STUDIES ON THYMUS FUNCTION

I. COOPERATIVE EFFECT OF THYMIC FUNCTION AND LYMPHOHEMOPOIETIC CELLS IN RESTORATION OF NEONATALLY THYMECTOMIZED MICE*

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(Received for publication 28 April 1970)

The presence of the thymus in mice is essential for proper development, maintenance, and function of the lymphoid system (1, 2). Thymic function results from the interaction of peculiar cellular migration patterns to and from the thymus (3–10) and from the inductive or expanding capabilities of thymic stroma that include humoral factors (11, 12).

In previous studies, we have shown that nonlymphoid thymomas induced restoration of immunological functions in neonatally thymectomized mice (13). When thymomas were grafted into allogeneic hosts, immunological restoration was mediated by lymphoid cells of host type (14). Thymomas and normal thymus were also capable of inducing restoration when enclosed in truly cell-impenetrable diffusion chambers (12). This evidence indicated that humoral inducing and/or expanding functions of thymus or thymomas mediate the restoration of the thymectomized hosts. Comparable results were obtained with free thymus grafts, and although restoration was mediated mainly by host-type cells, a thymus-derived cell population could be detected (6–10).

When the treatment of neonatally thymectomized hosts with thymomas or thymus grafts was delayed after neonatal thymectomy, a decrease in the restorative influence was observed (15). These results indicate that a population of cells in the thymectomized hosts capable of responding to the action of thymus or thymomas decreased progressively with time after neonatal thymectomy in absence of thymic function, when the mice were nursed in a conventional environment (15). This decrease was interpreted as a result of physiological attrition of cells differentiated prior to thymus extirpation (15).

* Aided by The National Foundation-March of Dimes, the U. S. Public Health Research Grant CA-10445, the American Cancer Society. Part of this work was presented at a Symposium of the American Society for Experimental Pathology, April 1968.

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The present report represents the attempts to characterize this population of cells by employing combined treatment of thymectomized animals with thymic function (thymoma or thymus) together with cells of various origins. Our present results indicate that a differentiated thymic-dependent cell is present in the lymphohemopoietic tissues of adult and newborn mice and is sensitive to the humoral action of the thymus.

Materials and Methods

Animals.—Inbred mice of the A, C3Hf, DBA/2, CBA/H, C57BL/1, and T6 strains and F1 hybrids of some of those strains were used. Animal care was as described in a previous paper (14). These mouse strains are derived from the colonies of Doctors J. J. Bittner and C. Martinez. A detailed description of the strains has been reported recently (16) and designed as University of Minnesota colony sublines (Um). All the adult cell donors were 60-day old females. Thymus graft donors were 20-day old. Newborn cell donors were less than 24 hr old.

Technical Procedures.—Techniques for neonatal thymectomy, thymoma grafting, skin grafting, and preparation of cell suspensions have all been described in previous publications (8, 14).

Bone marrow cells were obtained by flushing cold buffered Ringer's solution through long bones with hypodermic syringes and 27-gauge needles, after clipping both bone ends with scissors.

Thoracic duct cells were obtained from the subdiaphragmatic thoracic duct by the method described by Mandel (17), and the 18-24 hr drainage was collected in siliconized glass tubes containing 10% fetal calf serum in Ringer's solution and 1 µg/ml of preservative free heparin. The total number of cells drained per mouse was 50-80 × 10^6 cells.

Blood leukocytes were obtained from buffy coats from heparinized microcentrifuge tubes after centrifugation at 2000 rpm.

Peritoneal cells were obtained after intraperitoneal injection of 5 ml of Ringer's solution as described by Goodman (18). With this technique the number of cells obtained per mouse is 2-4 × 10^6 cells, and approximately 60% are lymphoid cells.

Peyer's patches were cut with scissors from the intestine and suspensions were prepared in glass homogenizers using a standard technique (7, 8). Since the patches were usually contaminated with intestinal contents, the fragments were repeatedly washed before preparation of the cell suspension, and all the procedure was performed using Ringer's solution with the addition of 100 units of penicillin and 0.1 mg streptomycin/ml.

Diffusion chambers were prepared with lucite rings and Millipore filters of 0.22 µ average pore size (Millipore Filter Corp., Bedford, Mass.). Details and descriptions of the chambers have been reported previously (12).

Graft-versus-host assays were performed using 10 million spleen cells from the A or C3Hf mice injected intraperitoneally into 8-day old (AxC57BL)F1 and (C3HxC57BL)F1 hybrids, respectively. Spleen indices were obtained 8 days later as described in previous publications, and the negative controls were injected with syngeneic F1 spleen cells (8, 14). Discriminant spleen assays (8, 14) were performed for C3H cells using (C3HxC57BL/1)F1 and (C3HxC56)F1 hybrids injected with 20 × 10^6 spleen cells from the test animals.

Tests for delayed hypersensitivity to sheep red cells were used, as described in a previous paper (12) and the footpad volume increase was measured as described by Axelrad (19).

Chromosome preparations were made as described in a previous paper (12, 20), 1 hr after the intraperitoneal administration of 0.02 ml/gm, body weight of a 0.004% solution of vincleukoblastine (Velban, Eli Lilly & Co., Indianapolis).
Thymomas.—Two functional thymomas (strain A thymoma and C3H thymoma No. 2) appearing 200 days after neonatal intrathymic application of 0.1 mg of 7,12-dimethylbenzanthracene were used. Histological characteristics, growth patterns, and functional studies have been described in previous papers (13, 14).

Restoration Criteria.—Neonatally thymectomized mice were considered restored when they fulfilled all the following criteria for restoration: (a) complete thymectomy at the termination of the experiment (microscopic analysis of the mediastinal contents); (b) 200 day survival; (c) capacity to reject DBA/2 skin in less than 15 days when grafted at 90–100 days of age (normal rejection time in C3Hf animals is 12.3 ± 1.3 and in A strain is 11.6 ± 1.5); (d) capacity to produce delayed hypersensitivity to sheep red cells when tested at 150–160 days of age (footpad volume increase of at least 15% when measured 48 hr after challenge with antigen); and (e) capacity of $10^6$ spleen cells to produce a positive graft-versus-host reaction when injected into appropriate F1 hybrids. This last test was not performed when F1 hybrid cells were used for restoration (see Tables II and VI). The presence of all five criteria was essential for considering a particular animal restored.

Experimental Design.—The basic experimental model consisted of neonatally thymectomized mice treated at 45 days of age. Treatment consisted of thymic function (either thymoma grafts or thymus within cell-impenetrable diffusion chambers) and the intraperitoneal injection of lymphohemopoietic cells of various types. Controls received either thymic function or cells alone. The injected cells, except in experiments where the contrary is indicated, were always syngeneic with the host. After treatment, the animal's testing for restoration was begun at 90–100 days of age, that is 45–55 days after treatment.

RESULTS

Association of Strain A Thymoma and Adult Lymphohemopoietic Cells.—Table I shows the incidence of restoration of 45-day old, neonatally thymectomized strain A and C3Hf mice treated with both strain A thymoma grafts and lymphohemopoietic cells of a variety of sources from 60-day old normal donors, syngeneic for the host. In both strains, thymoma grafts or cells alone were ineffective, or only low percentages of restoration were observed with the cell dosages tested. Restoration was observed in small numbers of animals, approximately 10%, treated with thymoma alone or with spleen, lymph node, or thoracic duct cells. Bone marrow cells were ineffective by themselves, even when 150 million syngeneic cells were used. For comparison it can be seen in Table I that the same number of syngeneic spleen cells produced high incidence of restoration. The other cell types tested were thymus, blood leukocytes, peritoneal cells, adult liver cells, and Peyer's patch cells. All these cell types were ineffective in producing significant restoration at the cell dosages employed.

On the contrary, the association of thymoma and cells, especially marrow, spleen, thymus, lymph node, thoracic duct, and blood leukocytes, was effective in favoring immunological restoration of the thymectomized hosts.

In the A strain mice (Table I, column 4), restoration was observed in 46 of 72 animals treated with thymoma plus spleen cells (63%), 22 of 48 treated with thymoma and bone marrow cells (45%), and 15 of 58 treated with thymoma and thymus cells (25%), expressed as combined results of the different cell dosages tested (50, 20, 10, and $5 \times 10^6$). Lymph node cells and blood leukocytes in
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### TABLE I

**Restoration of 45-day Old, Neonatally Thymectomized A and C3Hf Mice with Strain A Thymoma and Lymphohemopoietic Tissues from Adult Donors Syngeneic to the Host**

| Tumor graft | Treatment* | Cells     | Cell type | A strain | C3Hf strain |
|-------------|------------|-----------|-----------|----------|-------------|
| None        | None       | —         | 0/20 (0)  | 0/23 (0) |
| A thymoma   | None       | 150 marrow| 0/20      | 0/25 (0) |
|             | None       | 50 marrow | 0/12      | 0/11 (0) |
|             | None       | 20 marrow | 0/13      | 0/15 (0) |
|             | None       | 10 marrow | 0/12      | 0/10 (0) |
|             | None       | 150 spleen| 8/10 (80%)| 9/10 (90%)|
|             | None       | 20 spleen | 2/20 (10%)| 1/12 (8%) |
|             | None       | 10 spleen | 1/12 (8%) | 1/16 (6%) |
|             | None       | 5 spleen  | 3/31 (9%) | 1/16 (6%) |
|             | None       | 50 thymus | 1/18 (5)  | 0/10 (0) |
|             | None       | 20 thymus | 0/12      | 1/12 (8) |
|             | None       | 10 thymus | 0/12      | 0/13 (0) |
|             | None       | 20 lymph node | 0/10 (0) | 1/10 (10) |
|             | None       | 20 thoracic duct | ND        | 1/21 (12) |
|             | None       | 20 blood leukocytes | ND        | 1/20 (5) |
|             | None       | 20 peritoneal cells | ND        | 0/10 (0) |
|             | None       | 20 liver cells | ND        | 0/12 (0) |
|             | None       | 20 Peyer's patches | ND        | 0/10 (0) |
| A thymoma   | 50 marrow  | 6/10 (60%)| 6/12 (50%)|
| A thymoma   | 20 marrow  | 6/12 (50%)| 4/12 (33%)|
| A thymoma   | 5 marrow   | 10/26 (37%)| 4/16 (25%)|
| A thymoma   | 20 spleen  | 14/20 (70%)| 22/40 (55%)|
| A thymoma   | 10 spleen  | 20/31 (64%)| 8/16 (50%)|
| A thymoma   | 5 spleen   | 12/21 (57%)| 11/27 (41%)|
| A thymoma   | 50 thymus  | 6/18 (33%) | 5/13 (38%) |
| A thymoma   | 20 thymus  | 6/18 (33%) | 10/30 (33%)|
| A thymoma   | 10 thymus  | 3/22 (16%) | 5/22 (22%) |
| A thymoma   | 20 lymph node | 6/10 (60%)| 20/40 (50%)|
| A thymoma   | 20 thoracic duct | ND        | 5/10 (50%)|
| A thymoma   | 20 blood leukocytes | 3/10 (30%)| 4/12 (33%)|
| A thymoma   | 20 peritoneal cells | 2/20 (10%)| 1/12 (8) |
| A thymoma   | 20 liver cells | ND        | 2/16 (12%)|
| A thymoma   | 20 Peyer's patches | ND        | 3/16 (18%)|

* Neonatally thymectomized A and C3Hf mice, treatment performed at 45 days of age. Tumor grafts as subcutaneous implantation of $1 \times 10^6$ cells. Cells as intraperitoneal injection simultaneous with tumor grafting. Donors were 60-day old A or C3Hf females respectively, syngeneic for the host strain. For restoration criteria see text.

Association with this strain A thymoma gave 60 and 44% restoration, respectively, while peritoneal cells were ineffective.

Comparable results were obtained with C3Hf thymectomized hosts treated with the association of strain A thymoma and lymphohemopoietic cells from...
normal adult C3Hf donors. Table I (column 5) shows that the association of thymoma and cells produced significant number of restorations. By contrast, animals treated with thymoma or cells alone were not restored. Restoration was observed in 41 of 83 mice treated with thymoma plus spleen cells (49%), 14 or 40 treated with thymoma and bone marrow cells (35%), and 20 of 65 treated with thymoma and thymus cells (30%). The majority of the restored C3Hf animals eventually rejected the strain A thymoma after its temporary growth, and only 11 of a total of 80 restored animals receiving tumor grafts and spleen, marrow, or thymus cells accepted the thymoma for 60 days or more after grafting. These results were similar to those previously reported (14). In this system

### TABLE II

Restoration of 45-day Old, Neonatally Thymectomized C3Hf Mice Treated with Strain A Thymoma and Spleen Cells from Adult F1 Hybrids*

| Tumor graft | Cell donors | Number restored per number treated | Acceptance of A thymoma† | Acceptance of A skin† |
|-------------|-------------|------------------------------------|--------------------------|----------------------|
| A thymoma   | —           | 1/12 (8)                           | 1/1                      | 0/1                  |
| None        | (C3H × A)F1 | 0/10                               | —                        | —                    |
| None        | (C3H × C57BL/1)F1 | 1/12 (8)                              | —                        | —                    |
| None        | (C3H × T6)F1 | 0/12                               | —                        | —                    |
| A thymoma   | (C3H × A)F1 | 9/20 (45)                           | 9/9                      | 9/9                  |
| A thymoma   | (C3H × C57BL/1)F1 | 12/25 (47)                             | 0/12                     | 0/12                 |
| A thymoma   | (C3H × T6)F1 | 11/20 (55)                           | 1/11                     | 0/11                 |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Subcutaneous implantation of 1 × 10⁶ tumor cells. Intraperitoneal injection, simultaneous with tumor grafting of 10 × 10⁶ cells. Donors were 60-day old F1 hybrid females.
† Acceptance for 20 days or more after grafting. Skin grafting performed at 90 days of age. Number of animals accepting graft per number grafted.

C3Hf lymph node and thoracic duct lymphocytes and blood leukocytes were also effective in producing restoration when they had been given to C3Hf recipients in association with the strain A thymoma. Peyer’s patches gave borderline effects, while peritoneal cells and adult liver cells were ineffective when given in association with the strain A thymoma.

Table II shows restoration of 45-day old, neonatally thymectomized C3Hf mice treated with strain A thymoma plus 10 × 10⁶ spleen cells from three different normal adult F1 hybrids. Restoration was 45, 47, and 55% respectively for mice treated with thymoma and spleen cells of (C3HxA)F1, (C3HxC57BL/1)F1, and (C3HxT6)F1 hybrids. As would be predicted if restoration is mediated by expansion of the injected spleen cell population, the mice restored with (C3HxA)F1 cells accepted the strain A thymoma and A strain skin, whereas animals restored with the other F1 hybrid combinations rejected strain A thymoma and skin grafts. Such tolerance was specific, since all restored animals
were capable of rejecting DBA/2 skin within normal time limits (see restoration criteria).

A special group of 45-day old, neonatally thymectomized C3Hf mice treated with strain A thymoma and spleen cells from (C3H×T6)F1 hybrids was studied, and chromosome preparations of their lymphhemopoietic tissues are shown in Table III. Chromosome preparations were made at 10, 30, 60, and 155 days after treatment, and all animals (with exception of the 10-day group) were capable of rejecting DBA/2 skin grafts within normal time limits when grafted 15 days after treatment. In this experiment, as well as in a similar experiment performed using newborn (C3H×T6)F1 hybrid liver cells, the only immunological parameter tested for restoration was the rejection of DBA/2 skin. The results of the 30-day group represent animals that had rejected a skin graft a few days before. Since there was small variation for the individual values for each group, the results are presented as pooled data from four animals. Table III shows that a substantial portion of cells, especially in lymph nodes, have the karyotype characteristics of the spleen-cell donor (positive for the small autosomal T6 marker). The per cent of spleen-type metaphases in different tissues was approximately 51–67 for spleen, 86–97 for axillary and cervical nodes, 42–96 for mesenteric node, 9–37 for mesenteric node, and 2–37 for the thymoma graft. If considered in relation to time after treatment, the per cent of spleen-type metaphases was fairly constant in spleen, marrow, axillary nodes, and Peyer’s patches and increased progressively in mesenteric nodes. The low percentage of donor cells in the marrow at 60 days (8%) is difficult to explain. The higher percentage of donor cells in axillary and cervical nodes

### Table III

| Days after treatment | Spleen donor type metaphases per total metaphases |
|---------------------|--------------------------------------------------|
|                     | Spleen | Bone marrow | Axillary nodes | Mesenteric nodes | Peyer’s patches | Thymoma |
| 10                  | 57/111 (51) | 10/32 (31) | 19/22 (86) | 6/14 (42) | 1/30 (3) | 1/40 |
| 30                  | 98/142 (67) | 9/32 (28) | 72/74 (97) | 20/33 (66) | 1/30 (3) | 3/8 |
| 60                  | 83/155 (53) | 8/91 (8) | 44/50 (88) | 5/16 (88) | 1/23 (4) | ND |
| 155                 | 60/106 (56) | 13/48 (27) | 26/27 (96) | 12/14 (96) | 2/22 (9) | ND |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age and consisted of subcutaneous injection of 1 \( \times 10^5 \) strain A thymoma cells and intraperitoneal injection of 10 \( \times 10^6 \) spleen cells from 60-day old, normal (C3H×T6)F1 hybrid females. Animals received DBA/2 skin grafts 15 days after treatment. Chromosome preparations made an hour after Velban administration. Four animals per group, and the results are expressed as combined data for each tissue. Per cent of spleen type per total metaphases in parentheses.
(97%) at day 30 must be related to skin graft rejection, since the grafts were applied on areas drained by those nodes. The high incidence (37%) of donor cells in the thymoma at day 30, although a small sample, may represent the events leading to ultimate rejection of the tumor graft. These results suggest that adoptive restoration of the thymectomized host by the injected spleen cells occurs, but the experiment does not differentiate dividing cells and immunologically competent cells.

The host-type immunological component (C3Hf) was assessed using a discriminating spleen assay described in previous publications (8, 14). Test animals were (C3HxC57BL/1)F1 and (C3HxT6)F1 hybrids injected with 20 $\times$ 10^6 spleen cells from the restored animals. Host-type competent cells were detected in one of 10 animals studied 40 days after treatment with strain A thymoma and (C3HxT6)F1 spleen cells (Table IV). In that only case, the host (C3Hf) cells were specifically tolerant to the (C3HxT6)F1 while they were detected in the (C3HxC57BL/1)F1. For comparison, nine animals were tested in which

**TABLE IV**

| Experimental groups* | Test litters and spleen indexes† |
|----------------------|---------------------------------|
|                      | (C3H $\times$ C57BL/1)F1 | (C3H $\times$ T6)F1 |
| A thymoma and (C3H $\times$ T6)F1 spleen cells (10) | 0.98, 1.01, 1.01, 1.03, 0.88, 1.12, 1.20, 1.01, 1.16, 1.02, 0.99, 1.00, 1.67, 1.02 |
| A thymoma and C3Hf spleen cells (9) | 1.55, 1.69, 1.80, 1.81, 1.88, 1.89, 1.90, 1.95, 2.02, 1.47, 1.88, 2.00, 1.66, 1.90, 1.66, 2.00, 1.79, 1.88 |
| A thymoma (10) | 0.90, 1.00, 1.01, 1.05, 1.07, 1.10, 1.11, 1.13, 1.35, 1.00, 1.10, 0.99, 1.12, 1.00, 1.03, 1.10, 1.17, 1.40, 1.69, 1.55 |
| Normal C3Hf (29)§ | 2.89 (2.10–3.00) | 2.00 (1.75–2.90) |

* Neonatally thymectomized C3Hf mice treated at 45 days of age with a subcutaneous thymoma graft and 10 $\times$ 10^6 spleen cells of (C3H $\times$ T6)F1 or C3Hf 60-day old, normal females. Discriminant test performed 40 days after treatment. Numbers in parentheses indicate number of animals tested. Results are paired for each individual mouse tested (i.e., 0.98 and 1.03 in the first group, etc.)
† Positive index higher than 1.30. All hybrid litters were 8 days old and given intraperitoneal injections of 20 $\times$ 10^6 spleen cells. Negative controls received syngeneic spleen cells.
§ Spleen indexes expressed as mean and range.
restoration was produced by the strain A thymoma plus spleen cells of C3Hf origin. The spleen index test revealed the C3H component in all the instances (Table IV). Table IV also shows that of 10 animals treated only with the strain A thymoma, the host (C3Hf) component was detected in only two animals, a result compatible with the incidence of restoration revealed in the earlier studies (Table I).

### TABLE V

| Treatment* | Cells | Number restored per number treated |
|------------|-------|-----------------------------------|
| None       | None  | 0/19                              |
| Bone marrow| 50    | 4/18 (50)                         |
| Bone marrow| 20    | 3/12 (25)                         |
| Bone marrow| 5     | 1/10 (10)                         |
| Spleen     | 20    | 6/10 (60)                         |
| Spleen     | 5     | 4/12 (33)                         |
| Thymus     | 50    | 4/12 (33)                         |
| Thymus     | 20    | 5/20 (25)                         |
| Thymus     | 5     | 2/20 (10)                         |
| Lymph node | 20    | 4/8 (50)                          |
| Thoracic duct| 20 | 4/8 (50)                         |
| Blood leukocytes| 20 | 3/10 (30)                        |
| Peritoneal cells| 20 | 1/10 (10)                        |
| Peyer's patches| 20 | 1/12 (8)                          |

* Neonatally thymectomized C3Hf mice treated at 45 days of age with a C3Hf thymus lobe from a 20-day old donor enclosed within a diffusion chamber (0.22 μ pore size) implanted intraperitoneally and injection of lymphohemopoietic cells from 60-day old syngeneic female donors.

These results support the view that the immunological restoration of 45-day old, neonatally thymectomized C3Hf mice treated with strain A thymoma plus adult spleen cells is adoptively mediated by expansion mainly of the spleen donor population.

**Association of Thymus Within Diffusion Chambers and Adult Lymphohemopoietic Cells.**—Table V shows the restoration of 45-day old, neonatally thymectomized C3Hf mice with syngeneic thymus within diffusion chambers and the association of lymphohemopoietic cells from syngeneic adult donors. As was shown in a previous publication (15), thymus grafts within cell-impenetrable chambers are incapable of restoring neonatally thymectomized mice when the treatment is performed at 45 days of age. On the other hand, the association of intraperitoneal diffusion chambers containing one thymus lobe of 20-day old syngeneic donors plus the intraperitoneal injection of cell suspensions from
syngeneic lymphohemopoietic organs was effective. The combined results of different cell dosages show that bone marrow produced 20% restoration (8 of 40), spleen 45% (10 of 22), and thymus 21% (11 of 52). As in the thymoma experiments (Table I) lymph node and thoracic duct cells were effective, producing 50% restoration, and blood leukocytes produced 30% restoration. Peritoneal cells and Peyer's patch cells produced some degree of restoration with 10 and 8%, respectively.

Table VI shows the effects of treatment with strain A thymoma and liver, spleen, or thymus cells from C3Hf newborn donors on restoration of 45-day old, neonatally thymectomized C3Hf mice. As in the experiments using cells derived from adults (Table I), restoration was observed only when thymoma grafts and

**Table VI**

| Treatment* | Number restored per number treated |
|------------|----------------------------------|
|            | Cell type | × 10⁶ | %          |
| None       | None      | — | 0/10 |
| A thymoma  | None      | — | 2/22 (10) |
| None       | 100 liver | 0/16 |
| None       | 50 liver  | 0/15 |
| None       | 20 liver  | 0/11 |
| None       | 5 liver   | 0/20 |
| None       | 100 spleen| 0/18 |
| None       | 50 spleen | 0/10 |
| None       | 20 spleen | 0/13 |
| None       | 5 spleen  | 0/12 |
| None       | 100 thymus| 0/10 |
| None       | 50 thymus | 0/12 |
| A thymoma  | 100 liver | 5/10 (50) |
| A thymoma  | 50 liver  | 13/23 (56) |
| A thymoma  | 20 liver  | 7/17 (41) |
| A thymoma  | 5 liver   | 4/20 (20) |
| A thymoma  | 100 spleen| 5/10 (50) |
| A thymoma  | 50 spleen | 4/9 (44) |
| A thymoma  | 20 spleen | 5/9 (55) |
| A thymoma  | 5 spleen  | 4/13 (31) |
| A thymoma  | 100 thymus| 20/30 (66) |
| A thymoma  | 20 thymus | 7/17 (41) |
| A thymoma  | 5 thymus  | 2/10 (20) |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Subcutaneous graft of 1 × 10⁶ tumor cells. Intraperitoneal injection, simultaneous with tumor grafting, donors were C3Hf mice less than 24-hr old.
cells were administered together. The combined results for the different cell dosages tested indicate that restoration was observed in 29 of 70 animals treated with hemopoietic liver cells (41%), 18 of 41 treated with spleen (43%), and 29 of 57 treated with newborn thymus cells (50%). If only one cell dosage is compared (50 × 10⁶), restoration was 66% for thymus, 56% for liver, and 44% for spleen. These results contrast with those using adult cells in combination with thymoma in which the most effective cells were spleen, and comparable dosages of marrow or thymus cells produced 50 and 38% restoration respectively (Table I). The combined results for all cell dosages of adult thymus cells plus thymoma grafts indicate 25% (15 of 58) restoration for the A strain.

Table VII

| Tumor graft | Cell donors | Number restored per number treated | Acceptance of A thymoma† | Acceptance of A strain† |
|-------------|-------------|-----------------------------------|--------------------------|------------------------|
| None        | (C3H × A)F₁ | 0/20                              | —                        | —                      |
| None        | (C3H × C57BL/1)F₁ | 0/22                             | —                        | —                      |
| None        | (C3H × T6)F₁ | 0/16                              | —                        | —                      |
| A thymoma   | (C3H × A)F₁ | 8/17 (47)                         | 8/8                      | 8/8                    |
| A thymoma   | (C3H × C57BL/1)F₁ | 14/32 (43)                     | 1/14                     | 0/14                   |
| A thymoma   | (C3H × T6)F₁ | 10/20 (50)                        | 1/10                     | 1/10                   |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Subcutaneous implantation of 1 × 10⁶ tumor cells and intraperitoneal injection simultaneous with tumor grafting of 50 × 10⁶ liver cells from newborn F₁ hybrid donors.
† Acceptance for 20 days or more after grafting. Skin grafting performed at 90 days of age. Number of animals accepting graft per number of grafted.

and 30% (20 of 65) for the C3Hf when treated with adult thymus (Table I). On the other hand, newborn thymus cells produced 50% (29 of 57) restoration (Table VI) even when smaller cell dosages were used (5 as opposed to 10 × 10⁶ cells as the lower dosage in the adult thymus experiments).

Table VII shows restoration produced by strain A thymoma and 50 × 10⁶ liver cells from newborn donors of (C3HxA)F₁, (C3HxC57BL)F₁, and (C3HxT6)F₁ origin. These results are comparable to the ones obtained with 10 × 10⁶ adult F₁ spleen cells (Table II) or 50 × 10⁶ syngeneic newborn liver cells (Table IV). As in the previous experiments (Tables II–IV), the results suggest that restoration is mediated by expansion of the donor cell population.

Chromosome analysis of the peripheral lymph nodes of the animals injected with (C3HxT6)F₁ liver cells 155 days after treatment indicated that 186 of 201 metaphases studied were of liver-donor type (combined results of eight animals).
In a separate group of six animals restored with (C3HxT6)F1 newborn thymus cells and thymoma graft, the peripheral lymph nodes contained 99 of 101 metaphases of thymus-donor type at 155 days after treatment. The number of donor-type metaphases in the bone marrow of the treated animals was 16 of 40 for the animals treated with newborn liver and 5 of 63 for the animals treated with newborn thymus cells in association with strain A thymoma. When these results are compared with the figures obtained 155 days after treatment with adult spleen cells (Table III), it can be seen that the proportions of donor cells in the peripheral axillary lymph nodes are comparable and higher than 90%. The results for bone marrow are different since the proportion of donor cells when the animals were treated with adult spleen was 27%, as opposed to 40% for the animals treated with newborn liver and 7% for the animals treated with newborn thymus. Other tissues were not studied.

Host (C3Hf) component was studied with a discriminant spleen index assay in (C3HxC57BL/1)F1 and (C3HxT6)F1 hybrids. As in the animals restored with adult F1 hybrid spleen cells, the majority of the mice restored with strain A thymoma and liver cells from newborn (C3HxT6)F1 hybrids did not show a detectable host component. When 10 such animals were tested 40 days after treatment, the C3Hf component was detected in only two animals. As in the animals restored with adult spleen (Table IV) the C3Hf component was specifically tolerant to (C3HxT6)F1 and was detectable only in the (C3HxC57BL/1)F1 hybrid.

The detection of cellular chimerism and the absence of a detectable immunocompetent component of host origin indicate that the restoration is mediated by the action of the thymoma on the injected donor cells.

Combination of Thymus with Bone Marrow or Liver Cells.—Table VIII shows the results obtained with the association of cell suspensions of thymus with adult bone marrow or newborn liver and subcutaneous grafts of strain A thymomas or C3Hf thymus grafts enclosed in cell-impenetrable chambers implanted intraperitoneally. Whether adult or newborn thymus cells were used in combination with adult bone marrow or newborn liver alone or in association with thymoma grafts or thymus within diffusion chambers, no synergism was observed. The percentages of restored animals were comparable to the ones observed with similar cell dosages by themselves or by the combined effect of both cell types (Table I, V, and VI).

DISCUSSION

Cellular and humoral components of thymus function (1–15) and synergism between thymus and cells of other origins (21, 22) have been demonstrated, but the significance of these phenomena under physiological conditions remains unknown. Despite these limitations the study of reconstituted animals, taken in
conjunction with previous work, offers information about the cellular basis of immunological development in the mouse and permits interpretations of these observations.

For the present discussion of our results, the term “humoral activity” of the thymus will be applied to the type of restorative capacity observed with functional thymoma grafts and thymus or thymomas housed within cell-impenetrable diffusion chambers. “Cellular activity” will be applied to the type of restoration capacity observed with viable thymus grafts and is meant to indicate the humoral activity and both traffic of cells to and from the thymus and the influence of the putative microenvironment within the thymus stroma which favors lymphoid maturation.

In a previous publication, we postulated the existence in the peripheral lymphohemopoietic tissues of the mouse of a population of cells capable of responding, probably by proliferation, to the humoral activity of the thymus (15). This population is thymus dependent in the sense that continuous presence of the thymus is required for its renewal in animals reared in a conventional environment. This population of cells decreases progressively with time in neonatally thymectomized mice in the absence of thymic function, probably through

### TABLE VIII

Attempts to Show Synergism Between Thymus and Bone Marrow or Newborn Liver Cells in Restoration of 45-day Old, Neonatally Thymectomized C3Hf Mice

| Treatment* | Cell numbers per number treated | Number restored | % |
|------------|---------------------------------|-----------------|---|
| Adult thymus + bone marrow | 20 + 20 | 0/16 |
| Adult thymus + newborn liver | 20 + 20 | 0/14 |
| Newborn thymus + bone marrow | 20 + 20 | 0/14 |
| Newborn thymus + newborn | 20 + 20 | 0/13 |
| Adult thymus + bone marrow | 20 + 50 | 0/12 |
| Adult thymus + bone marrow | 50 + 50 | 0/10 |
| Adult thymus + bone marrow and strain A thymoma | 20 + 20 | 7/14 (50) |
| Adult thymus + bone marrow and C3Hf thymus in D.C. | 20 + 20 | 11/27 (41) |
| Newborn thymus + bone marrow and strain A thymoma | 20 + 20 | 4/12 (33) |
| Newborn thymus + bone marrow and C3Hf thymus in D.C. | 20 + 20 | 4/12 (33) |
| Newborn thymus + newborn liver and strain A thymoma | 20 + 20 | 3/6 (50) |
| Newborn thymus + newborn liver and C3Hf thymus in D.C. | 20 + 20 | 3/6 (50) |
| Adult thymus + newborn liver and strain A thymoma | 20 + 20 | 2/6 (33) |
| Adult thymus + newborn liver and C3Hf thymus in D.C. | 20 + 20 | 3/8 (37) |

* Neonatally thymectomized C3Hf mice treated at 45 days of age with intraperitoneal injection of syngeneic thymus or marrow-liver cells derived from 60-day old females or newborn mice. Thymoma grafts were subcutaneous and thymus were implanted intraperitoneally within diffusion chambers (D.C.) of 0.22 μ pore size.
physiological attrition and/or immunological commitment. This renewal process may be related to the differentiative activity of the thymus, mediated through its cellular traffic.

Our present experiments indicate that when adult lymphohemopoietic cells are used in cooperation with thymic humoral activity, restoration of 45-day old, neonatally thymectomized mice can be achieved with cell numbers that will be ineffective by themselves. If a particular cell dosage is analyzed (i.e., $2 \times 10^7$, see Table I), the effectivity for restoration when given in association with functional thymoma grafts in decreasing order is the following: spleen, lymph nodes, thoracic duct, bone marrow, thymus, blood leukocytes, and Peyer's patch cells.

When the same comparison was made for the association of cells with thymus within impenetrable chambers for the same dosage ($2 \times 10^7$) of cells (see Table V), effectiveness in restoration in decreasing order was: spleen, lymph node, thoracic duct, blood leukocytes, bone marrow, thymus, peritoneal cells, and Peyer's patch cells.

It can be proposed and supported by the present data that a certain population of lymphoid cells widely distributed in the lymphohemopoietic tissues of young adult mice is sensitive to humoral expansion by the thymus. Our results indicate that this cell population is present in different concentrations in the tissues studied with largest numbers in the peripheral lymphatic tissues (spleen, lymph nodes, and thoracic duct). Cell numbers are estimated indirectly on a functional basis, assuming that restorative effect of the cells in association with thymic function is proportional to the number of “sensitive” cells present in the inoculum. Bone marrow contains not only this population of cells, but also the cell type capable of thymus traffic in the adult mouse (5), which has been considered the true prethymic precursor (23). Our results also indicate that bone marrow does not contain competent cells capable of restoring thymectomized animals by themselves in absence of thymus. Even $1.5 \times 10^8$ syngeneic marrow cells were ineffective when tested in 45-day old, neonatally thymectomized A or C3Hf mice (Table I). These results contrast with the effectiveness of spleen and other lymphoid tissues in restoring neonatally thymectomized mice (8, 20).

The presence in the blood and thoracic duct of cells capable of cooperative effect when associated with thymic function (Table I) suggests that they can be mobilized according to demands. Preliminary work indicates an increase of this circulating cell in blood following 500 R of whole body irradiation (Stutman, unpublished observations). On the other hand, the cell that is sensitive to humoral activity of the thymus is absent from peritoneal and adult liver cell populations.

When Peyer's patch cells, containing mainly small lymphocytes, were tested, the results were borderline (Tables I and V) and could be accounted for by contamination of the patch cells with blood (as could the small responsiveness
of peritoneal cells seen in Table V). Two other interpretations can be proposed for the relative lack of influence of Peyer's patch cells in this regard. One is that, if their lymphoid cells are comparable to the avian bursa (24), they might contain precursors for the antibody-producing series and not for the population responsible for cell-mediated immunity that was analyzed in the present work. On the other hand, this population could also represent highly immunized peripheral lymphoid tissue, already committed immunologically and incapable of further differentiation (25). Preliminary work suggests that the latter possibility may indeed be correct since spleen cells from adult animals hyperimmunized with brucella antigen are less effective using the present model, as compared to nonimmunized spleen cells (Stutman, unpublished observations).

The mechanism of this cooperation between cells and the thymic humoral activity in producing restoration of thymectomized mice seems to be related to proliferative expansion of the injected cells. Tables II–IV indicate the results obtained with F₁ hybrid cells in association with thymoma grafts. These results strongly support the view that restoration is achieved by establishment of the transplanted population of cells and its proliferative expansion. This is indicated by the specific tolerance for grafts of the other parental type (Tables II and VII), by chimerism reflected by chromosome analysis showing spleen-donor type cells throughout the lymphatic tissues of the host in numbers greater than could be attributed to the original inoculum (Table III), and by absence of significant immunocompetence attributable to the host in graft-versus-host assays (Table IV).

Comparable results were obtained when similar experiments were performed using cells from newborn animals. Newborn spleen, hemopoietic liver or thymus cells in association with the strain A thymoma, produced significant restoration of neonatally thymectomized 45-day old recipients. If a particular cell dosage is compared (2 X 10⁷), the effectiveness in restoration was 55, 41, and 41% respectively for spleen, liver, and thymus. When compared to the same dosage of adult cells, newborn thymus had greater activity than that of the adult (Tables I and VI). These results suggest that the newborn thymus contains higher numbers of possible competent cells than does the adult thymus, cells that are probably in the process of acquiring the ability to migrate to the lymphohemopoietic tissues but are already sensitive to the humoral activity of the thymus. Hemopoietic newborn liver was slightly more effective than was adult bone marrow, while the results for spleen were comparable. This last point is of interest because it indicates that the content of cells capable of cooperative restoration when associated with humoral thymic function is similar in newborn and adult spleen, while it is known that the content of mature competent cells capable of producing graft-versus-host reactions is almost absent from newborn spleen (26). Comparable results have been obtained when newborn cell inocula were associated with thymus grafts enclosed in diffusion chambers (27, 28).
When F₁ hybrid newborn liver cells were used (Table VII) the results indicated that proliferative expansion of the injected population of cells mediated the restoration. Since a peculiar synergism between thymus and bone marrow cells has been described for some immune responses (21, 22), the effect of combining dispersed thymus cells and bone marrow or newborn liver cells was studied. Table VIII shows that no particular synergism between these cell types was observed when the cells were given in association with either strain A thymoma or thymus grafts in diffusion chambers. An absence of such synergism for graft-versus-host cell-mediated immunity has previously been described when thymus-bone marrow combinations (29, 30) or thymus-fetal liver combinations were used (31). On the other hand, fetal liver can act synergistically with thymus cells for the humoral response to sheep red cells (32).

In previous publications, we proposed a model that fits with the present data (15, 27). We postulated that the population of cells present in the lymphohemopoietic tissues of newborn or adult animals, capable of cooperative restoration when associated with humoral activity of the thymus, represents a population of cells that has received thymic influence before thymectomy was performed. The humoral activity of the thymus can expand solely such a population of "postthymic" cells in the peripheral tissues of the host. The word "expand" is used here to indicate proliferation and/or also a differentiative process eventually leading to a larger number of fully competent cells. At the present writing the words "humoral," "expander," and "inducer" are used as operational terms, since the evidence for a true hormonal lymphopoietic effect of the thymus is still unclear (33).

We also suggested that the population of postthymic cells was incapable of self-renewal in absence of the thymus and that the process by which this population was replaced included traffic of hemopoietic stem cells to the thymus (15). We have shown that embryonic hemopoietic tissues do not contain postthymic cells capable of cooperative restoration when associated with humoral activity of the thymus (27, 28). The embryonic hemopoietic tissues, however, contain a cell population that we termed "prethymic," population characterized by capacity to traffic to thymus and bone marrow (34) and which is insensitive to the humoral activity of the thymus (27, 28).

The ontogeny of lymphoid development may be interpreted as a result of both processes: first a build up of the postthymic population by traffic of hemopoietic stem cells through thymic stroma during the final stages of embryonic development and first days after birth, and further expansion of the postthymic population by humoral activity of the thymus. The maintenance of the postthymic population seems to be dependent on a continuing traffic of bone marrow cells to thymus, as well as the expanding influence of the thymic humoral function.
SUMMARY

Immunological restoration of 45-day old, neonatally thymectomized C3Hf mice by treatment with humoral thymic function (thymoma grafts, thymus or thymoma in diffusion chambers) ranges from 0 to 12% and is difficult to achieve. When small numbers (5–20 × 10⁶) of young adult lymphohemopoietic cells, ineffective by themselves, are given in association with humoral thymic function, a cooperative effect is observed and restoration ranges from 30 to 60%. With a particular cell dosage (20 × 10⁶), effectiveness for cooperation with thymic function was the following in decreasing order: spleen, lymph nodes, thoracic duct cells, bone marrow, blood leukocytes, thymus, and Peyer's patch cells. Comparable results were obtained using spleen, thymus, and hemopoietic liver from newborn donors in association with thymic function. For similar cell dosages, newborn thymus cells were more effective than adult thymus in their cooperative effect with thymic function. Dispersed thymus cells in association with young adult bone marrow or newborn hemopoietic liver cells showed no synergism for the cooperative effect with thymic function in the present model.

Using hemiallogeneic cells (F₁ hybrid into parent) it was possible to show that restoration was mediated by proliferative expansion of the injected cells. This was indicated by specific tolerance to tissues of the other parental strain and by cellular chimerism, especially of lymphoid tissues, as indicated by chromosome markers and absence of significant numbers of immunocompetent cells of host origin.

A population of paritally differentiated cells of hemopoietic origin, termed postthymic, sensitive to humoral activity of the thymus and present in the lymphohemopoietic tissues of adult and newborn mice is postulated to explain our results. These cells are postthymic and thymus dependent in the sense that they already received thymic influence, probably through traffic, and are incapable of self-renewal in absence of the thymus. Sensitivity to humoral activity of the thymus is characterized by proliferative expansion and/or a differentiative process eventually leading to larger numbers of competent cells.

The authors wish to thank M. J. O'Connor, J. M. Smith, A. Hanson, and C. Baker for their able assistance.

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