STUDIES ON THE ROLE OF THE THYMUS IN IMMUNOBIOLOGY

Reconstitution of Immunologic Capacity in Mice Thymectomized at Birth*

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(Received for publication, August 12, 1963)

It has recently been established that the thymus plays a fundamental role in developmental immunobiology (1–3), a role that is most important in early life, before birth and immediately thereafter, but continues into adult life (4).

Mice thymectomized immediately after birth are extremely defective in rejecting allogeneic skin or tumor grafts (2, 4, 5) and even xenogeneic skin (6). Their responsive antibody formation is also greatly impaired (6–8), although not necessarily for all antigens (8). These animals often develop a wasting syndrome, with failure of body growth and early mortality (6, 8–10), and are extremely susceptible to the runt disease produced by administration of allogeneic lymphoid cells (9–11). Furthermore, the lymphoreticular cells derived from neonatally thymectomized mice are defective with respect to their capacity to elicit graft *versus* host reactions (12, 13). These animals also have a low level of circulating lymphocytes, a depletion of lymphocytes in the tissues, and, to a somewhat lesser degree, a depletion of plasma cells (2, 6, 8–11, 13). The immunologic defect, lymphoid cell depletion, and growth failure of these animals can be prevented by grafting a newborn thymus into the subcutaneous tissue (11) or by administration, shortly after thymectomy, of adult lymph node cells from syngeneic donors (13, 14).

Some of the characteristics of the mice thymectomized at birth have also been described in other species subjected to neonatal thymectomy: the rat (15–17), rabbit (3, 10, 18), and hamster (19–22). Particularly interesting are the findings in the thymectomized hamster in which a wasting syndrome associated with hypogammaglobulinemia has been observed (19).

Thymectomy after the immediate neonatal period does not cause wasting in mice, although it still has a significant effect on the development of immunologic reactivity as the animal matures. In certain strain combinations, isogenic at the H-2 histocom-

* Aided by grants from the United States Public Health Service (grants HE-02085, AI-00798, and CA-03511), the American Cancer Society, Minnesota and American Heart Associations, and The National Foundation.

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patibility locus, thymectomy as late as 30 to 40 days of age permits prolonged survival and even permanent takes of allogeneic skin and tumor grafts (4, 10, 23), and in certain F_1 hybrid mice thymectomy at this age increases the susceptibility to graft *versus* host reaction induced by administration of lymphoid cells from parental strain animals (10). It has also been reported that mice (24) and rabbits (25, 26) thymectomized as adults and given a sublethal dose of whole body x-irradiation show decreased ability to recover from the immunologic impairment imposed by the irradiation. Finally, it has recently been found that mice thymectomized at 3 to 4 weeks of age and given 900 roentgens of x-irradiation at 10 weeks show markedly deficient recovery of lymphoid structure of the spleen as compared to normal irradiated controls (27).

In studies reported by Miller (11, 13, 14), attempts to reconstitute neonatally thymectomized mice with thymic extracts or free thymus cells have failed, but allogeneic thymic grafts have provided both physiologic and immunologic restoration. Using T6 chromosome marker systems, as well as immunologic parameters, it has been shown that the cellular reconstitution which is accomplished by the allogeneic thymus grafts is primarily of host origin (14).

It is the purpose of this paper to present additional experiments seeking a better understanding of the immunologic and developmental deficiencies encountered in mice thymectomized at an early age. It will be shown that lymphoreticular cells from mice thymectomized between the 1st and 35th days of life are deficient in immunologic function as revealed in studies of the graft *versus* host reaction, and that the degree of deficiency is inversely correlated with age at thymectomy. The deficiency of the cells from animals thymectomized on the 1st day of life is extreme; however, there is a residuum of immunologic activity, evident in the runting model when the dosage of cells is quadrupled. Further, it will be shown that spleen cells from adult mice thymectomized immediately after birth are capable of inducing immunologic tolerance upon injection into allogeneic newborn recipients without inducing graft *versus* host reactions. We will also show that the wasting syndrome which regularly develops in neonatally thymectomized mice can be prevented by administration of suspensions of syngeneic viable thymus or spleen cells, or by transplanting newborn thymus tissue. Immunologic capacity can be restored by grafting thymus tissue from neonatal or adult donors, and, as assayed in the graft *versus* host reaction, full immunologic competence is restored by transplantation of neonatal thymus as well as by intravenous injection of large numbers of syngeneic adult spleen cells. Finally, we will demonstrate that immunologic restoration of neonatally thymectomized mice achieved by grafting *allogeneic* thymus acts primarily through development of the host's own lymphoid cells, with a relatively small component of the lymphoid cells of thymus donor origin presumably derived from the thymus transplant. By contrast, the lesser degree of restoration of immunologic function achieved by allogeneic spleen grafts is attributable instead to the donor tissue of the transplant.

Materials and Methods

Mice.—Inbred mice of the A, C_3H/Bi, $C_{57}Bl/1$, and DBA/2 strains, and the (A × $C_{57}Bl/1$), (C_3H × $C_{57}Bl/1$), and (C_3H × DBA/2)F_1 hybrids were used in these experiments. All were separated from the mouse colonies of the late Dr. John J. Bittner in 1956, and have been maintained in our own colonies since that time by rigorous inbreeding procedures. The details of housing and care of the animals were given in an earlier paper (10).

Thymectomy.—The thymus was removed, under ether anesthesia, by a method similar to that of Dischler and Rudali (28). In preparing sham-operated mice, the thorax was opened but the thymus left intact.

Thymus Grafting.—Mice thymectomized on the 1st day of life were grafted subcutaneously 2 days later with thymus from either 2-day-old or 2-month-old donors. One thymic lobe from the newborn donors or an equivalent amount of tissue from the 2-month-old donors was sliced into 3 or 4 pieces and introduced into the subcutaneous tissue of each axillary region of the recipients. In other experiments spleen from 2-month-old donors was grafted in the same way into neonatally thymectomized mice.

Skin Grafting.—Full thickness abdominal skin was placed on the back of the tested animals by the technique employed as routine in this laboratory (29). Survival of the grafts was judged by gross inspection.

Cell-Free Tissue Extracts.—Thymus or spleen taken from 2- to 4-week-old C_3H mice was mechanically disrupted by a glass tissue homogenizer (Potter-Elvehjem) with a tight fitting pestle. Approximately 15 ml of Ringer's lactate saline solution per gm of tissue was used. The homogenate was then centrifuged at 3000 RPM for 15 minutes, and the supernate stored at −20°C for a few days until used. In one experiment a thymus extract, described by Szent-Gyorgyi *et al.* (30) and designated "promine,"[1] was employed.

Cell Suspensions.—Suspensions of cells from thymus, spleen, and lymph nodes were prepared in Ringer's lactate saline solution, again using a Potter-Elvehjem homogenizer but with a loose fitting pestle. Further dissociation of the cells was completed by passing the suspension gently in and out of a 27 gauge needle. Suspensions to be injected intravenously were washed once with Ringer's lactate saline solution employing low speed centrifugation (500 RPM) for 15 minutes in an International No. 2 centrifuge. Cell counts were made and dilutions prepared so that 10 million nucleated cells were contained in 0.05 to 0.10 ml. The suspensions were injected into the orbital branch of the anterior facial vein in the newborn mice and into the tail vein of the 40-day-old animals.

Assay of Graft versus Host Activity.—In one group of experiments the spleen assay of graft *versus* host reaction of Simonsen *et al.* (31) was used. This assay assesses the spleen enlargement induced in young F_1 hybrid recipients of lymphoid cells from one of the parent strains. Litters of 6 to 8 hybrid mice were injected at 8 days of age with spleen or lymph node cells from: (*a*) syngeneic mice, (*b*) sham-thymectomized or non-operated mice of one of the parent strains, or (*c*) thymectomized mice of the same parent strain used in *b*. Eight days after cell administration the animals were sacrificed, their body and spleen weights recorded, and the relative spleen weight (mg/10 gm body weight) determined. Finally, the "spleen index" was calculated by dividing the mean relative spleen weight of the animals receiving parent strain cells from non-thymectomized mice or from thymectomized animals by the mean relative spleen weight of the mice injected with cells from syngeneic donors.

In another group of experiments, the mortality assay of the graft *versus* host reaction, described by Billingham and Brent (32) and by Siskind and Thomas (33) was employed. For this purpose newborn mice were injected with spleen cells derived from allogeneic thy-

[1] The authors are indebted to Dr. Albert Szent-Gyorgyi who generously provided this material.

mectomized or sham-operated animals, and the number of runts as well as the number of animals dying between the 5th and 30th days after cell administration was recorded.

Discriminant Assay of Cell Chimerism.—Adult mice thymectomized at birth and subsequently grafted with allogeneic thymus or spleen tissue were studied by the discriminating spleen assay of graft *versus* host activity described by Simonsen and Jensen (34) to assess the immunologic capacity of their spleen and lymph node cells and to determine the immunogenetic composition of these tissues. At 2 to 4 months of age, the spleen or lymph nodes from the mice bearing allogeneic thymus grafts were made into suspensions, and the host and donor components studied in appropriate 6- to 8-day-old F_1 hybrid mice. In thymectomized A mice grafted with C_3H lymphoid tissues, the host component (A) was assayed in the $(A \times C_{57}Bl)F_1$ hybrid and the donor component (C_3H) in the $(C_3H \times C_{57}Bl)F_1$ hybrid. Only litters of 6 to 8 animals were employed, and in each experiment negative controls were prepared by injecting some of the F_1 members with cells from mice syngeneic with a component of the chimera that was expected to be rejected by the hybrid; *e.g.*, A into $(C_3H \times C_{57}Bl)F_1$. The dosage of cells in the controls was always the same as that used to assay the components of the chimera: in most cases 10 million cells, but in some instances 20 to 30 million cells, as indicated in the data. Two positive controls of the litter were given 10 million cells from normal animals syngeneic with the component of the chimera that was expected to be accepted by and to react against the F_1 hybrid; *e.g.*, A into $(A \times C_{57}Bl)F_1$. Test animals were sacrificed 8 days later, and their body and spleen weights determined. A relative spleen weight was then calculated (mg/10 gm body weight) and a mean value determined for each group (experimental, positive control, and negative control). The "experimental spleen index" was calculated by dividing the mean relative spleen weight of the experimental group by the mean relative spleen weight of the negative controls. In the same manner, the "control spleen index" was calculated by dividing the mean value of positive controls by the mean value of the negative controls.

RESULTS

Spleen Assay of Graft versus Host Activity of Spleen Cells Derived from Mice Thymectomized at Different Ages.—

In the first group of experiments, the spleen assay of graft *versus* host reaction was employed to compare the immunologic impairment produced by thymectomy performed at different ages. C_3H mice thymectomized at 1, 6, 14, 25, and 35 days of age were sacrificed 2 or 6 months following thymectomy. A cell suspension was prepared from the spleen of each animal, and 10 million of the cells administered intraperitoneally to $(C_3H \times DBA/2)F_1$ hybrid recipients which were killed 8 days later. Spleen cells from normal C_3H mice sacrificed at 14 days, 35 days, and 2 to 3 months of age were also tested and served as controls.

It will be seen in Fig. 1 that, as measured in this way, spleen cells from unthymectomized mice of the C_3H strain show progressive development of immunologic capacity during the first 3 months of life. The older the C_3H mice the greater the splenomegaly produced by injection of their spleen cells into $(C_3H \times DBA/2)F_1$ recipients. Very little splenomegaly followed the injection of the spleen cells from 14-day-old donors. The splenomegaly was greater when the spleen cells were taken from 35-day-old animals, and a 2- to 3-fold increase in the spleen index was observed in hybrid recipients of cells from 2- to 3-month-old donors approximating the characteristic activity of adult cells.

These results also show clearly that thymectomy on the 1st, 6th, and 14th

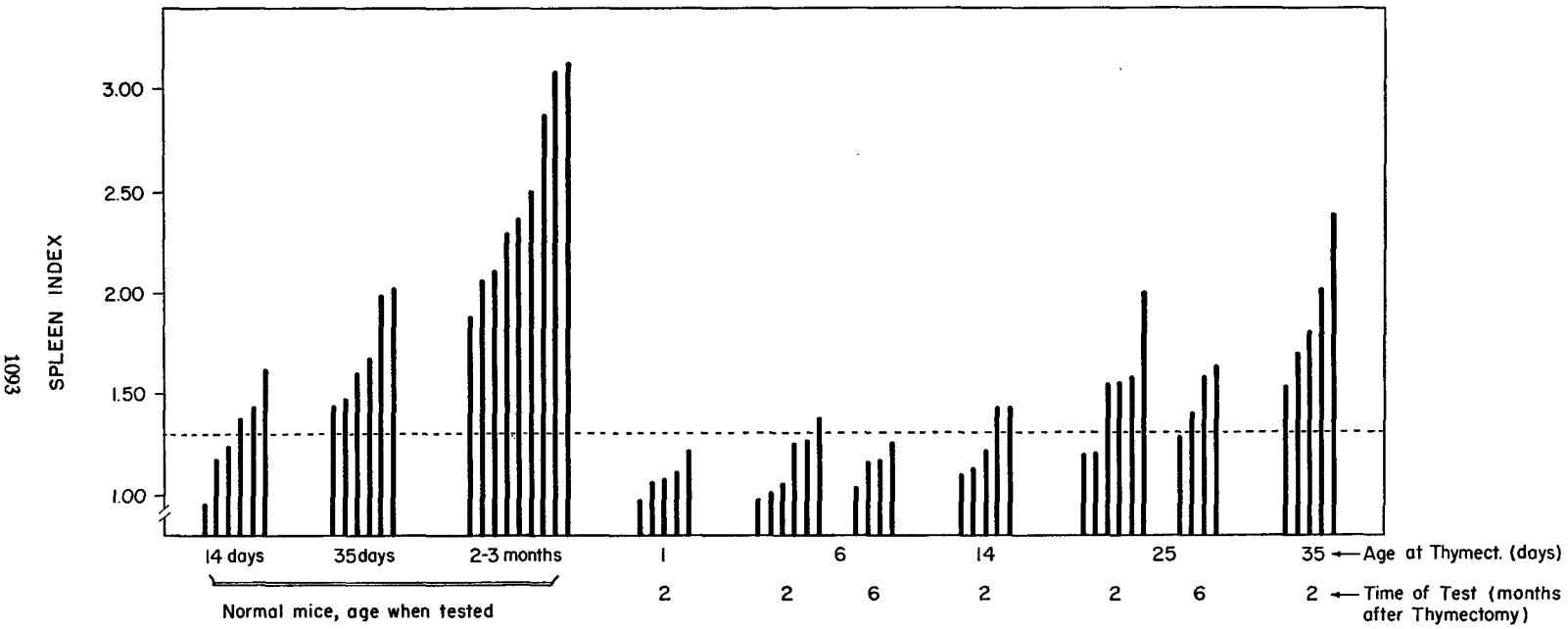

FIG. 1. Effect of thymectomy at different times after birth on development of immunologic capacity of the spleen. It will be seen in the figure that capacity of the spleen cells to induce graft *versus* host reactions increases progressively after birth. Thymectomy as late as 14 days after birth almost completely inhibits development of immunologic capacity as measured in this way, and thymectomy as late as 35 days of age arrests development of immunologic capacity at approximately the level which had been achieved prior to thymectomy.

day of life virtually abolishes the capacity of the spleen cells of the C_3H animal to produce graft *versus* host reactions in this model. Definite suppression of immunologic reactivity was also observed when cells from mice thymectomized as late as 25 or 35 days of age were injected into the hybrid recipients. Both of these groups, tested at about 3 months of age, showed a marked deficiency when compared to unoperated mice 2 to 3 months of age; rather, their cells had immunologic activity in the Simonsen assay approximating that of unthymectomized control animals sacrificed at 35 days of age.

Another point of interest in Fig. 1 is the finding that cells from mice thymectomized 6 days after birth were incompetent in the Simonsen graft *versus* host assay when tested both 2 and 6 months after thymectomy. This observation indicates that the completely thymectomized mouse of this strain does not recover immunologic capacity over a 6 month period when the capacity for immune reaction is tested in this way. Fig. 1 also shows that the spleen cells of mice thymectomized at 25 days of age have greater immunologic capacity than those thymectomized at 1, 6, or 14 days, and that this greater immunologic reactivity is present whether the animals are tested at 2 or 6 months after thymectomy. Thus, when thymectomy is carried out during development but after the establishment of a degree of immunologic competence of the peripheral lymphoid tissues, the attained level of immune capability remains where it was at the time of thymectomy: this degree of competence is not lost, but neither do the animals "catch up" to the unthymectomized control animals in the maturation of immunologic function of the peripheral lymphoid tissues. The effect of thymectomy has a degree of permanence in mice, and the level of immune activity of the splenic tissue of such animals reflects the age at which thymectomy was performed.

Effect of Thymectomy on Capacity of Spleen Cells to Produce Immunologic Runt Disease.—

In the second group of experiments, the presence of immunologically competent cells in the spleens of mice thymectomized at 1 to 24 hours of age was studied by determining the incidence of immunologic runting among newborn mice injected with allogeneic adult spleen cells obtained from neonatally thymectomized donors at 2 months of age. Two strain combinations were used: $C_{57}Bl$ donors and A hosts, and A strain donors and C_3H hosts. In the basic study 5 million cells were administered intravenously. When this dosage failed to produce runt disease, an additional study was performed involving administration of 20 million cells from $C_{57}Bl$ donors to newborn A recipients. The results are recorded in Table I.

It will be seen in this table that, in both strain combinations, 5 million spleen cells from neonatally thymectomized animals failed to produce runting in the newborn recipients. To the contrary, 5 million spleen cells from 2-month-old donors not subjected to neonatal thymectomy regularly produced runt disease

and early death in the neonatal recipients. That this defect in the spleen cells of neonatally thymectomized animals is quantitative rather than qualitative is shown in the experiments involving a larger inoculum of cells. When 20 million cells from 2-month-old $C_{57}Bl$ mice that had been thymectomized at birth were administered intravenously to newborn A hosts, 5 of 11 recipients developed characteristic evidence of runt disease.

We conclude from these experiments that the peripheral lymphoid tissues (spleen) of mice thymectomized in the newborn period are markedly defective in their ability to produce graft *versus* host reactions. The defect is relative,

TABLE I

Effect of Neonatal Thymectomy on Capacity of Spleen Cells to Produce Runt Disease in Allogeneic Mice

Recipient strain	Donor strain	Previous treatment of donor	No. of cells injected (million)	Incidence of runt disease*
A	$C_{57}Bl/1$	Sham-thymectomy	5	27/28
		Neonatal thymectomy	5	1/29
		Neonatal thymectomy	20	5/11
		—	0	0/18
C_3H		Sham-thymectomy	5	13/31
		Neonatal thymectomy	5	1/61
		—	0	0/36

* Immunologic runt disease indicated by characteristic runting syndrome followed by death between days 5 and 30 after cell administration. Numerator equals number of animals that died, and denominator the number of animals in the group.

however; if a sufficiently large inoculum is employed, some immunologic reactivity of spleen cells from neonatally thymectomized animals can be demonstrated.

Induction of Immunologic Tolerance with Spleen Cells from Mice Thymectomized at Birth.—

The surviving mice from the experiments in the preceding section (A mice injected with $C_{57}Bl$ spleen cells, and C_3H mice receiving A strain spleen cells) were grafted at 6 to 7 weeks of age with skin taken from normal animals of the same strain as the cell donors. The results are shown in Table II.

The mice of the A strain, recipients of spleen cells from $C_{57}Bl$ mice at birth, were not tolerant of $C_{57}Bl$ skin. In fact, with the exception of 2 mice that kept the grafts for 55 to 60 days, the rejection time was about the same as that of non-injected control animals.

On the other hand, in the C_3H mice injected at birth with spleen cells from either normal or thymectomized A strain mice, long lasting tolerance (survival of skin for 6 months or more) was achieved in a similar proportion of both groups, 61 per cent of the mice receiving cells from normal donors and 58 per cent of the animals receiving cells from thymectomized donors.

Attempts to Reconstitute Neonatally Thymectomized Mice with Cell-Free Extracts.—Mice thymectomized at birth are frequently defective in growth, assume a hunched posture, and die at an early age. As indicated earlier, when the

TABLE II

Induction of Immunologic Tolerance with Spleen Cells from Mice Thymectomized at Birth

Recipient strain	Donor strain	Previous treatment of donor	No. of cells injected (million)	Incidence of tolerance*
A	$C_{57}Bl/1$	None	5	0/1‡
		Neonatal thymectomy	5	1/18§
		Neonatal thymectomy	20	1/5§
		—	0	0/18
C_3H	A	None	5	11/18
		Neonatal thymectomy	5	33/57
		—	0	0/34

* Tolerance indicated by the number of mice accepting skin graft from the strain of mice donating the cells at birth.

‡ Twenty-seven of 28 A strain animals injected with $C_{57}Bl$ spleen cells soon died of runt disease.

§ These 2 animals kept the homografts for 55 to 60 days and then the grafts were finally rejected.

thymectomy has been complete, such mice accept skin grafts across both weak and strong histocompatibility barriers. The observations reported above establish that the peripheral lymphoid tissues (spleen) of mice thymectomized as late as 35 days of age are defective with respect to capacity to induce graft *versus* host reactions.

To determine whether treatment with cell-free thymic or splenic extracts might reconstitute neonatally thymectomized animals, three experiments were performed. In the first experiment, mice were given repeated subcutaneous injections (3 times a week for 6 weeks beginning at 3 days of age) of an aqueous thymic extract, and the incidence of wasting disease compared with that of untreated neonatally thymectomized animals. In the second experiment, groups of mice thymectomized at 6 and 14 days of age were treated with aqueous extracts of either thymus or spleen, given 6 days a week for 6 weeks beginning at 1 month of age, and the graft *versus* host activity of their spleen cells compared with that of untreated animals thymectomized at the same ages. Finally, 6 mice thymectomized at 6 days of age were given "pro-

mine," an extract of thymus prepared by Szent-Gyorgyi and associates, in a dosage of 0.25 ml daily for 15 consecutive days beginning at 1 month of age, followed, after 10 days, by a test of graft *versus* host activity of their spleen cells compared to that of appropriate controls. The results of these experiments are summarized in Tables III and IV.

TABLE III

Effect of Aqueous Extract of Thymus on Wasting Syndrome in Mice

Group	Incidence of wasting disease
C_3H mice, thymectomized at birth, given aqueous thymic extract*	11/14 (78 per cent)‡
C_3H mice, thymectomized at birth, not treated	17/21 (81 per cent)
C_3H mice, sham-operated	0/24

* Complete thymectomy in neonatal period, 0.1 ml saline extract of thymus from syngeneic mice, begun at 3 days of age and continued 3 times per week for 2 weeks, and then 0.25 cc given subcutaneously 3 times per week over next 4 weeks.

‡ Mice showed growth failure, hunched posture, and died before 4 months of age.

TABLE IV

Effect of Aqueous Extract of Thymic Tissue and Promine on Immunologic Capacity of Peripheral Lymphoid Tissue of Thymectomized C_3H Mice

Group	Treatment	Immunologic activity of spleen cells in Simonsen's graft *versus* host reaction*
days		
Thymectomy, 6	Thymus extract‡	0/4
Thymectomy, 14	Thymus extract	0/4
Thymectomy, 6	Spleen extract	0/4
Thymectomy, 14	Spleen extract	0/4
Thymectomy, 6	Promine§	0/6
Thymectomy, 6	None	0/10
Thymectomy, 14	None	0/5
Sham-operated	—	10/10

* Mice were tested for graft *versus* host reactions by the spleen assay technique of Simonsen. Animals were sacrificed 10 days following the last injection of aqueous spleen extract and spleen cells tested in $(C_3H \times DBA/2)F_1$ animals. A positive spleen index for these experiments are litters with a spleen index of 1.40 or greater; mice whose spleen index falls below this value are considered negative.

‡ Subcutaneous injections of aqueous extracts of thymus or spleen from mice 2 to 3 weeks of age were given in a dosage of 0.25 ml 6 times a week for a period of 6 weeks.

§ Injections of "promine" (Szent-Gyorgyi) were begun at 1 month of age and given subcutaneously in a dosage of 0.25 ml for 15 consecutive days.

As shown in Table III, C_3H mice thymectomized at birth and treated with an aqueous thymic extract for 6 weeks showed an incidence of wasting and early death (before 4 months of age) comparable to control animals thymectomized at birth but not given thymus extract. Sham-operated C_3H mice kept under identical laboratory conditions did not show wasting or early death.

On Table IV are recorded observations which indicate that aqueous extracts of splenic or thymic tissue did not restore immunologic capacity of the spleen cells of mice thymectomized at 6 or 14 days of age. It is also apparent that Dr. Szent-Gyorgyi's compound, promine, shown to be a growth-promoting factor present in high concentration in the thymus, did not restore immunologic reactivity of the splenic tissue of mice thymectomized at 6 days of age.

Effect of Syngeneic Thymus Grafting and Administration of Syngeneic Lymphoid Cell Suspensions on Mice Thymectomized in the Neonatal Period.—

Several additional studies were performed in an effort to reconstitute mice thymectomized during the first 24 hours after birth. Among the methods used were: intraperitoneal injection at 2 days of age of 10 million viable cells from the thymus of 2-day-old donors; intraperitoneal injection at 2 days of age of 10 million viable thymus cells from 2-month-old donors; intraperitoneal injection at 2 days of age of 10 million spleen cells from 2-month-old donors; intravenous injection at 40 days of age of 100 million spleen or thymus cells from 2-month-old donors; and subcutaneous grafting at 2 days of age with thymus from 2-day-old donors. Three comparisons were made: survival to 8 months of age, rejection of allogeneic skin grafts, and capacity of the animal's spleen cells to induce splenomegaly after intraperitoneal injection into $(C_3H \times DBA/2)F_1$ hybrids.

The results of these experiments are summarized in Table V. It will be seen that of the three standards of reconstitution, the one most uniformly affected by treatment was longevity; indeed, every method used in these experiments contributed significantly toward prolongation of life in the neonatally thymectomized mice, and three of the treatments: intraperitoneal injection of thymus cells from 2-day-old donors at 2 days of age, intraperitoneal injection of spleen cells from 2-month-old mice at 2 days of age, and intravenous administration of spleen cells from 2-month-old donors at 40 days of age, were as effective in increasing the life span as was subcutaneous grafting of thymus in the neonatal period.

With respect to the second criterion of recovery, rejection of allogeneic (DBA/2) skin grafts, the two uniformly effective methods were grafting of neonatal thymus in the neonatal period and administration of spleen cells from 2-month-old donors either at 2 or 40 days of age. Injections of suspensions of viable thymic cells from neonatal or 2-month-old donors appeared to provide very little restoration of capacity to reject skin grafts.

Finally, reconstitution of capacity of the spleen cells of the thymectomized mice to exercise a graft *versus* host reaction in hybrid hosts was assessed. By this standard, grafting of syngeneic thymus and injection at 40 days of 100 million spleen cells from 2-month-old donors proved to be most efficient. Much less consistent evidence of restoration of this function was evidenced after administration of thymus cells, whether from newborn or 2-month-old donors, and whether administered at 2 or 40 days of age. Intermediate in effect, but

definitely effective in restoring immunologic activity of spleen cells, was intraperitoneal injection at 2 days of age of spleen cells from 2-month-old animals.

Thus, we conclude that subcutaneous grafting of a syngeneic thymus, intraperitoneal injection of syngeneic adult spleen cells at an early age, or intravenous administration of large numbers of syngeneic adult spleen cells at 40 days of

TABLE V

Effects of Syngeneic Cells and Thymus Grafts on C_3H Mice Thymectomized in the Neonatal Period

Treatment	Age when treated	Survivors at 8 months		DBA/2 skin grafts*	Spleen assay of graft *versus* host reaction‡ (spleen index)
	days		*per cent*		
Injection (i.p.) of 10 million 2-day-old thymus cells....	2	9/10	90	4/7	1.00 1.03 1.21 1.45
Injection (i.p.) of 10 million 2-month-old thymus cells..	2	17/26	73	6/12	0.98 0.99 1.05 1.22 1.60
Injection (i.p.) of 10 million 2-month-old spleen cells...	2	19/20	95	0/12	1.04 1.32 1.45 1.53 1.88 2.0
Grafting (subcut.) of 2-day-old thymus.....	2	12/14	86	0/6	1.60 1.97 2.15 2.48 2.63
Injection (i.v.) of 100 million 2-month-old thymus cells.....	40	6/11	55	2/5	0.91 1.14 1.19 1.98
Injection (i.v.) of 100 million 2-month-old spleen cells.....	40	12/13	92	0/6	1.52 1.76 2.14 2.37
Non-injected thymectomized controls.....	—	2/15	13	6/8	0.95 to 1.25 (10 mice)
Non-injected unthymectomized controls.....	—	15/16	94	0/15	1.85 to 3.08 (10 mice)

* Number of mice accepting DBA/2 skin graft for 30 days or more/number of mice grafted.

‡ Spleen assay performed when animals were 2 to 4 months old by injecting 10 million spleen cells into 8-day-old ($C_3H \times DBA/2)F_1$ hybrids. Mice were sacrificed 8 days later and spleen index calculated as described in Material and Methods.

age, will restore the immunologic capabilities of neonatally thymectomized mice by the three criteria used in this evaluation.

Immunologic Characteristics of Neonatally Thymectomized Mice Bearing Allogeneic Thymus Grafts.—

In this experiment, A and C_3H mice were thymectomized or sham-operated on the 1st day of life. Two days later, the A animals were grafted with thymus from 2-day-old C_3H donors, and the C_3H mice were grafted with thymus from 2-month-old A donors. Thymectomized C_3H and sham-operated A and C_3H animals served as controls.

As will be seen in Table VI, the thymectomized A strain mice grafted with neonatal C_3H thymus accepted skin grafts from both A and C_3H donors at 35 days of age, but regularly rejected skin from DBA/2 animals. Similarly, C_3H mice bearing thymus grafts from 2-month-old A donors accepted skin from both A and C_3H donors and rejected third party skin from DBA/2 animals in every instance. By contrast, control C_3H mice thymectomized in the neonatal period accepted skin grafts from both A and DBA/2 donors. As is to be expected, intact C_3H and A strain mice rejected the allogeneic grafts across the H-2 barrier in every instance.

From these observations, it appears that mice thymectomized in the immediate neonatal period can be restored to a state of normal or nearly normal immunologic reactivity with respect to transplantation immunity by allogeneic grafts of thymic tissue, but that the animals become chimeric and thus capable of accepting skin grafts from the same strain as that of the thymus donor.

TABLE VI

Skin Homograft Survival in Mice Thymectomized at Birth and Grafted Subcutaneously 2 Days Later with Thymus Gland from an Allogeneic Strain

Groups	Skin grafts*		
	A	C_3H	DBA/2
Thymectomized A, grafted with 2-day-old C_3H thymus	8/8	7/8	1/7
Thymectomized C_3H, grafted with 2-month-old A thymus	8/8	9/9	0/5
Thymectomized C_3H, no thymus graft	7/11	—	6/8
Sham-thymectomized C_3H, no thymus graft	0/11		0/11
Sham-thymectomized A, no thymus graft		0/10	0/10

* Number of mice taking the graft for 30 days or more/number of mice grafted.

Immunologic Analysis of the Chimeric Lymphoid Tissue of Neonatally Thymectomized Mice Bearing Allogeneic Thymus Grafts.—

The next group of experiments was undertaken to determine whether the immunologic recovery of neonatally thymectomized mice grafted with allogeneic thymus tissue reflects recovery of host lymphoid cells or seeding of thymic cells to the spleen and lymph nodes where proliferation and immunologic function might occur.

Thymectomy was performed in the neonatal period on a large group of mice of the A and C_3H strains. Each animal received a thymus graft from either a newborn or 2-month-old donor of the other strain. Others were grafted with splenic tissue from 2-month-old allogeneic donors, and still others received xenogeneic thymus grafts from 2-day-old rat donors. The spleens of

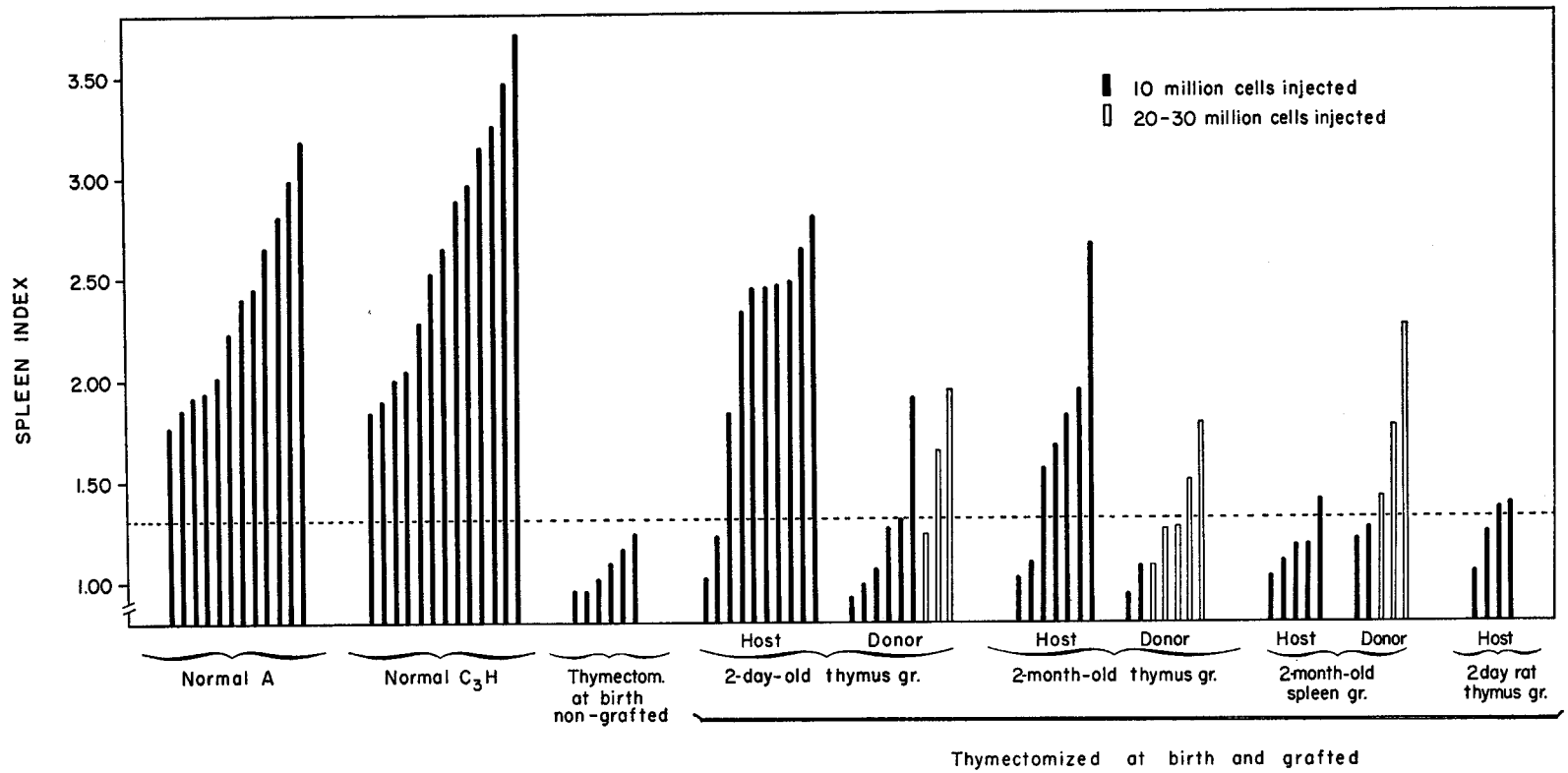

FIG. 2. Reconstitution by thymus and spleen transplants of immunologic capacity in mice thymectomized at birth. Neonatal thymectomy abolishes capacity of spleen cells to exercise graft *versus* host reactions. Homotransplantation of thymus from 2-day-old or 2-month-old donors reconstitutes immunologic capacity of the neonatally thymectomized mouse. The reconstitution obtained is attributable to both donor and host components, with quantitatively greater capacity being due to the host component. Spleen grafts are less effective than thymus grafts, but reconstitute with the donor component.

the 2- to 4-month-old animals were assayed for donor and host components (host component only of mice receiving rat cells) of the presumed chimera using the discriminating spleen assay of Simonsen and Jensen (34). As indicated in the section on methods, the cell recipients were 8-day-old hybrid mice, (A × $C_{57}Bl$)F_1 for the crucial host component of A mice bearing

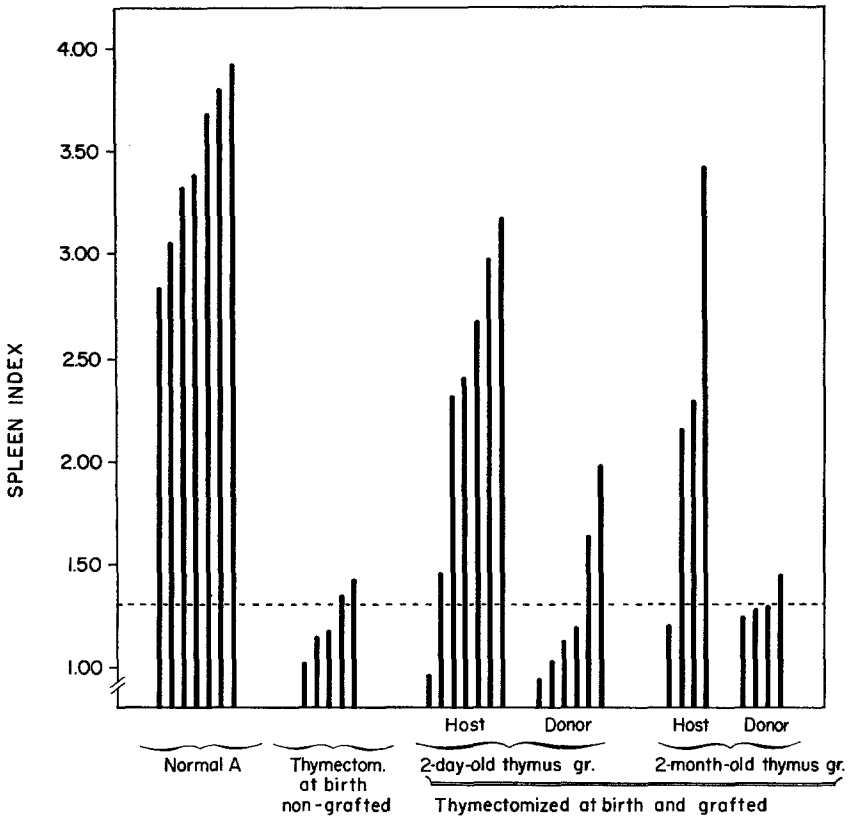

FIG. 3. Reconstitution of immunologic capacity of lymph node cells of neonatally thymectomized mice with allogeneic thymus transplants. Here, too, the thymus transplant reconstitutes primarily the host-type immunologic capacity.

C_3H thymuses, and (C_3H × $C_{57}Bl$)F_1 for the host component of C_3H mice bearing A strain thymus tissue. The spleen index was calculated for each group.

The results are summarized in Fig. 2. The spleen index of normal adult A and C_3H spleen cells in the appropriate F_1 hybrid was always near 2 or greater. By contrast, mice thymectomized in the neonatal period and not treated further had spleen indexes of less than 1.3 in every instance. The neonatally thymectomized mice grafted in the newborn period with thymus from 2-day-old allogeneic donors regularly showed significant restoration of immunologic func-

tion. In the discriminating spleen assay, we found that the host component was predominant, but that a donor component was also demonstrable in some of the animals. When the transplant was taken from a 2-month-old donor, restoration of splenic immunologic activity was also accomplished, but the cell activity was less vigorous. Again the restoration was attributable primarily to development of host cells, although some development of donor cell activity was also noted.

When allogeneic spleen tissue from 2-month-old donors was transplanted to neonatally thymectomized mice, less restoration of immunologic competence occurred, but it was attributable almost entirely to donor cells.

Finally, thymus tissue from 2-day-old rats was transplanted to neonatally thymectomized mice. Although these grafts took at first, became vascularized, and survived for a time, they failed to restore immunologic competence of the host component as determined by the discriminating spleen assay.

In the experiments summarized in Fig. 3, the discriminating spleen assay was again used to determine the relative prominence of the host component in the lymph nodes of neonatally thymectomized mice reconstituted with allogeneic thymus grafts from either 2-day-old or 2-month-old donors. As will be seen in the figure, a marked degree of immunologic restoration occurred, attributable largely to activity of host cells. However, in 2 of 6 animals receiving thymus tissue from 2-day-old donors, a significant donor component could be demonstrated.

DISCUSSION

The first group of experiments reported in this study, using the Simonsen assay technique, shows that thymectomy performed even as late as 35 days after birth in mice interferes with development of full immunologic competence as the animals get older. Indeed, from these data, it seems likely that thymectomy interferes with the development of the peripheral lymphoid tissues beyond the phase of maturation reached by the time the surgery is carried out. Looking at these results in another way, it can be reasoned, in support of the earlier observations of Makinodan and Peterson (35), that the immunologic development of the lymphoid tissues in mice continues for a prolonged period after birth and that such development is, at least in part, thymus-dependent.

It also seems clear from the observations presented here that, as revealed in the graft *versus* host assay, mice do not show appreciable recovery of immonologic competence following thymectomy even though they are studied as long as 6 months later. In this manner mice differ from rabbits. Archer *et al.* (36) have observed recovery from the morphologic deficit produced by neonatal thymectomy in rabbits, a recovery they attribute to the function of the appendix in this animal.

The demonstration in the initial group of experiments that large inocula of

spleen cells from neonatally thymectomized animals will induce runting in appropriate F_1 recipients indicates, as might be inferred from earlier work on the antibody producing capacity of these animals (8), that the defect produced by neonatal thymectomy in the mouse is quantitative and not qualitative. Further, the data show clearly that, although thymectomy at birth interferes with the development of immunologic capacity of splenic tissue, this procedure does not interfere demonstrably with the capacity of the splenic tissue of A strain mice to produce immunologic tolerance in neonatal C_3H mice. It has not been possible heretofore to induce immunologic tolerance by injecting neonatal A strain mice with $C_{57}Bl$ spleen cells. Instead, a high incidence of severe runt disease regularly follows such cell administration, and any animals surviving are not tolerant of $C_{57}Bl$ skin (32, 37).

With the availability of neonatal thymectomy as a means of reducing the capacity of spleen cells from $C_{57}Bl$ mice to react against the A strain hosts, we were hopeful that finally tolerance might be produced in these recalcitrant strain combinations. The results were unexpected and perplexing: under the conditions of our study, the A strain mice injected at birth with spleen cells from adult $C_{57}Bl$ mice thymectomized at birth did not develop immunologic tolerance. Thus, for reasons as yet unclear, induction of tolerance of $C_{57}Bl$ skin in A mice is still not possible in our laboratories.

In the studies reported here, numerous attempts to reconstitute the mice thymectomized at birth have been carried out. Thus far, none of the cell-free materials used has been effective in preventing runt disease and early death, or in reinstating immunologic competence. However, such negative results are not of great moment, and attempts to find a non-cellular substance of this nature in the thymus, which might reveal an endocrinologic role for this organ, must be continued.

Our studies of syngeneic thymus grafts and injection of isolated syngeneic cells from thymus and spleen indicate that significant reconstitution of thymectomized mice can be achieved by several methods. Thymus cells from 2-day-old or 2-month-old donors, spleen cells from 2-month-old donors, and grafted neonatal thymus all made possible regular survival of neonatally thymectomized mice to 8 months. However, restoration of capacity to reject allogeneic skin grafts was accomplished by only two methods: transplantation of thymus from newborn syngeneic donors and intravenous or intraperitoneal injection of spleen cells from adult donors of the same strain. The findings regarding the efficacy of neonatal thymus grafts and of neonatal administration of syngeneic peripheral lymphoid cells parallel those of Miller (11, 14). He used the $C_{57}Bl$ strain, rather than the C_3H, and administered syngeneic lymph node cells, in a dosage of 5 to 10 million cells, on the 5th day of life. Sixty per cent of the animals escaped the early death of wasted animals, and all the survivors had adequate homograft immunity. Lymphoid cells given after the first week of life had no

effect. The appreciably higher rate of survival and the effectiveness of late (40 days) administration of spleen cells in our studies probably reflects strain differences in the severity of the wasting process. The data of Parrott and East (8) indicate that animals of both the C_3H/Bi and $C_{57}Bl$ strains consistently develop wasting symptoms after neonatal thymectomy, but that death occurs earlier and more precipitately in the $C_{57}Bl$. Parrott and East (8) have prevented wasting disease in 11 of 12 C_3H/Bi mice by administration of 4 to 5 million spleen cells intravenously 1 or 2 days after thymectomy. In addition, they restored all 7 neonatally thymectomized mice of this strain grafted with whole syngeneic thymus in the kidney capsule in the newborn period.

Finally, in studies of the reconstitution of the capacity of the spleen cells of the neonatally thymectomized mice to produce graft *versus* host reactions, only two of the treatments appeared to give full restoration of function: transplantation of syngeneic newborn thymus at an early age and intravenous injection of a large dose of syngeneic spleen cells from an immunologically mature animal prior to the onset of wasting disease in the thymectomized recipient. Some restoration, of lesser degree, was evident in animals receiving spleen cells from a mature donor intraperitoneally at 2 days of age.

The observations, particularly the efficacy of peripheral lymphoid cells in restoring immunologic capacity, are compatible with the concept that the deficiencies of neonatally thymectomized mice reflect a deficit of peripheral lymphoid cells and do not necessarily imply endocrinologic effects or effects other than those attributable to failure of normal development of the lymphoid tissue. They would not, however, be inconsistent with the possible operation of an endocrinologic function of the thymus.

Our observations on allogeneic thymus transplantation to neonatally thymectomized mice agree substantially with those of Miller (11). It is shown that neonatally thymectomized mice bearing allogeneic thymus grafts are chimeric and able to accept skin grafts from the strain of the thymus donor. The same animals are fully capable of rejecting skin grafts from a third strain. Thus, allogeneic thymus grafts appear to be capable of producing full immunologic tolerance toward the strain donating the thymus and restoring immunologic capacity in the thymectomized host.

Finally, perhaps the most interesting observations of this series are those employing the discriminant spleen assay of Simonsen and Jensen (34) to study the immunologic characteristics of the spleen and lymph node cells of mice thymectomized at birth and transplanted with allogeneic thymus grafts from 2-day-old donors. These results again establish that thymus transplants can reconstitute immunologic reactivity of both spleen and lymph nodes of the thymectomized mouse.

In keeping with Miller's observations (11, 13), using the T6 marker system, we found that, quantitatively, the primary reconstitution occurs with respect to

the host component in the peripheral lymphoid tissues. However, in some animals there is an appreciable donor component. By contrast, grafts of spleen from 2-month-old donors were less effective in restoring the immunologic capabilities of neonatally thymectomized mice; however, when such restoration occurred it was attributable entirely to the donor component of the chimera. These observations suggest, certainly, that cells of thymic origin can "peripheralize" to the spleen and lymph nodes, as was first suggested by Beard (38) and subsequently reiterated by several investigators (39–41). On the other hand, and quantitatively more important, the allogeneic thymus graft appears to be essential to development of lymphoid cells which have the immunologic characteristics of the host. This finding could reflect a distant effect of the thymus transplant, such as might be expected of an endocrine organ, or it could be a consequence of direct cell-to-cell influence, either by the thymus cells acting upon splenic mesenchymal cells of the host or by an influence of the mesenchymal cells of the host on some of the "peripheralizing" thymic cells. Scientific demonstration of any of these several possibilities would be most contributory to the understanding of a number of basic biologic processes in mammals.

SUMMARY

The immunologic competence of spleen cells of mice, as assessed by their graft *versus* host capabilities, increases to 35 days of age and beyond. Thymectomy at any point along this continuum of development produces "immunologic arrest:" the peripheral lymphoid tissues of such mice do not show significant increases in activity as the animals mature, nor is there appreciable loss of activity up to 6 months after surgery.

Adult spleen cells from mice thymectomized at 1 to 24 hours of age have a greatly reduced ability to induce runt disease. Five million spleen cells from immunologically mature animals will uniformly cause fatal runt disease in neonatal recipients, but this same number of cells from neonatally thymectomized animals produces almost no runt disease. When the dosage of cells from neonatally thymectomized C_{57}Bl mice is increased to 20 million, about half of the A recipients develop runt disease. Thus, the defect is a quantitative one.

Spleen cells from neonatally thymectomized mice will induce tolerance of skin grafts from the donor strain. In one recalcitrant strain combination, C_{57}Bl to A, use of spleen cells from neonatally thymectomized donors as the tolerance-inducing inoculum permits survival of the recipients, which usually die with severe runt disease, but does not induce tolerance.

Cell free extracts of spleen and thymus tissue, including "promine" of Szent-Gyorgyi *et al.*, did not affect the runting syndrome or the immunologic reactivity of neonatally thymectomized mice.

When syngeneic thymic tissue is grafted into neonatally thymectomized mice, or the animals are given viable syngeneic spleen or thymus cells, the majority of the animals escape the early mortality characteristic of this group. Administration of syngeneic spleen cells from adult donors and grafting of syngeneic neonatal thymus provide restoration of homograft immunity and graft *versus* host reactivity of the peripheral lymphoid tissues in most of the neonatally thymectomized animals. Thymus cells rarely provide significant restoration of these parameters.

Allogeneic thymus grafts also restore neonatally thymectomized mice. Such animals are chimeric: the immunologically competent cells of their peripheral lymphoid tissues are chiefly of host origin as determined by the discriminant spleen assay, but in many instances a significant donor component is also demonstrable in this system. These chimeric animals accept skin grafts from both donor and host strains.

A degree of reconstitution has also been attained by grafting of allogeneic adult spleen in neonatally thymectomized animals. The discriminant spleen assay indicates that cells of the donor strain predominate in the peripheral lymphoid tissues of such mice.

BIBLIOGRAPHY

1. MacLean, L. D., Zak, S. J., Varco, R. L., and Good, R. A., Thymic tumor and acquired agammaglobulinemia—a clinical and experimental study of the immune response, *Surgery*, 1956, **40**, 1010.
2. Miller, J. F. A. P., Immunological function of the thymus, *Lancet*, 1961, **2**, 748.
3. Archer, O., and Pierce, J. C., Role of thymus in development of the immune response, *Fed. Proc.*, 1961, **20**, 26.
4. Martinez, C., Kersey, J., Papermaster, B. W., and Good, R. A., Skin homograft survival in thymectomized mice, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109**, 193.
5. Martinez, C., Dalmasso, A., and Good, R. A., Acceptance of tumour homografts by thymectomized mice, *Nature*, 1962, **194**, 1289.
6. Miller, J. F. A. P., Effect of neonatal thymectomy on the immunological responsiveness of the mouse, *Proc. Roy. Soc. London, Series B*, 1962, **156**, 415.
7. Papermaster, B. W., Dalmasso, A. P., Martinez, C., and Good, R. A., Suppression of antibody forming capacity with thymectomy in the mouse, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111**, 41.
8. Parrott, D. M. V., and East, J., Studies on a fatal wasting syndrome of mice thymectomized at birth, *in* The Thymus in Immunobiology, (R. A. Good and A. E. Gabrielsen, editors), New York, Hoeber-Harper, 1964, in press.
9. Parrott, D. M. V., Strain variation in mortality and runt disease in mice thymectomized at birth, *Transplant. Bull.*, 1962, **29**, 102.
10. Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O. K., Pierce, J. C., and Papermaster, B. W., The role of the thymus in development of immunologic capacity in rabbits and mice, *J. Exp. Med.*, 1962, **116**, 773.

11. Miller, J. F. A. P., Role of the thymus in transplantation immunity, *Ann. New York Acad. Sc.*, 1962, **99,** 340.
12. Dalmasso, A. P., Martinez, C., and Good, R. A., Failure of spleen cells from thymectomized mice to induce graft versus host reactions, *Proc. Soc. Exp. Biol. and Med.*, 1962, **110,** 205.
13. Miller, J. F. A. P., Marshall, A. H. E., and White, R. G., The immunological significance of the thymus, *Advances Immunol.*, 1962, **2,** 111.
14. Miller, J. F. A. P., The thymus and immunity, *Lancet*, 1963, **1,** 43.
15. Jankovic, B. D., Waksman, B. H., and Arnason, B. G., Role of the thymus in immune reactions in rats. I. The immunologic response to bovine serum albumin (antibody formation, Arthus reactivity, and delayed hypersensitivity) in rats thymectomized or splenectomized at various times after birth, *J. Exp. Med.*, 1962, **116,** 159.
16. Arnason, B. G., Jankovic, B. D., Waksman, B. H., and Wennersten, C., Role of the thymus in immune reactions in rats. II. Suppressive effect of thymectomy at birth on reactions of delayed (cellular) hypersensitivity and the circulating small lymphocyte, *J. Exp. Med.*, 1962, **116,** 177.
17. Waksman, B. H., Arnason, B. G., and Jankovic, B. D., Role of the thymus in immune reactions in rats. III. Changes in the lymphoid organs of thymectomized mice, *J. Exp. Med.*, 1962, **116,** 187.
18. Archer, O. K., Pierce, J. C., Papermaster, B. W., and Good, R. A., Reduced antibody response in thymectomized rabbits, *Nature*, 1962, **195,** 191.
19. Sherman, J. D., and Dameshek, W., "Wasting disease" following thymectomy in the hamster, *Nature*, 1963, **197,** 469.
20. Sherman, J. D., Adner, M. M., Costea, N., Schwartz, R., Lewis, F. B., and Dameshek, W., The function of the thymus gland in the golden hamster, *Fed. Proc.*, 1963, **22,** 600.
21. Defendi, V., Roosa, R. A., and Koprowski, H., Effect of thymectomy at birth on response to tissue, cells and virus antigens, *in* The Thymus in Immunobiology, (R. A. Good and A. E. Gabrielsen, editors), New York, Hoeber-Harper, 1964, in press.
22. Roosa, R. A., Wilson, D., and Defendi, V., Effect of neonatal thymectomy in hamsters, *Fed. Proc.*, 1963, **22,** 599.
23. Martinez, C., Dalmasso, A. P., and Good, R. A., Homotransplantation of normal and neoplastic tissue in thymectomized mice, *in* The Thymus in Immunobiology (R. A. Good and A. E. Gabrielsen, editors), New York, Hoeber-Harper, 1964, in press.
24. Miller, J. F. A. P., Immunological significance of the thymus of the adult mouse, *Nature*, 1962, **195,** 1318.
25. Archer, O. K., Kelly, W. D., Papermaster, B. W., and Good, R. A., Further morphological and immunological studies of the role of the thymus in immunobiology, *Fed. Proc.*, 1963, **22,** 599.
26. Archer, O. K., Papermaster, B. W., and Good, R. A., Thymectomy in rabbit and mouse: consideration of time of lymphoid peripheralization, *in* The Thymus in Immunobiology, (R. A. Good and A. E. Gabrielsen, editors), New York, Hoeber-Harper, 1964, in press.

27. Auerbach, R., Thymus: its role in lymphoid recovery after irradiation, *Science*, 1963, **139**, 1061.
28. Dischler, W., and Rudali, G., La thymectomie totale chez le souriceau nouveau né, *Rev. franç. etudies clin. et biol.*, 1961, **6**, 88.
29. Martinez, C., Smith, J. M., Aust, J. B., and Good, R. A., Acquired tolerance to skin homografts in mice of different strains, *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 736.
30. Szent-Gyorgyi, A., Hegyeli, A., and McLaughlin, J. A., Constituents of the thymus gland and their relation to growth, fertility, muscle, and cancer, *Proc. Nat. Acad. Sc.*, 1962, **48**, 1439.
31. Simonsen, M., Engelbreth-Holm, J., Jensen, E., and Poulsen, H., A study of the graft-*versus*-host reaction in transplantation to embryos, F_1 hybrids, and irradiated animals, *Ann. New York Acad. Sc.*, 1958, **73**, 834.
32. Billingham, R. E., and Brent, L., Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease, *Phil. Tr. Roy. Soc. London, Series B*, 1958, **242**, 439.
33. Siskind, G. W., and Thomas, L., Studies on the runting syndrome in newborn mice, *in* Biological Problems of Grafting, (F. Albert and G. Lejeune-Ledant, editors), Springfield, Illinois, Charles C. Thomas, 1959, 176.
34. Simonsen, M., and Jensen, E., The graft *versus* host assay in transplantation chimaeras, *in* Biological Problems of Grafting, (F. Albert and G. Lejeune-Ledant, editors), Springfield, Illinois, Charles C. Thomas, 1959, 214.
35. Makinodan, T., and Peterson, W. J., Relative antibody-forming capacity of spleen cells as a function of age, *Proc. Nat. Acad. Sc.*, 1962, **48**, 234.
36. Archer, O. K., Sutherland, D. E. R., and Good, R. A., Appendix of the rabbit: a homologue of the bursa in the chicken?, *Nature*, in press.
37. Good, R. A., Martinez, C., and Gabrielsen, A. E., Progress toward transplantation of tissues in man, *Advances Pediat.*, in press.
38. Beard, J., The development and probable function of the thymus, *Anat. Anz.*, 1894, **9**, 476.
39. Ruth, R. F., Ontogeny of the blood cells, *Fed. Proc.*, 1960, **19**, 579.
40. Auerbach, R., Genetic control of thymus lymphoid differentiation, *Proc. Nat. Acad. Sc.*, 1961, **47**, 1175.
41. Burnet, F. M., Role of the thymus and related organs in immunity, *Brit. Med. J.*, 1962, **2**, 807.