The ability of thymus to restore the immune deficiency produced by neonatal thymectomy in mice decreases with the delay of treatment after thymectomy (1). Eventually a state is reached when thymus grafts become ineffective, especially when the animals develop the postthymectomy-wasting syndrome (2, 3). These results were interpreted as indicating that a population of cells in the host, capable of responding to the action of thymus, decreased progressively with time in absence of thymus (1). Under these conditions, and using as model the 45-day old, neonatally thymectomized mouse, we have shown that lymphohemopoietic cells of adult and newborn origin can act in cooperation with thymus function in restoring such a host (4). This cooperative effect was observed especially with cells from spleen, lymph node, thoracic duct, thymus, bone marrow, or newborn hemopoietic liver (4). The cooperative effect of the adult or newborn cells was observed whether thymus and thymoma grafts or thymus and thymoma within diffusion chambers were used (4).

The present report is a comparison of the cooperative effect of hemopoietic cells from newborn or embryonic liver when associated with thymic function. Our results indicate that while newborn cells are effective when associated with humoral activity of the thymus (thymoma grafts or thymus in diffusion chambers), embryonic cells are effective only in association with viable free thymus grafts.

Since we have shown that hemopoietic liver cells are capable of traffic to thymus (5), we interpret the present results as indicating that a differentiative process including thymus traffic may be responsible for the development of
the "postthymic" cells present in the newborn and adult tissues that are sensitive to the humoral activity of the thymus.

Materials and Methods

The animals used and the majority of the techniques have been described in a previous publication (4, 6).

The dating of fetal development was based on timed matings of hormonally primed immature females, with the first day of gestation being counted from the morning after mating (8).

The techniques for intraperitoneal and subcutaneous thymus grafting have been described in a previous publication (3), and thymus grafting under the left kidney capsule was performed as described by Dukor et al. (7). Thymic lobes were cut in half, and both fragments were implanted under the kidney capsule. Thymus graft donors were 20-day old females.

Cell suspensions of hemopoietic liver from embryos or newborn mice were prepared with glass homogenizers with loose fitting pestles as described in previous publications with cells of other sources (2, 3). The whole uteri, each fetus, and then the excised livers were repeatedly rinsed in large volumes of ice-cold buffered Ringer's solution to eliminate contamination from the mother's blood.

As in the previous papers, diffusion chambers were prepared with Millipore filters of 0.22 μm mean pore size (6).

The thymomas and restoration criteria of the neonatally thymectomized animals have been described in previous publications (1, 4, 6).

Experimental Design.--The experimental model used was described in a previous paper (4). Basically the model consisted of 45-day old, neonatally thymectomized C3Hf mice injected intraperitoneally with variable numbers of hemopoietic liver cells from newborn or embryonic C3Hf donors and grafted with one of the following types of tissues: (a) strain A thymoma, subcutaneous (Table I); (b) C3Hf thymus, subcutaneous, intraperitoneal, or under the kidney capsule (Table II); (c) intraperitoneal diffusion chambers containing thymus or thymoma (Table III); and (d) subcutaneous thymus grafts from allogeneic or hemiallogeneic origin (Table IV). In one experiment (Table V), the animals were injected intraperitoneally with the combination of dispersed thymus cells and newborn or embryonic liver cells.

RESULTS

Table I shows the attempts to restore 45-day old, neonatally thymectomized A or C3Hf mice with strain A thymoma in association with hemopoietic liver cells from 17- to 18-day old embryos syngeneic to the host. With cell dosages ranging from 20 to 500 × 10⁶, the embryonic liver cells were ineffective when associated with the strain A thymoma, whether in the syngeneic (A) or allogeneic (C3Hf) combination. The grouped results show 8 of 91 animals of the A strain to be restored and 7 of 80 in the C3Hf groups. These results are essentially similar to the results obtained with thymoma alone. Within the cell dosages tested, embryonic liver cells were ineffective by themselves in producing restoration of the thymectomized hosts. On the other hand, when subcutaneous thymus grafts were used in the A strain mice in association with syngeneic embryonic liver cells restoration was observed in 25 of 45 treated animals (55%) as opposed to 5 of 22 (23%) for the group treated with a subcutaneous thymus graft only.

Table II shows the restoration of 45-day old, neonatally thymectomized
TABLE I
Attempts to Restore 45-day Old, Neonatally Thymectomized A or C3Hf Mice with Strain A Thymoma and Embryonic Liver Cells Syngeneic to the Host

| Treatment* | Number restored per number treated |
|------------|----------------------------------|
|            | Graft Cells | A strain | C3Hf |
|            | × 10⁶ | % | % |
| A thymoma | None | 4/30 (13) | 2/19 (10) |
| None   | 20 | 0/10 | 0/10 |
| None   | 50 | 0/10 | 0/12 |
| None   | 100 | 0/10 | 0/17 |
| None   | 200 | ND | 0/10 |
| None   | 400 | ND | 0/5 |
| A thymoma | 20 | 3/37 (8) | 1/12 (8) |
| A thymoma | 50 | 1/19 (5) | 1/12 (8) |
| A thymoma | 100 | 2/14 (14) | 2/23 (9) |
| A thymoma | 200 | 1/12 (8) | 1/10 (10) |
| A thymoma | 400 | 1/9 (11) | 1/9 (11) |
| A thymoma | 500 | ND | 1/14 (7) |
| A thymus | None | 5/22 (23) | ND |
| A thymus | 50 | 10/20 (50) | ND |
| A thymus | 100 | 15/25 (60) | ND |

* Neonatally thymectomized A and C3Hf mice, treatment performed at 45 days of age. Grafted with 1 × 10⁶ subcutaneous thymoma cells or a subcutaneous graft of 20-day old, strain A thymus. Cells injected intraperitoneally, simultaneous with grafting. All cell donors were 17- to 18-day old, A or C3Hf embryos, syngeneic to the host.

TABLE II
Restoration of 45-day Old, Neonatally Thymectomized C3Hf Mice with Syngeneic Thymus Grafts in Association with Newborn or Embryonic Syngeneic Hemopoietic Cells*

| Type of thymus graft | Number restored per number treated |
|----------------------|----------------------------------|
|                      | No cells | Newborn cells | Embryonic cells |
|                      | % | % | % |
| No graft             | 0/39 | 0/16 | 0/19 |
| Subcutaneous         | 3/30 (10) | 22/32 (69) | 25/38 (66) |
| Intraperitoneal       | 8/30 (26) | 8/10 (80) | 23/30 (76) |
| Under kidney capsule  | 3/29 (10) | 6/12 (50) | 11/20 (55) |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Thymus grafts from 20-day old C3Hf females. Each recipient grafted with one thymus except the grafts under the kidney capsule that were of only one lobe. The animals were also injected with 100 × 10⁶ viable nucleated liver cells derived from less than 24 hr newborn or 17- to 18-day old embryos of C3Hf origin.

C3Hf mice treated with 100 × 10⁶ liver cells from 17- to 18-day old embryos or newborn C3Hf mice and the association with C3Hf thymus grafts. Thymus grafts were placed either subcutaneously, intraperitoneally, or under the renal capsule, and the cells were always injected intraperitoneally. The results indi-
cate that both cell types, embryo or newborn, were equally effective when associated with syngeneic thymus grafts, especially when the grafts were placed intraperitoneally. Restoration when thymus grafts were associated with cells ranged from 50 to 80%, while thymus grafts alone restored only 10–26% of the animals. As in previous work, intraperitoneal thymus grafts were the most effective restorative treatment of the thymectomized hosts (3).

The previous experiments indicate that embryonic hemopoietic liver cells can produce significant restoration of thymectomized hosts when associated with thymus grafts but not with functional thymomas, while newborn liver cells produce equal restoration when associated with thymus or thymoma grafts.

Table III shows the restoration of 45-day old, neonatally thymectomized C3Hf mice with thymomas or thymus within diffusion chambers in association with newborn or embryonic liver cells of C3Hf Origin.

| Thymic function | Number restored per number treated | None   | Newborn liver cells | Embryonic liver cells |
|-----------------|------------------------------------|--------|---------------------|-----------------------|
|                 | %                                  | %      | %                   | %                     |
| C3Hf thymus     | 1/40 (2)                           | 10/31 (32) | 1/41 (2)            |                       |
| 5 C3Hf thymii   | 2/39 (5)                           | 10/30 (30) | 1/39 (2)            |                       |
| A thymus        | 0/16                               | 5/22 (22)   | 0/19                |                       |
| C3H thymoma No. 2 | 0/15                           | 6/28 (21)  | 0/40                |                       |
| A thymoma       | 2/30 (7)                           | 32/70 (46) | 2/39 (5)            |                       |
| Empty chamber   | 0/12                               | 0/12     | 0/10                |                       |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Intraperitoneal implantation of diffusion chambers (lucite rings and Millipore filters 0.22 μm pore size) containing thymus from 20-day old normal donors or 2 × 2 mm. fragments of thymomas. Liver cells (100 × 10⁶) were injected intraperitoneally after chamber implantation and were derived from newborn or 17- to 18-day old embryos of C3Hf origin.

Table IV shows the effect of association of allogeneic or hemiallogeneic sub-
cutaneous thymus grafts with newborn or embryonic (17–18-day old) C3Hf liver cells on the restoration of 45-day old, neonatally thymectomized C3Hf hosts. Restoration with thymus grafts alone ranged from 5 to 8%. When thymus grafts were associated with newborn liver cells restoration ranged from 21 to 41%. When thymus grafts were associated with embryonic liver cells restoration ranged from 16 to 20% and in one combination it was ineffective. These results are in contrast with similar experiments performed with the completely syngeneic system (see Tables I and II) in which restoration usually surpassed 50–60%. In a previous report, we have shown that traffic of liver cells to thymus grafts is more effective if cells and graft are syngeneic, indicating that histocompatibility differences play a role in this type of traffic (5).

Table V shows attempts to restore 45-day old, neonatally thymectomized C3Hf mice by the intraperitoneal injection of dispersed newborn or 20-day old thymus cells in association with newborn or embryonic liver cells or adult bone marrow cells. None of the cell combinations was effective in producing significant restoration of the thymectomized hosts, indicating that synergism between thymus and hemopoietic cells (liver or marrow) is not observed in the present experimental condition and that a viable thymus graft is essential for the expression of the restorative capacities of embryonic and newborn liver.

In an attempt to determine the time when hemopoietic liver cells became capable of restoring 45-day old, neonatally thymectomized C3Hf animals in association with strain A thymoma, experiments were performed using donors of ages ranging from 12 days of embryonic life to 10 days after birth. Table VI shows that hemopoietic liver cells from embryos of ages ranging from 12–14 to

### Table IV

| Thymus grafts* | Number restored per number treated |
|----------------|-----------------------------------|
|                | No cells | Newborn liver | Embryonic liver |
|                | %        | %            | %              |
| A              | 1/20 (5) | 10/30 (33)   | 2/16 (16)      |
| A              | —        | 13/31† (41)  | 1/5‡ (20)      |
| C57BL/1        | 0/12     | 3/14 (21)    | 0/4            |
| CBA/H          | 1/16 (6) | 12/30 (40)   | 1/5 (20)       |
| (C3HxA)F1      | 1/12 (8) | 12/30 (40)   | 2/10 (20)      |
| (C3HxT6)F1     | 1/18 (5) | 5/20 (25)    | 1/6 (16)       |

* Neonatally thymectomized C3Hf mice, treatment performed at 45 days of age. Thymus grafts from 20-day old female donors, placed subcutaneously. Cells were injected intraperitoneally after thymus grafting and each animal received 100 × 10⁶ viable cells from newborn or 17–18-day old embryos of C3Hf origin.

† 200 × 10⁶ cells.

‡ 200 × 10⁶ cells.
**TABLE V**

*Attempts to Restore 45-day Old, Neonatally Thymectomized C3Hf Mice with Dispersed Syngeneic Thymus Cells and Newborn or Embryonic Liver Cells*

| Treatment* | Cells | Number restored per number treated |
|------------|-------|-----------------------------------|
|            | × 10⁶ |                                    |
| Thymus     | 50    | 1/16                              |
| Thymus     | 100   | 2/13                              |
| Newborn thymus | 50 | 0/12                              |
| Newborn thymus | 100 | 0/12                              |
| Thymus + newborn liver | 50 + 50 | 0/14                          |
| Thymus + newborn liver | 50 + 100 | 0/10                          |
| Thymus + newborn liver | 100 + 100 | 1/14                        |
| Newborn thymus + newborn liver | 100 + 100 | 0/16                        |
| Thymus + embryonic liver | 50 + 50 | 0/13                        |
| Thymus + embryonic liver | 100 + 100 | 0/9                          |
| Thymus + adult marrow | 100 + 100 | 2/14                        |

* Neonatally thymectomized C3Hf mice treated at 45 days of age with intraperitoneal injection of the cells. Thymus cells from 20-day old females; newborn, less than 24 hr and embryos were 17-18 days old.

**TABLE VI**

*Attempts to Restore 45-day Old, Neonatally Thymectomized C3Hf Mice with Strain A Thymoma and C3Hf Liver Cells of Different Ages*

| Treatment* | Number restored per number treated |
|------------|-----------------------------------|
| Tumor graft | Age of cell donor | %        |
| A thymoma   | None              | 2/19 (10) |
| A thymoma   | 12-14             | 1/13 (7)  |
| A thymoma   | 15-16             | 1/13 (7)  |
| A thymoma   | 17-18             | 1/14 (7)  |
| A thymoma   | 19-21             | 4/14 (28) |
| A thymoma   | Birth             | 7/13 (54) |
| A thymoma   | 1                 | 7/13 (54) |
| A thymoma   | 3                 | 6/12 (50) |
| A thymoma   | 6                 | 4/12 (33) |
| A thymoma   | 10                | 1/13 (7)  |

* Neonatally thymectomized C3Hf mice treated at 45 days of age. Subcutaneous injection of 1 × 10⁶ tumor cells. Intraperitoneal injection simultaneous with tumor grafting of 50 × 10⁶ liver cells from C3Hf donors of different ages, ranging from 12-14 embryonic days to 10 days after birth.
17–18 days were ineffective. Cells from 19 to 21-day old embryos gave some degree of restoration (28%) and liver from newborn, 1, and 3 days after birth gave substantial restoration, usually higher than 50%. Liver cells from 6-day old animals were less effective (33%), and cells from 10-day old animals ineffective, as were adult liver cells (see Table I). This last result appears to be the consequence of a decrease of hemopoietic elements in liver occurring during the first week after birth (9). The capacity to restore thymectomized animal when hemopoietic liver cells are given in association with functional thymomas appears shortly before birth and is maintained during the first days after birth during the period when the liver is a major source of hemopoietic elements.

On the other hand, when comparable experiments were performed using thymus grafts in association with hemopoietic embryonic liver of various ages, the results were different. Table VII shows that all ages tested, ranging from 10 to 19 days of embryonation, were equally effective in the cooperative restoration.

**DISCUSSION**

As in a previous paper (4), the term “humoral activity” of the thymus will be applied to the type of restorative capacity observed with functional thymoma grafts and with thymus or thymomas within cell-impenetrable diffusion chambers. “Cellular activity” will be applied to the type of restoration observed
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with viable thymus grafts and is meant to indicate the humoral activity and both the cellular traffic of cells to and from the thymus and the influence of the thymic microenvironment that favors lymphoid differentiation.

Embryonic liver cells (17–18-day old) were unable to cooperate with syngeneic or allogeneic functional thymoma grafts to produce restoration of 45-day old, neonatally thymectomized mice (Table I). This absence of effect was observed within a wide range of cell dosages (20–500 × 10⁶). In a previous paper, and using a similar model, we have shown that newborn hemopoietic liver cells are effective in restoring thymectomized animals when associated with functional thymoma grafts (4). On the other hand, embryonic hemopoietic liver cells were as effective as were newborn cells when given in association with syngeneic thymus grafts (Tables I and II). The cooperative effect in producing restoration was observed with embryonic liver cells and viable thymus grafts placed either in subcutaneous tissue, intraperitoneally, or under the kidney capsule.

The discrepancies between the cooperation of embryonic hemopoietic liver cells and thymus grafts as opposed to thymoma grafts suggested that the normal thymic stroma and perhaps direct contact within stroma played an important role. This idea has been supported by previous work showing the existence of a traffic of hemopoietic liver cells to thymus grafts (5). Present studies also suggest that newborn hemopoietic tissues already contain cells capable of responding to the humoral influence of the thymus.

To test the possibility of direct traffic, embryonic or newborn liver cells were given in association with allogeneic or syngeneic thymus or thymomas enclosed in cell-impenetrable diffusion chambers implanted intraperitoneally (Table III). The results showed that as would be predicted from the thymoma studies, newborn cells were effective in producing restoration of neonatally thymectomized hosts, while embryonic cells were ineffective. These results support the view that a step including direct contact of embryonic hemopoietic cells and thymus stroma may be involved.

Since we have shown that histocompatibility differences are of importance in regulating the traffic of hemopoietic liver cells to thymus grafts (5), the next set of experiments consisted of the association of embryonic or newborn liver cells and allogeneic or hemiallogeneic-free subcutaneous thymus grafts (Table IV). The prediction was that the allogeneic grafts will exert the humoral activity thus cooperating with the newborn cells, while they will not allow good traffic and produce less effective restoration when associated with embryonic cells. The results indicate that the assumption was correct, since embryonic cells were 50% less effective than newborn cells when associated with allogeneic thymus grafts. Indeed, in one strong H-2 allogeneic difference they seemed to be completely ineffective.

The possibility that intact thymic structure is essential for the cooperation
of thymus and embryonic hemopoietic cells was tested by combining the liver cells with fully dispersed thymus cells (Table V). No cooperative effect was observed within the cell dosages tested. These findings support previous work indicating the absence of synergism between thymus and bone marrow cells (10) or liver cells (11) in the development of thymus-dependent immunities.

The possibility of a trephocytic function of the thymocytes (12) has been considered, but the present experiments seem to indicate that embryonic hemopoietic liver cells require intact thymus stroma to develop into a population capable of restoring thymectomized hosts.

The time of appearance in liver of the cells sensitive to the humoral activity of the thymus, termed “postthymic” cells in previous papers (4, 13), indicated that such cells can be detected as early as the 19th–21st day of embryonation. The number of these cells increases at birth and in the immediate postnatal period, while the liver is the major source of hemopoietic elements. To explain these results, we have to accept the assumption that postthymic cells that originated from “prethymic” hemopoietic precursors are capable of returning to the liver, perhaps as part of the process by which the mouse develops the adult type of hemopoiesis in marrow and spleen (9). Similarly, we have to accept that postthymic cells can return to the adult bone marrow, as shown in our previous experiments (4, 14). The adult bone marrow would contain a combination of prethymic cells capable of thymus traffic and postthymic cells, sensitive to the humoral activity of the thymus, that received already thymic influence through traffic. Using direct intrathymic labeling techniques, the utilization of thymic DNA by bone marrow cells has been demonstrated, suggesting such traffic patterns (15).

Hemopoietic fetal liver contains cellular elements capable of thymus repopulation after lethal irradiation (16) and of producing immunological recovery of irradiated hosts (17). Using injection of embryonic cells of parental type into sublethally irradiated F₁ hybrids and then retransplanting the tissues of these animals into second irradiated hosts, it has been shown that cells present in embryonic liver mature within the primary host and can produce graft-versus-host reactions when transplanted to secondary hosts (18). This maturation process is thymus dependent, since it does not occur if the primary host are thymectomized in adult life and then irradiated (19, 20). Thymus grafts but not thymus within diffusion chambers were capable of producing maturation of the embryonic liver cells in the adult thymectomized-irradiated primary host (21). Similarly, immunological competence is not acquired in vitro by embryonic liver cells unless they are cultured in combination with thymus tissue (22). When comparable studies using double transfer technique were employed in the adult mouse, bone marrow was the source of these potentially immunocompetent cells (23). Thymus grafts and thymus within diffusion chambers in association with bone marrow cells produce maturation of these bone marrow
derived cells (19, 24). Embryonic liver also contains precursors of antibody-forming cells (17) and a thymus-independent cell population of precursors of antibody-producing cells has been described (25). This population can mature and respond immunologically in absence of the thymus (26) and may represent the population capable of direct traffic to bone marrow observed in our previous experiments (5).

Although using different models, the latter experiments are concordant with our present findings. The embryonic liver experiments are compatible with the interpretation that only prethymic cells are present in embryonic liver, whereas late embryonic or newborn liver and bone marrow contain populations of both pre- and postthymic cells.

From our previous and present studies, it can be postulated that postthymic cells are mainly present in lymphoid tissues of newborn and young adult mice, while the prethymic cells are mainly present in the hemopoietic tissues of both fetal, newborn and young adult mice. Embryonic hemopoietic tissues contain only prethymic cells while hemopoietic tissues from newborn and young adult mice contain mixtures of both pre- and postthymic cells.

Whether the results of our studies reflect accurately normal ontogenic development cannot be ascertained from the present experiments. One can only conclude that embryonic liver contains cells that are potentially competent to restore immune functions of thymectomized animals, and that these cells require thymic influence, represented as a viable free thymus graft, for maturation.

The notion of the pre- and postthymic precursors present in the lymphohemopoietic tissues of the mouse, the ontogenic development of these cell populations, their different requirements for thymic grafts, and their relative sensitivity to humoral function of the thymus may help define thymic function under physiological conditions.

SUMMARY

Significant immunological restoration of 45-day old, neonatally thymectomized C3Hf mice was obtained by the cooperation of syngeneic newborn or embryonic hemopoietic liver cells with thymic function. Thymic function or cells alone are almost ineffective or restore approximately 10% of the animals.

Newborn liver cells are effective in association with thymus grafts or humoral thymic function (thymoma grafts and thymus or thymomas in diffusion chambers). Embryonic liver cells are ineffective, even in large numbers, when associated with humoral thymic function. On the other hand, embryonic liver cells are effective in the cooperative effect only in association with viable thymus grafts, preferably syngeneic, whether the grafts were placed subcutaneously, intraperitoneally, or under the kidney capsule. Dispersed viable thymic cells are ineffective in association with embryonic liver cells. Cells capable of co-
operating with humoral thymic function start to appear in embryonic liver by
day 19–21 of gestation and are detectable until day 5–6 postbirth. Embryonic
hemopoietic liver cells from 12 to 18 days of gestation contain cells capable of
cooperation only with viable free thymus grafts and not with humoral thymic
function.

A prethymic cell population of partially differentiated cells of hemopoietic
origin, insensitive to humoral activity of the thymus but requiring thymic
stroma and traffic through the thymus is postulated to explain our results. This
population of prethymic cells can become postthymic through this process and
eventually develop into competent cells. Postthymic cells are characterized by
their sensitivity to humoral activity of the thymus and by their wide distribu-
tion in the lymphohemopoietic tissues of newborn and young adult mice.

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