrease in over-all size of the patch.

B. *Partially depleted:* increase in size of splenic follicles with germinal centers uniformly present; depletion of lymphocytes in periarteriolar area present but to a lesser degree than in stage A; aggregates of small lymphocytes in depleted intermediate zone, and an increase in

numbers of lymphocytes surrounding the germinal centers in the cortex of the lymph nodes; increase in numbers of lymphocytes in, and size of, Peyer's patch.

C. *Normal or nearly normal:* abundant lymphocytes present in the areas described as depleted in spleen, lymph node, or Peyer's patch; large confluent lymphoid follicles in spleen; increase in over-all size of lymph node and Peyer's patch; possibly a few areas in serial sections of tissues where slight lymphoid depletion can be found.

To give a semiquantitative picture of the over-all presence or absence of lymphoid restoration, we recorded the grade of each tissue and obtained the sum of tissues at a particular grade for each experimental group.

The morphologic staging of the regenerating remnant grafts was as follows: Stage 0, no graft found. Stage 1, graft composed of epithelial reticular cells only. (All remnant grafts were assumed to be in this stage at the time of grafting.) Stage 2, epithelial cells in clusters with scattered, small, dark lymphoid cells surrounding them and in blood vessels adjacent to clusters. Stage 3, graft composed mainly of large lymphoblasts with many mitoses. (Epithethelial reticular cells obscured by these cells.) Stage 4, microscopic morphology of normal thymus with a well-defined cortex and medulla. The number of grafts in each stage was recorded for each experimental group.

Antibody Titration.—The blood was obtained from the retroorbital sinus or by section of the axillary vessels 7 days after the intraperitoneal injection of 0.2 ml of a 20% suspension of SRBC. Hemagglutinin titers were performed in microtiter trays, serial twofold dilutions of serum were made in 0.9% sodium chloride, beginning with a dilution of 1:2. Agglutination was expressed as $\log_2$ of the reciprocal of the last dilution of serum which showed agglutination. For measurement of secondary response, sheep erythrocytes were given 14 days after the primary dose and serum was obtained 7 days later.

Chromosome Preparation.—These studies were carried out as described in a previous study (6). The lymphoid tissues of neonatally thymectomized mice grafted with epithelial remnants or whole thymus from CBA/H-T6T6 mice were studied for the presence of T6 chromosome markers in their dividing cells.

RESULTS

Short-term Thymus Remnant Grafts, 3 wk of Age.—C3H/HeJ mice were neonatally thymectomized and grafted at 3 wk of age with AKR thymus remnants under the kidney capsule. The grafts were then removed after 7 and 14 days, and the animals were sacrificed for study between 80 and 110 days of age. 42 mice had grafts in place for 7 days and 33 animals had grafts in place for 14 days. The majority of the animals died of wasting disease 1–3 wk after the grafts were removed. There were only 19 mice surviving after 80 days. A few of them were underweight, but all of them appeared vigorous and healthy. 15 of the survivors had 7-day grafts and four had 14-day grafts. Seven of these animals had the characteristic depletion of lymphoid tissues; eight had partially depleted tissues; and four could be classified as nearly normal. Germinal centers were absent in the tissues of five of the depleted animals. 11 of the surviving mice had low primary responses to SRBC. 15 animals had a secondary response tested. It was found to be impaired in six. There was no relationship of the appearance of the grafts at the time of removal to the ultimate fate of the animal, i.e., animals that died early with wasting disease or the 19 survi-

vors. All stages of lymphoid repopulation were found in the grafts removed from both groups of mice.

Six thymectomized 3-wk old mice were grafted with epithelial remnants under the kidney capsule. The grafts were left in place until the animals were sacrificed at 90 days of age. All were of normal weight and appearance, and had a normal microscopic morphology of their lymphoid tissues. Four of six animals had normal primary immune responses to SRBC, and all had normal secondary responses. The grafts resembled a normal thymus.

50 control mice were neonatally thymectomized and not grafted. 48 of these

TABLE I

Short-Term Kidney Capsule Thymus Remnant Allografts in Neonatally Thymectomized C3H/HeJ mice

Time graft *in situ*	No. mice	Morphology of graft stage*					No. deaths from wasting day 27–70	No. survivors 90 days	Lymphoid tissue morphology grade*			Log_2 SRBC agglutinins Mean (range)		Blood lymphocytes Mean ± S.D.	Marrow lymphocytes Mean ± S.D.
		0	1	2	3	4			A	B	C	Prim.	Second.		
days														mm^3	%
7	42	0	2	13	0	4	27	15	32	10	15	4(1–6)	5(2–10)	1892 ± 1462	7.2 ± 5
14	33	1	0	0	1	2	29	4	7	2	4	4, 4, 4, 5	3, 6, 7, 8		
72–80	6	0	0	0	0	6	0	6	0	0	24	5(4–7)	7(7–8)	3268 ± 1656	18.1 ± 7
Thymectomized controls	50	–	–	–	–	–	48	2	8	0	0	4, 4	0, –	540, 650	1, 2
Sham-grafted intact controls	17	–	–	–	–	–	0	17	0	0	44‡	5(3–8)	8(7–11)	5123 ± 1812	18.1 ± 3

* See Materials and Methods section for grading and staging criteria.

‡ Tissues studied in 11 mice of this group.

animals died of wasting disease at 28–70 days of age. Only two mice survived and were sacrificed at 90 days. These animals were wasted, had depleted lymphoid tissues, and an impaired immune response.

A second control group consisted of 17 intact mice sham-grafted with blank Millipore membranes under the kidney capsule which were removed by nephrectomy at 7 and 14 days. All of these animals were grossly and microscopically normal. The primary response to SRBC was low in 2 of 11 mice and the secondary response was normal in all animals. The results of this experiment are summarized in Table I. It can be seen in this table that values for blood and marrow lymphocytes were lower in the short-term-grafted animals than in those with 72–80 day grafts or the sham-grafted controls.

Thymus Remnant Grafts Immediately Replacing Host Thymus.—To determine if restoration could be obtained by immediate replacement of thymus

epithelial cells, we subcutaneously placed grafts at the time of neonatal thymectomy and removed them after 7 and 14 days. The animals were sacrificed at 30–40 days. The recipient animals in these experiments were C3H/HeJ mice. Some of the animals received remnant isografts, others received allografts of AKR epithelial remnants. The results from the various groups were similar and were pooled. Of 45 grafted mice, 10 died with wasting before definitive studies could be performed. The findings in these animals with 7- and 14-day subcutaneous remnant grafts differed little from those in 16 neonatally thymectomized control mice which were examined at 40 days of age. There was a characteristic depletion of lymphoid tissues, impaired response to SRBC, and blood and marrow lymphopenia.

TABLE II

Subcutaneous Thymus Remnant Grafts Immediately Replacing Host Thymus in C3H/HeJ Mice

	No. Mice	Lymphoid tissue morphology grade*			Log_2 primary SRBC agglutinins Mean ± S.D. (range)	Blood lymphocytes Mean ± S.D.	Marrow lymphocytes Mean ± S.D.
		A	B	C			
						mm^3	%
Neonatal thymectomy graft day 1–7 or 1–14	35	111	27	2	2.5 ± 1.3 (0–5)	1078 ± 861	8 ± 6
Neonatal thymectomy no graft	16	58	7	0	2.4 ± 1.4 (0–4)	1308 ± 589	6 ± 6
Thymectomy day 7	19	0	21	55	3.7 ± 1.1 (1–5)	2136 ± 875	12.5 ± 7
Normal C3H/HeJ	30	0	0	32‡	5.6 ± .7 (5–7)	3974 ± 1340	29.3 ± 9

* See Materials and Methods section for grading criteria.

‡ Tissues studied on eight animals in this group.

All of the grafts were composed of viable cells at the time of their removal and were approximately 1–4 mm in size. 14 of the 7-day grafts were stage 1, two were stage 2. Of the 14-day grafts five were stage 1; two, stage 2; eight, stage 3; and three, stage 4.

These observations were in striking contrast to those in animals in which the thymus was left *in situ* for the first 7 days of life and then thymectomized. These animals grew and developed normally and were of normal body weight and vigorous health when sacrificed at 34 and 90 days of age. There were 19 mice so studied. The microscopic appearance of the lymphoid tissues was normal in a majority of the animals. Normal agglutinin responses to SRBC were found in six mice. This study is summarized in Table II, and the results are compared with findings in intact C3H/HeJ mice of a similar age.

A Comparison of Thymus Remnants and Whole Thymus Grafts Left in situ for 14 Days.—Because of the demonstrated relative ineffectiveness of short-term

thymus remnant grafts in restoring the deficits of neonatally thymectomized mice, because of evidence that restoration occurred with lymphoid reconstituted remnants left *in situ* for 80 days, and because wasting and severe lymphoid and immunologic defects did not develop in animals thymectomized at 7 days, an experiment was initiated making a comparison of short-term thymus remnant grafts with those of the intact organ. All of the recipients in this study were neonatally thymectomized mice of the C3H/HeJ strain. The animals were grafted with thymus and thymus remnants from syngeneic as well as

TABLE III

Comparison of Epithelial Remnants with Intact Thymus Grafts Left in Situ for 14 Days

	No. mice	Wt.	Lymphoid tissue morphology*			Log_2 SRBC agglutinins Mean ± S.D. (range)	Morphology of graft*					Leukocytes Mean ± S.D.	Lymphocytes Mean ± S.D.	Marrow lymphocytes Mean ± S.D.
		Mean ± S.D.	A	B	C		0	1	2	3	4			
		g										mm^3	mm^3	%
Remnant	19	15.3 ± 2.8	56	19	1	2.7 ± .9 (1–4)	2	3	1	2	12	2868 ± 1715	1416 ± 657	12.2 ± 8
Intact thymus	38	17.5 ± 2.3	30	47	66	4.2 ± 1.3 (1–6)	0	0	0	0	38	3775 ± 1880	1922 ± 1017	19.0 ± 7
Thymectomy no graft	18	13.3 ± 2.3	72	0	0	2.1 ± 1.1 (0–4)	—	—	—	—	—	4760 ± 1680	1354 ± 695	6.6 ± 6
Nonthymectomized controls	30	15.4 ± 1.7	0	0	32‡	5.6 ± .7 (5–7)	—	—	—	—	—	5800 ± 1860	3974 ± 1340	29.3 ± 9

* See Materials and Methods section for grading.

‡ Tissues studied on eight control animals

CBA/H-T6T6 donors. Using studies for chromosome markers, we included these latter grafts to determine any contribution to the peripheral lymphoid tissues of cells from the thymus grafts. All of the animals were thymectomized within 24 hr of birth and grafted at 10 days of age under the kidney capsule. The grafts were removed by nephrectomy after 14 days. Sacrifice was between 34 and 40 days of age. Because there is evidence that the postneonatal thymectomy–wasting syndrome is precipitated by factors of infectious origin, i.e. it does not occur in animals raised in a pathogen-free or germfree environment (7), the condition of the animals in this experiment differed from those in the preceding two in that all of the animals were kept in cages with filter tops from 1 wk of age until sacrifice. Even thymectomized animals housed in this manner did not develop wasting. A total of 19 animals received thymus epithelial remnant grafts, 7 had C3H/HeJ remnants, and 12, T6 grafts. The similar results in the two groups were pooled. The animals all appeared vigorous

at the time of sacrifice. Their body weights were comparable to intact control animals of the same age. The grafts showed varying degrees of lymphoid restoration, and 12 of the 19 grafts had regained the microscopic morphology of thymus. The findings in this group were similar to those of remnant-grafted animals in the preceding studies. The agglutinin response to SRBC was impaired, and there was minimal evidence of restoration of the depleted lymphoid tissues with lymphopenia in the peripheral blood and reduction of lymphocytes in the bone marrow.

There was a total of 38 animals receiving grafts of intact neonatal thymus, 20 animals with C3H/HeJ grafts, and 18 animals with T6 thymus. The similar results in these two groups were combined. The animals appeared vigorous and healthy, and of normal body weight at the time of sacrifice. All of the grafts had the microscopic morphology of normal thymus at the time of removal. The agglutinin responses to SRBC in this group of animals was only slightly impaired. Morphologically, the lymphoid tissues showed a greater

TABLE IV

Marker Chromosome Studies in Neonatally Thymectomized C3H/HeJ Mice Given 14-Day Epithelial Remnant and Whole Thymus Grafts from CBA/HT6T6 Mice

Graft	No. of cells with thymus donor characteristics/No metaphases counted		
	Spleen	Lymph node	Peyer's patch
Thymus remnant	0/147	3/89	3/312
Whole thymus	0/346	23/334	3/519

degree of restoration than that found in the remnant-grafted animals. The lymphocytes in the peripheral blood were reduced, but the marrow lymphocytes were present in greater numbers than those in the remnant-grafted mice. There was some evidence of regeneration in all but one of the lymphoid tissues examined in this group. The results of this study are summarized in Table III, in which the animals in the two grafted groups are compared with thymectomized and intact controls sacrificed at 34–40 days of age.

Table IV presents the results of the chromosome studies of the tissues of animals bearing thymus grafts with marker chromosomes. It can be seen that neither the remnant nor whole thymus grafts contributed to any great extent to the dividing cell population of the lymphoid tissues. There was, however, a small graft-derived population in the lymph nodes of the animals which had received whole thymus grafts.

DISCUSSION

These experiments show that when left *in situ* for 7 and 14 days, epithelial cell grafts of thymus in neonatally thymectomized mice have little effect in restoring immunologic responsiveness as measured by the development of

agglutinins to SRBC. The grafts are ineffective in protecting hosts from wasting, in restoring the appearance of histologic normality to the lymphoid tissues, and in promoting normal lymphocyte levels in the blood and marrow. There is little difference in the observed results whether the animals are grafted immediately after thymectomy or at 3 wk of age. Similar results are found when animals are studied within a few weeks or as long as 90 days after grafting. A trend toward normality of the parameters measured in these studies occurs when (*a*) kidney capsule remnant grafts are left in place for 90 days, (*b*) thymectomy is delayed until 7 days of age, and (*c*) when short-term grafts of intact thymus are used. These results suggest that histologic and immunologic recovery are associated with a normal lymphocyte population of the thymus, i.e., thymus lymphocytes which have reached maturity in the thymus epithelial cell environment. Previous studies carried out in this laboratory comparing thymus remnant with intact thymus grafts in the thymectomized-irradiated adult host have shown similar findings (8).

Miller and Mitchell (9, 10) have presented the concept that the thymus supplies antigen-reactive cells which leave the organ and interact with cells in other lymphoid tissues to produce cells capable of antibody formation. The results of the present studies would suggest that such lymphocytes ultimately develop in the thymus grafts under the direction of the epithelial reticular cells, and that they are immediately available in grafts of the intact thymus.

The experiments reported herein imply a role of mature thymus lymphocytes rather than a direct effect of epithelial reticular cells in the recovery of neonatally thymectomized mice. Migration of lymphocytes to lymphoid tissues and to areas specifically depleted in neonatally thymectomized animals has been demonstrated by the use of radioactive labeling techniques (11, 12). The presence of thymus-derived dividing cells has been shown in lymph nodes of thymus-grafted, neonatally thymectomized mice (13) as well as in the spleen and lymph nodes of thymus-grafted, thymectomized irradiated mice (14). Miller et al. (15) have presented evidence of the partial recovery of immunologic function in neonatally thymectomized animals with short-term intact thymus grafts. The same study shows that thymus irradiated in vitro with 2000 R and grafted for 1 or 2 wk loses the ability to restore the neonatally thymectomized host. In their inability to restore neonatally thymectomized hosts, the short-term remnant grafts in the present studies resemble the 2000 R irradiated graft. That is, there are either remnant grafts initially without lymphocytes but with intact epithelial reticular cells or grafts heavily irradiated with a dose that destroys lymphocytes but not epithelial cells (16); these two grafts during a 14 day period *in situ* have no effective thymus lymphocyte population which can result in restoration. The lymphocytes found in the remnant grafts at the time of removal may not have reached functional maturity, and the irradiated graft is not populated at 14 days. It is shown in this same study (15) that grafts

preirradiated with 500 R, a dose which produces a graft initially lymphoid depleted and repopulated at the same rate as the 2000 R graft, restores immune responsiveness of the neonatally thymectomized host. An explanation of these observations could be as follows. The results with short-term intact thymus grafts in the present study and in that of Miller et al. (15) demonstrate that thymus lymphocytes play a vital role in the restoration of the neonatally thymectomized host. Intact thymus placed in a cell-impermeable chamber (5), as well as thymus extracts (17, 18), also effect some degree of restoration of neonatally thymectomized mice. Therefore, one can conclude that viable lymphocytes per se may not be necessary, but that subcellular products of these cells which can permeate the pores of the chambers and exist in the extracts could be active in the restorative processes. It might then be argued that these hypothetical materials could be present in the grafts receiving 500 R but inactivated by a higher dose of irradiation. They are, of course, not present in the epithelial remnant grafts, having been lost during the preparation of these grafts in the diffusion chambers.

The viable epithelial cells of the remnant grafts, and the grafts receiving both irradiation doses, are fully capable of restoring a new functional thymus by recruiting lymphocyte precursor cells from the host (8, 19). Thymus epithelial reticular cell function, then, is to promote maturation of thymus lymphocytes but has no direct effect on restoring immunocompetence or a normal lymphocyte population to the defective host.

The thymus lymphocytes, on the other hand, leave the thymus to circulate (20) and to enter the lymphoid tissues (12, 13); and also many lymphocytes may die within the thymus (11, 21). These cells could then interact with other lymphoid cells or, in death, liberate products which too are effective in restoring normal immunologic functions to their hosts. The small numbers of thymus-derived cells seen in this and other studies (11–13) in the peripheral tissues does not correlate well with the rather large microscopic deficit of lymphocytes consistently found in the thymus-dependent areas in neonatally thymectomized animals (4). This is another argument for the presence of a thymus-derived factor which stimulates production of lymphocytes in the nodes, spleen, and Peyer's patch. The results of this study would suggest that this factor is from the lymphocytes and not from epithelial reticular cells of the thymus.

SUMMARY

Neonatally thymectomized mice were implanted with thymus grafts composed of epithelial reticular cells for periods of 7 and 14 days. Regardless of whether the grafts were placed immediately after thymectomy, or at 3 wk of age, there was little recovery of the lymphocyte depletion and impaired immunologic responsiveness, characteristically found in a neonatally thymectomized host. The findings were similar in animals studied at 2 months or 2

wk after graft removal. Many of the short-term remnant grafts were populated with lymphocytes and had attained the morphologic appearance of thymus by 14 days.

A lesser degree of lymphocyte depletion and impaired responsiveness to SRBC occurred if thymectomy was delayed until 7 days of age, if remnant grafts were removed after 2 months, and if intact neonatal thymus was used for the short-term grafts. Complete normality was found in some of the animals in all of these groups.

These observations suggest a direct role for mature thymus lymphocytes in reconstituting the neonatally thymectomized host and indicate that epithelial cell function is to direct the maturation of cells that ultimately behave as thymus lymphocytes.

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