STUDIES ON THE DIFFERENTIATION OF THYMUS-DERIVED LYMPHOCYTES

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There is substantial evidence that stem cells migrate from yolk sac and fetal liver in embryonic life (1, 2) and from bone marrow in adult life (3, 4) into the thymus. Within the thymic environment, these migrant cells proliferate and differentiate into thymus lymphocytes (thymocytes). Although an unknown number of thymus lymphocytes may die in situ (5), cell-marker experiments have shown that some of them do migrate to peripheral lymphoid tissues where they are appropriately termed "thymus-derived" lymphocytes (6-12).

A number of distinct cell-surface alloantigens have been described on mouse lymphocytes (13, 14). Alloantigens such as θ, TL, Ly-A, and Ly-B show variable representation on bone marrow cells, thymocytes, and peripheral lymphocytes (15, 16) which suggests that they may prove useful as surface markers of cell differentiation. In this paper we describe experiments in which θ and TL alloantigens were used to investigate the differentiation pathway leading to the maturation of thymus-derived lymphocytes.

Thymic rudiments were removed from mouse embryos at a stage (14 days' gestation) when they had received migrant stem cells but before these cells had differentiated to lymphocytes. In a first series of experiments, 14 day rudiments were placed in diffusion chambers and cultured in the nutritive environment provided by the chick embryo chorioallantois. This allowed changes in representation of surface alloantigens (θ and TL) during the differentiation of stem cells to be studied in a "closed system" and the complication of further inflow of cells, which might themselves have surface alloantigens, could be avoided.

In a second series of experiments, thymus from CBA.H embryo donors was placed under the kidney capsules of thymectomized, irradiated, bone marrow-reconstituted AKR hosts. Since there are two θ alloantigen specificities, θAKR present in AKR and a few related strains of mice, and θC3H in all other strains tested, peripheral (thymus-derived) lymphocytes originating from the graft (θC3H) could be identified and studied in host lymphoid tissues (θAKR).

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Materials and Methods

Thymic rudiments were dissected from mouse embryos of CBA.H, BALB/c, and A strains using a stereomicroscope and fine cataract knives. Gestational stage was determined by noting the appearance of vaginal plugs (counted as day 0). Cytotoxicity tests for \( \theta \) alloantigen were carried out in CBA.H and BALB/c strains (\( \theta C 3 H \) specificity) and for TL alloantigen in A strain (TL 1, 2, 3 specificities).

Diffusion Chamber Cultures of Embryonic Thymus.—The technique used was the same as that previously described for the culture of embryonic thymus (2) and a variety of other cell types (17, 18). In brief, chambers were constructed by cementing Millipore (type TW) filter discs (Millipore Filter Corp., Bedford, Mass.) to one side of Plexiglas diffusion chamber rings (14 mm diameter) with Millipore MF cement. Thymic rudiments were carefully placed on the floor of each chamber, which was left open. Diffusion chambers were placed onto the chorioallantois of 7 or 8 day chick embryos through a hole cut in the shell. No fluid media was added since the chambers fill with nutritive fluid from the chorioallantoic membrane. Finally, the eggs were sealed and incubated in a horizontal position with the chambers lying on the “collapsed” chorioallantois.

In most experiments 14-day thymic rudiments were cultured for periods of 4 days and experiments were designed so that one thymic lobe from each embryo in a litter was cultured, while the other lobe was used for immediate cytotoxic testing. Cell suspensions were prepared by teasing tissues with fine knives in veronal-buffered saline containing 0.1% bovine serum albumin (VBS).1

Embryonic Thymus Grafts to Irradiated Hosts.—AKR mice were thymectomized at 6-8 wks of age and 10 days later were irradiated (800 R from a Cobalt 60 source) and injected with AKR bone marrow cells. Thymic rudiments were removed from 14 day CBA.H embryos and were placed under the kidney capsules of AKR hosts within 3 days of irradiation.

At various intervals after transplantation, mice were killed and thymus grafts were removed. The relative proportions of host and donor lymphocytes in grafts were determined by cytotoxicity tests with anti-\( \theta C 3 H \) and anti-\( \theta AKR \) sera. At the same time, these antisera were used in cytotoxicity tests on host lymph node lymphocytes and blood lymphocytes. Cells were obtained from pooled lymph nodes (mesenteric, inguinal, axillary, brachial, and cervical) and blood lymphocytes were obtained by the following procedure. Defibrinated blood was mixed 1:1 with plasmagel (Laboratoire Roger Bellon, Seine, France). Erythrocytes were allowed to sediment for 15-30 min at room temperature, after which the supernatant was removed and the cells contained in it were washed in medium 199 containing 15% fetal calf serum (FCS) and incubated in a glasswool column for 30 min at 37°C. Finally, lymphocytes were eluted from the column with fresh medium 199 and FCS.

Antisera and Cytotoxicity Testing.—Anti-\( \theta C 3 H \) serum was prepared by immunizing AKR mice with CBA thymocytes, and anti-\( \theta AKR \) serum by immunizing CBA mice with AKR thymocytes (13). The antisera were heat inactivated at 56°C for 30 min. The cytotoxic activity of the antisera could be completely absorbed by brain, which suggests its anti-\( \theta \) specificity (19).

Anti-TL serum (against specificities TL 1, 2, 3) was kindly supplied by Dr. L. J. Old, Sloan-Kettering Institute for Cancer Research, New York, and was prepared by injecting ASL1 leukemia cells (a transplantable A strain leukemia) into A (TL-negative congenic) mice (15). Trypan blue dye-exclusion cytotoxicity testing was carried out by the method of Boyse, Old, and Chouroulinkov (20), but incorporating the modification described by Schlesinger (21, 22). In brief, cells at a concentration of 0.5-1 \( \times 10^6 \) ml were first incubated in antisera at room temperature for 30-45 min; they were then washed in VBS and finally were incubated

1 Abbreviations used in this paper: FCS, fetal calf serum; VBS, veronal-buffered saline containing 0.1% bovine serum albumin.
in complement at 37°C for a further 30 min. Hamster serum, absorbed with mouse liver and
spleen to remove its natural toxicity for thymocytes, was used at a final concentration of 1:7
as a source of complement for tests on thymus cells. Guinea pig serum, absorbed with mouse
erthrocytes, was used at a final concentration of 1:15 as a source of complement for tests on
peripheral lymphocytes.

After treatment with complement, cells were spun down and the supernatant was discarded.
The cells were resuspended in a small quantity of trypan blue in saline and the suspension

![Graph](image)

Fig. 1. Cytotoxicity tests showing the development of θC3H alloantigen on 14 day CBA
embryo thymus cells after 4 days culture in diffusion chambers on chick embryo chorioallantois.
One thymic lobe from each embryo in a litter was cultured while the other lobe was used for immediate cytotoxicity testing. In further experiments, θC3H alloantigen was found
to develop on CBA thymus cultured in diffusion chambers placed intraperitoneally in AKR
hosts (θAKR), thus showing that the developing alloantigen could not be of host origin.

was examined in a hemocytometer chamber using a thin cover slip and phase contrast optics.
This technique allows stained (dead) and unstained (live) cells to be clearly distinguished and,
in addition, lymphocytes can be separately identified from red blood cells.

Each cell suspension was tested with a series of antiserum dilutions beginning at 1:4 in
the case of thymus cells and 1:2 for peripheral lymphocytes, and each test included a comple-
ment control (containing complement but no antiserum) or an antiserum control (containing
antiserum but no complement). The values of the percentage of stained cells in experimental
tubes were corrected by subtracting the percentage of dead cells in control tubes (c) and multi-
plying by a factor of 100/100-c (22). This correction is based on the assumption that alloanti-
gen-bearing cells are proportionally represented among dead cells in control tubes.
RESULTS

Development of θ Alloantigen on Embryonic Thymus-Stem Cells.—Fig. 1 shows the results of cytotoxicity tests to demonstrate θC3H alloantigen on 14 day CBA embryo thymus cells both before and after a period of 4 days' culture in diffusion chambers. It is clear that at the end of the culture period the cells show a sensitivity to the cytotoxic action of anti-θC3H serum and complement which is not found in 14 day embryo thymus cells before culture. Indeed, the proportion of cells killed after culture corresponds with the proportion killed in suspensions of normal 18 day CBA embryo thymus treated with anti-θC3H serum and complement (Fig. 2). This result was confirmed in four further experiments in which 14-day CBA or BALB/c thymus rudiments were cultured for 4 days (Table I).

The histological appearances of 14 day embryo thymus before and after a 4 day culture period are shown in Figs. 3 and 4, and cells in suspensions on which cytotoxicity tests were carried out are shown in Figs. 5 and 6. There are obvious morphological changes occurring in the cell population during culture. Thus, the predominant cell type in suspensions of 14 day embryo thymus is a large basophilic cell, whereas the predominant cell in suspensions after 4 days' culture is a small lymphoid cell. Since many mitotic figures can be seen in sections of thymus at the end of culture and since thymus rudiments show a considerable

![Graph showing results of cytotoxicity tests](image-url)
increase in size during the culture period, cell proliferation must accompany the morphological and surface-alloantigen changes found.

In further experiments, 14 day thymic rudiments were cultured in diffusion chambers for only 2 days. The results of cytotoxicity tests with anti-θC3H serum and complement show that at this stage a maximum of 77 and 62% of cells were killed in suspensions from cultures of two CBA litters and 60% of cells from cultures of a BALB/c litter. In each case, no cells were killed in cytotoxicity tests on 14 day thymus cells before culture, so it is clear that some differentiation of θ alloantigen takes place in the first 2 days of culture. The level of differentiation at this stage compares favorably with that seen in normal 16 day embryo cells (Fig. 2).

**Development of TL Alloantigen on Embryonic Thymus-Stem Cells.**—The differentiation of TL alloantigen was examined in 14 day A strain embryo thymus cultured in diffusion chambers. The results are shown in Fig. 7. While a maximum of 61% of cells were killed by TL antiserum and complement following a period of 4 days' culture, no cells were killed in cytotoxicity tests on 14 day A thymus. Although this result demonstrates substantial development of TL alloantigen on the surfaces of cells following the 4 day culture period, the proportion of cells killed is not as large as that killed in cytotoxicity tests on normal 18 day A embryo thymus (Fig. 8). This contrasts with the results obtained on θ development where comparable proportions of cells were killed in suspensions of 14-day CBA thymus cultured for 4 days and normal 18 day CBA embryo thymus.

It is of interest that when parallel cytotoxicity tests with TL and θC3H antisera were carried out on the same suspension of 14 day A embryo thymus cells cultured for 4 days, the proportion of cells killed by anti-θC3H serum (88%) was considerably greater than that killed by anti-TL serum (62%). This suggests that the difference noted between θ and TL development in culture
is a property of the alloantigens themselves and is not due to strain differences between the mice used.

*Peripheral Lymphocytes Derived from Embryonic Thymus Grafts.*—14 day CBA embryo thymus grafts grow extremely well when placed under the kidney

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**Fig. 3.** Section of 14 day CBA embryo thymus showing large cells with darkly staining, basophilic cytoplasm. There is evidence that these cells, which are scattered among the paler-staining epithelial cells of the thymic rudiment, are stem cells of yolk sac or fetal liver origin (2). Giemsa Stain; × 1300.

**Fig. 4.** Section of 14 day CBA embryo thymus after 4 days' culture. A large number of small lymphoid cells are now present. Giemsa Stain; × 1300.

**Fig. 5.** Cells in suspensions prepared from 14 day embryo thymus for cytotoxicity tests. The cells which are released into suspension are the large basophilic cells seen in Fig. 3. These cells are θ and TL-negative. Cytocentrifuge preparation. Giemsa Stain; × 2200.

**Fig. 6.** Cells in suspensions prepared from 14 day thymus after 4 days' culture (θ and TL-positive). The majority of cells are small lymphoid cells and few cells are present which approach the size of those seen in 14 day thymus before culture (Fig. 5). Cytocentrifuge preparation. Giemsa Stain; × 2200.
capsule of AKR hosts (CBA and AKR mice are H2 compatible). Thus, two thymus lobes (containing about 50,000 cells) from a single embryo reach a weight of about 20 mg at 4 wk after transplantation. Histological examination of embryonic thymus removed at short intervals after transplantation shows

![Graph showing cytotoxicity tests](image)

**Fig. 7.** Cytotoxicity tests showing the development of TL alloantigen on 14 day A strain thymus cells cultured for 4 days. The maximum percentage of cells killed is not as great as that found with thymus cells removed from 18 day embryos (Fig. 8).

![Graph showing cytotoxicity tests](image)

**Fig. 8.** Cytotoxicity tests demonstrating TL alloantigen on A strain thymus cells of embryos of various gestational stages.

that they implant without undergoing necrosis (Fig. 9). This result is in sharp contrast with the extensive necrosis which is known to take place in newborn thymus after transplantation (12). No doubt the difference is related to graft size.
The repopulation of thymic grafts by host cells was examined by carrying out cytotoxicity tests with anti-θAKR sera on cells of grafts removed at various intervals after transplantation (Table II). At periods up to 16 days, grafts are populated by donor cells (θC3H) while, thereafter, an increasing proportion of host cells (θAKR) are detected, and by 28 days the great majority of lymphoid cells in the grafts are of host origin.

The results of studies on the distribution of θ-bearing cells in peripheral lymphoid tissues of AKR mice carrying CBA thymus grafts are presented in Table III. It is clear that relatively few θ-bearing cells of either θC3H or θAKR type can be detected in blood or lymph nodes during the first 3 wk after thymus transplantation. However, after 4 wk a significant proportion of cells derived from the thymus graft (θC3H) are present in blood (25%), lymph nodes (31%), and spleen (20%). At later stages, the proportion of θC3H cells in blood and lymph nodes remains fairly constant, but the proportion of θAKR cells increases. When considering these percentages, it is relevant to take into account the increase in total blood lymphocyte number during the period of study (Table III). This provides a parameter of the lymphoid restoration taking place in various tissues following irradiation.

One further point is of considerable interest. When lymph node cells removed from AKR mice 28 days after thymus implantation were incubated with various
dilutions of anti-θC3H serum and guinea pig complement, the proportion of cells killed fell rapidly with antiserum dilution so that at a dilution of 1:64 no cells were killed above control levels (Fig. 10). In fact the cytotoxic curve is identical with that seen in tests on peripheral lymphocytes from normal CBA mice and contrasts with the sensitivity of thymocytes to anti-θ sera where a majority of cells are killed by antiserum diluted to 1:1024. Thus, although a proportion of lymph node cells are derived from the CBA thymus graft as shown by the presence of θC3H alloantigen on their surfaces, they clearly resemble peripheral θ-positive lymphocytes rather than thymus lymphocytes in their sensitivity to anti-θC3H serum.

### DISCUSSION

A considerable body of information has been obtained in the mouse about the role of thymus-derived lymphocytes as both antigen-reactive and effector cells in "cell-mediated" immune responses (23). In addition, thymus-derived cells, while not secreting antibody themselves, have been shown to co-operate with bone marrow precursors of antibody-forming cells in immune responses involving humoral antibody production (24–26). The differentiation of thymus-derived lymphocytes from more primitive cell types is, therefore, a topic of considerable relevance to an understanding of the immune response in general.

Recent progress made in the study of alloantigens on mouse lymphocytes

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**TABLE II**

*Repopulation of 14 Day CBA Embryo Thymus Grafts in Irradiated, Reconstituted AKR Mice*  

| Days after transplantation | Cells killed with θC3H antiserum (donor cells) | Cells killed with θAKR antiserum (host cells) |
|---------------------------|-----------------------------------------------|-----------------------------------------------|
|                           | (%)                                           | (%)                                           |
| 15                        | 98                                            | 0                                             |
| 15                        | 96                                            | 3                                             |
| 16                        | 94                                            | 0                                             |
| 21                        | 52                                            | 43                                            |
| 21                        | 33                                            | 61                                            |
| 21                        | 21                                            | 72                                            |
| 28                        | 4                                             | 94                                            |
| 29                        | 4                                             | 93                                            |
| 29                        | 5                                             | 94                                            |
| 35                        | 0                                             | 98                                            |
| 35                        | 0                                             | 95                                            |
| 42                        | 2                                             | 94                                            |
| 42                        | 4                                             | 93                                            |

* AKR mice thymectomized at 6–8 wk of age, irradiated (800 R) and injected with AKR bone marrow cells prior to thymus grafting.
† Values corrected to account for dead cells in control tubes; see Materials and Methods.
and, in particular, the demonstration that some alloantigens are expressed on only a few cell types (15, 16) suggests that they should prove valuable as surface markers in studies on the interaction of different cell populations (27–29) and for investigating cell transitions in a differentiation pathway. In the experiments presented in this paper, we have attempted to analyze the steps involved in the differentiation of thymus-derived lymphocytes using $\theta$ and TL alloantigens as markers of cell differentiation.

The development of alloantigens on embryonic thymus cells can be studied by carrying out cytotoxicity tests on thymuses removed from embryos at successive stages of gestation (30, Figs. 2 and 8). However, it cannot be concluded from these data that alloantigen-positive cells found at later stages of

| Days after thymus implantation | Tissue examined | Cells killed: C3H antiserum (cells from thymus graft) | Cells killed: AKR antiserum (host cells) | Total WBC × 10⁶/mm³ (% mononuclear cells) |
|-------------------------------|----------------|-----------------------------------------------------|------------------------------------------|----------------------------------------|
| 14                            | Blood          | 5                                                   | 10                                       | --                                     |
| 14                            | Blood          | 4                                                   | 18                                       | --                                     |
| 14                            | Blood          | 14                                                  | 24                                       | --                                     |
| 14                            | Blood          | 0                                                   | 0                                        | --                                     |
| 15                            | Blood          | 0                                                   | 0                                        | 1.4 (35%)                              |
|                               | Lymph nodes    | 0                                                   | 0                                        | --                                     |
| 15                            | Blood          | 0                                                   | 20                                       | 3.4 (34%)                              |
|                               | Lymph nodes    | 0                                                   | 0                                        | --                                     |
| 21                            | Blood          | 0                                                   | 0                                        | --                                     |
|                               | Blood          | 25                                                  | 6                                        | --                                     |
| 28                            | Lymph nodes    | 31                                                  | 5                                        | 2.5 (53%)                              |
|                               | Spleen         | 20                                                  | 10                                       | --                                     |
| 35                            | Blood          | 20                                                  | 8                                        | 4.2 (58%)                              |
| 42                            | Blood          | 22                                                  | 19                                       | 8.6 (59%)                              |
|                               | Lymph nodes    | 18                                                  | 25                                       | --                                     |
| 42                            | Blood          | 38                                                  | 14                                       | 6.2 (63%)                              |
|                               | Lymph nodes    | 11                                                  | 8                                        | --                                     |
| 60                            | Blood          | 17                                                  | 23                                       | --                                     |
|                               | Lymph nodes    | 14                                                  | 13                                       | --                                     |
| 63                            | Blood          | 23                                                  | 35                                       | --                                     |
|                               | Lymph nodes    | 26                                                  | 32                                       | --                                     |

* Thymectomized, irradiated AKR mice injected with AKR bone marrow cells and grafted with a 14 day embryo thymus.
† Values corrected for dead cells in control tubes; see Materials and Methods.
gestation are necessarily derived from alloantigen-negative cells of earlier stages, since an inflow of alloantigen-bearing cells could take place during the period of study. The embryonic thymus can be isolated from cell inflow in diffusion chambers (Fig. 11) and in this situation we have shown that stem cells of 14 day embryo thymus, which are θ and TL negative, will during a period of 4 days' culture give rise to a population of lymphoid cells the majority of which are θ positive and, in A strain thymus, TL positive also (Figs. 1 and 7).

This acquisition of surface alloantigens by developing thymus-stem cells marks the first differentiation step in the maturation of thymus-derived lymphocytes. Whether a comparable differentiation step takes place in stem cells of the adult thymus is less certain. However, there is some evidence that certain alloantigens are not expressed in bone marrow-stem cells from which thymocytes of the adult are derived. Thus, bone marrow suspensions treated with antisera against various alloantigens in the presence of complement are able to repopulate thymus of irradiated recipients with lymphocytes bearing the respective alloantigens (16). Also, Schlesinger and Hurvitz (22) have shown that thymic grafts are first repopulated by TL negative cells while later TL positive cells can be detected. Studies in the rat have also demonstrated the differentiation of surface antigens on cells repopulating the irradiated thymus (31).

Evidence has been presented in a previous paper that the large, basophilic cells seen in sections (Fig. 3) and cell suspensions (Fig. 5) of early embryo
thymus are migrant stem cells probably of fetal liver or yolk sac origin (2). Little is known about the nature and potentialities of these stem cells or of the cells which migrate to adult thymus (which may be a different type of cell [32, 33]). For example, it is not known whether the thymus-stem cell is the same cell which, perhaps under the maturing influence of some other organ comparable to the bursa of Fabricius of birds, becomes an antibody-secreting cell.

Fig. 11. Diagram showing experiments designed to study maturation of embryonic thymus stem cells. Stem cells, which are negative for \(\theta C3H\) alloantigen in cytotoxicity tests, become strongly positive (\(\theta C3H+++\)) on culture in diffusion chambers or in grafts to thymectomized, irradiated, reconstituted AKR mice. These grafts are later repopulated by host cells (\(\theta AKR+++)\). Lymphocytes bearing \(\theta C3H\) alloantigen can be detected in the lymph nodes and blood of thymus-grafted mice. These cells are less sensitive to anti-\(\theta\) serum than thymus cells and so probably have less \(\theta\) alloantigen on their surfaces (\(\theta C3H^+\)).

or, alternatively, whether there are separate classes of stem cell for both mature cell types. In more general terms, the relationship of lymphoid precursor cells to other hemopoietic precursor cells is uncertain, although there is evidence in the embryo (34) and adult (35, 36) for the presence of a pluripotential stem cell type with myeloid and lymphoid capabilities.

Whatever the answer to these problems, the results presented here show that once stem cells have entered the thymic environment their differentiation rapidly proceeds in a lymphoid direction with concomitant changes in gross
morphology and surface structure. The stimulus for this differentiation is presumed to originate from epithelial cells of the thymus cytoreticulum. However, it is not known whether stem cells must enter the thymus for differentiation to take place or whether this can occur in other locations under the influence of hormone secreted by the thymus (37–39). Even if it is shown that stem cells must enter the thymus, it is possible that thymic humoral factors may influence further maturation of thymus-derived lymphocytes in peripheral organs (40, 41).

In a second series of experiments, studies were made on embryonic CBA thymic grafts and peripheral lymphocytes derived from them in irradiated, reconstituted AKR hosts (Fig. 11). Despite the small number of cells in 14 day thymus grafts at the time of transplantation and the considerable rate of growth they undergo, cytotoxicity tests with anti-θC3H and anti-θAKR sera showed that no significant host-cell repopulation takes place before 21 days (Table II). This rate of repopulation is the same as that found in 14 day CBA embryo thymus grafts to kidney capsules of adult (CBA × AKR) F1 hybrids using anti-θAKR serum to detect host cells and to CBA T6T6 hosts using the T6 marker chromosome to detect host cells.2 If a majority of thymus lymphocytes are replaced every 3 or 4 days in the mouse (5, 42), then cells present in 14 day embryo thymus must have a considerable capacity for self-renewal. This we feel justifies our referring to them as stem cells.

Peripheral lymphocytes bearing θC3H alloantigen were found in substantial numbers in lymphoid tissues of AKR mice from 28 days after transplantation of CBA thymus grafts onwards (Table III). Studies on the quantitative representation of θ alloantigen on thymus lymphocytes as compared to peripheral lymphocytes have shown that the alloantigen covers a greater area of the surface of thymocytes than peripheral cells (15). When cytotoxicity tests were carried out on lymph node cells of thymus-grafted AKR mice with various dilutions of anti-θC3H sera, the titration curve obtained (Fig. 10) was characteristic of peripheral lymphocytes rather than thymus lymphocytes. Thus, following the first differentiation step from embryonic stem cell to thymus lymphocyte, a second differentiation step must take place from thymus lymphocyte to peripheral (thymus-derived) lymphocyte and this second step must also involve a reorganization of surface structure. Since peripheral lymphocytes are TL negative in those strains of mice whose thymus lymphocytes are TL positive, the second step is probably accompanied by loss of TL alloantigen (16).

Recognition of the stage in this differentiation pathway at which immunological responsiveness is acquired is clearly of considerable importance. Recent studies have shown that a small proportion of thymus lymphocytes are TL negative in those strains of mice where a majority of thymocytes are TL.

2 M. A. Ritter and J. J. T. Owen. Unpublished results.
positive, and that it is these TL negative cells which are able to mount graft-versus-host reactions (43) and which migrate to lymph nodes, following transfusion, like peripheral lymphocytes (44). This suggests that the second differentiation step takes place before cells migrate from the thymus and that immunocompetence is gained at this stage.

Two further points arising from the results obtained in thymus-grafted mice are of interest. First, since few graft-derived cells of donor type (\(\theta C3H\)) were found in host tissues before 4 wk after transplantation, it is difficult to avoid the conclusion that the large number of lymphocytes produced within the graft during this period never leave it but perhaps die in situ. Second, \(\theta AKR\)-bearing cells were found in increasing numbers in peripheral tissues with time after irradiation and reconstitution. Some of these host cells might be cells which have survived irradiation, but at later stages it seems likely that they are cells which have passed through the thymus graft. The possibility remains that \(\theta\) alloantigen could mature on stem cells under the influence of the thymus but without these cells passing through it (45). We feel, however, that at the moment it is reasonable to assume from the evidence presented here and elsewhere (27) that those peripheral lymphocytes which bear \(\theta\) alloantigen are not only thymus dependent but also thymus derived.

**SUMMARY**

The development pathway from embryonic thymus-stem cell to peripheral thymus-derived lymphocyte has been demonstrated using the alloantigens \(\theta\) (theta) and TL as surface markers of cell differentiation. On the basis of cytotoxicity tests carried out on CBA.H or A embryo thymus cultured in diffusion chambers and on CBA.H embryo thymus grafts and peripheral lymphocytes derived from them in AKR hosts, it has been concluded that two differentiation stages take place during the maturation of thymus-derived cells, namely a first step from stem cell to thymocyte and a second step from thymocyte to peripheral lymphocyte.

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