

Thymic Selection and Cell Division

By Bettina Ernst, Charles D. Surh, and Jonathan Sprent

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Summary

Cell division during thymic selection was studied with a system in which purified populations of T cell antigen receptor (TCR)⁻ CD4⁺8⁺ (double-positive [DP]) cells and fetal thymic epithelial cells (TEC) were reaggregated in tissue culture. In this system, immature DP cells differentiate into mature single-positive (SP) CD4⁺8⁻ and CD4⁻8⁺ TCR^{hi} cells within 3–4 d, indicative of positive selection. By adding the DNA precursor, bromodeoxyuridine, to the cultures and staining cells for bromodeoxyuridine incorporation, T cell division in reaggregation cultures was found to be high on day 1, low on day 2, and high on days 4–5. Cell separation studies established that cell division on day 1 was restricted to DP blast cells. In the absence of blast cells, small DP cells failed to proliferate and differentiated into SP cells without cell division, thus indicating that proliferation is not an essential component of positive selection. This applied to SP cells generated within the first 2–3 d. Surprisingly, the SP cells generated later in culture showed a high rate of cell division; the proliferating SP cells were TCR^{hi} and included both CD4⁺8⁻ and CD4⁻8⁺ cells. Turnover of TCR^{hi} SP cells was also prominent in the normal neonatal thymus and in TEC reaggregation cultures prepared with adult lymph node T cells. We speculate that division of mature SP cells in the perinatal thymic microenvironment is driven by stimulatory cytokines released from TEC. Such proliferation could be a device to expand the mature T cell repertoire before export to the periphery.

Generation of mature T cells in the thymus involves a combination of positive and negative selection and leads to the production of a postthymic repertoire with “physiological” specificity for self MHC molecules (1–5). Positive selection operates at the level of TCR^{lo} CD4⁺8⁺ (double-positive [DP])¹ cells, through TCR contact with peptides bound to MHC molecules on cortical epithelial cells, T cells with low-to-intermediate affinity for self MHC are signaled to survive, to up-regulate TCR expression, and to differentiate into mature T cells, i.e., into TCR^{hi} CD4⁺8⁻ and CD4⁻8⁺ single-positive (SP) cells. The bulk (>95%) of DP cells have negligible affinity for the MHC molecules on thymic epithelial cells (TEC), and these cells die rapidly in the cortex from “neglect” (6). Other T cells have high affinity for thymic MHC molecules and are eliminated via negative selection.

The question of whether thymic selection involves cell division is controversial. Proliferation of thymocytes is

prominent among double-negative (DN) precursor cells but ceases when DN cells differentiate into small DP cells (7). Although most DP cells are small resting cells, a sizeable proportion (10–20%) of DP cells are cycling blast cells. Some researchers argue that small DP cells are a dead-end population and that positive selection is restricted to DP blast cells (8, 9). However, other groups have shown that small DP cells are capable of differentiating into SP cells after intrathymic injection (10, 11). Irrespective of whether positive selection affects blasts or resting cells, cell division during differentiation of selected TCR^{hi} DP cells into mature SP cells is thought to be minimal (12–14). However, it is notable that a significant proportion of CD69⁺ TCR^{hi} DP cells, i.e., cells showing early signs of positive selection, are in cell cycle (9). Proliferation is also apparent at the level of mature SP cells in the medulla. Thus, although most medullary T cells are resting cells, 2–10% of these cells are in S phase (9, 13, 15).

To seek direct evidence on cell division during thymic selection, we used the system of Jenkinson et al. (16), in which dispersed populations of purified immature DP cells and fetal TEC are reaggregated *in vitro*. In this system, TCR⁻ DP cells switch to TCR^{hi} SP cells in 3–4 d, indicative of positive selection. We show here that cell division in reaggregation cultures is extensive and occurs at both early and late stages of culture.

¹Abbreviations used in this paper: BrdU, bromodeoxyuridine; DN, double negative; DP, double positive; FSC, forward scatter; HSA, heat-stable antigen; SP, single positive; SSC, side scatter; TdT, terminal deoxynucleotidyl transferase; TEC, thymic epithelial cells; TUNEL, TdT-mediated dUTP nick-end labeling.

Materials and Methods

Animals. C57BL/6J (B6) mice were obtained from the breeding colony of The Scripps Research Institute (La Jolla, CA) and time-bred by checking for vaginal plugs.

Antibodies. mAbs reactive to the following markers were previously described (17, 18): CD3 (C363.29B, rat IgG), CD4 (RL172, rat IgM), CD8 (3.168.8, rat IgM), I-A^b (28-16-8s, mouse IgM), and heat-stable antigen (HSA) (J11d, rat IgM). mAbs reactive to K^bD^b (28-8-6s, mouse IgG) (19) and to mouse fibroblasts (2F7, hamster IgG) (gift of Dr. W. Havren, The Scripps Research Institute, La Jolla, CA) were also used. Biotinylated mAbs to TCR- β (H57-597, hamster IgG), CD44 (KM201, rat IgG), CD45 (30F11.1, rat IgG), CD69 (H1.2F3, hamster IgG), and HSA (J11D) were purchased from Pharmingen (San Diego, CA). FITC-conjugated mAbs specific for CD4 (H129.19, rat IgG) (GIBCO BRL, Gaithersburg, MD), bromodeoxyuridine (BrdU) (B44, mouse IgG) (Becton Dickinson & Co., San Jose, CA), PE-conjugated anti-CD4 (GK1.5, rat IgG) (Becton Dickinson & Co.), and RED613-conjugated anti-CD8 (53-6.72, rat IgG) (GIBCO BRL) or Cy-chrome-conjugated anti-CD8 (53-6.72) (Pharmingen) were also used. Biotinylated normal rat IgG was used as a negative control.

Cell Purification. TCR⁻ CD4⁺ CD8⁺ thymocytes were purified from newborn (1–3 d) or adult (5–6 wk) B6 thymuses by treatment with cytotoxic anti-CD3 (C363.29B) plus anti-K^bD^b (28-8-6s) mAbs plus C followed by positive panning on anti-CD4 (RL172)-coated plates (20). TCR⁻ DP cells were further fractionated by centrifugation on stepwise Percoll (Pharmacia LKB, Uppsala, Sweden) density gradients at 1,400 *g* for 30 min (21); cells from the 1.07, 1.08, and 1.90 density layers were designated as large, medium, and small cells, respectively. Small TCR⁻ DP cells were also sorted with a FACStar[®] (Becton Dickinson & Co.) after staining with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs and gating on CD4⁺8⁺ cells with forward scatter (FSC).

TEC were obtained by trypsin digestion of deoxyguanosine-treated day 15–16 fetal thymic lobes (16). TEC were depleted of fibroblasts and CD45⁺ cells by sequential incubation, between washes, with a mixture of antifibroblast (2F7) and biotinylated anti-CD45 mAbs, biotinylated anti-hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), FITC-streptavidin (Jackson ImmunoResearch Laboratories, Inc.), and biotinylated micromagnetic beads (Miltenyi Biotec Inc., Sunnyvale, CA) followed by passage through magnetic columns (MiniMACS; Miltenyi Biotec, Inc.).

Preparation of Reaggregation Cultures. Each thymic reaggregation culture (16) was established by centrifuging a mixture of 0.5×10^6 TEC and 1×10^6 TCR⁻ DP thymocytes and pipetting the pellet onto a nucleopore filter placed on a foam sponge (16) in standard medium (see below); in some experiments 0.5×10^6 TEC were mixed with 0.3×10^6 purified LN CD4⁺ cells (20). The slurry of cells reaggregated and formed a lobe within 12–18 h of culture. Single-cell suspension from reaggregated lobes was obtained by teasing the lobes with needle points. Cell yields were estimated by counting viable lymphocytes under a phase-contrast microscope.

Culture Conditions and BrdU Labeling. Reaggregation cultures or cell suspensions were cultured in standard RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, L-glutamine, penicillin, streptomycin, and gentamicin. LN T cells were stimulated with 2.5 μ g/ml Con A (20) (Sigma Chemical Co., St. Louis, MO) for 3 d. Where indicated, cultures were supplemented with BrdU (Sigma Chemical Co.) at final concentra-

tions of 5, 25, or 125 μ g/ml. For in vivo labeling, newborn (1–2 d) and adult (5–6 wk) mice were intravenously injected with 0.1 or 0.8 mg BrdU in PBS, respectively.

Flow Cytometry Analysis. For surface staining, cells were incubated with various mixtures of biotinylated and/or fluorochrome-conjugated mAbs using conventional methods (17). For example, three-color analysis of thymocytes was performed by incubation with a mixture of biotinylated anti-TCR- β , FITC-conjugated anti-CD4, and RED613-conjugated anti-CD8 mAbs, washed, incubated with PE-conjugated streptavidin (GIBCO BRL), and analyzed on a FACScan[®] (Becton Dickinson & Co.); propidium iodide was used to exclude dead cells (17). For BrdU-labeled cells, cells were first stained for surface markers, fixed with ethanol and paraformaldehyde, treated with DNase, and then incubated with FITC-conjugated BrdU as previously described (22). For four-color analysis, thymocytes were incubated with a cocktail of biotinylated anti-TCR- β , PE-conjugated anti-CD4, and Cy-chrome anti-CD8 mAbs, washed, incubated with Texas red-conjugated streptavidin (GIBCO BRL), washed, fixed in ethanol followed by paraformaldehyde, digested with DNase I (Sigma Chemical Co.), and incubated with FITC-conjugated anti-BrdU. Four-color-stained cells were analyzed on a FACS[®] Vantage (Becton Dickinson & Co.); FSC and side scatter (SSC) gating was used to eliminate dead cells and debris.

To detect viable (nonapoptotic) BrdU⁺ cells, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method (18) was used. Thymocytes were fixed in 70% ethanol, washed in PBS, fixed in 1% paraformaldehyde, washed once with PBS and then with TdT buffer, and incubated with 5 μ M biotin-21-dUTP (CLONTECH, Palo Alto, CA) plus 5 U TdT at 37°C for 30 min. The cells were washed in PBS, incubated with PE-conjugated streptavidin, washed in PBS, incubated with 50 Kunitz U DNase I (Sigma Chemical Co.) for 10 min (22), washed with PBS, and incubated with FITC-conjugated anti-BrdU. Cells were analyzed in a FACScan[®].

Results

Experimental Approach. Immature DP thymocytes were set up in reaggregation cultures with purified fetal TEC by the method of Jenkinson et al. (16). In brief, thymocytes from newborn or adult mice were first treated with a mixture of cytotoxic anti-CD3 and anti-class I mAbs plus C at 37°C (which killed ~60% of the cells) and then positively panned at 4°C on plates coated with anti-CD4 mAb. After panning, the cells consisted almost entirely (99%) of CD4⁺8⁺ cells (Fig. 1 *a*). TCR- β expression on these cells was negative to low. As expected, culturing these TCR⁻ DP cells in vitro overnight (without TEC) led to up-regulation of TCR- β expression to the intermediate level (Fig. 1 *a*). Significantly, the cultured cells maintained their DP phenotype; contamination with SP CD4⁺8⁻ and CD4⁻8⁺ cells and DN CD4⁻8⁻ cells was extremely low (Fig. 1 *a*). TEC were prepared by culturing fetal thymus lobes with deoxyguanosine for 5–6 d; after dispersing the lobes with trypsin, contaminating fibroblasts and CD45⁺ cells were removed by magnetic bead separation.

In agreement with Jenkinson et al. (16), culturing fresh TCR⁻ DP cells with fetal TEC in reaggregation cultures generated substantial numbers of SP cells (Fig. 1, *a* and *b*).

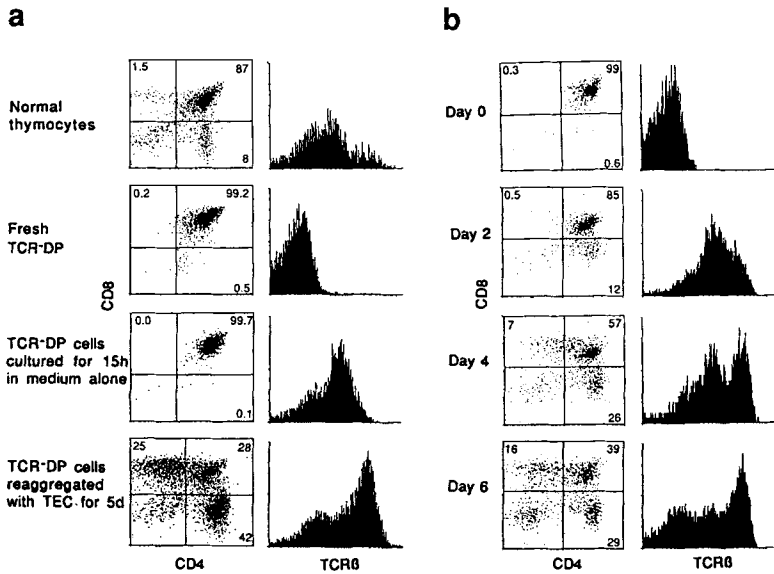


Figure 1. Differentiation of TCR⁻ DP cells in TEC reaggregation cultures. Purified TCR⁻ DP cells prepared from newborn thymus were set up in reaggregation cultures with purified fetal TEC (Materials and Methods). After harvest, the cells were three-color stained for expression of CD4, CD8, and TCR-β; staining was analyzed on a FACScan®. In *a*, TCR⁻ DP cells were stained immediately after preparation, after culture for 15 h in vitro, or after reaggregation with TEC for 5 d; staining of normal thymocytes is shown as a control. In *b*, cells were stained after reaggregation with TEC for 0, 2, 4, or 6 d. The data are from two different experiments.

CD4⁺8⁻ cells appeared as early as day 2 of culture, whereas CD4⁻8⁺ cells were not prominent until day 4. Most of the SP cells were TCR^{hi} cells (Fig. 1, *a* and *b*, and see below). At late stages of culture, variable numbers of DN cells were found. These cells appeared to be a heterogeneous population contaminated with TEC and dying lymphoid cells and will not be discussed further.

The above results confirm the data of Jenkinson et al. and indicate that the transition of TCR⁻ DP cells to TCR^{hi} SP cells in TEC reaggregation cultures is a valid model for positive selection. With this system we examined the extent of cell division during thymic selection.

T Cell Yields from Reaggregation Cultures Prepared from Small vs Large TCR⁻ DP Cells. With newborn TCR⁻ DP cells as input cells, total yields of viable lymphoid cells from TEC reaggregation cultures declined progressively during the first 3 d of culture but then stabilized at ~20% of the input number by day 4–5 (Fig. 2 *a*); at this stage, 40–70%

of the lymphoid cells were SP cells (Fig. 1). In cultures prepared from adult TCR⁻ DP cells, cell yields on day 5 were much lower, i.e., ~5% of the input number (Figure 2 *a*).

On the basis of FSC and SSC, fresh neonatal TCR⁻ DP cells contained ~20% blast cells (Fig. 3 *a*, top). To examine the contribution of blast cells to cell yields in reaggregation cultures, newborn TCR⁻ DP cells were separated into populations enriched for large, medium, or small cells on Percoll gradients (Fig. 3 *a*, top). When these cell fractions were cultured with BrdU for 9 h in vitro in the absence of TEC, double staining for BrdU incorporation vs TUNEL (to detect apoptotic cells) indicated that cycling (BrdU⁺) cells were prominent with the large cell fraction, less common with the medium cells, and rare with the small cells (Fig. 3 *a*, middle). Apoptotic (TUNEL⁺) cells were more common with the small cells than with the other fractions, and most of the apoptotic cells were BrdU⁻.

With each cell fraction, culturing the TCR⁻ DP cells for

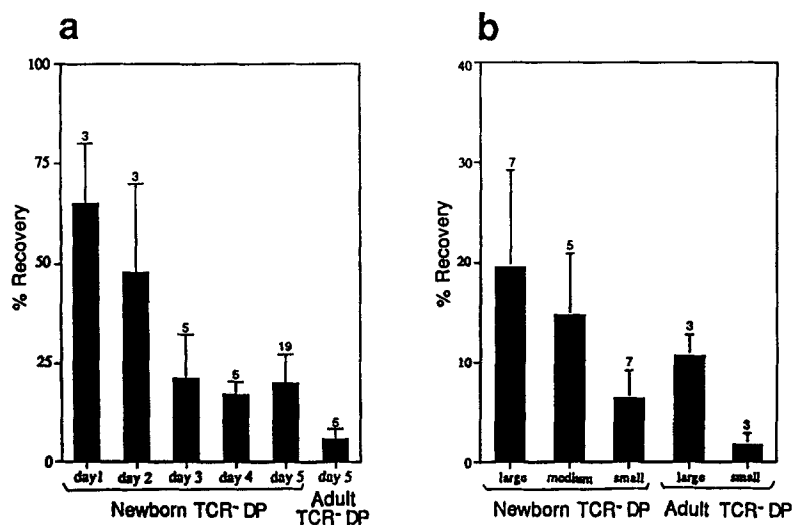


Figure 2. Percent recovery of thymocytes obtained from reaggregation cultures of TEC and neonatal vs adult TCR⁻ DP cells. In *a*, the input TCR⁻ DP cells were cultured with TEC for the times indicated. In *b*, before culture with TEC, the input DP cells were fractionated on Percoll gradients to prepare populations enriched for small, medium, or large cells (Materials and Methods); cells were counted on day 5 of culture. In *a* and *b*, the data refer to the mean percentage of recovery of viable lymphoid cells (\pm SD) relative to the number of input T cells; the number of experiments involved is shown above the SD bars. Cells with the morphology of viable lymphoid cells were counted by phase-contrast microscopy.

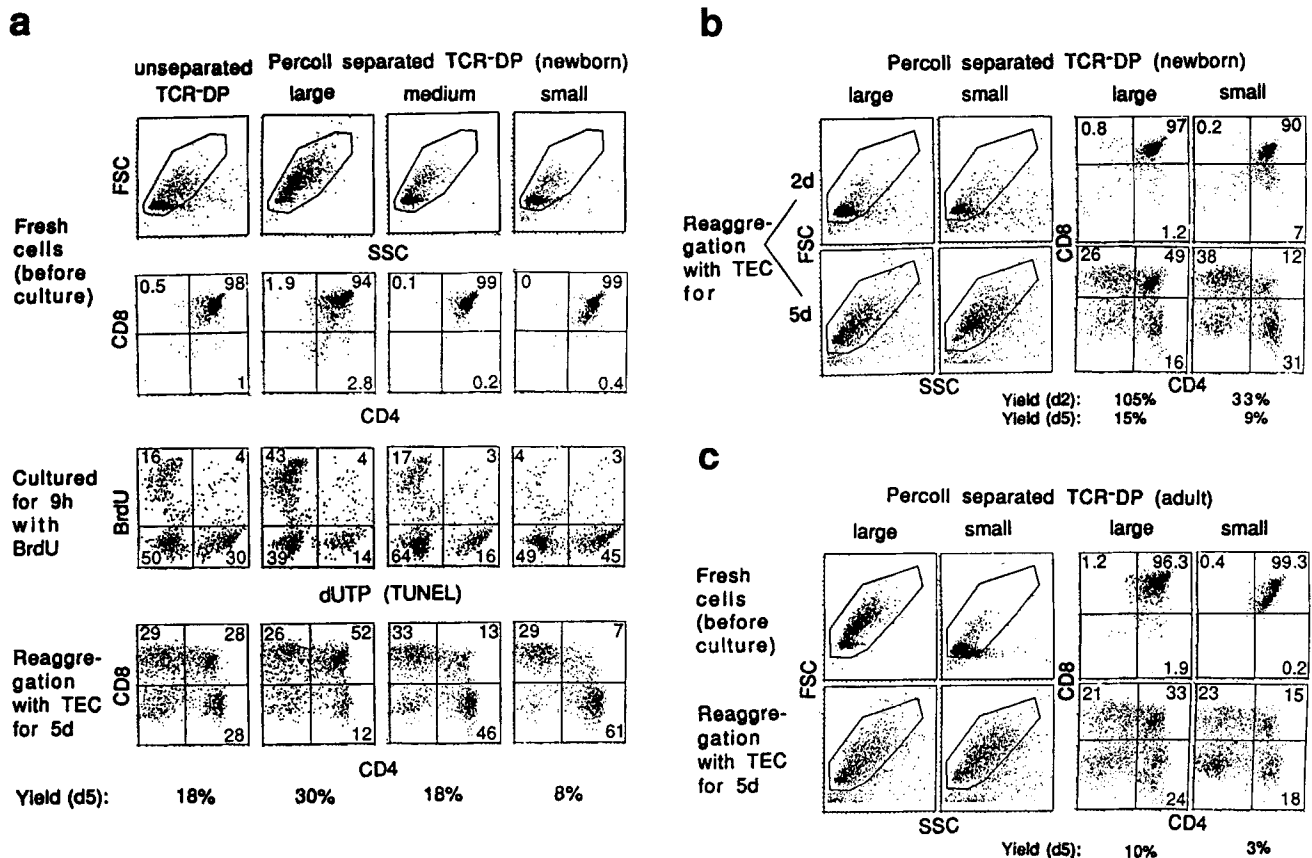


Figure 3. Differentiation of Percoll-separated TCR⁻ DP cells in TEC reaggregation cultures. Populations of small, medium, or large cells were prepared by Percoll separation of TCR⁻ DP cells from newborn (*a* and *b*) or adult (*c*) thymocytes. (*a*, top) FSC/SSC analysis and CD4/CD8 staining show the size and purity of the Percoll-separated cells. (*a*, middle) The turnover and viability of the separated cells was measured by placing the cells in tissue culture (without TEC) with BrdU (25 μ g/ml) for 9 h and then, after fixation, double staining the cells with anti-BrdU antibody vs incorporation of nucleotide (dUTP) in the presence of TdT (TUNEL staining) (Materials and Methods). (*a*, bottom) Cells recovered after 5 d in reaggregation cultures were analyzed for CD4 vs CD8 expression using two-color staining. In *b* and *c*, cells recovered from reaggregation cultures were analyzed for size using FSC/SSC analysis and for CD4 vs CD8 expression. The data in *a*, *b*, and *c* were from three separate experiments; percent recoveries of T cells from the cultures (relative to the input number of DP cells) are shown for each experiment.

5 d with TEC in reaggregation cultures induced a marked DP \rightarrow SP switch (Fig. 3 *a*, bottom). Switching to SP cells was more pronounced with small DP cells (70–90% SP cells on day 5) than with large DP cells (\sim 40% SP cells on day 5) (Fig. 3, *a* and *b*). However, total cell yields were substantially higher with the large DP fraction than with the small cells (30 vs 8%, see Fig. 3 *a*, bottom); in addition, the proportion of CD4⁺8⁺ cells at day 5 was much higher in cultures prepared from large DP cells than from small cells (52 vs 7%; see also Fig. 3 *b*). The large DP cells also led to higher yields of cells on day 2 of culture (105%, Fig. 3 *b*). Interestingly, FSC/SSC analysis of the cells harvested on day 2 revealed that, by this stage, the large DP cells had reverted to small cells, but with minimal differentiation into SP cells (Fig. 3 *b*). By contrast, cultures prepared with the small DP fraction generated appreciable numbers of CD4⁺8⁻ cells (but not CD4⁺8⁺ cells) by day 2 of culture (Fig. 3 *b*). (The significance of the increase in cell size in day 5 cultures will be considered later.)

The above data indicate that the high yields of SP cells in reaggregation cultures are at least partly a reflection of divi-

sion and expansion of blast precursors, presumably at the DP stage and before day 2 of culture. The higher yields of SP cells generated from large DP cells than from small DP cells were observed in several different experiments and applied to both newborn and adult DP cells (Figs. 2 *b* and 3, *b* and *c*). For each fraction studied, total cell yields from the cultures were appreciably lower with adult than with newborn DP cells (Figs. 2 *b* and 3, *b* and *c*).

Since FSC analysis revealed that Percoll-separated fractions of small DP cells were contaminated with a few blast cells and cycling cells (Fig. 3 *a*), the SP cells generated from the small DP fraction could have been derived from contaminating large cells. To examine this possibility, TCR⁻ DP cells from newborn mice were FACS[®] separated into small cells on the basis of FSC (Fig. 4 *a*). The sorted cells were highly pure and were virtually devoid of cycling cells; i.e., <1% of the cells incorporated BrdU after overnight culture (data not shown). When reaggregated with TEC, these purified small DP cells generated SP cells in the same numbers and with the same kinetics as Percoll-separated small cells. Thus, CD4⁺8⁻ cells were evident by day 2 of

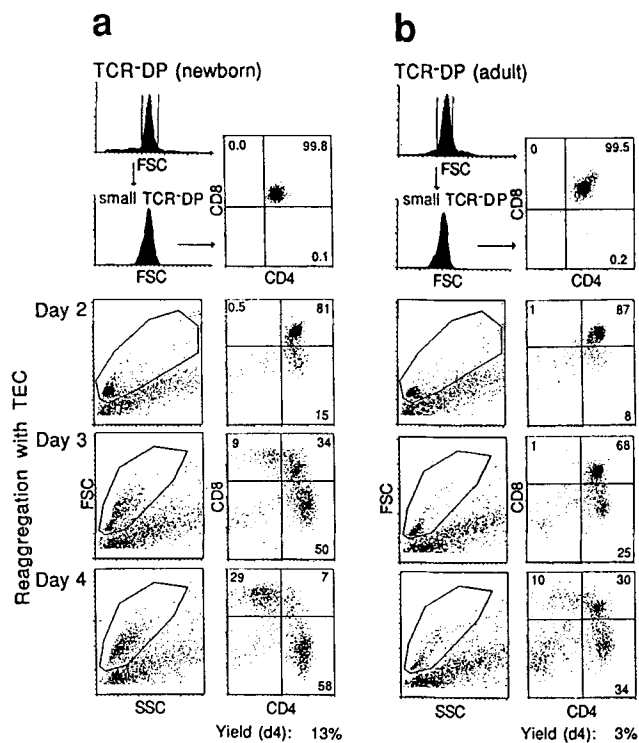


Figure 4. Differentiation of FACS[®]-separated small TCR⁻ DP cells in TEC reaggregation cultures. TCR⁻ DP cells prepared from newborn (*a*) or adult (*b*) mice were FACS[®] separated to obtain purified populations of small DP cells (*top*) (Materials and Methods). These populations were then placed in TEC reaggregation cultures and analyzed for size (FSC/SSC) and CD4/CD8 expression after 2, 3, or 4 d. Cell yields from the cultures on day 4 are shown at the bottom.

culture, whereas CD4⁻8⁺ cells appeared 1 d later (Fig. 4 *a*). Comparable results were obtained with FACS[®]-sorted small DP cells prepared from adult thymus (Fig. 4 *b*); total cell yields, however, were much lower with adult than with newborn cells, and CD4⁻8⁺ cells were rare before day 4.

Cell Division in Reaggregation Cultures. To study cell division during culture, reaggregation cultures were supplemented with BrdU continuously from day 0–5 (Fig. 5 *a*). In the initial experiments shown in Fig. 5, the cultures were prepared with unseparated (non-Percoll-separated) DP cells. With adult TCR⁻ DP cells, the percentage of BrdU labeling of SP cells on day 5 was surprisingly high: 66% for CD4⁺8⁻ cells and 93% for CD4⁻8⁺ cells (Fig. 5 *a*). Newborn TCR⁻ DP cells generated even higher labeling of SP cells: 95% for CD4⁺8⁻ cells and 99% for CD4⁻8⁺ cells (Fig. 5 *a*). To examine the kinetics of BrdU incorporation, cultures prepared from unseparated newborn TCR⁻ DP cells were pulsed with BrdU at different stages of culture and harvested 1 d after BrdU addition. When the cultures were harvested at the end of day 1, 52% of the cells were BrdU⁺ (Fig. 5 *b*). BrdU labeling was much lower on day 2 and day 3 (8 and 10%, respectively) but then increased to high levels on day 4 (59%). The increase in the percentage of BrdU labeling on day 4 was as-

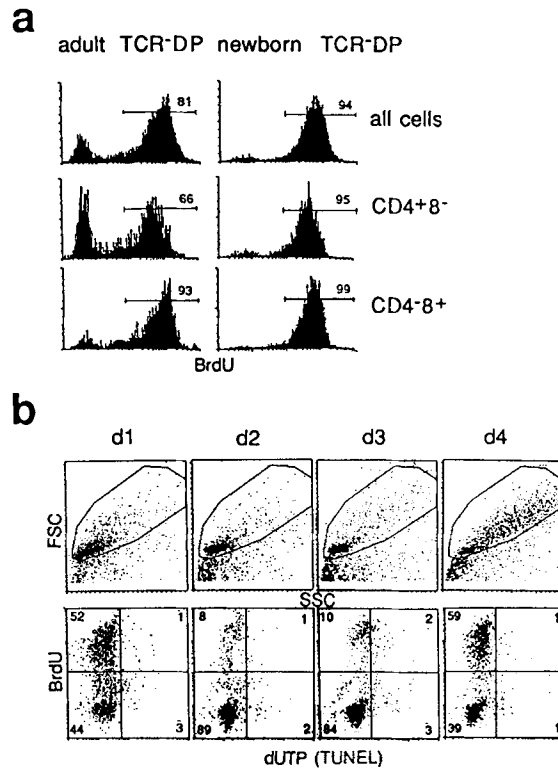


Figure 5. BrdU incorporation by TCR⁻ DP cells cultured with TEC in the presence of BrdU. In *a*, TCR⁻ DP cells prepared from adult (*left*) or newborn (*right*) mice were placed in TEC reaggregation cultures supplemented with BrdU (5 μ g/ml). After 5 d of continuous exposure to BrdU, the cells were harvested and three-color stained for BrdU incorporation and CD4/CD8 expression. The histograms show the percentage of BrdU incorporation and CD4/CD8 expression by total lymphoid cells, CD4⁺8⁻ cells, and CD4⁻8⁺ cells; the horizontal marker to define BrdU⁺ cells was set relative to control cultures not supplemented with BrdU (see Fig. 6). In *b*, TCR⁻ DP cells from newborn mice were placed in TEC reaggregation cultures and harvested after 1, 2, 3, or 4 d; BrdU (25 μ g/ml) was added during the last 24 h of culture. After harvest, the cells were stained for BrdU incorporation vs TUNEL as for Fig. 3 *a*.

sociated with a substantial increase in cell size (Fig. 5 *b*) (see also Figs. 3 and 4). At each stage of culture, >95% of cells in the FACS[®] gates examined were viable (TUNEL⁻) (Fig. 5 *b*).

The above data indicate that cell division in reaggregation cultures prepared from unseparated TCR⁻ DP cells is extensive and skewed to the early and late stages of culture. The features of cells proliferating at these two stages are discussed below.

Cell Division during Early Culture. Three-color staining showed that most of the cells proliferating on day 1 of culture were TCR⁻ DP cells (data not shown) and were presumably derived from the blast cells in the input population. To study cell division in the absence of blast cells, reaggregation cultures were prepared from FACS[®]-sorted small DP cells, using newborn thymocytes. BrdU was added at the initiation of culture, and cells were harvested at daily intervals; the cells were thus exposed to BrdU continuously (compared with the 1-d pulse used in Fig. 5 *b*).

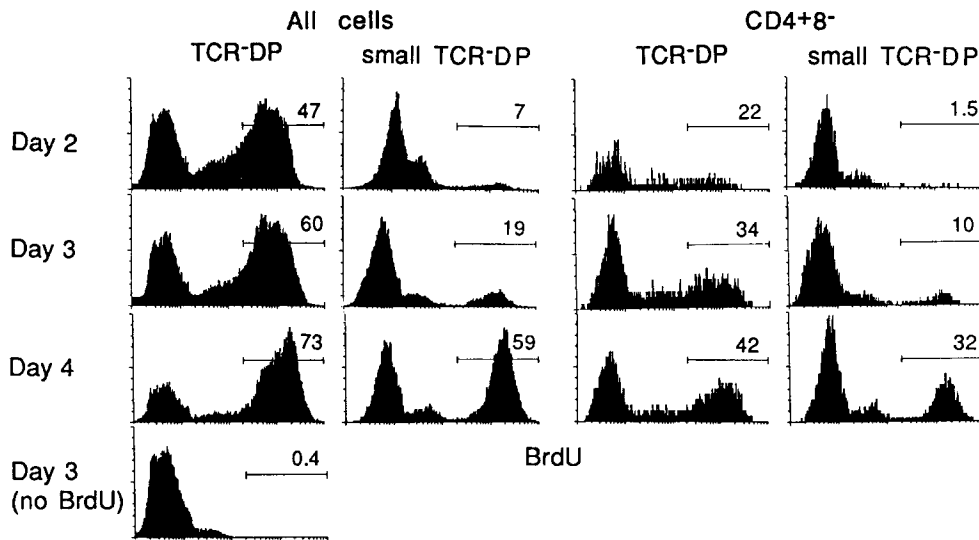


Figure 6. BrdU incorporation by FACS[®]-separated small TCR⁻ DP cells cultured with TEC in the continuous presence of BrdU. FACS[®]-separated small DP cells were prepared from newborn TCR⁻ DP cells and placed in TEC reaggregation cultures. BrdU (5 μg/ml) was added at the initiation of culture, and cells were harvested after 2, 3, or 4 d. The cells were then stained for BrdU incorporation vs CD4/CD8 expression. The data show the percentage of BrdU incorporation by all lymphoid cells (cells expressing either CD4 or CD8 or both) and by CD4⁺8⁻ cells. BrdU staining of cells from reaggregation cultures not supplemented with BrdU is shown as a negative control (*bottom left*).

As expected, prior removal of blast cells virtually abolished proliferation in the early stages of culture. BrdU incorporation was almost undetectable on day 1 of culture (data not shown), and only 7% labeling was found on day 2 (compared with 47% for cultures prepared from unseparated TCR⁻ DP cells) (Fig. 6). These data refer to total cells. Gating on SP cells revealed negligible labeling of CD4⁺8⁻ cells on day 2 (2%) and low labeling on day 3 (10%) (Fig. 6). At later stages of culture, however, labeling of SP cells increased considerably and reached high levels by day 4: 32% for CD4⁺8⁻ cells (Fig. 6) and 82% for CD4⁺8⁺ cells (data not shown). CD4⁺8⁺ cells were rare before day 4 in this experiment; of the few cells recovered on day 3, 60% were BrdU⁺.

The above results indicate that prior removal of blast cells prevented the early phase of proliferation and allowed small DP cells to switch to SP cells (CD4⁺8⁻ cells) in the absence of cell division. It is notable, however, that removal of blast cells failed to prevent the high rate of cell turnover found on day 4. Cell division during the late stages of culture is discussed below.

Cell Division during Late Culture. The high rate of cell division observed for both CD4⁺8⁻ and CD4⁻8⁺ cells on day 4 of culture could indicate that cell division is required for the late generation of SP cells from DP cells. Alternatively, cell division on day 4 may occur after the switch to SP cells. To examine this second possibility, reaggregation cultures prepared from unseparated TCR⁻ DP cells were pulsed for 4 h with BrdU at different stages of culture and harvested immediately after the pulse period. The short pulse of BrdU labeled only a small proportion (6%) of the early wave of CD4⁺8⁻ cells found on day 3 (Fig. 7 *a*). At days 4–6, however, the percentage of BrdU labeling was quite high: 18–21% for CD4⁺8⁻ cells and 29–37% for CD4⁻8⁺ cells. The high labeling of SP cells pulsed with BrdU at late stages of culture was observed in several different experiments and applied irrespective of whether the in-

put TCR⁻ DP cells were from adult or newborn mice or were prepared from large or small DP cells (Fig. 7 *b*); the percentage of labeling was always low on days 2–3 of cul-

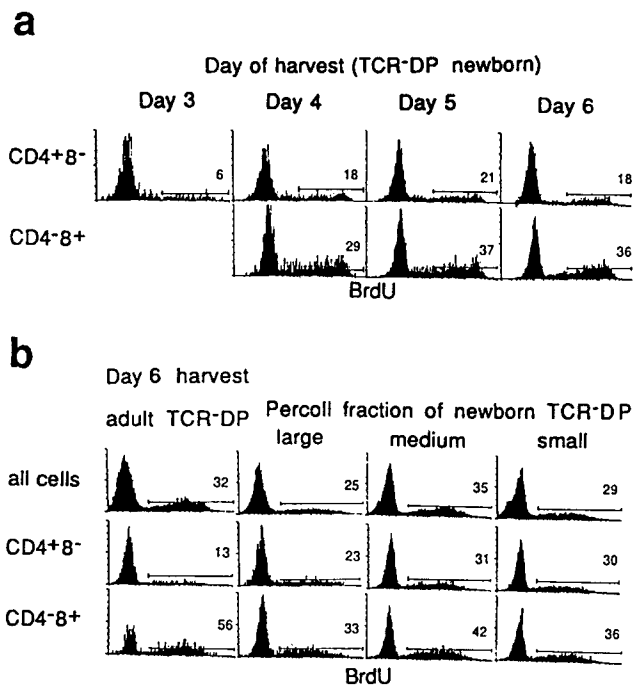


Figure 7. Effects of pulsing reaggregation cultures with BrdU for 4 h at different stages of culture. In *a*, TEC reaggregation cultures were prepared from newborn TCR⁻ DP cells and harvested after 3, 4, 5, or 6 d; BrdU (125 μg/ml) was added at the beginning of the days indicated, and cells were harvested 4 h later. In *b*, TEC reaggregation cultures were prepared from adult TCR⁻ DP cells or from Percoll-separated fractions of newborn TCR⁻ DP cells; BrdU was added on day 6 of culture, and cells were harvested 4 h later. In both *a* and *b*, the harvested cells were three-color stained for expression of BrdU, CD4, and CD8. The data show the percentage of BrdU incorporation by CD4⁺8⁻ and CD4⁻8⁺ cells. As for Figs. 5 and 6, the marker used to define BrdU⁺ cells was set relative to control cultures not pulsed with BrdU. The data in *a* and *b* were from different experiments.

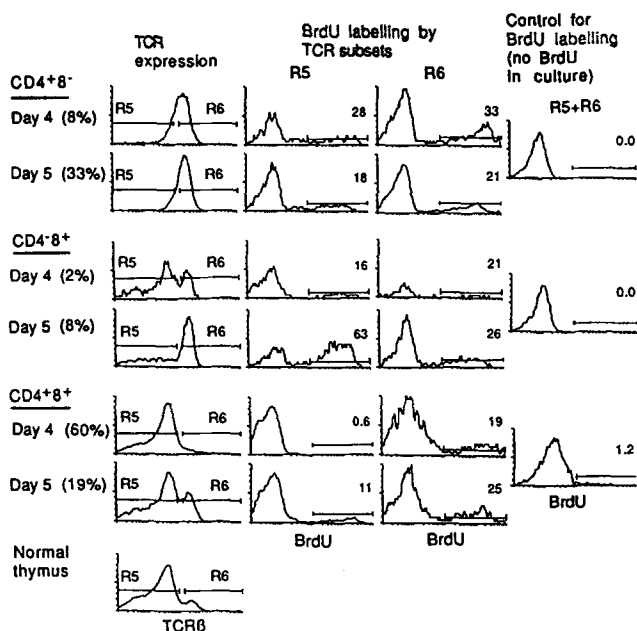


Figure 8. BrdU incorporation by TCR^{hi} cells in TEC reaggregation cultures pulsed for 4 h with BrdU on day 4 or day 5. TCR⁻ DP cells from newborn mice were set up in TEC reaggregation cultures; BrdU (125 μ g/ml) was added at the beginning of day 4 or 18 h later at the end of day 4 (called day 5 for simplicity), and cells were harvested 4 h later. The harvested cells were four-color stained for BrdU, TCR- β , CD4, and CD8. TCR- β expression on CD4⁺8⁻, CD4⁻8⁺, and CD4⁺8⁺ cells is shown at the left of the figure; the percentage of cells in each subset is shown in parenthesis; TCR- β staining of normal thymus (left, bottom) was used to define TCR^{hi} cells. Percent BrdU incorporation by TCR^{lo-to-int} (R5) and TCR^{hi} (R6) cells is shown in the middle of the figure; the lack of BrdU staining when cultures were not pulsed with BrdU is shown at the right.

ture (<5% in other experiments).

The above data indicate that the SP cells found at late stages of culture showed a surprisingly high rate of cell division; since the BrdU pulse was brief, i.e., 4 h, cell division

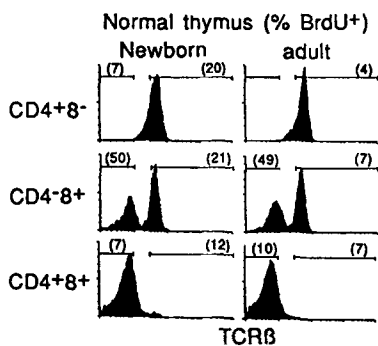


Figure 9. BrdU incorporation of TCR^{hi} thymocytes from neonatal vs adult mice pulsed with BrdU in vivo. Normal neonatal and adult mice were injected intravenously with BrdU (0.1 mg for neonates, 0.8 mg for adults) and killed 3 h later. Thymocytes were four-color stained for BrdU, TCR- β , CD4, and CD8 expression. The data show TCR- β expression on CD4⁺8⁻, CD4⁻8⁺, and CD4⁺8⁺ cells; the percentages of BrdU⁺ cells in TCR^{lo-to-int} and TCR^{hi} cells (mean of four mice/group) are shown in parenthesis; a distinct peak of TCR^{hi} DP cells is not evident for adult thymus in the experiment shown but was discernible in other experiments.

presumably occurred after (rather than during) the DP \rightarrow SP switch. To examine the TCR density on the dividing cells, reaggregation cultures prepared from newborn TCR⁻ DP cells (unfractionated) were pulsed with BrdU for 4 h at daily intervals (as for Fig. 7) and were then four-color stained for expression of CD4, CD8, TCR- β , and BrdU. Data on cells recovered on days 4 and 5 are shown in Fig. 8. Gating on CD4⁺8⁻, CD4⁻8⁺, and CD4⁺8⁺ cells revealed that each of these subsets contained TCR^{hi} cells. Gating on these TCR^{hi} cells to examine BrdU incorporation showed that BrdU labeling of TCR^{hi} (R6) cells was high and quite similar (19–33%) for each of the three CD4/8-defined subsets, both on day 4 and day 5 (Fig. 8). TCR^{lo-to-int} (R5) cells were rare in the CD4⁺8⁻ subset but were clearly detectable in CD4⁻8⁺ cells (especially on day 4) and prominent in CD4⁺8⁺ cells. BrdU labeling of these TCR^{lo-to-int} cells was variable, ranging from <1% for DP cells on day 4 to 63% for CD4⁻8⁺ cells on day 5 (Fig. 8).

The above data indicate that cell division in late culture affected a spectrum of cell types, including TCR^{hi} cells. To exclude artifact resulting from cell culture, comparable experiments were performed on normal thymus tested directly ex vivo. Newborn and adult mice were given an intravenous injection of BrdU and were then killed 3 h later to prepare thymocytes. TCR- β expression on CD4⁺8⁻, CD4⁻8⁺, and CD4⁺8⁺ cells is shown in Fig. 9; the percentage of BrdU labeling of TCR^{hi} (R6) and TCR^{lo-to-int} (R5) cells is illustrated in parenthesis in the histograms. In the case of newborn thymocytes, it is evident that the percentage of BrdU labeling of TCR^{hi} cells was prominent: 20% for CD4⁺8⁻, 21% for CD4⁻8⁺ cells, and 12% for CD4⁺8⁺ cells. These percentages are in surprisingly close accord with the above data for day 4–5 reaggregation cultures (compare Figs. 8 and 9). The high turnover of TCR^{hi} cells found in reaggregation cultures thus seemed to be real rather than artifactual and closely mirrored the turnover of TCR^{hi} cells in the normal neonatal thymus. Turnover of TCR^{hi} cells was substantially lower in the adult thymus: 4%

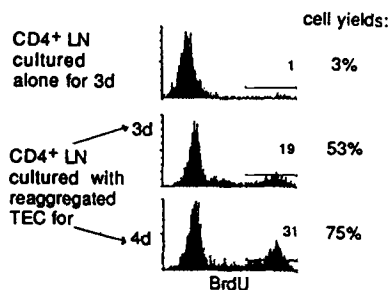


Figure 10. BrdU incorporation by LN CD4⁺ cells in LN/TEC re-aggregation cultures. Purified CD4⁺ cells from LN of normal adult mice were placed in TEC reaggregation cultures for 2 (not shown), 3, or 4 d and pulsed with BrdU (25 μ g/ml) for 22 h before harvest; LN CD4⁺ cells cultured in medium alone were used as a control. After harvest, the cells were two-color stained for BrdU incorporation and CD4 expression. The data show the percentage of BrdU incorporation by CD4⁺ cells; total yields of viable lymphoid cells (relative to the input number of CD4⁺ cells) are shown on the right.

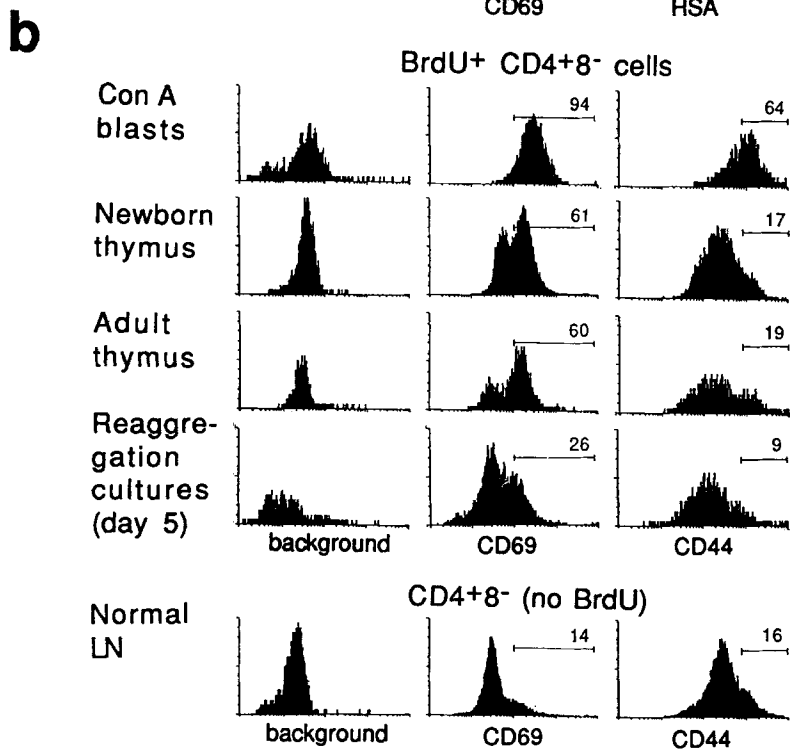
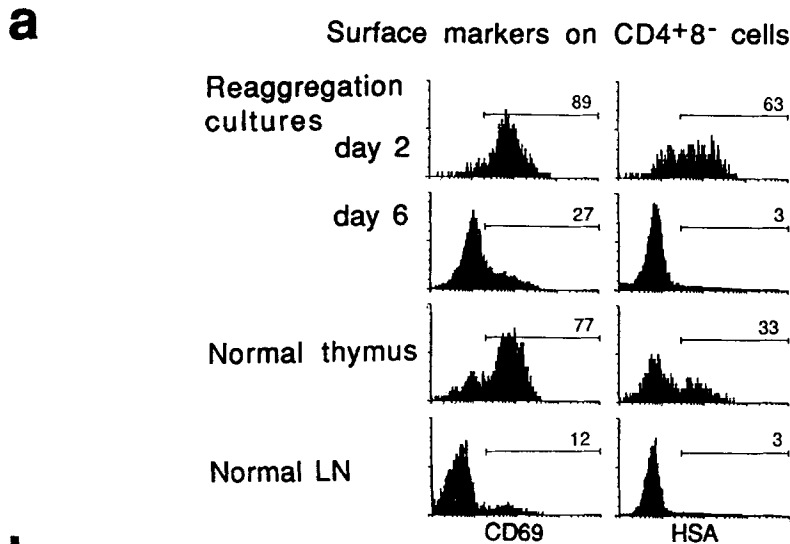


Figure 11. Surface markers on SP cells generated from neonatal TCR⁻ DP cells in reaggregation cultures. In *a*, cells from reaggregation cultures harvested on day 2 or day 6 were three-color stained for expression of CD69 or HSA vs CD4 and CD8. The data show CD69 and HSA expression on CD4⁺8⁻ cells; expression of these markers on CD4⁺8⁻ cells prepared from adult thymus and LN is included as a control. In *b*, reaggregation cultures were pulsed with BrdU (125 μg/ml) on day 5 of culture and were harvested 4 h later. As controls, cultures of Con A blasts were pulsed with BrdU (125 μg/ml) on day 3 and were harvested 4 h later; as additional controls, thymocytes were prepared from normal neonatal or adult mice injected intravenously with BrdU 4 h before (see Fig. 9 for BrdU dose). All four populations of cells were treated with anti-CD8 mAb plus C and then three-color stained for expression of BrdU, CD4, and CD69 or CD44. The data show CD69 and CD44 expression on BrdU⁺ CD4⁺8⁻ cells. The percentage of cells defined as being CD69⁺ or CD44^{hi} is marked on the histograms. Background staining (second Ab only) is shown on the left. The markers for CD69 and CD44^{hi} expression were set on the basis of staining of normal LN CD4⁺ cells (*bottom*).

BrdU labeling for CD4⁺8⁻ cells and 7% for CD4⁻8⁺ cells (Fig. 9).

The simplest explanation for the above data is that TEC from fetal and newborn mice release factors, e.g., cytokines, that induce mature T cells to divide. To examine this question, reaggregation cultures were prepared from a mixture of fetal TEC and normal LN CD4⁺8⁻ cells. The cultures were harvested at days 2–4 and pulsed with BrdU for 1 d before harvest. In marked contrast to LN CD4⁺8⁻ cells cultured alone, the percentage of BrdU labeling of CD4⁺8⁻ cells in LN/TEC cultures increased progressively with time and reached 30% by day 4 with high T cell recoveries (Fig. 10); labeling on day 2 was low (5%, data not shown). Extensive division of mature T cells in LN/TEC cultures was observed in several different experiments and

applied to both CD4⁺8⁻ and CD4⁻8⁺ cells. With Percoll-separated LN cells, proliferation was more marked with large cells than with small cells.

Surface Markers on Cells in Late Culture. Positive selection is known to cause rapid up-regulation of CD69 followed by gradual down-regulation of CD69 and HSA (1). In agreement with this scheme, the early wave of CD4⁺8⁻ cells found on day 2 in DP/TEC reaggregation cultures was predominantly CD69⁺ HSA⁺ (Fig. 11 *a*). Thereafter, the expression of these markers on CD4⁺8⁻ cells gradually decreased, and the majority of CD4⁺8⁻ cells were CD69⁻ HSA⁻ by day 6 (Fig. 11 *a*).

Since some of the cells in late culture retained CD69 expression, this phenotype might denote dividing cells. To examine this question, day 5 reaggregation cultures were

pulsed with BrdU for 4 h and then stained for expression of CD69 and CD44 (Pgp-1). Thymocytes from BrdU-injected (4 h) neonatal and adult mice were used as a control; as a further control, LN Con A blasts were stained after a pulse of BrdU on day 3 of culture. All four populations of cells were treated with anti-CD8 mAb plus C to remove CD4⁻8⁺ and CD4⁺8⁺ cells, thereby enriching for TCR^{hi} CD4⁺8⁻ cells, and were then three-color stained. Gating on CD4⁺(8⁻) BrdU⁺ cells revealed that the majority of the dividing cells in the control population of Con A blasts were CD69⁺ CD44^{hi}, i.e., the typical phenotype of TCR-activated T cells (Fig. 11 *b*). BrdU⁺ CD4⁺8⁻ cells from newborn and adult thymus showed a high frequency of CD69⁺ cells, but most of these cells were CD44^{lo} (Fig. 11 *b*). Interestingly, the bulk of the BrdU⁺ cells from day 5 re-aggregation cultures were CD69⁻ CD44^{lo} (Fig. 11 *b*); this also applied to the BrdU⁻ cells (data not shown). The CD4⁺8⁻ cells proliferating in the late stages of re-aggregation cultures thus did not display the phenotype of T cells responding to TCR-mediated signals.

Discussion

T cell proliferation in DP/TEC re-aggregation cultures was extensive and occurred at two distinct stages, i.e., in the early and late stages of culture. The first wave of proliferation was maximal on day 1 and reflected division and expansion of DP blast precursors. This was apparent from cell separation studies. Thus, T cell proliferation on day 1 was high in cultures prepared with DP blast cells but almost undetectable when blast cells were removed.

The absence of early proliferation in cultures prepared from small DP cells made it possible to test whether positive selection, i.e., differentiation of DP cells into SP cells, involves cell division. The key finding was that the vast majority of CD4⁺8⁻ cells generated from small DP cells during the first 3 d of culture failed to incorporate BrdU, despite the continuous presence of BrdU in the cultures. This observation provides direct support for the view that positive selection takes place in the absence of cell division (13, 14). It should be emphasized, however, that our data rest on studies with CD4⁺8⁻ cells. The data on CD4⁻8⁺ cells were less clear cut. In agreement with intrathymic injection experiments (10, 11), generation of SP cells in re-aggregation experiments was appreciably slower for CD4⁻8⁺ than for CD4⁺8⁻ cells. Thus, CD4⁺8⁻ cells were clearly apparent by day 2 of culture, especially when generated from purified small DP cells, but CD4⁻8⁺ cells were rare before days 3–4. Determining whether CD4⁻8⁺ cells arose from DP cells without division was complicated by the late wave of proliferation in the cultures (see below). Nevertheless, when BrdU was added at day 0 to cultures prepared from small DP cells, ~40% of the small numbers of CD4⁻8⁺ cells recovered on day 3 remained BrdU⁻. This finding suggests that at least a proportion of CD4⁻8⁺ cells arises from DP cells without division.

The onset of the second wave of T cell proliferation occurred at days 4–5 of culture. At this stage, proliferation was most prominent among TCR^{hi} cells, including the small subset of TCR^{hi} DP cells, but was undetectable at the level of TCR^{lo} DP cells. At later stages of culture, proliferation affected all T cell subsets, including TCR^{lo} cells. Late proliferation in culture was not unique to thymocytes because high T cell turnover was evident in LN/TEC cultures.

Although the late wave of T cell proliferation in re-aggregation cultures could be a tissue culture artifact, it is striking that near-identical proliferation was apparent in the normal neonatal thymus. Thus, pulsing neonatal mice with BrdU 3–4 h before removing the thymus revealed extensive turnover of all T cell subsets, including TCR^{hi} cells. Marked proliferation of CD4⁺8⁻ cells in the neonatal thymus was reported previously (15), and we used four-color staining to show that such proliferation also affects TCR^{hi} CD4⁻8⁺ and TCR^{hi} CD4⁺8⁺ cells. These findings on the neonatal thymus thus suggest that the proliferation seen in re-aggregation cultures is real and not artifactual.

Why T cell turnover is so prominent in the neonatal thymus and the late stages of re-aggregation cultures is unclear. The possibility that the cells proliferate as a manifestation of positive selection seems unlikely because many of the proliferating cells were TCR^{hi} SP cells, the phenotype of mature postselected T cells. If proliferation involved TCR signaling, one would expect the proliferating cells to display the typical phenotype of activated/memory T cells. Surprisingly, however, most of the proliferating TCR^{hi} cells found in late re-aggregation cultures were CD69⁻ CD44^{lo}, which contrasted markedly with the CD69⁺ CD44^{hi} phenotype of Con A blasts. In view of these findings, our working hypothesis is that the proliferation of postselected T cells in the perinatal thymus is not driven by TCR-mediated signals but by cytokines released from TEC, e.g., IL-7 (23). One way to test this idea is to examine whether proliferation can occur in the absence of TCR signals, by culturing postselected T cells (TCR^{hi} SP cells or LN T cells) in re-aggregation cultures prepared with normal vs MHC-deficient (MHC⁰) TEC. In fact, preliminary work has shown strong proliferation of T cells in LN/MHC⁰ TEC cultures.

Proliferation of mature T cells in the perinatal thymus could be a device to expand the mature T cell repertoire before export to the periphery. Alternatively, activation of postselected T cells in the thymus may be required for inducing the expression of “thymic export” receptors, i.e., receptors enabling mature medullary T cells to penetrate veins or lymphatics at the cortico-medullary junction. Whatever the explanation, it is notable that proliferation of mature T cells was also detectable in the adult thymus, albeit at a low level. Thus, labeling of adult thymocytes after a brief BrdU pulse *in vivo* affected 4% of TCR^{hi} CD4⁺8⁻ cells and 7% of TCR^{hi} CD4⁻8⁺ cells. In adults, proliferation of mature SP thymocytes is usually attributed to local responses to exogenous antigen (14) or back-migration of activated T cells to the thymus from the periphery (24).

However, it remains possible that the proliferation of mature T cells in the adult thymus is antigen independent and is controlled by the same factors operating in the neonatal thymus. A priori, this idea could be tested by culturing DP thymocytes with TEC prepared from adult thymus. Because of the difficulty of preparing purified TEC from adult thymus, however, we have been unable to conduct this experiment.

As a final point, it is worth noting that generation of SP cells in reaggregation cultures was substantially higher with neonatal than with adult DP cells. This also applied to purified small DP cells. Taking into consideration the total cell yields on day 4–5 culture (Fig. 2) and the proportion of SP cells in the cultures (70–90% on day 5 for neonatal cells and 40–50% for adult cells; see Figs. 3 and 4), the percentage of

recovery of SP cells relative to the input number of (small) DP cells was 4–6% for neonatal cells and ~1% for adult cells. Although this difference could reflect a higher proportion of dead-end DP cells in the adult thymus, it may be relevant that, unlike newborn thymocytes, adult thymocytes show high expression of TdT, the enzyme responsible for N-region diversity (25). In this respect, it is intriguing that generation of SP cells in the adult thymus is augmented in TdT “knockout” mice (26), perhaps because the lack of TCR N regions facilitates TCR contact with MHC molecules and thereby promotes positive selection (26). Hence, the finding that neonatal DP cells generate higher yields of SP cells in reaggregation cultures than adult DP cells could simply reflect that neonatal cells have low TdT expression.

We thank Barbara Marchand for preparation of the manuscript, and Jonathan Kaye, David Lo, and David Tough for their comments.

This work was supported by grants CA38355, CA25803, AI32068, and AI21487 from the United States Public Health Service. C.D. Surh is a Special Fellow of the Leukemia Society of America. This work is publication number 9255-IMM from The Scripps Research Institute.

Address correspondence to Jonathan Sprent, M.D., Ph.D., Department of Immunology, IMM4, The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

Received for publication 17 April 1995 and in revised form 1 June 1995.

References

1. von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell*. 76:219–228.
2. Nossal, G.J.V. 1994. Negative selection of lymphocytes. *Cell*. 76:229–239.
3. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell*. 76:17–27.
4. Ashton-Rickardt, P.G., A. Bandeira, J.R. Delaney, L. Van Kaer, H.P. Pircher, R.M. Zinkernagel, and S. Tonegawa. 1994. Evidence for an avidity model of T cell activation in the thymus. *Cell*. 73:1041–1049.
5. Sebzda, E., V.A. Wallace, J. Mayer, R.S.M. Yeung, T.W. Mak, and P.S. Ohashi. 1994. Positive and negative thymocytes selection induced by different concentrations of a single peptide. *Science (Wash. DC)*. 263:1615–1618.
6. Surh, C.D., and J. Sprent. 1994. T-cell apoptosis detected *in situ* during positive and negative selection in the thymus. *Nature (Lond.)*. 372:100–103.
7. Shortman, K., M. Egerton, G.J. Sprangrude, and R. Scollay. 1990. The generation and fate of thymocytes. *Semin. Immunol.* 2:3–12.
8. Gidos, C.J., I.L. Weissman, and B. Adkins. 1989. Intrathymic maturation of murine T lymphocytes from CD8⁺ precursors. *Proc. Natl. Acad. Sci. USA*. 86:7542–7546.
9. Lucas, B., F. Vasseur, and C. Penit. 1994. Production, selection, and maturation of thymocytes with high surface density of TCR. *J. Immunol.* 153:53–62.
10. Swat, W., H. von Boehmer, and P. Kisielow. 1994. Small CD4⁺8⁺ TCR^{low} thymocytes contain precursors of mature T cells. *Eur. J. Immunol.* 24:1010–1012.
11. Lundberg, K., and K. Shortman. 1994. Small cortical thymocytes are subject to positive selection. *J. Exp. Med.* 179:1475–1483.
12. Egerton, M., R. Scollay, and K. Shortman. 1990. Kinetics of mature T-cell development in the thymus. *Proc. Natl. Acad. Sci. USA*. 87:2579–2582.
13. Shortman, K., D. Vremec, and M. Egerton. 1991. The kinetic of T cell antigen receptor expression by subgroups of CD4⁺8⁺ thymocytes: delineation of CD4⁺8⁺3⁺ thymocytes as post-selection intermediates leading to mature T cells. *J. Exp. Med.* 173:323–332.
14. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell*. 66:533–540.
15. Ceredig, R. 1990. Intrathymic proliferation of perinatal mouse $\alpha\beta$ and $\gamma\delta$ T cell receptor-expressing mature T cells. *Int. Immunol.* 2:859–867.
16. Jenkinson, E.J., G. Anderson, and J.J.T. Owen. 1992. Studies on T cell maturation on defined thymic stromal cell populations *in vitro*. *J. Exp. Med.* 176:845–853.
17. Kosaka, H., C.D. Surh, and J. Sprent. 1992. Stimulation of

- mature unprimed CD8⁺ T cells by semiprofessional antigen-presenting cells in vivo. *J. Exp. Med.* 176:1291–1302.
18. Kishimoto, H., C.D. Surh, and J. Sprent. 1995. Upregulation of surface markers on dying thymocytes. *J. Exp. Med.* 181: 649–655.
 19. Ozato, K., and D.H. Sachs. 1981. Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigens of the H-2^b haplotype reveal genetic control of isotype expression. *J. Immunol.* 126:317–321.
 20. Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068–2088.
 21. Nathanson, S.D., P.L. Zamfirescu, S.I. Drew, and S. Wilbur. 1977. Two-step separation of human peripheral blood monocytes on discontinuous density gradients of colloidal silica-poly-vinylpyrrolidone. *J. Immunol. Methods.* 18:225–234.
 22. Tough, D.F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127–1135.
 23. Ceredig, R., and C. Waltzinger. 1990. Neonatal mouse CD4⁺ mature thymocytes show responsiveness to interleukin 2 and interleukin 7: growth in vitro of negatively selected Vβ6- and Vβ11-expressing CD4⁺ cells from (C57BL/6 × DBA/2)F₁ mice. *Int. Immunol.* 2:869–877.
 24. Agus, D.B., C.D. Surh, and J. Sprent. 1991. Reentry of T cells to the adult thymus is restricted to activated T cells. *J. Exp. Med.* 173:1039–1046.
 25. Rothenberg, E., and D. Triglia. 1983. Clonal proliferation unlinked to terminal deoxynucleotidyl transferase synthesis in thymocytes of young mice. *J. Immunol.* 130:1627–1633.
 26. Gilfillan, S., C. Waltzinger, C. Benoist, and D. Mathis. 1994. More efficient positive selection of thymocytes in mice lacking terminal deoxynucleotidyl transferase. *Int. Immunol.* 6: 1681–1686.