# POST-THYMIC T LYMPHOCYTE MATURATION DURING ONTOGENESIS*

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Newborn (NB)[1] and young (10–15-d-old) mice are unable to produce a strong humoral response to antigens (1, 2). The cellular basis of this unresponsiveness has been attributed to various peculiarities, among which are an excess of suppressor T cells (1) and an incompetence of macrophages in their antigen-presenting function (3). Evaluation of the functional capacity of the peripheral T lymphocytes from mice shortly after birth has rested mainly on comparisons of the performance of their spleen cells (1, 2, 4–6). However, we have recently found that this approach is not adequate because of the enormous difference in the cellular composition of these organs; not only is the content of their T cells very different (~1 and 30% of the 5-d-old and adult spleen cells, respectively), but also the spleen of young mice is essentially an hematopoietic organ, which contains large numbers of erythroblasts and other precursors (7). Among these are monoblasts, whose proliferation and activation during cultures exert a marked suppressive effect on the humoral response of co-cultured adult spleen cells (7).

In this study, we took advantage of the rather similar compositions of adult and NB lymph node cells (LNC), both of which are devoid of hematopoiesis and which have a proportion of T lymphocytes of ~60 and 80% of the cells, respectively, to compare the various T cell functions on a per T cell basis. We found that the peripheral T lymphocytes of 5-d-old mice have some phenotypic features of cortical thymocytes (peanut lectin binding, high frequency of cells in S phase), and that many of the functional characteristics of LNC from 5-d-old mice are also comparable to those of cortical thymocytes: lectin responsiveness, capacity to induce a lethal graft vs. host reaction (GVHR), and ability to help B lymphocytes in a humoral response. In contrast, the in vitro induction of cytotoxic T lymphocytes (CTL) from NB LNC was comparable in magnitude to that of adult LNC. These results are relevant not only for the understanding of the immunoincompetence of the NB and young mice but also for the maturation of T lymphocytes during ontogenesis.

## Materials and Methods

*Mice.* CBA/Ca, BALB/c, and C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and bred in our laboratory under conventional conditions. "B" mice

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocytes; CTL-P, CTL precursors; FCS, fetal calf serum; GVHR, graft vs. host reaction; IF, immunofluorescence; LNC, lymph node cells; MLC, mixed lymphocyte culture; MLR, mixed lymphocyte reaction; NB, newborn; PFC, plaque-forming cells; PHA, phytohemagglutinin; PNL, peanut lectin; SRBC, sheep erythrocytes.

used as recipients for the transfer of NB T lymphocytes were prepared by adult thymectomy, lethal irradiation (850 rad delivered from a $^{60}$Co source for 4–6 min), and reconstitution by an intravenous injection of isologous bone marrow cells treated with an anti-Thy-1 alloantiserum and an agarose-absorbed rabbit serum as a complement source; cells were first incubated at $10^8$/ml with the alloantiserum at 4°C and then diluted with the rabbit serum (1:10 final dilution) to $10^7$/ml and incubated for 45 min at 37°C. $F_1$ mice used for the GVHR were irradiated with 700 rad and injected intravenously within 24 h with $2 \times 10^6$–$3 \times 10^6$ isologous bone marrow cells and the parental cells being tested. Irradiated animals drank water supplemented with neomycin sulfate (0.1 mg/ml) for the following 3 wk.

*Cells.* Thymocytes, LNC, and spleen cells were prepared as described previously (8). LNC from NB mice were collected, using a cataract knife and fine forceps, with the aid of Zeiss binocular lenses (Carl Zeiss, Inc., New York, N. Y.). Peripheral (axillary, cubital, iliac, and mesenteric) LNC were collected in Hanks' balanced salt solution containing 5% fetal calf serum (FCS), teased in a glass homogeneizer (Bellco Glass, Inc., Vineland, N. J.), and filtered through nylon wool and washed. Approximately $5 \times 10^5$ living (trypan-blue-excluding) cells could be thus collected from a 4–6-d-old mouse. T-depleted spleen cells were prepared by treatment of spleen cells with an anti-Thy-1 alloantiserum plus complement (agarose-absorbed rabbit serum) and the success of the treatment was controlled by the abolition of the response to phytohemagglutinin (PHA; 9). Adult T cells were purified from LNC using a nylon wool column (10). Irradiated cells received 1,000 rad from a $^{60}$Co source.

*Cultures.* Responsiveness to PHA and concanavalin A (Con A) was tested as described previously (11) using flat-bottomed microwells and 0.2 ml of Click's medium supplemented with 0.5% mouse serum and $5 \times 10^{-5}$ M 2-mercaptoethanol (12). Mixed lymphocyte reaction (MLR) was performed under the same conditions using $4 \times 10^5$ responding and $5 \times 10^5$ irradiated spleen cells. The proliferative response to mitogen or alloantigen was measured by a 2- or 4-h pulse of [$^3$H]TdR on the 3rd or 4th day of culture, respectively (11). Generation of CTL was assayed in flat-bottomed wells (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) with $3–5 \times 10^6$ responding cells and $5 \times 10^6$ irradiated target cells, in 2 ml Click's medium supplemented with 5% FCS and 2-mercaptoethanol. CTL were tested on EL4 or P815 target cells maintained in culture (13). Response to sheep erythrocytes (SRBC) was assayed in flat-bottomed microwells in 0.1 ml-cultures, containing $4 \times 10^5$ T-depleted spleen cells, $10^5$ SRBC in Click's medium supplemented with 10% FCS and 2-mercaptoethanol ($5 \times 10^{-5}$ M) (14).

*Local Hemolysis in Gel Assay.* The number of plaque-forming cells (PFC) was determined by the slide modification of the method of Jerne et al. (15).

*Immunofluorescence (IF) and Autoradiography.* Cells in DNA synthetic S phase were labeled in vitro by a 60-min pulse of [$^3$H]TdR (1 μCi/ml, sp act 1 Ci/mmol) and then processed for IF staining and autoradiography (16). T lymphocytes were detected with a rabbit anti-Thy-1 antiserum, conjugated with rhodamine. The preparation and specificity of this serum, which recognizes only the Thy-1 antigen on the surface of radioiodinated murine lymphoid cells, has been previously described (17). Peanut lectin (PNL)-binding T cells were identified by double IF; after an incubation in the cold with 2 μg/ml PNL (18), followed by washing and mild fixation with 0.4% formaldehyde and cytocentrifugation, the methanol-fixed smears were then stained with a fluorescein-conjugated rabbit anti-PNL antiserum (18), applied with a rhodamine-labeled rabbit anti-Thy-1 antiserum. Use of the anti-PNL antiserum without prior incubation of the cells with PNL did not result in staining of the T cells. Peripheral T cells cannot be sharply demarcated into PNL$^+$ and PNL$^-$ cells, as there is a continuous gradation of the staining intensity. Cells considered as PNL$^+$ were the most strongly stained, i.e., those whose staining approached that of thymocytes. Lyt-1 and Lyt-2 antigens were detected using monoclonal antibodies produced by hybridoma cell lines provided by The Salk Cell Distribution Center, San Diego, Calif. (clone 53.7.3 for anti-Lyt-1 and clone 53.6.7 for anti-Lyt-2 [19]). The frequencies of T cells bearing Lyt-1 and Lyt-2 were determined by double IF. Cell suspensions were incubated in the cold with the anti-Lyt antibodies, followed after washing by incubation with a fluorescein-labeled mouse anti-rat Ig antiserum. Without prior incubation with anti-Lyt antibodies, this serum did not give any staining. After cytocentrifugation and methanol fixation, the Thy-1$^+$ cells were revealed with the rhodamine-labeled rabbit anti-Thy-1 antiserum. The

present exploration was limited to the expression of Lyt antigen among Thy-1$^+$ cells. A few cells (<2% of the spleen cells) had some fluorescein after staining for Lyt, but were Thy-1$^-$.

*[$^3$H]TdR-labeling of Thymocytes In Situ.* Adult or 5–6-d-old mice received [$^3$H]TdR (0.02–0.1 μCi in 2–10 μl, 2 Ci/mmol), injected into a thymic lobe (20) and were killed 2 d later. Peripheral lymphoid cells were stained for PNL, processed for autoradiography and exposed for 2–3 mo. Labeled cells had >10 grains, whereas in the control (mice injected with the same dose intraperitoneally), no spleen cells bearing more than three grains could be detected.

*Evaluation of the Pool of Peripheral T Cells.* This was accomplished by counting the number of cells and the percentage of Thy-1$^+$ cells, detected by IF, in the spleen and in a sample of peripheral LNC (axillary, cubital, inguinal, and mesenteric). The value obtained (~$10^8$ for adult mice) represents an obvious underevaluation, as a number of compartments such as the blood, the bone marrow, and a number of LNC, are not included. Compared with other methods (21, 22), this might correspond to approximately half the real pool of peripheral T cells.

## Results

*Phenotypic Differences between Thymocytes and Peripheral T Cells from Adult and NB Mice.* To study T lymphocytes shortly after birth, 4–6-d-old mice were selected as a compromise, because 1–2-d-old animals do not have enough T cells in their lymphoid organs for functional studies (~$3 \times 10^4$ and $3 \times 10^5$ Thy-1 cells in their spleen and peripheral LNC, respectively), and 10-d-old mice, which contain ~$1 \times 10^6$ and $2.5 \times 10^6$ T cells in their spleen and LNC, are more "mature" according to the phenotypic and functional criteria discussed below. Spleen lymphocytes or LNC from 4–5-d-old mice differ from adult peripheral T lymphocytes, and resemble cortical thymocytes, either adult or young (Table I), in having a high percentage of PNL$^+$ cells and an increased frequency of cells in the DNA synthetic S phase (Table I). In contrast, the frequencies of T cells bearing Lyt-1 or Lyt-2 antigens were similar in peripheral adult and NB T cells (Table I), either in the spleen or the LNC. Approximately 40% of the peripheral T cells bear the Lyt-2, and most, i.e., >80%, bear the Lyt-1 marker, but because of the gradation of the staining intensity, it was difficult to determine whether there really existed Lyt-1$^-$ cells, as opposed to cells bearing very low amounts of

TABLE I

*Phenotypic Differences between Thymocytes and Peripheral T Cells from NB and Adult Mice*

| Marker | Thymocyte (adult or 4–5-d-old) | Thy-1$^+$ spleen cells | | Thy-1$^+$ LNC | |
|---|---|---|---|---|---|
| | | 4–5-d-old | Adult | 4–5-d-old | Adult |
| | % | % | | % | |
| DNA S phase | 11 | 12 | 0.9 | 4 | 0.3 |
| PNL | 85 | 70 | 25 | 50 | 15 |
| Lyt-1 | 94 | 85 | 80 | 90 | 85 |
| Lyt-2 | 75 | 36 | 38 | 38 | 42 |

Results are the mean of three to five experiments performed with either CBA or BALB/c cells. The frequency of Thy-1$^+$ cells in 4–6-d-old and adult mice was 1 and 30% for spleen and 82 and 58% for LNC, respectively. The results refer to the percentage of all thymocytes or of Thy-1$^+$ spleen or LNC (detected by IF with a rhodamine-labeled anti-Thy-1 antiserum) bearing either [$^3$H]TdR grains (combination of IF and radioautography), or Lyt-1 or Lyt-2 antigens, detected by double IF (see Materials and Methods).

Lyt-1. This situation differs from that in the thymus, and has been described in detail by others concerning adult peripheral T lymphocytes (23).

The possibility had to be considered that the Thy-1$^+$, PNL$^+$ cells observed in the spleen or LN of NB mice were not mainly post-thymic. We have indeed observed, in the spleen of nude mice and in the fetal liver or in the adult bone marrow of normal mice, Thy-1$^+$ cells that can be considered "pre-thymic," and are also characterized by the fact that they are in the majority PNL$^+$ and have a high rate of division (P.-F. Piguet, C. Irle, and P. Vassalli, manuscript in preparation). However, most of these "pre-thymic" Thy-1$^+$ cells bear very little or no Lyt-1 or Lyt-2 antigens, in contrast to the peripheral T cells of NB mice. To show more directly that Thy-1$^+$ cells recently emigrated from the thymus can be PNL$^+$, a group of 5–6-d-old mice received an intra-thymic injection of [$^3$H]TdR, and their spleen cells were studied by a combination of radioautography and IF 2 d later. Only a small fraction of spleen cells were labeled, but the majority of these cells, whose thymic origin was thus directly demonstrated, were PNL$^+$, suggesting that at least a fraction of the Thy-1$^+$ cells observed in the spleen have left the thymus as PNL$^+$ cells (Table II). This does not seem to be a situation peculiar to the neonatal period because the same observation was made in adult mice (Table II).

*Lectin Responsiveness.* To compare the responses of NB and adult LNC in conditions where the response to the mitogen is proportional to the number of responding cells, various numbers of LNC (five successive twofold dilutions, starting at $4 \times 10^5$/culture) were diluted with a constant number of irradiated thymocytes and the numbers of T lymphocytes required for a given increase in [$^3$H]TdR incorporation after 3 d of culture were determined (Fig. 1). T lymphocytes from 5-d-old mice responded very weakly to PHA because in four experiments, one of these being represented as an example on Fig. 1, an average of sevenfold higher numbers of 5-d-old T cells were required to obtain a response of magnitude comparable to that of adults (210 and 31 $\times 10^3$ 5-d-old and adult T cells for a response of 5,000 cpm, respectively). When the response to Con A was explored with this titration technique, thymocytes, 5-d-old, and adult T cells required $120 \times 10^3$, $110 \times 10^3$, and $23 \times 10^3$ cells, respectively, for a comparable response after 3 d of culture. This indicates that peripheral T cells from 5-d-old mice display a marked deficit in their responsiveness to both PHA and Con A. This was not due to the presence of a population of cells with a strong suppressive activity, because addition of various amounts ($5 \times 10^4$–$40 \times 10^4$) LNC from 4–6-d-old mice to $2 \times 10^5$ adult LNC did not show any significant diminution of the response to PHA (compared with the addition of similar numbers of adult LNC).

TABLE II

*PNL Phenotype of Spleen T Cells of Recent Thymic Origin*

| Age | Spleen cells | |
|---|---|---|
| | Frequency of [$^3$H]TdR$^+$ | PNL$^+$/[$^3$H]TdR$^+$ |
| *d* | | % |
| 5–6 | $\sim 10^{-4}$ | 44/65 (67) |
| 25–30 | $\sim 4 \times 10^{-5}$ | 27/38 (71) |

Thymocytes from CBA or CBA × C57 $F_1$ mice were labeled *in situ* with [$^3$H]-TdR and the mice were killed 2 d later. Results were obtained from seven adult and eight 4–5-d-old mice.

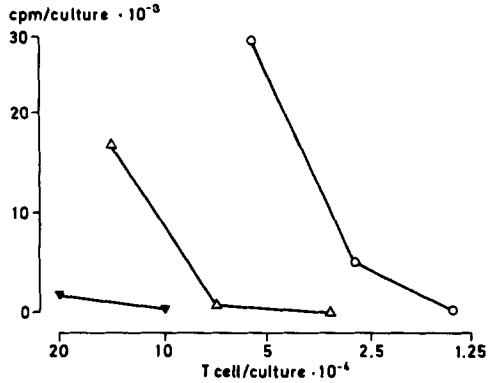


FIG. 1. Proliferative response to PHA, as judged on the 3rd d of culture by [$^3$H]TdR incorporation, of thymocytes (▲), LNC from a 5-d-old (△), and LNC from an adult mouse (○). Various numbers of viable cells (the number of T cell/culture of LNC being determined by IF) were tested in the presence of a constant number ($4 \times 10^5$) of irradiated thymocytes.

Furthermore, the response of 5-d-old LNC was markedly enhanced when the cultures were performed with irradiated adult LNC as feeder layer cells instead of thymocytes, or if the 24-h supernate of a culture of PHA-stimulated spleen cells was added (data not shown), as we have found for cortical thymocytes (8). Although it has not been precisely evaluated whether in the presence of supernate 5-d-old LNC behave as adult cells (whose titration curve is also modified by the addition of supernate), it appears, however, that the poor response of 5-d-old T lymphocytes, similar to that of thymocytes, is at least in part related to a suboptimal production of the lymphokine(s), which play a critical role in T cell activation.

*GVHR.* Thymocytes and 5-d-old and adult LNC were compared in a mortality assay for GVHR, which is sensitive and precise enough to detect the effect of a very small number ($2 \times 10^4$–$4 \times 10^4$) of adult immunocompetent T cells (Table III, experiment 1). In this assay, although $6 \times 10^4$ adult LNC killed almost all the recipients, a 10-fold higher number of LNC from 5-d-old mice killed only a minority of recipients (Table III). The LNC of 10-d-old mice were more efficient than those of 5-d-old mice, but still much less than adult cells. That this was not related to "suppressor" cells was shown by the observation that NB T cells did not inhibit the GVHR of adult T cells (Table III, experiment 2). $2 \times 10^7$ spleen cells from 5-d-old mice were totally ineffective compared with a similar number of adult LNC or to $3 \times 10^5$ adult spleen cells, which were competent to kill all the recipients. When 5-d-old spleen cells were injected together with adult T cells, they seemed to have a slight inhibitory effect on the GVHR, because $4 \times 10^5$, instead of $1 \times 10^5$ adult LNC were required to kill the recipients when injected together with $2 \times 10^7$ spleen cells from 5-d-old mice (Table III, experiment 3). We do not consider it likely that this inhibition is due to suppressive Thy-1$^+$ cells, as proposed by Skowron-Cendrzak and Ptak (24), because this would assume that, in 5-d-old mice, the spleen T cells are extremely suppressive, and LNC are not suppressive at all. It is more likely that this inhibition is related to the presence of hematopoietic precursors within the NB spleen, which facilitates the resistance of the irradiated $F_1$ mice to the aggression of the parental T cells. This interpretation is supported by the finding that injection of $10^7$ anti-Thy-1

TABLE III

*Comparison of Adult and NB T Cells in a Mortality Assay for GVHR*

| Experiment | Parental cells | T cells* | Percent survivors after | | |
|---|---|---|---|---|---|
| | | | 20 d | 40 d | 80 d |
| 1 | None | | 100 | 97 | 93 (45)‡ |
| | Thymocytes, $4 \times 10^7$ | $4 \times 10^7$ | 89 | 66 | 53 (15) |
| | 5-d-old LNC, $1 \times 10^6$ | $8 \times 10^5$ | 100 | 91 | 84 (13) |
| | 10-d-old LNC, $1 \times 10^6$ | $7 \times 10^5$ | 85 | 38 | 27 (11) |
| | Adult LNC, $1 \times 10^5$ | $6 \times 10^4$ | 34 | 9 | 6 (32) |
| 2 | Adult LNC, $6 \times 10^4$ | $3.6 \times 10^4$ | 100 | 55 | 33 ( 9) |
| | 5-d-old LNC, $4 \times 10^5$ | $3.2 \times 10^5$ | 100 | 89 | 77 ( 9) |
| | Adult ($6 \times 10^4$) + 5-d-old LNC ($4 \times 10^5$) | — | 77 | 33 | 11 ( 9) |
| 3 | 5-d-old spleen, $2 \times 10^7$ | $4 \times 10^5$ | 100 | 100 | 100 ( 6) |
| | 5-d-old spleen, $2 \times 10^7 + 10^5$ adult LNC | — | 100 | 100 | 100 ( 6) |
| | 5-d-old spleen, $2 \times 10^7 + 4 \times 10^5$ adult LNC | — | 100 | 33 | 33 ( 6) |

In experiment 1, the results are pooled from several experiments performed with two strain combinations; CBA → CBA × C57 $F_1$, and BALB/c → CBA × BALB/c $F_1$, which showed no significant differences in the number of parental T cells required to kill the $F_1$ host. Experiments 2 and 3 were performed with CBA cells and CBA × C57 $F_1$ recipient mice.

* Approximate number of Thy-$1^+$ cells, as determined by IF.

‡ The number of $F_1$ recipient mice studied are in parentheses.

TABLE IV

*Helper Effects of NB and Adult T Cells In Vivo*

| T cell reconstitution | Number of mice studied | PFC/$10^6$ spleen cells* |
|---|---|---|
| None | 8 | 5 (5) |
| $5 \times 10^5$ adult LNC | 7 | 51 (44)‡ |
| $5 \times 10^5$ 6–7-d-old LNC | 6 | 10 (8)‡ |
| $2 \times 10^6$ adult LNC | 3 | 208 (122)§ |
| $2 \times 10^6$ 6-d-old LNC | 3 | 37 (6)§ |

Recipient B mice were injected intravenously with SRBC and nylon wool-filtered adult LNC or LNC from 6-d-old mice, 4 wk after lethal irradiation and bone marrow protection. Results are the mean (± SD) of the number of PFC detected 7 d after immunization and T cell reconsititution, in two different experiments.

* The number of PFC developed with an anti-mouse Ig antiserum (indirect PFC) was not significantly above that of direct PFC.

‡ These values were different at $P < 0.1$.

§ These values were different at $P < 0.06$.

plus complement-treated $F_1$ bone marrow cells similarly increased the resistance of $F_1$ hybrid mice to the aggression of $10^5$ parental LNC.

*Helper Activity in Antibody Response to SRBC.* Adult and 6-d-old LNC were compared for their ability to help a primary response to SRBC in vivo. B mice (thymectomized, lethally irradiated, and bone marrow-protected 4 wk earlier) were injected intravenously with $10^6$ SRBC and either reconstituted with adult nylon wool-filtered or 6-d-old LNC. The number of anti-SRBC PFC observed in the spleen 7 d later showed that comparable numbers of NB or adult T cells differ in their ability to help a primary response (Table IV).

Adult, 5-d-old, and 10-d-old LNC and thymocytes were also compared for their ability to provide a nonspecific allogeneic helper effect in vitro. Exploration of the allogeneic helper effect was chosen, because in our experience, with the type of microassay and the mouse strain used, it is much more sensitive and reliable than the syngeneic helper effect. In this system too, 5-d-old and 10-d-old T cells were much less competent than adult T cells, and only slightly more effective than adult thymocytes (Fig. 2). The possibility that the poor performance of 4–6-d-old LNC in helper assays was due to a high percentage of T cells with a suppressor activity was not tested in the systems described above, because we have recently shown (7) that in a culture system exploring the response of adult spleen cells to SRBC, the addition of 4–6-d-old LNC had no suppressive effect whatever, whereas that of 4–6-d-old spleen cells strongly decreased the response.

*Reactivity in Mixed Lymphocyte Culture (MLC) and in the Generation of CTL.* Responsiveness in MLC as judged by the proliferative response in the presence of allogeneic or semiallogeneic irradiated stimulator cells was explored in parallel with LNC of 5-d-old, 10-d-old, or adult mice (Table V). 5-d-old LNC proliferated three to four times less in the presence of semiallogeneic cells than adult LNC, whereas 10-d-old LNC did not show any significant difference with adult LNC. The proliferation of 5-d-old LNC was stronger, and thus the difference with adult LNC was less marked, when irradiated, fully allogeneic cells were used as stimulators. This suggests that the weak response of NB T cells is enhanced by mediators produced by the allogeneic cells, because these cells, contrary to the semiallogeneic cells, are also stimulated in the reaction. The generation of CTL was explored on the 5th d of MLC. In contrast to the low responsiveness of 5-d-old LNC in MLR, no significant difference was observed between the cell-mediated cytotoxicity of cultures performed with LNC of 5-d-old or of adult mice (Table VI).

*In Vivo Expansion and Maturation of the Peripheral T Lymphocytes after Removal of the Thymus at an Age of 5 d.* To explore whether the maturation of NB T lymphocytes

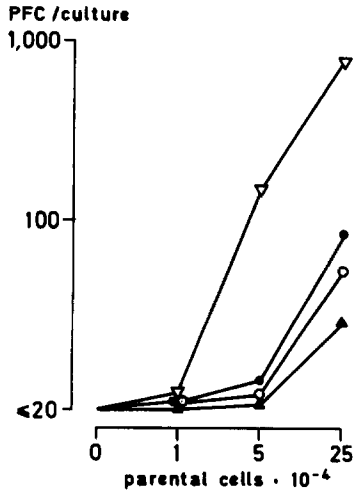


FIG. 2. Allogeneic helper effect in vitro. $F_1$ T-depleted spleen cells and SRBC were added with irradiated parental cells, which were: (▲), thymocytes; (○) LNC from 5-d-old mice; (●) LNC from 10-d-old mice; (△), adult LNC. Results are the mean of three experiments.

TABLE V
*MLR with Adult or NB LNC*

| MLC: responder cell, BALB/c | Age | Proliferative response in the presence of irradiated target cells | | | |
|---|---|---|---|---|---|
| | | BALB/c × C57F$_1$ | | C57 | |
| | | SI | cpm/culture | SI | cpm/culture |
| | *d* | | | | |
| Thymocyte | 30 | 1.0 (1) | 55 | 2.0 (2) | 162 |
| LNC | 5–6 | 5.0 (5) | 880 | 14.0 (13) | 2,840 |
| LNC | 10 | 15.0 (12) | 1,380 | 47.0 (14) | 5,590 |
| LNC | 40 | 18.0 (9) | 5,500 | 31.0 (14) | 8,100 |

Results are the mean values of four experiments in each group. The stimulation index (SI) refers to the ratio of the cpm in the culture added with allogeneic to that added with syngeneic irradiated cells; the SD is indicated in parentheses. Proliferation is statistically different only for 5-d-old and adult LNC cultured with semiallogeneic irradiated cells ($P < 0.05$).

TABLE VI
*Generation of CTL from NB or Adult LNC*

| MLR: BALB/c vs. BALB/c × C57 F$_1$ | | Cell-mediated lympholysis: percent specific lysis for | | | | | |
|---|---|---|---|---|---|---|---|
| | | EL4 | | | | P815 | LU/culture‡ |
| Responders | Cell yield | 100* | 33 | 11 | 4 | 100* | |
| | % | | | | | | |
| Thymocyte | 2 | 2 | 0 | 0 | 0 | — | 1 |
| LNC, 5-d-old | 42 | 79 | 49 | 34 | 19 | 8 | 106 (82) |
| LNC, adult | 71 | 88 | 59 | 40 | 21 | 11 | 153 (30) |

Results are the mean of four experiments performed with adult thymocytes and 5-d-old and adult LNC.
* Effector:target cell ratio. Target cells were EL4 cells (C57) with control performed with $H_2$ syngeneic P815 cells (DBA/2).
‡ Lytic units (LU)/culture, with SD in parentheses.

could proceed in the peripheral lymphoid organs in the absence of the thymus, mice were thymectomized 4–6 d after birth (i.e., at an age when the number and immunological competence of their peripheral T cells had been explored, as described above) and then killed either at 40–50 d or at 4–6 mo of age. The pool of peripheral T cells of these thymectomized mice showed a remarkable expansion, the number of T cells in the spleen and LN increasing from about $1 \times 10^6$ at the time of thymectomy up to about $30 \times 10^6$ at 6 mo of age (Table VII).

Evaluation in the GVHR indicated a considerable maturation because the adult peripheral T cells of these mice thymectomized at an age of 5 d were considerably more efficient than those of 5-d-old mice and about as competent as those of a normal adult, the difference observed in the number of T cells required to kill the F$_1$ host ($10 \times 10^4$ vs. $5 \times 10^4$) being statistically insignificant (Table VII).

TABLE VII

*Expansion and Maturation In Vivo of the Peripheral T from NB Mice after Thymectomy at 4–6 d of Age*

| Age at thymectomy | Age at sacrifice | Number of mice | T cells/mouse ($\times 10^{-6}$)* | GVHR T cells $\times 10^{-4}$‡ |
|---|---|---|---|---|
| *d* | *d* | | | |
| — | 4–6 | 4 | 1.6 (0.7) | 57 (37) |
| 4–6 | 40–50 | 3 | 9.0 (8.0) | 11 (2.0) |
| 4–6 | 120–180 | 4 | 30.0 (11.0) | 10 (7.0) |
| — | 120–180 | 4 | 83.0 (30.0) | 4.5 (3.9) |

Mean values of experiments performed with CBA and CBA × C57 $F_1$ mice, with the SD indicated in parentheses.

* Number of Thy-1$^+$ cells within the spleen cells and LNC as determined by the total cell number and the percentage of T cells detected by IF.

‡ Number of Thy-1$^+$ cells from the LNC needed to kill, in the GVHR assay, >50% of the recipients within 80 d, with SD in parentheses.

## Discussion

The present study shows that peripheral T lymphocytes from NB (4–6-d-old) mice represent a peculiar stage in the T lymphocyte maturation pathway, intermediate between immature cortical thymocytes and peripheral T cells of the adult, as indicated both by their phenotype and functional characteristics.

Phenotypically, NB peripheral T cells display features of cortical thymocytes, because they contain a high percentage of PNL$^+$ cells and of cells in the S phase. On the other hand, the distribution of the Lyt-1 and Lyt-2 antigens is similar to that of adult T cells, ~40% being Lyt-2 and almost all bearing various amounts of Lyt-1, in agreement with other investigations performed by IF (25).

The functional capacity of peripheral T lymphocytes from NB animals was assessed mainly by the use of LNC, in contrast to the use of spleen cells by other investigators (1, 2, 4–6). As already mentioned, the use of LNC was considered essential for the functional comparison of NB and adult peripheral T cells because it allows a comparison on a per T cell basis without interference by unrelated nonlymphoid cells. This comparison showed that 4–6-d-old peripheral T cells are only slightly more mature than thymocytes and thus very different from adult peripheral T cells with respect to their PHA and Con A responsiveness, their ability to induce a GVHR and proliferate in an MLR, and their helper capacity for a humoral response. LNC from 10-d-old mice were more mature than those of 5-d-old mice, although in some assays, notably the GVHR, they were still markedly less competent than adult cells. Because NB and adult peripheral T cells differ in their antigenic experience (adult peripheral T cells probably contain cells already primed against a variety of antigens), one might wonder if these differences are not more related to prior immunological experience than to real immunological maturity, i.e., to the existence in the adult of a large number of cross-reacting memory cells, reacting more efficiently to antigens than NB "virgin" T cells. However, the poor performance of NB T cells in assays independent of antigen recognition, such as the response to PHA or Con A (explored in conditions where accessory cells were provided in equal numbers to adult and NB T cells), indicates that NB peripheral T cells are indeed incompletely mature in some of their functions.

The possibility that NB peripheral T cells contain a high percentage of suppressor cells, explaining their weak reactivity, appears to be ruled out by the observation that, in the in vitro response to lectins, the in vivo response to GVHR and, as we have recently shown (7), the in vitro response of adult spleen cells to SRBC, the addition of 4–6-d-old LNC to the adult cells does not show any evidence of a suppressive effect. In contrast, NB spleen cells display strong suppressive effects in various in vitro systems, but we have shown that these effects are unrelated to NB spleen T cells, an interpretation also supported by the observation that NB spleen cells of athymic nude mice are as suppressive in anti-SRBC response in vitro as NB spleen cells of normal mice (6).

A striking exception to this poor reactivity of 4–6-d-old peripheral T cells concerns the generation of CTL. Previous studies have shown that the spleens of 5-d-old mice could generate detectable cell-mediated cytotoxicity (4, 5). Our experiments show that, on a per T cell basis, 5-d-old and adult T cells are comparable, despite the poor proliferative response of NB T cells in the MLR. The development of CTL is generally considered to require the help of Lyt-$1^+$,Lyt-$2^-$ cells, and thus one can wonder how NB peripheral T lymphocytes are capable of generating CTL. Recent evidence, however, suggests that help for the maturation of CTL can also be provided by Lyt-$2^+$ cells (26). This adult level of cytotoxic cells among the NB T lymphocytes after MLC may be correlated with the evaluation of CTL-precursors (CTL-P) among NB thymocytes, which shows that CTL-P appear in the thymus immediately after birth and are present in concentrations as great as in adult thymocytes (27). This contrasts with the progressive rise of CTL-P in the spleen, which can be related to the slow increase in percentage of T cells that we have observed in this organ after birth (27, 28). Because CTL are T lymphocytes recognizing K and D determinants of the MHC, whereas the GVHR, the assays of helper activity, and the MLC seem to explore the function of T lymphocytes recognizing Ia determinants (29), our observations might correspond to an asynchrony in the maturation of these two subclasses of T lymphocytes, which might correspond to Lyt-$2^+$ and Lyt-$2^-$. In this respect, it is interesting that NB mice are easily tolerized to Ia-encoded determinants of the MHC, but not to K/D (30), which might correspond precisely to a delay in the maturation of the subset of T cells recognizing Ia determinants.

Finally, the following general conclusions can be drawn from the phenotypic and functional characteristics of the NB peripheral T lymphocytes found in the present work, as well as from the observations made on adult mice thymectomized shortly after birth.

First, these results strongly support the concept that the traffic and ontogenic maturation of T lymphocytes take place, at least in part, directly from $PNL^+$ immature cortical thymocytes to the peripheral lymphoid organs. This contradicts the still commonly held view that all $PNL^+$ cortical thymocytes have to migrate to the thymic medulla and to become $PNL^-$ cortical thymocytes before migrating to the periphery. Our experiments with NB and adult thymocytes labeled with [$^3$H]TdR *in situ* and subsequently recovered in the spleen as $PNL^+$ cells offer a direct demonstration of this thymic cortex-peripheral lymphoid organs pathway. However, it cannot presently be decided if this is an exclusive pathway, or if medullary $PNL^-$ thymocytes also contribute to the seeding of peripheral lymphoid organs, because the minority of $PNL^-$ cells found among the labeled cells of thymic origin might have rapidly lost

their $PNL^+$ nature in the peripheral organs, or migrated out of the thymus as $PNL^-$, medullary thymocytes.

Second, the observation that the peripheral T lymphocytes of adult mice thymectomized at 5 d of age have undergone not only considerable numerical expansion but also maturation allows two conclusions. (*a*) Maturation of T cells is indeed completed outside the thymus within the peripheral lymphoid organs, as proposed by Stutman (31). This is a corollary of the thymic migration described above, and rules out other interpretations for the immaturity of the NB peripheral T cells, such as the escape from an immature thymus, of cells that would never become competent in the periphery. (*b*) This post-thymic maturation and expansion of the T cells can take place in the absence of a thymic humoral influence, at least after 5 d of age. The latter conclusion raises a number of interesting questions that can be experimentally explored, for example, whether the maturation and expansion of immature post-thymic lymphocytes result from the help of a small number of fully mature, antigenically stimulated T lymphocytes, acting perhaps through the release of lymphokines, and whether the antigenic repertoire of a T cell population considerably amplified in the absence of a thymus shows evidence of being significantly restricted.

## Summary

Peripheral T lymphocytes from newborn (4–6-d-old) mice, isolated from the spleen or lymph nodes, show phenotypic features of immature cortical thymocytes, such as high frequencies of proliferating cells and of peanut lectin-binding cells. These are features of peripheral T cells of recent thymic origin, as shown by *in situ* labeling of thymocytes and subsequent observation of the migrants to the spleen, which were mainly peanut lectin-binding cells. The function of newborn peripheral T cells was compared, on a per T cell basis, with that of thymocytes and of fully mature peripheral T cells of the adult, using preparations of newborn lymph node cells containing ~80% of T lymphocytes. They were strikingly (about 10-fold) less competent than adult T cells in their phytohemagglutinin responsiveness, their capacities to induce a graft vs. host reaction, to proliferate in the mixed lymphocyte reaction, and to help B lymphocytes in a humoral response in vivo and in vitro. In contrast, newborn T lymphocytes were comparable to those of adults in their capacity to generate cytotoxic T lymphocytes. No suppressive effect of newborn T lymphocytes could be demonstrated in several of these assays. These results argue for an asynchronous maturation of two T cell subsets during ontogeny and demonstrate that at least some T lymphocytes leave the thymus as immature T cells resembling cortical thymocytes and further mature at the periphery. Investigation of mice submitted to thymectomy at 5 d of age showed that these incompetent post-thymic T lymphocytes are capable of considerable expansion and maturation in the peripheral lymphoid organs in the absence of a thymic influence.

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