STUDIES ON THYMUS FUNCTION

III. DURATION OF THYMIC FUNCTION*†

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Immunological functions can be restored in neonatally thymectomized mice with transplants of allogeneic or hemiallogeneic thymus (1–5) or functional thymoma grafts (7). In certain strain combinations, the thymectomized hosts eventually reject the restoring grafts after a period of temporary growth (5–7). Since these mice, although immunologically reconstituted, have no thymic function, long-term studies on the persistence of the thymus-dependent immune capacities were performed. It was predicted that in the absence of the thymus the restored immune functions should decay with time, mimicking the influence of adult thymectomy on immune capacity (8–10). A decrease with time of thymus-dependent immune functions was indeed observed after restoration in the absence of thymus. Whether the animals rejected spontaneously the restoring allogeneic graft or were subjected to surgical excision of syngeneic thymus grafts after a period of temporary growth, a marked decline of immune functions was found when the animals were tested at 200–600 days of age. This decrease of the immune functions was more profound than the physiological decay of immunity observed in normal animals of similar age. Our findings indicate that the continuous presence of the thymus is essential for the long-term maintenance of the immunological apparatus responsible for the thymus-dependent immune responses.

Materials and Methods

Mice.—Inbred mice of C3Hf, A, CBA, and C57BL/1 strains and their F1 hybrids were used. These mice are derived from the colony of the late Doctors J. J. Bittner and C. Martinez and have been rigorously inbred. Details on the strains and of animal care have been described in previous publications (7).

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Procedures.—Neonatal and adult thymectomies and subcutaneous grafting of thymomas were performed according to techniques previously described (7). Since recovery of intraperitoneal thymus grafts is somewhat difficult (5), our previous technique was modified for the present experiments. Thymuses from 10-day old donors were attached to a 7 × 5 mm fragment of Millipore filter (mean pore size 0.45 μ, Millipore Corp., Bedford, Mass.) with a drop of syngeneic mouse plasma and implanted intraperitoneally as described in a previous publication (5). Thymus grafts could be located easily and surgical extirpation was performed by removing the filter fragment with its attached graft.

Skin grafting was performed using full-thickness tail skin placed in the backs of the recipients (11). Skin graft size was 10 × 7 mm in all experiments. Skin-grafted animals were kept in individual cages until grafts healed completely and were observed daily beginning 4 days after grafting.

The graft-versus-host (GVH) assay was performed in 8-day old (C3H × C57BL/1)F1 mice injected intraperitoneally with 10 × 10^6 spleen cells from C3Hf experimental donors and the spleen indices were calculated after sacrifice of the animals 8 days later, as described in a previous publication (7).

Sensitivity to mouse hepatitis was tested by intraperitoneal injection of 0.5 ml of a 10% liver suspension in buffered saline, prepared from infected livers from 10-day old C3Hf mice (12) that had been injected 5 days previously with 0.2 ml of 10% liver suspension derived from a liver which in turn had macroscopic lesions that appeared spontaneously in a 40-day old neonatally thymectomized C3Hf mouse (13). The present mouse hepatitis virus (MHV), although not completely characterized, has infectious behavior comparable to that of MHV-1 virus (13). The hepatitis virus used produces a lethal or severe liver infection in newborn C3Hf mice or such mice less than 20 days of age. It produced a very mild disease when administered to animals 30 days of age or older.

The response to phytohemagglutinin (PHA) was performed using spleen cells and using the same technique reported in a previous publication (14). The cultures consisted of 10 × 10^6 spleen cells cultured for 48 hr with PHA-P (Difco Laboratories, Inc., Detroit, Mich.) and the results were expressed as mean thymidine-3H incorporation expressed in counts per minute, in cultures pulse labeled during the final 12 hr of culture.

At the end of each experiment the animals were sacrificed by ether inhalation and the absence of mediastinal thymic tissue was verified under the dissecting microscope and by microscopic examination of histological preparations of the mediastinal contents. Animals with residual thymus were excluded from consideration in the final analysis.

Experimental Design.—Immunological functions of the different experimental groups were measured by the capacity to reject first and second skin allografts, capacity of their spleen cells to produce graft-versus-host reactions, resistance to mouse hepatitis virus infection, and response of spleen cells to PHA. Since long-term experiments were planned, the experimental mouse strain selected was the C3Hf (free of mammary tumor virus) the mice of which have a very low incidence of spontaneous tumors. The experimental groups included: (a) normal male and female C3Hf mice of different ages; (b) neonatally thymectomized C3Hf mice grafted at 15 days of age with a syngeneic thymus by the intraperitoneal route; (c) same as group b but the thymus graft was removed surgically 10, 20, or 30 days after grafting; (d) neonatally thymectomized C3Hf mice grafted subcutaneously at 15 days of age with 1 × 10^8 cells from a strain A functional thymoma; only mice that rejected the thymic tumor after temporary growth were selected for further study; (e) C3Hf mice thymectomized at 30 days of age.

Details of origin and transplantation characteristics of the strain A thymoma have been reported in a previous publication (7). Groups c and d represented animals exposed to thymic function for a limited and variable period.

Abbreviations used in this paper: GVH, graft-versus-host; MHV, mouse hepatitis virus; PHA, phytohemagglutinin.
Immunological functions of the different groups were tested at 100, 200, 300, 400, 500, and sometimes at 600 days of age. The life-span of the C3Hf mice in our colony is 590 ± 16 days (mean and se) for males and females combined. This data is derived from observations on both breeders and virgin stock. The experimental design for evaluation of immune functions was somewhat different for each test.

For skin allograft rejection, the different experimental groups were separated at random at weaning, and grafted with CBA (Table I) or A strain (Table II) skin at the different ages indicated. For the groups grafted at 500 days of age all of the animals were sacrificed 50 days after grafting, and many animals were killed with grafts in place as is indicated in Tables I-III. This fact accounts for the lower rejection times in these groups.

For second-set skin allografts (Table III), all of the animals were immunized with \(20 \times 10^6\) spleen cells from A donors, injected intraperitoneally and twice subcutaneously in the back, at weekly intervals, so that the last immunization was 10 days before grafting A skin at the different ages indicated.

For testing graft-versus-host reactivity, the experiments were performed using animals selected at random from the four experimental groups at the different ages studied. The selected animals were sacrificed and their spleens were used for the graft-versus-host assay. In one experiment (Table IV) three additional groups were used: (a) animals grafted with a C57BL thymus intraperitoneally at 15 days of age; these animals reject the restoring thymus in the majority of the cases (6); (b) animals grafted at 15 days of age with C3Hf thymus within a diffusion chamber (DC); chambers were constructed with 0.22 μm pore size filters as described previously (15). (c) mice injected intraperitoneally at 15 days of age with \(200 \times 10^6\) spleen cells from syngeneic 60-day old female donors.

In another set of experiments (Table VI), individual animals of different experimental groups were tested twice for graft-versus-host (GVH) reactivity at 100 and 400 days of age, by performing partial splenectomy for the first test. Partial splenectomy was performed through a left lateral incision by attrition of the spleen in two halves with a surgical silk suture.

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**TABLE I**

Effect of Age and Thymic Function on Allograft Rejection

| Experimental group* | Age  | No. of mice | Mean rejection ± se |
|---------------------|------|-------------|---------------------|
| Sham thymectomized  |      |             |                     |
| 100                 | 9    | 12.1 ± 1.6  |
| 200                 | 5    | 12.1 ± 1.7  |
| 300                 | 7    | 12.5 ± 1.8  |
| 400                 | 8    | 12.4 ± 1.9  |
| 500                 | 9    | 12.9 ± 1.9  |
| Neonatally thymectomized, rejected A thymoma | 100 | 7 | 12.3 ± 1.8 |
| 200                 | 8    | 16.0 ± 4.6  |
| 300                 | 8    | 32.7 ± 15.8 |
| 400                 | 8    | 53.4 ± 22.0 |
| 500                 | 6    | 42.0 ± 12.6*|

* All mice were C3Hf/Umc mice grafted at different ages (indicated under "Age") with allogeneic skin from CBA mice. The neonatally thymectomized mice were grafted with strain A thymoma at 15 days of age and rejected the tumor after 27 days of temporary growth (12–38 days).

† Sacrificed 50 days after grafting and some animals were killed with accepted skin grafts. Actual rejection times for the group were 22, 30, 50, 50, 50, and 50 days.
For testing susceptibility to hepatitis infection (Table VII), animals were selected at random from the different experimental groups and infected at 100, 200, 400, or 500 days of age. Susceptibility to the virus was indicated by spontaneous death within 10 days after infection or by the presence of macroscopic liver lesions at sacrifice, 10 days later.

The capacity of spleen cells to respond to PHA in vitro was also measured (Table VIII) and the ages of the animals tested ranged from 40 to 500 days of age. The experimental groups studied consisted of thymectomized untreated (tested only at 40 and at 100 days of age due
to poor survival) mice, sham-thymectomized mice, and thymectomized mice exposed to a thymus graft for 20 days (these mice were grafted at 15 days of age with one syngeneic thymus implanted intraperitoneally and removed surgically 20 days later).

In a special experiment, neonatally thymectomized C3Hf mice grafted with a syngeneic thymus graft or exposed to a thymus graft for only 20 days were studied at 400 days of age (Table IX). Their spleen cells were tested for response to PHA while the lymph node cells from the same animal were tested for their capacity to produce GVH reactions in (C3H × C57BL)F1 hybrids injected with $5 \times 10^6$ lymph node cells.

### TABLE II

| Experimental group* | Age | No. of mice | Mean rejection ± sD |
|---------------------|-----|-------------|---------------------|
| Normal or sham thymectomized† | 100 | 13 | 12.4 ± 1.8 |
|                      | 300 | 13 | 12.6 ± 1.8 |
|                      | 400 | 13 | 12.8 ± 2.3 |
|                      | 500 | 13 | 13.4 ± 1.9 |
|                      | 600 | 13 | 13.4 ± 2.1 |
| Neonatally thymectomized and syngeneic thymus graft | 100 | 7 | 13.0 ± 2.5 |
|                      | 300 | 8 | 13.4 ± 2.3 |
|                      | 400 | 9 | 13.4 ± 2.9 |
|                      | 500 | 9 | 15.1 ± 2.6 |
| Neonatally thymectomized and syngeneic thymus removed after 20 days | 100 | 6 | 12.0 ± 1.5 |
|                      | 200 | 7 | 14.9 ± 3.8 |
|                      | 300 | 8 | 26.6 ± 9.9 |
|                      | 400 | 12 | 46.6 ± 23.5 |
|                      | 500 | 6 | 39.0 ± 13.1§ |
| Thymectomy at 30 days of age | 100 | 6 | 12.5 ± 1.7 |
|                      | 300 | 7 | 14.0 ± 2.6 |
|                      | 400 | 7 | 19.3 ± 7.5 |
|                      | 500 | 7 | 24.1 ± 8.6 |

* All animals were C3Hf/Umc mice, and were grafted at different ages (indicated under "Age") with allogeneic skin from A mice. For details on the experimental groups see Material and Methods.
† Group data of seven normals and six shams, no difference between groups.
§ Sacrificed 50 days after grafting; actual rejection times were 19, 30, 35, 50, 50, and 50 days. Animals were killed with accepted grafts.
RESULTS

Effect of Age and Thymic Function on Allograft Skin Rejection.—In a previous report, subcutaneous grafts of a strain A functional thymoma were rejected after temporary growth by 76% of neonatally thymectomized C3H mice when the grafting was performed at 15 days (7). In the present study neonatally thymectomized C3H mice that rejected the strain A thymoma after temporary growth showed mean rejection time of 27.5 days with a range of 12-38 days. These animals were selected for long-term studies, and their capacity to reject skin allografts was determined at 100, 200, 300, 400, and 500 days of age. Since there were no differences between the results obtained in virgin males or females, the results for both sexes are combined for all of the experiments reported.

Table I shows the mean rejection times of CBA skin grafts in sham-thymectomized controls and the neonatally thymectomized C3H mice that had rejected

### TABLE III

| Experimental group* | Age | No. of mice | Mean rejection ± SD |
|---------------------|-----|-------------|---------------------|
| Normal              |     |             |                     |
|                     | 100 | 6           | 9.1 ± 1.3           |
|                     | 300 | 6           | 9.3 ± 1.5           |
|                     | 400 | 6           | 9.3 ± 1.5           |
|                     | 500 | 6           | 10.0 ± 0.9          |
|                     | 600 | 6           | 11.3 ± 1.8          |
| Thymectomized and syngeneic thymus graft | 100 | 8 | 9.0 ± 1.1 |
|                     | 300 | 6 | 10.0 ± 0.9 |
|                     | 400 | 8 | 10.9 ± 2.1 |
|                     | 500 | 8 | 11.1 ± 1.7 |
| Thymectomized and syngeneic thymus graft removed after 20 days | 100 | 6 | 9.2 ± 1.3 |
|                     | 300 | 6 | 10.4 ± 2.0 |
|                     | 400 | 11 | 25.2 ± 8.8 |
|                     | 500 | 6 | 35.1 ± 16.5 |
| Thymectomized, rejected A thymoma | 100 | 7 | 9.5 ± 1.2 |
|                     | 300 | 6 | 11.0 ± 2.1 |
|                     | 400 | 11 | 34.2 ± 15.0 |
|                     | 500 | 7 | 37.8 ± 16.5 |

* All groups were immunized with A cells, injected intraperitoneally (IP) and subcutaneously, twice, at weekly intervals, 10 days before grafting with A skin.
† Sacrificed 50 days after grafting, and some mice were killed with accepted skin grafts. Actual rejection times were 15, 15, 26, 30, 30, and 50 days.
‡ Sacrificed 50 days after grafting. Actual rejection times were 15, 15, 35, 50, 50, 50, and 50 days.
**TABLE IV**

**Effect of Age and Thymic Function on Graft-Versus-Host Capacity**

| Experimental groups* | Age  | Spleen indices (mean and range)* | No. positive per number tested |
|----------------------|------|---------------------------------|------------------------------|
|                      | days |                                 |                              |
| Normal C3Hf          | 100  | 2.16 (1.59–3.01)                | 14/14                        |
|                      | 300  | 2.10 (1.45–3.00)                | 10/10                        |
|                      | 400  | 2.09 (1.49–3.10)                | 12/12                        |
|                      | 500  | 2.01 (1.37–2.67)                | 12/12                        |
|                      | 600  | 1.86 (1.28–2.30)                | 4/5                          |
| Sham-thymectomized C3Hf | 100 | 2.30 (1.66–3.10)                | 8/8                          |
|                      | 400  | 2.00 (1.46–2.99)                | 7/7                          |
|                      | 500  | 1.97 (1.40–2.11)                | 8/8                          |
|                      | 600  | 1.50 (1.31–1.66)                | 3/3                          |
| Thymectomized C3Hf grafted with syngeneic thymus | 100  | 1.73 (1.39–2.06)                | 12/12                        |
|                      | 300  | 1.80 (1.37–3.00)                | 7/7                          |
|                      | 400  | 1.86 (1.35–2.99)                | 6/6                          |
|                      | 500  | 1.79 (1.37–2.10)                | 6/6                          |
|                      | 600  | 1.49 (1.39–1.59)                | 2/2                          |
| Thymectomized C3Hf rejected A thymoma | 100  | 1.76 (1.32–2.12)                | 6/6                          |
|                      | 200  | 1.60 (1.20–1.95)                | 4/5                          |
|                      | 300  | 1.55 (1.27–1.87)                | 4/5                          |
|                      | 400  | 1.28 (1.05–1.59)                | 3/6                          |
|                      | 500  | 1.13 (1.01–1.29)                | 0/6                          |
| Thymectomized C3Hf rejected C57BL thymus | 100  | 2.86 (2.56–3.00)                | 5/5                          |
|                      | 300  | 2.60 (1.29–2.99)                | 5/6                          |
|                      | 400  | 1.66 (1.01–1.87)                | 3/6                          |
|                      | 500  | 1.31 (1.17–1.42)                | 2/6                          |
|                      | 600  | 1.20                            | 0/1                          |
| Thymectomized C3Hf + C3Hf thymus in DC | 100  | 1.60 (1.45–2.36)                | 6/6                          |
|                      | 300  | 1.10 (0.99–1.25)                | 0/6                          |
|                      | 400  | 1.12 (0.86–1.22)                | 0/6                          |
| Thymectomized C3Hf + syngeneic adult spleen cells | 100  | 1.90 (1.67–3.00)                | 7/7                          |
|                      | 200  | 1.60 (1.25–3.00)                | 4/5                          |
|                      | 300  | 1.59 (1.28–2.66)                | 4/5                          |
|                      | 400  | 1.33 (1.19–1.69)                | 3/6                          |
|                      | 500  | 1.19 (0.90–1.21)                | 0/6                          |

* Neonatal thymectomy, treatment performed at 15 days of age. Treatments were as follows: intraperitoneal implantation of one thymus from 20-day old syngeneic females, subcutaneous implantation of 1 million A thymoma cells, IP implantation of C57BL thymus from 17–19 day old embryo, IP implantation of diffusion chamber containing C3H thymus, and IP injection of 200 million spleen cells from 60-day old syngeneic females.

† 8 days after intraperitoneal injection of 10 million spleen cells into 8-day old (C3Hf × C57BL/1) F1 hybrids.
the strain A thymoma after a period of subcutaneous growth. Both skin donor and recipient share the same H2k, although they differ in non-H-2 histocompatibility loci (16). The rejection times for the sham-thymectomized animals are comparable, regardless of the age when the grafting was performed. Thus the C3Hf mice represent a strain in which aging produces only a mild immunological incompetence, measured as a lengthening of the time for skin allograft rejection. Similar results were reported using a different skin grafting technique (17).

A more detailed study of the decline with age of the restored immune function in neonatally thymectomized animals after temporary reconstitution in the absence of thymic function is described in Table II. Here the animals were tested with allogeneic strain A skin, a donor strain that differed from C3Hf at the H-2 locus (16). The experimental groups included: normal or sham-thymectomized controls (results are combined, since no differences were observed between sham and nonoperated animals); neonatally thymectomized animals restored with an intraperitoneal thymus graft; neonatally thymectomized animals grafted with a syngeneic thymus graft that was removed after 20 days; and animals thymectomized at 30 days of age. The results indicated: (a) that a slight increase in rejection times occurred with age in the normal controls (when t tests were applied to the data, the difference between the 100- and the 500-day groups

| Experimental group* | Graft excised | Age at test | Spleen indices (mean and range)† | No. of positive tests |
|---------------------|---------------|------------|---------------------------------|----------------------|
| Neonatally thymectomized C3Hf mice grafted at 15 days of age with syngeneic thymus, IP | days | days | |
| 10 | 100 | 1.60 (1.37–2.10) | 6/6 |
| 200 | 1.50 (1.10–1.72) | 4/6 |
| 300 | 1.33 (1.07–1.78) | 3/6 |
| 400 | 1.12 (1.10–1.39) | 2/6 |
| 500 | 1.10 (0.99–1.23) | 0/6 |
| 20 | 100 | 1.79 (1.33–3.01) | 6/6 |
| 300 | 1.50 (1.10–1.80) | 3/6 |
| 400 | 1.30 (1.10–1.47) | 3/6 |
| 500 | 1.01 (0.96–1.12) | 0/6 |
| 30 | 100 | 1.68 (1.35–2.05) | 6/6 |
| 200 | 1.72 (1.20–2.10) | 4/5 |
| 300 | 1.52 (1.12–1.91) | 3/5 |
| 400 | 1.21 (1.07–1.42) | 3/6 |
| 500 | 1.05 (0.97–1.19) | 0/6 |

* Neonatal thymectomy and intraperitoneal implantation of one thymus from 20-day old syngeneic donors. Thymus grafts were removed 10, 20, or 30 days after grafting.
† 10 million spleen cells injected into 8-day old (C3H × C57BL/1)F1 mice.
gave a $P$ value of 0.15); (b) that the same suggestive increase in rejection time may be observed in the thymectomized group that had been restored with a syngeneic thymus graft (difference between 100 and 500 days gave a $P$ value of more than 0.10 and less than 0.05 and the same $P$ value was obtained if the 500-day groups were compared between normal mice and mice thymectomized and grafted with thymus graft at birth); (c) that a marked inability to reject allogeneic skin grafts occurred in the group exposed for only 20 days to thymic function: the animals were still restored immunologically when tested at 100 days of age but were profoundly incompetent when tested at 200, 300, 400, or 500 days of age; (d) that the effect is less marked, although significant especially at 400 and 500 days of age, when the animals thymectomized as adults were tested;

**TABLE VI**

**Effect of Age and Thymic Function on GVH Capacity**

| Experimental groups* | Spleen indices† | | | | |
|----------------------|-----------------|---|---|---|
|                      | 100 days        | 400 days | | |
| Normal               | 1.57, 1.90, 2.70 (3/3) | 1.68, 1.77, 1.92 (3/3) | | |
|                      | Mean 2.05       | Mean 1.79 | | |
| Sham thymectomized   | 1.60, 1.89, 2.10 (3/3) | 1.90, 1.42, 1.67 (3/3) | | |
|                      | Mean 1.86       | Mean 1.66 | | |
| Thymectomy + thymus  | 1.47, 1.60, 1.73 (3/3) | 1.40, 1.33, 1.99 (3/3) | | |
| graft                | Mean 1.60       | Mean 1.57 | | |
| Thymectomy + thymus  | 1.33, 1.50, 1.53 (6/6) | 1.20, 1.32, 1.30 (2/6) | | |
| graft excised 20 days | 1.77, 1.77, 1.92 | 1.01, 1.44, 1.30 | | |
|                      | Mean 1.65       | Mean 1.26 | | |

* Neonatal thymectomy and intraperitoneal implantation of one thymus from 20-day old syngeneic donors; group 4, thymus grafts were removed 20 days postgrafting; hemi-splenectomy at 100 days for GVH.
† 10 million spleen cells injected into 8-day old (C3H X C57BL/1)F1 mice. Spleen indices are paired for each individual mouse (i.e., 1.57 at 100 days and 1.68 at 400 days).

this decline of immunologic vigor is comparable to the results obtained by Davis and Cole with skin grafts (18) and by others with humoral antibody responses (8-10) and graft-versus-host reactions (8, 10).

Table III presents the results with preimmunized animals when second-set graft rejection was studied. Again the ability to become immunized to allogeneic antigens and reject skin grafts in a second-set fashion was slightly impaired in the aging normal controls or in the older thymectomized mice restored with a syngeneic thymus graft. On the other hand, a marked decline in this function was observed in the animals exposed to thymic function for a limited period only, especially when they were tested at 400 and 500 days of age.

During the immunization procedure, using adult allogeneic A spleen cells, several deaths were observed in the 400-500-day old age groups, but only in the experimental groups exposed temporarily to thymus function, suggesting a
graft-versus-host reaction in those incompetent animals. Indeed a graft-versus-host reaction was produced with the immunizing spleen cell injection in three of nine mice of the 500-day old neonatally thymectomized mice that had previously had a thymus graft implanted and removed 20 days later. These animals had the clinical symptoms of graft-versus-host reaction and a weight loss of

TABLE VII
Effect of Age and Thymic Function on Resistance to Hepatitis

| Experimental groups | Days | Hepatitis$ | |
|---------------------|------|------------|---|
| Normal              | 100  | 0/12       | 0/12 |
|                     | 200  | ND         | 0/12 |
|                     | 400  | 0/12       | 0/12 |
|                     | 500  | 0/12       | 0/12 |
| Sham thymectomized  | 100  | 0/12       | 0/10 |
|                     | 200  | ND         | 0/12 |
|                     | 400  | 0/12       | 0/12 |
|                     | 500  | 0/12       | 1/12 |
| Thymectomized + syngeneic | 100 | 0/12 | 0/10 | 0/12 | 2/12 |
| thymus | | | | |
| Thymectomized + syngeneic | 100 | 1/12 | 2/10 | 7/12 | 12/12 |
| thymus removed after 20 days | | | | |

* Neonatal thymectomy and treatment at 15 days of age.

† Mortality and/or macroscopic liver lesions, 30 days after intraperitoneal injection of 0.5 ml of 10% liver suspension; number positive animals per number infected.

TABLE VIII
Effect of Age and Thymic Function on Response of Spleen Cells to Phytohemagglutinin

| Age at test | Sham thymectomized | Thymectomized, untreated | Thymectomized exposed to thymus for 20 days |
|-------------|---------------------|--------------------------|-------------------------------------------|
| days        |                     |                          |                                           |
| 40          | 86, 660/4, 112 (8)  | 6, 021/4, 332 (8)        | 86, 585/6, 101 (7)                        |
| 100         | 86, 444/4, 250 (7)  | 9, 335/8, 873 (1)        | 70, 910/5, 110 (6)                        |
| 200         | 85, 370/9, 130 (6)  | ND                       | 73, 744/10, 360 (6)                       |
| 300         | 92, 810/11, 120 (5) | ND                       | 70, 170/12, 000 (5)                       |
| 400         | 103, 250/13, 104 (4)| ND                       | 50, 112/23, 120 (4)                       |
| 500         | 111, 549/12, 120 (4)| ND                       | 15, 779/15, 603 (5)                       |

Results are expressed as mean thymidine-$H$ incorporation in counts per minute from triplicate culture tubes containing $10 \times 10^6$ cells cultured for 48 hr with 10 $\mu$ of PHA. Results of PHA stimulated/unstimulated cultures, numbers in parentheses, indicate the number of animals tested for each group. ND, not done. Standard errors of the means ranged from 46 to 229 for means below 20,000 and from 156 to 1,102 for higher means.

approximately 10%. Sacrificed as moribund, they showed a marked lymphoid atrophy and the reticular cell proliferation characteristic of the graft-versus-host reaction.

Effect of Age and Thymic Function on Graft-Versus-Host Reactivity.—Table IV shows the effects of age and thymic function on graft-versus-host reactivity. Normal, sham-thymectomized, or neonatally thymectomized restored mice
with a syngeneic thymus graft showed only a slight decrease of GVH capacity with age, especially at 600 days. On the other hand, all of the experimental groups that were exposed temporarily to thymic function showed marked impairment of GVH reactivity that could be detected at 300–500 days of age. This was observed in the animals that had rejected a functional strain A thymoma, rejected a C57BL thymus graft, had received a thymoma graft within a cell-impermeable chamber, or were adoptively restored by competent spleen cells. Several points deserve comment. In the case of the animals that rejected

### TABLE IX

| Experimental group* | Response to PHA of spleen cells† | GVH capacity of lymph node cells§ |
|---------------------|----------------------------------|-----------------------------------|
| Thymectomized syngeneic thymus | 71, 090/3, 205 (67, 885) | 2.30 |
| 89, 480/6, 001 (83, 449) | 2.65 |
| 102, 544/8, 711 (93, 833) | 2.33 |
| 110, 612/11, 001 (99, 611) | 1.99 |
| Thymectomized, exposed to thymus for 20 days | 82, 309/4, 566 (77, 793) | 1.26 |
| 49, 301/3, 729 (45, 572) | 1.10 |
| 50, 899/9, 006 (41, 893) | 1.19 |
| 111, 678/16, 305 (95, 373) | 1.23 |
| 78, 005/21, 078 (56, 927) | 1.33 |
| 69, 320/9, 007 (60, 313) | 1.19 |

* For details on strain, age at treatment, and type of treatment see text. All mice were studied at 400 days of age. The results for PHA with spleen cells and GVH with lymph node cells are paired for each individual animal studied.

† Results are expressed as thymidine-³H incorporation in counts per minute from triplicate culture tubes containing 10 X 10⁶ cells cultured for 48 hr with 10 μl of PHA versus the counts obtained in unstimulated cultures. Net counts per culture stimulated minus unstimulated values are given in parentheses.

§ Results are expressed as spleen indices obtained 8 days after intraperitoneal injection of 5 X 10⁶ lymph node cells into 8-day old (C3H X C57BL)F1 mice.

C57BL thymus graft, the spleen indices reflect the response of preimmunized cells, since the test hybrids used were (C3H X C57BL) F1 and still the immunological demise was observed. These results are then comparable to the experiments with second-set grafts (Table III). The group that received the thymus in cell-impermeable diffusion chambers can also be considered as exposed temporarily to thymic function, since the chambers remain in good functioning order for a limited period of time only (15). Finally, the group adoptively restored with competent syngeneic spleen cells, in the absence of thymic function, also showed marked incompetence when tests were performed at 400–500 days of age. In this case the restoring spleen cells were exposed to thymic function in the normal donor environment and had been transplanted to a thymus-free host.
The loss of ability to produce GVH reactions appeared with aging in all of the animals exposed only temporarily to thymic function, irrespective of the restorative procedure used.

Table V shows a study of these phenomena, under more controlled conditions. In this experiment the thymectomized animals were grafted intraperitoneally at 15 days of age with a syngeneic thymus graft, but the graft was excised at 10, 20, or 30 days after grafting. The capacity of spleen cells derived from these animals to produce GVH reactions was then measured at 100, 200, 300, 400, or 500 days of age. Again, as in the previous experiments, approximately half of these animals were incompetent by 300 days and all were incompetent when tested at 500 days of age. By contrast, all animals of the group were still competent when tested at 100 days. No differences in the magnitude of the response were observed between the different groups, whether the thymus had been in place for 10, 20, or 30 days. These experiments therefore show that restoration can be obtained by short exposure to thymus function (19). This of course is not a permanent immunological restoration if the restorative influence is removed.

The fate of the thymus grafts was studied in a special group of animals and serial histological sections were done from thymus grafts recovered at 3, 5, 6, 7, 8, 9, and 10 days after intraperitoneal grafting. The sequential changes of lymphoid depletion and repopulation described for subcutaneous grafts were also observed (20) but vascularization was rapid and by day 5–8 the grafts were vascularized and by day 7–9 had the features of a normal thymus with well-defined cortex and medulla.

It was of interest to see if the decline of immunologic function after thymus removal actually occurred in animals that had been shown previously to be immunologically competent. Table VI shows experiments designed to address this question. Individual animals were tested twice, at 100 and 400 days of age, for their ability to produce GVH reactions. It is clear that normal, sham-thymectomized, and thymectomized mice that had been treated with a thymus graft showed good reactivity whether tested at 100 or 400 days of age. Conversely, the group of thymectomized animals exposed to thymic function for only 20 days were all competent when tested at 100 days, while only two of those same six animals showed even weakly positive reaction when the same study was carried out at 400 days of age. The rest were entirely negative. These results support the view that temporary exposure to thymic function produces temporary immunological restoration, and indicates that the thymus plays a permanent role in the maintenance of immunological functions.

Effect of Age and Thymic Function on Resistance to Hepatitis Infection.—Resistance to mouse hepatitis virus in certain strains is a function of age (12) and is also thymus dependent (21). Infection of thymectomized animals regardless of age produces the same lethal or severe disease that is observed when the virus is injected into young normal mice. Table VII shows the results observed in
animals exposed temporarily to thymic function, when they were infected with mouse hepatitis virus at 100, 200, 400, or 500 days of age. In some of the animals tested at 500 days from the sham-thymectomized group or the group of thymectomized mice grafted with thymus, a few focal macroscopic lesions were observed (3 of 24 mice in the combined groups). On the other hand, significant numbers of animals had severe hepatitis (many focal lesions showing coalescence with generalized liver disease or bright yellow liver background showing both focal lesions and hemorrhagic areas) when exposed only temporarily to thymic function and tested by infection with hepatitis virus at 400 or 500 days of age. The experimental animals exposed temporarily to thymic function had been thymectomized at birth and treated with a syngeneic thymus graft that was removed after 20 days. Severe liver lesions developed upon exposure to the hepatitis virus in 7 of 12 animals tested at 400 days and in 12 of 12 tested at 500 days.

Effect of Age and Thymic Function on Response of Spleen Cells to Phytohemagglutinin.—The response to phytohemagglutinin by lymphoid cells has been shown to be depressed in neonatally thymectomized (22) or in adult thymectomized and lethally irradiated mice (14). Table VIII shows the results of PHA responses of spleen cells from sham-thymectomized mice, neonatally thymectomized, untreated animals, and neonatally thymectomized mice exposed to a thymus graft for 20 days (grafts were implanted intraperitoneally at 15 days of age). Small numbers of animals from these experimental groups were tested at ages ranging from 40 to 500 days (the untreated thymectomized animals were tested at 40 days and only one survivor could be tested at 100 days). The results indicate that: (a) the PHA response of spleen cells remains the same in C3Hf sham-thymectomized animals during the time period studied, the only change detected being the increase of thymidine uptake in unstimulated cultures derived from older mice; (b) the PHA response in the untreated thymectomized mice was substantially reduced; (c) the PHA response of spleen cells from mice exposed only for 20 days to a thymus graft was comparable to the sham-thymectomized controls when the mice were studied at 40, 100, 200, and 300 days of age, showed a moderate decrease when tested at 400 days, and was markedly reduced when studied at 500 days. These results suggest, however, that the response to PHA of spleen cell suspensions is a less sensitive test to demonstrate the decline of thymic-dependent functions in absence of thymus function by comparison with skin graft rejection or graft-versus-host reactivity (see Tables II, III, and V).

To study these differences in more detail, experiments were performed in which the capacity of spleen cells to respond in vitro to PHA was compared with the capacity of lymph node cells from the same animal to produce GVH reactions in vivo. Table IX shows these results for neonatally thymectomized C3Hf mice treated with a thymus graft or exposed temporarily for 20 days to the function of a thymus graft. All the animals were tested at 400 days of age. It is clear
that the six mice exposed temporarily to thymus function had a marked impairment of their capacity to produce GVH reactions (one positive out of six animals) while the PHA response was still detectable and only slightly decreased in comparison to the responses in the animals with thymus function (the mean net counts of the thymus-grafted group being 86.194 versus 62.970 for the group exposed temporarily to thymus).

**DISCUSSION**

From the present results it is clear that neonatally thymectomized mice exposed temporarily to thymic function are indeed reconstituted immunologically, even after only 10 days of exposure to thymus, but that the immunological reconstitution is not self-sustaining in the absence of thymus. After a variable period of time, usually at 200–300 days of age, the immune functions tested (first- and second-set skin graft rejection, graft-versus-host reactivity of spleen cells, resistance to mouse hepatitis infection, and capacity of cells to respond to PHA) showed a marked deficiency. This extreme deficiency was more pronounced than the mild involution of immune functions observed in the C3Hf mice with normal aging and was observed in all the different models used, regardless of the type of thymic function to which the animals were exposed. These treatments were mainly situations in which the restored thymectomized animal eventually rejects the restoring thymus or thymoma graft (this occurs with high frequency especially in certain graft-host strain combinations) or surgical ablation at different intervals of the restoring of the thymus graft. In one set of experiments the actual decline of the restored immune functions was demonstrated (Table VI) because the animals were tested twice in their lifetime for their capacity to produce GVH reactions, one time early after thymus exposure, when the animals indeed showed normal immune functions, and a second time after temporary exposure to thymic function, when the same animals proved to be immunologically incompetent.

Of all the tests studied, the response of spleen cells to PHA was to be preserved longest in the animals exposed only temporarily to thymic function (Table VIII). When the capacity to respond to PHA and the capacity to produce graft-versus-host reactions were compared in the same animals at 400 days of age, the deficit of graft-versus-host capacity was shown to be extreme while the response to PHA was only moderately decreased and two of six mice had responses comparable to normal controls (Table IX). Although for most purposes the cells responding to PHA can be considered to be thymus dependent (14, 22), it is likely that in mice other cell types may also be stimulated to blast transformation by PHA. Perhaps such cells are increased in thymectomized animals exposed temporarily to thymus function as a result of prolonged antigenic stimulation in a conventional environment. Indeed, an increase in the background uptake of thymidine was observed with increasing age even in the control mice (Table VIII).
The present results are in accordance with our interpretation of previous data pertaining to the characteristics of postthymic cells (23). In our previous experiments we noticed that if treatment with thymus grafts or thymus in diffusion chambers was delayed after neonatal thymectomy, the treatment was very effective if performed within the 1st 3 wk, and progressively became less effective if delayed. Eventually the animals became refractory to restoration by thymic grafts or thymus tumors (24). The interpretation of these results was that a peculiar cell population present in the peripheral tissues of these mice was declining with time in the absence of thymic function, that this population is incapable of self-renewal, and that it is thymus dependent for its existence and is thus termed postthymic (23). We also obtained evidence that the postthymic pool is produced by traffic of hemopoietic stem cells to the thymus and subsequent export of these cells to the periphery as postthymic cells (25, 26). The postthymic population is not necessarily competent immunologically but is sensitive to the humoral activity of the thymus and to antigenic stimulation (27). The present data suggest a decline of the postthymic pool in the absence of thymus as the mechanism underlying the immune deficiencies detected in the experimental animals.

It is possible that the temporary exposure of the thymectomized animals to thymic function permits the development of a substantial pool of postthymic cells capable of further differentiation into competent cells but that this pool is incapable of self-renewal in the absence of the thymus and progressively declines with time through physiological attrition and immunological commitment to antigens in the environment. It can be postulated that the postthymic pool is thymus dependent for its development and renewal but thymus independent for its immune functions in the peripheral lymphoid tissues. Further differentiation in the periphery is proposed for the postthymic cells, and perhaps some direct influence of the thymic humoral factors, but most probably antigen is the main driving force in this differentiative process.

From the present data, some indications of the time required for these events can be inferred. It seems that a rather short exposure to thymus is required. 10 days was the shortest period studied and since vascularization of the intraperitoneal thymus grafts occurs within a period of 5–7 days, it may be assumed that only 3 days of effective action with fully developed vascular supply of the thymus graft is sufficient to build a pool of competent cells capable of being expanded and lasting at least 100 days. It is also clear that in the absence of thymus the immunological competence of this thymus-derived population in the peripheral tissues is not self-sustaining and that ultimately a loss of this population occurs. In the experiments in which the animals were grafted at 15 days of age and exposed only for 10 days (Table V) it can be seen that incompetence in graft-versus-host reactions is detected at 200 days of age (175 days after removal of the thymus graft).

The present observations and interpretations accord with the delayed effects
on immune reactivity observed after thymectomy in the adult mouse (8–10, 18) or with the effect of low dose-rate gamma irradiation (18) or ALS treatment in mice thymectomized as adults (28). These situations can be interpreted as a decline of the postthymic population with time and as evidence of the impossibility of replacement of that population in the absence of thymic function.

Our present observations also appear to have clinical relevance since evidence of rejection of the restoring thymus graft in the treated humans affected with the DiGeorge syndrome has been obtained (W. W. Cleveland, personal communication). Two such children were apparently restored by the thymus grafting (29, 30) and from findings here it may be predicted that the restoration will not be permanent in the absence of thymic function if the restoring grafts were indeed rejected. Perhaps a second grafting of thymus will be required. This possibility has to be considered since we showed that neonatally thymectomized animals which have already developed a wasting syndrome can be saved by two successive treatments with thymus grafts (5).

Estimates of the duration of the effects of thymus-dependent lymphoid functions after loss of thymus in man are difficult. The question can be stated, however, in general terms. What is the true human equivalent of 200 days in the life of the mouse which has a full life-span of some 700 days? No highly probable answer to this question can be given at the moment. Continued comparative studies may provide the data necessary for resolving important decisions concerning the treatment of human immune deficiencies. This estimate should be made and may be of great importance, particularly since complete restoration of thymus-dependent function, once it is completely lost, requires a well-established functional thymus graft. Substitution of the indirect, long-range humoral influence of the thymus by thymus in diffusion chambers, or stromal epithelial thymoma, will not be effective after the postthymic population has been lost (24).

Experiments in progress indicate that, in the present experimental model, immune capabilities can be restored by treating the animals at 300–400 days of age with thymus grafts, provided small numbers of adult spleen or bone marrow cells are also included (24). Thymus grafts by themselves are not sufficient, suggesting that even the prethymic population in bone marrow may be depleted (or deviated into thymus-independent differentiation) in aging animals which lost thymic function. Another possibility is that the buildup of a postthymic population by traffic through thymus is deficient in the aging mouse or that its achievement requires a longer time than in the younger animals. Further detailed study of this important relationship is required.

SUMMARY

The immune functions of neonatally thymectomized C3Hf mice exposed only temporarily to thymus function show a progressive decay with time in the absence of the thymus. The immune responses studied at different ages in the
range of 100–600 days were: first-set rejection of H-2-compatible and incompatible skin allografts, second-set rejection of skin allografts, capacity of spleen cells to produce graft-versus-host reactions in F₁ hybrids, resistance to infection with mouse hepatitis virus, and response of spleen cells to phytohemagglutinin in vitro. These long-term studies had the purpose of determining the duration of the restoration induced by thymus function when the mice were exposed only temporarily to it. Different models were used but the two basic ones were: (a) mice grafted intraperitoneally at 15 days of age with a syngeneic thymus that was removed surgically at 10, 20, or 30 days after grafting, and (b) mice grafted at 15 days of age with allogeneic strain A thymoma or C57BL thymus, these representing situations in which there is spontaneous rejection of the restoring graft. In all the experimental models used, the animals were restored when tested at 100 days of age, but progressively became immunologically incapacitated at 200–300 days of age. From the more controlled experiments in which the restoring thymus graft was removed surgically, the following conclusions can be drawn. (a) A short exposure to a thymus graft can produce restoration of immune functions in neonatally thymectomized mice, but this restoration is not self-sustaining in the absence of the thymus and declines progressively with age. The decline usually starts at 200–300 days of age. (b) This was especially clear in experiments in which the same animal was tested twice in its lifetime for capacity to produce graft-versus-host reactions; these animals were competent at 100 days and became incompetent at 400 days of age. (c) The shortest period of thymic exposure studied was 10 days; if vascularization of the graft is taken into account, 2–3 days of thymic function are sufficient to produce restoration. (d) The immune decay observed in the thymectomized animals exposed temporarily to thymus was more profound than the physiological decay of immunity observed in control animals of similar age. (e) Of all the tests studied, the response of spleen cells to phytohemagglutinin was to be preserved the longest in animals exposed only temporarily to thymic function.

The present results were interpreted in accordance with our previous findings indicating that a population of postthymic cells can be developed by temporary exposure of neonatally thymectomized animals to thymic function, but that this population is not self-sustaining in the absence of thymus and progressively decays by physiological attrition.

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