

# Turnover of Naive- and Memory-phenotype T Cells

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## Summary

On the basis of their surface markers, T lymphocytes are divided into subsets of "naive" and "memory cells". We have defined the interrelationship and relative life spans of naive and memory T cells by examining the surface markers on murine T cells incorporating bromodeoxyuridine, a DNA precursor, given in the drinking water. Three findings are reported. First, using a new method we show that the release of newly formed naive T cells from the unmanipulated thymus is very low (confirming the findings of others with surgical approaches). Second, in thymectomized mice, T cells with a naive phenotype remain in interphase for prolonged periods; however, some of these cells divide and retain (or regain) their "naive" markers. Third, most T cells with a memory phenotype divide rapidly, but others remain in interphase for many weeks. Collectively, the data indicate that long-lived T cells have multiple phenotypes and contain a mixture of memory cells, naive (virgin) cells, and memory cells masquerading as naive cells.

On the basis of their surface markers, T cells are divided into two broad groups of cells. In young life, most CD4<sup>+</sup> and CD8<sup>+</sup> T cells express high molecular weight isoforms of CD45 (CD45RA/B/C) (1–7), a low density of CD44 (Pgp-1) (8, 9), and a high density of L-selectin (MEL-14) (10, 11). These cells are thought to be naive (virgin) cells that have remained in interphase since their release from the thymus (12, 13). Another population of T cells displays a reciprocal phenotype, i.e., CD45R<sup>lo</sup> CD44<sup>hi</sup> L-selectin<sup>lo</sup>. Since this phenotype is shared by T cells carrying memory to defined antigens (1–4, 8, 11), the T cells with this phenotype in normal animals are considered to be memory cells primed to various environmental antigens. T cells with a memory phenotype are rare in young life but are conspicuous in old age (14–17).

Although it is well accepted that memory T cells arise from naive precursor cells (2, 18, 19), the interrelationship and relative life spans of these two types of T cells is controversial. In the case of naive T cells in rodents, many groups regard these cells as short-lived cells that either die within a few weeks of leaving the thymus or switch to memory cells (11). By contrast, other workers view naive T cells as potentially long-lived cells that can remain in interphase for many months (20). Similar uncertainty applies to the life span of memory cells. Until recently, it was generally assumed that memory to defined antigens is carried by a population of resting long-lived lymphocytes (21). Now, however, there is increasing evidence that memory cells are short-lived unless the cells retain contact with residual antigen (22).

In view of this controversy, the question arises whether the subsets of naive- and memory-phenotype T cells in normal

animals differ in their rate of turnover. Direct evidence on this topic is sparse. Thus, although it is well established that T cells divide infrequently *in vivo* at a population level (13, 23), information on the relative turnover rates of naive (CD45R<sup>hi</sup> CD44<sup>lo</sup> L-selectin<sup>hi</sup>) and memory (CD45R<sup>lo</sup> CD44<sup>hi</sup> L-selectin<sup>lo</sup>) T cells is still fragmentary. In sheep, it is reported that CD44<sup>lo</sup> T cells in lymphatic vessels are resting cells, whereas most CD44<sup>hi</sup> cells divide rapidly over a period of days (24). In humans, chromosome marker studies on irradiated patients indicate that CD45RA<sup>lo</sup> cells divide more frequently than CD45RA<sup>hi</sup> cells (25). The turnover of human CD45RA<sup>lo</sup> cells is quite slow, however, because most of these cells can remain in interphase for months (compared to years for CD45RA<sup>hi</sup> cells). In mice, the relative turnover rates of naive and memory cells has not been studied, although there is one report that most CD44<sup>hi</sup> cells are in G<sub>1</sub> (9).

We have defined the turnover of T cell subsets in mice by administering a DNA precursor, bromodeoxyuridine (BrdU),<sup>1</sup> in the drinking water (12, 26–28), and then examining the surface markers on BrdU-labeled cells. The results confirm that most T cells with a memory phenotype divide much more rapidly than naive-phenotype cells. Nevertheless, some memory-phenotype cells can remain in interphase for prolonged periods. In addition, evidence is presented that some T cells divide without losing their naive phenotype, implying that some memory cells can masquerade as naive cells.

<sup>1</sup> Abbreviations used in this paper: ATx, adult thymectomized; BrdU, bromodeoxyuridine; STx, sham thymectomized.

## Materials and Methods

**Mice.** Male C57BL/6 (B6), BALB/c, and C.B-17 SCID mice were obtained from the breeding colony of The Scripps Research Institute. Where indicated, mice underwent a thymectomy or a sham operation at 5–6 wk of age (29). After surgery, the mice were given drinking water containing antibiotics (25  $\mu$ g/ml neomycin sulfate, 13  $\mu$ g/ml polymyxin B sulfate) and left for 1 mo before being used experimentally. For BrdU-incorporation studies, mice were given sterile drinking water containing BrdU (Sigma Chemical Co., St. Louis, MO) at 0.8 mg/ml, which was made fresh and changed daily. For experiments examining the disappearance of BrdU-labeled cells, mice were given BrdU-containing drinking water for 9 d and then transferred to normal drinking water. For cortisone treatment, mice were injected intraperitoneally with 25 mg cortisone acetate (Sigma Chemical Co.) in PBS.

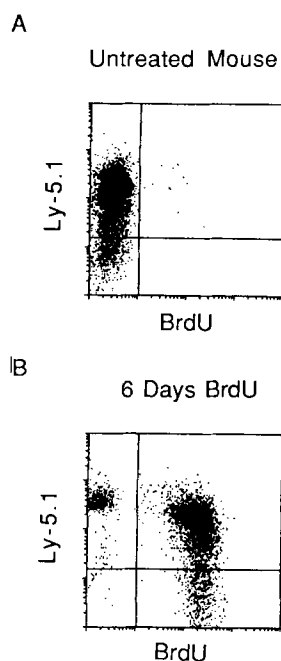
**Preparation of LN, Spleen, and Thymus Cells.** LN cells were pooled from cervical, axillary, inguinal, and mesenteric nodes. Cell suspensions were prepared with a tissue homogenizer. Mature thymocytes were isolated by treating thymocytes with anti-heat stable antigen (HSA) (J11d) mAb plus complement (30).

**Flow Cytometry.** mAbs used to stain cell surface antigens were: biotinylated-anti-Ly 5.1 (104-2.1, mouse IgG), biotinylated-anti-Pgp-1 (Pharmingen, San Diego, CA), biotinylated-anti-CD45RB (23G2) (Pharmingen), biotinylated-anti-L-selectin (MEL-14) (Pharmingen), PE-conjugated anti-CD4 (Becton Dickinson & Co., Mountain View, CA), PE-conjugated anti-CD8 (GIBCO BRL, Gaithersburg, MD), and RED613-conjugated anti-CD8 (GIBCO BRL). Bound biotinylated antibodies were detected with RED613-streptavidin (GIBCO BRL). After surface staining by conventional techniques (30), cells were washed, resuspended in cold 0.15 M NaCl, and fixed by dropwise addition of cold 95% ethanol. Using a modification of published methods (28), the cells were incubated for 30 min on ice, washed with PBS, then incubated with PBS containing 1% paraformaldehyde and 0.01% Tween 20 for 1 h. Cells were pelleted, then incubated with 50 Kunitz units DNase I (Sigma Chemical Co.) in 0.15 M NaCl, 4.2 mM MgCl<sub>2</sub>, pH 5, for 10 min. After washing, cells were incubated with FITC-conjugated anti-BrdU mAb (Becton Dickinson & Co.) and analyzed on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co.) using three-color analysis.

**Enumeration of Recent Thymic Emigrants.** At the indicated time points after the initiation of BrdU treatment, the total number of BrdU<sup>lo</sup> T cells (CD4<sup>+</sup> and CD8<sup>+</sup> cells) present among pooled LN and spleen cells from adult thymectomized (ATx) mice was subtracted from the number present in control sham thymectomized (STx) mice.

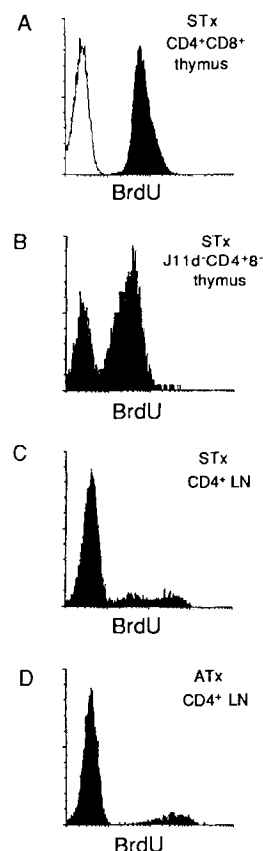
## Results

**Experimental Approach.** To seek information on the turnover of T cell subsets, groups of young adult B6 or BALB/c mice were given BrdU (0.8 mg/ml) in the drinking water for various periods. Using mAbs, lymphoid cells from the labeled mice were first stained for expression of various surface markers (see Materials and Methods). After fixation, the cells were then stained with an anti-BrdU mAb. Stained cells were detected by flow cytometry. At the dose used, BrdU in the drinking water for 6–9 d was sufficient to label >98% of Ly 5<sup>-</sup> (and most Ly 5<sup>+</sup>) bone marrow cells (Fig. 1 B) and >98% of CD4<sup>+</sup>8<sup>+</sup> thymocytes (Fig. 2 A). These populations are known to have a rapid rate of turnover. With re-



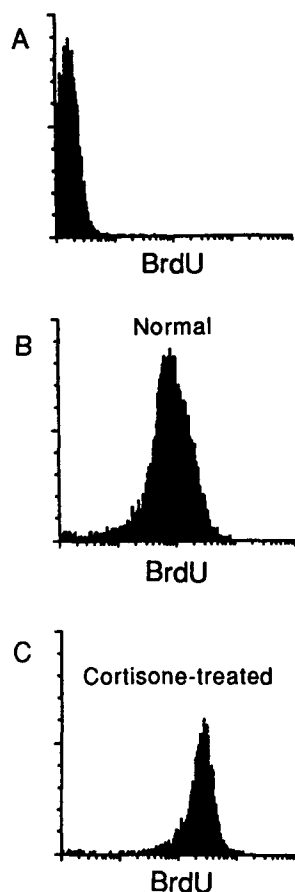
**Figure 1.** In vivo incorporation of BrdU by bone marrow cells. BrdU labeling and Ly 5 staining of bone marrow cells from (A) an untreated B6 mouse or (B) a B6 mouse given BrdU for 6 d. The small subset of unlabeled Ly 5<sup>+</sup> cells is probably contaminating mature T and B cells. These cells enter the marrow from the bloodstream and have a slow turnover (31).

gard to toxicity, continuous administration of BrdU for up to 5 wk caused no detectable thymic atrophy (data not shown). The data discussed below refer to young adult B6 mice; comparable data were obtained with BALB/c mice.



**Figure 2.** BrdU labeling of T cells from B6 sham thymectomized (STx) vs. adult thymectomized (ATx) mice. The data show BrdU labeling of (A) CD4<sup>+</sup>8<sup>+</sup> thymocytes from STx mice; (B) mature (J11d<sup>-</sup>) CD4<sup>+</sup>8<sup>-</sup> thymocytes from STx mice; (C) CD4<sup>+</sup>8<sup>-</sup> (CD4<sup>+</sup>) LN cells from STx mice; and (D) CD4<sup>+</sup> LN cells from ATx mice. STx and ATx mice were given BrdU water for 9 d. Staining of CD4<sup>+</sup>8<sup>+</sup> thymocytes from an untreated mouse not placed on BrdU water is shown by the open histogram (A).

**Different Intensities of BrdU Labeling in Thymus vs. Spleen and LN.** In accordance with the findings of others (32, 33), 9 d on BrdU water labeled about 70% of mature CD4<sup>+</sup>8<sup>-</sup> thymocytes (Fig. 2 B). Labeling of CD4<sup>+</sup> cells in LN (Fig. 2 C) and spleen (data not shown) was much lower, i.e., 15–20%. It is interesting to note that the density of BrdU labeling divided the labeled cells in LN and spleen into two discrete subsets of BrdU<sup>lo</sup> and BrdU<sup>hi</sup> cells. In the thymus, however, the labeled CD4<sup>+</sup>8<sup>-</sup> cells consisted solely of BrdU<sup>lo</sup> cells with virtually no BrdU<sup>hi</sup> cells. This finding suggested that the BrdU<sup>lo</sup> cells in LN/spleen might represent recent thymic emigrants. If so, removing the thymus before BrdU labeling would be expected to generate only BrdU<sup>hi</sup> cells in LN/spleen. Studies with ATx vs. STx mice confirmed this prediction (Fig. 2, C vs. D). Two possibilities could explain the uniform low density of BrdU labeling in the thymus: (a) the thymus has a poor blood supply; (b) massive death of thymocytes with local breakdown of DNA leads to cold target competition for BrdU use. To assess this second possibility, we examined two situations in which continuous cell death in the thymus is presumably lower than normal: (a) the regenerating thymus of cortisone-treated mice (left for 3 d to allow clearance of dead cells); and (b) the atrophic thymus of SCID mice (which consists solely of CD4<sup>-</sup>8<sup>-</sup> stem cells). In both situations, the intensity of BrdU labeling in the thymus was substantially higher than in the normal

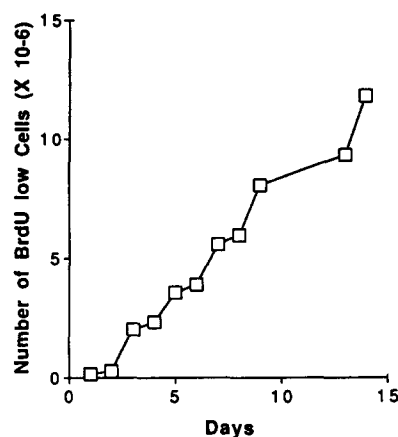


**Figure 3.** BrdU labeling of thymocytes from cortisone-treated mice vs. normal mice. The data show BrdU labeling of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from (A) an untreated B6 mouse; (B) a normal mouse given BrdU for 4 d, and (C) a mouse injected with cortisone acetate, rested for 3 d, and then given BrdU for 4 d.

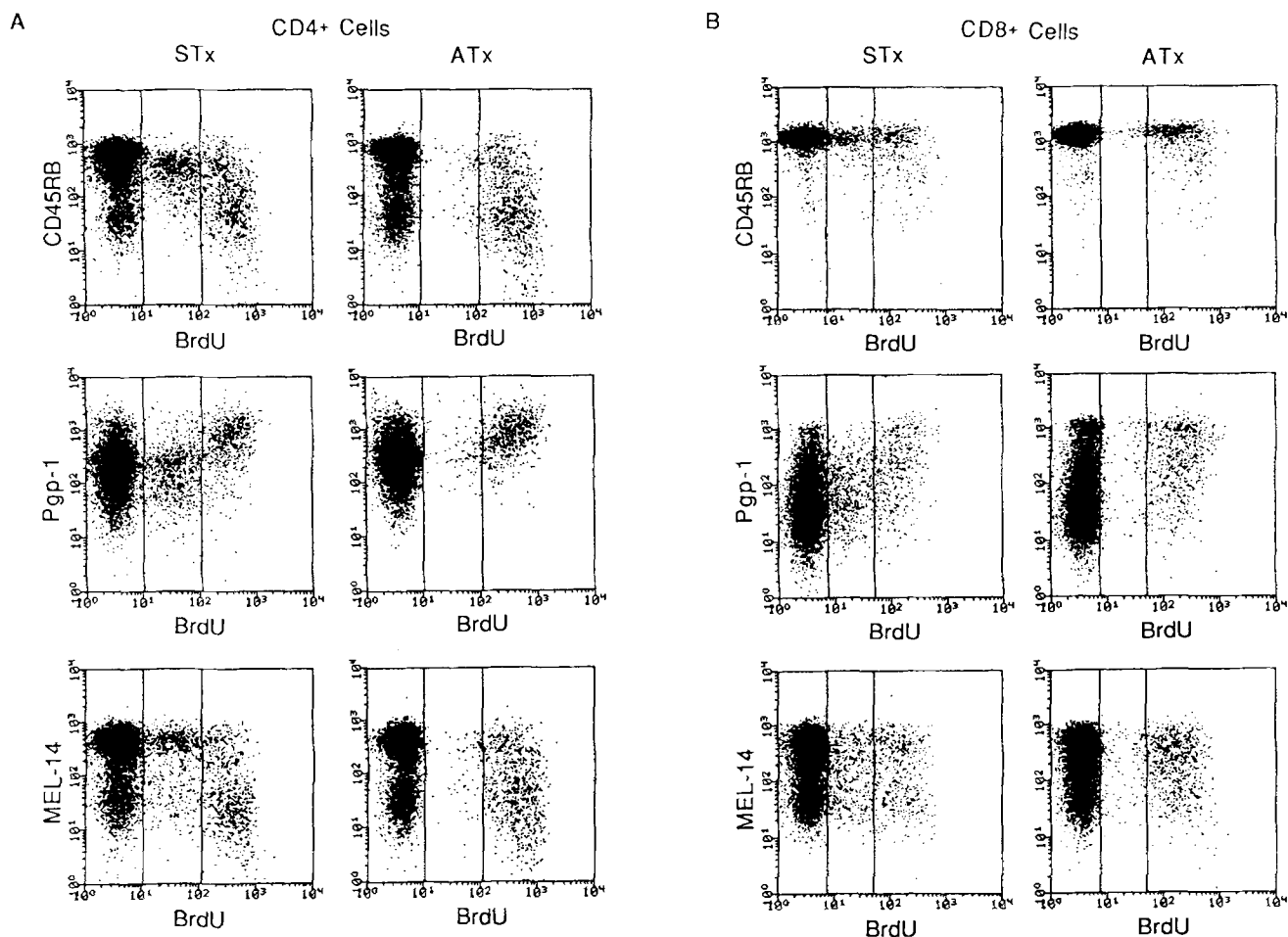
thymus (Fig. 3; data for the SCID thymus is not shown). This finding favors the notion that low BrdU labeling in the thymus is a reflection of local cold target competition.

**Quantitation of Recent Thymic Emigrants.** The observation that the BrdU<sup>lo</sup> cells in LN/spleen are recent thymic emigrants provided us with an approach to quantitate the release of T cells from the thymus of unmanipulated mice. The existing estimates of thymus release rates are based on studies in which the murine thymus was injected with FITC (or other labels) under anesthesia (34–37). We quantitated the appearance of BrdU<sup>lo</sup> cells in spleen plus pooled LN of STx vs. ATx mice during a 14-d labeling period; the small numbers of BrdU<sup>lo</sup> cells generated in the control ATx mice were subtracted from the data. In STx mice, BrdU<sup>lo</sup> cells appeared in the periphery after day 2 and then increased linearly during the period studied (Fig. 4). In the 8-wk-old mice tested, the accumulation of BrdU<sup>lo</sup> cells was  $\sim 0.9 \times 10^6$ /d. This estimate for thymus production rate is in close agreement with previous estimates (35).

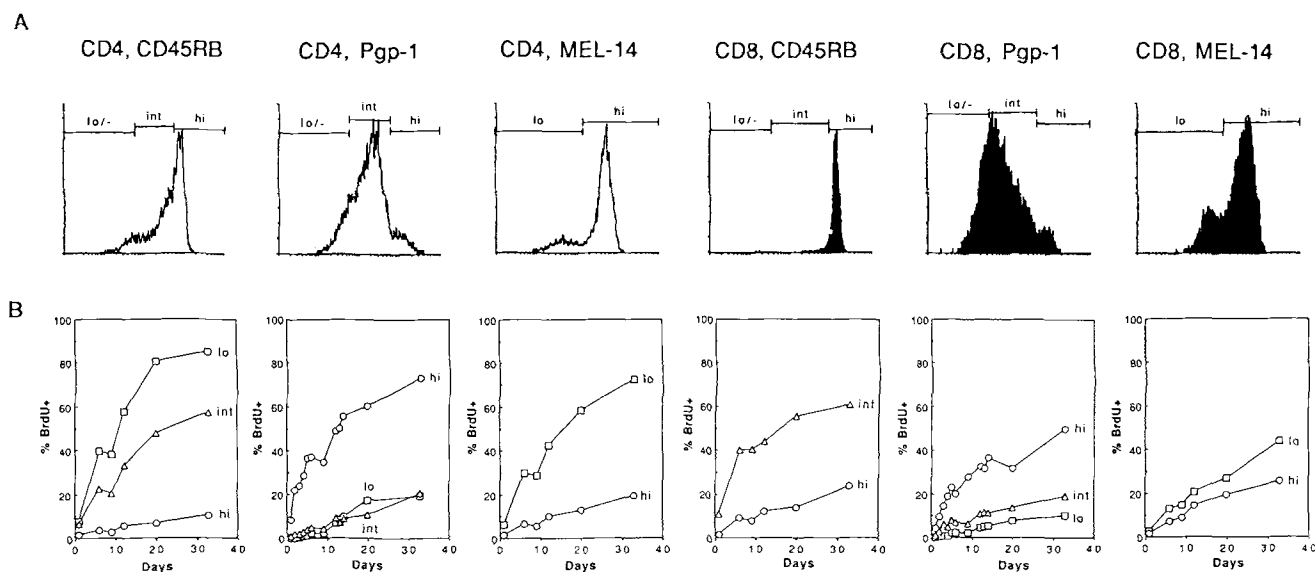
**Phenotype of Recent Thymic Emigrants.** In line with the BrdU<sup>lo</sup> cells being recent thymic emigrants, nearly all the BrdU<sup>lo</sup> cells in LN/spleen expressed the typical CD45RB<sup>hi/int</sup> Pgp-1<sup>lo/int</sup> MEL-14<sup>hi</sup> phenotype of naive T cells (although some cells were MEL-14<sup>lo</sup>; see below). This applied to both CD4<sup>+</sup> cells (Fig. 5 A) and CD8<sup>+</sup> cells (Fig. 5 B). The definition of high (hi), intermediate (int), and low (lo) levels of surface marker expression is shown in Fig. 6 A. Two points may be noted. First, in the case of CD4<sup>+</sup> cells, the density of CD45RB on recent thymic emigrants (BrdU<sup>lo</sup> cells) was a little lower than on the major population of unlabeled (BrdU<sup>-</sup>) mature CD4<sup>+</sup> cells. This finding is in agreement with the observation of others (36, 38, 39) that CD45R expression on recent thymic emigrants does not reach maximal levels until several days after export from the thymus. Second, in contrast to CD4<sup>+</sup> cells a surprisingly high proportion of



**Figure 4.** Accumulation of total BrdU<sup>lo</sup> cells (recent thymic emigrants) in spleen plus pooled LN of STx B6 mice. The total number of BrdU<sup>lo</sup> CD4<sup>+</sup> plus CD8<sup>+</sup> T cells found in pooled LN and spleen of STx mice, minus the number of these cells found in ATx mice, was determined at various times after initiation of BrdU treatment. The data show the values obtained for one to three mice per time point.



**Figure 5.** Surface markers on  $\text{BrdU}^{\text{lo}}$  and  $\text{BrdU}^{\text{hi}}$  T cells in the periphery of STx and ATx mice after 9 d on BrdU water. Using three-color staining, pooled LN cells were first stained for (a) CD45RB, Pgp-1, or MEL-14, then for (b) CD4 or CD8 followed, after fixation, by (c) nuclear staining for BrdU incorporation. The data show the expression of CD45RB, Pgp-1, and MEL-14 on BrdU-labeled  $\text{CD4}^+$  cells (A) and  $\text{CD8}^+$  cells (B).



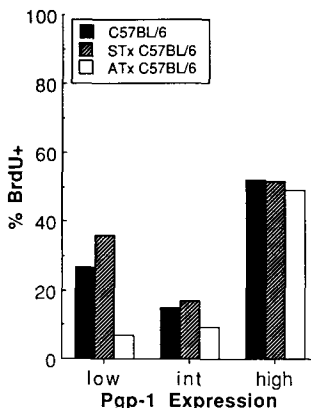
**Figure 6.** Turnover of T cells expressing naive vs. memory markers. Groups of ATx mice were placed on BrdU water for various periods. Pooled LN cells from these mice were then stained for expression of surface markers (see Fig. 5). (A) Staining profile of  $\text{CD4}^+$  and  $\text{CD8}^+$  LN T cells stained for CD45RB, Pgp-1, or MEL-14. The arbitrary definition of high (hi), intermediate (int), and low (lo) staining is shown. (B) Percent BrdU labeling of  $\text{CD4}^+$  and  $\text{CD8}^+$  LN T cells expressing lo, int, and hi levels of the markers shown in A. Data represent two to four mice per point. BrdU labeling refers to total ( $\text{BrdU}^{\text{lo}}$  plus  $\text{BrdU}^{\text{hi}}$ ) labeling.

CD8<sup>+</sup> recent thymic emigrants (40–50%) was MEL-14<sup>lo</sup> (see Discussion).

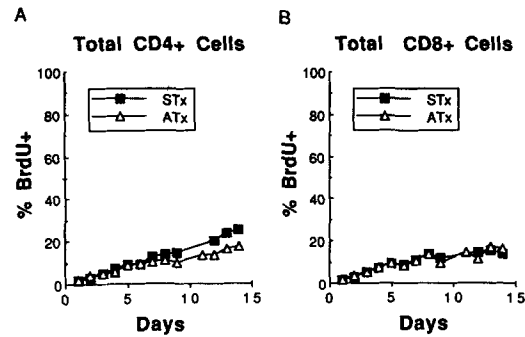
**Kinetics of BrdU Labeling of T Cells in ATx Mice.** Unlike the BrdU<sup>lo</sup> cells, most of the BrdU<sup>hi</sup> cells in LN/spleen of both ATx and STx mice expressed a memory (CD45RB<sup>lo</sup> Pgp-1<sup>hi</sup> MEL-14<sup>lo</sup>) phenotype. This applied after either 1 (data not shown) or 9 d (Fig. 5) on BrdU water. Expression of naive markers on the BrdU<sup>hi</sup> cells was limited at day 1 but was clearly apparent at day 9 (Fig. 5); at this stage a sizeable proportion of the labeled cells, especially CD8<sup>+</sup> cells, was CD45RB<sup>hi</sup> Pgp-1<sup>int</sup> MEL-14<sup>hi</sup>. This suggested that some of the mature T cells dividing in LN/spleen were reverting to resting cells and reacquiring some of the markers of naive cells.

To address this question, we examined the kinetics of BrdU labeling in ATx mice (thereby excluding the problem of recent thymic migrants). The data for LN (Fig. 6 B) and spleen (data not shown) were nearly identical. Three points emerge from the data. First, cells with a memory phenotype (CD45RB<sup>lo</sup> Pgp-1<sup>hi</sup> MEL-14<sup>lo</sup>) had a much faster rate of proliferation than naive-phenotype (CD45RB<sup>hi</sup> Pgp-1<sup>lo</sup> MEL-14<sup>hi</sup>) cells; this was more pronounced for CD4<sup>+</sup> cells than for CD8<sup>+</sup> cells. Second, despite their rapid rate of division, an appreciable proportion of memory-phenotype cells (20–30% of CD4<sup>+</sup> cells, 40–60% of CD8<sup>+</sup> cells) excluded BrdU over a 5-wk period, implying that these cells were non-dividing. Third, BrdU labeling of naive-phenotype cells was slow but reached significant levels after 5 wk (10–20% for both CD4<sup>+</sup> and CD8<sup>+</sup> cells). These data refer to ATx mice. When euthymic (STx or normal) mice were examined (Fig. 7), the turnover of naive-phenotype cells was much higher, presumably reflecting the accumulation of newly formed T cells released from the thymus. With regard to total turnover rates of CD4<sup>+</sup> and CD8<sup>+</sup> cells (Fig. 8), BrdU labeling of both subsets was slow and linear; percent labeling after 2 wk was 15–20%. For CD4<sup>+</sup> cells, labeling was slightly faster in STx than in ATx mice.

**Kinetics of Disappearance of BrdU-labeled T Cells.** Since BrdU is not reused, it was possible to do pulse-chase experiments. Groups of ATx mice were placed on BrdU water for 9 d and then transferred to normal water to examine the rate of decay of labeled cells. In the case of CD4<sup>+</sup> cells, the percent



**Figure 7.** BrdU labeling of CD4<sup>+</sup> T cell subsets from normal, STx, and ATx mice. The data show BrdU<sup>lo+hi</sup> incorporation by Pgp-1<sup>hi,int,lo</sup> subsets of LN CD4<sup>+</sup> cells from normal (■), STx (▨), or ATx (□) mice given BrdU for 12 d. Data represent three ATx, two STx, and two normal mice.



**Figure 8.** BrdU labeling of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells in normal, STx, and ATx mice. The data show BrdU<sup>hi+lo</sup> incorporation by total LN CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) cells. Data represent two to four mice per point.

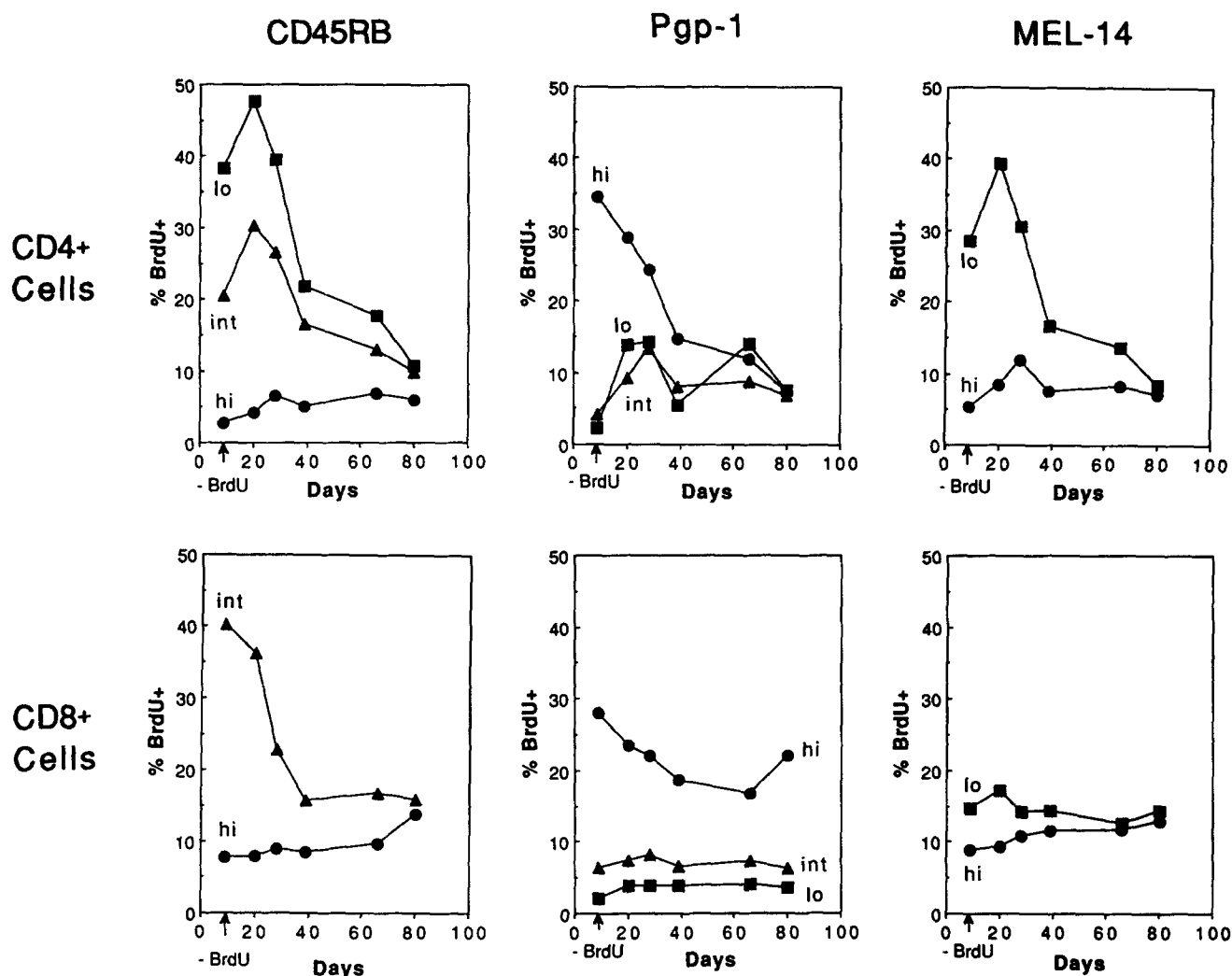
labeling of memory-phenotype cells declined rapidly during the first 4 wk but more slowly thereafter (Fig. 9). Similar kinetics applied to CD8<sup>+</sup> cells, but only for CD45RB expression. Thus, in marked contrast to CD4<sup>+</sup> cells, there was little or no decline in the percent labeling of Pgp-1<sup>hi</sup> CD8<sup>+</sup> cells or MEL-14<sup>lo</sup> CD8<sup>+</sup> cells throughout the 10-wk chase period. These findings apply to T cells expressing memory markers. For naive-phenotype cells, percent labeling of CD4<sup>+</sup> and CD8<sup>+</sup> cells remained constant or gradually increased (depending on the marker examined).

The above data refer to total BrdU<sup>lo+hi</sup> cells. When the density of BrdU labeling was examined after a 71-d chase period, three points were apparent (Fig. 10, A and B). First, there was a marked switch from BrdU<sup>hi</sup> to BrdU<sup>lo</sup> cells, implying extensive division (with dilution of label) during the chase period. Second, some of the cells remained BrdU<sup>hi</sup>, indicating very limited division of these cells during the chase period. Third, both the BrdU<sup>hi</sup> and BrdU<sup>lo</sup> cells comprised a mixture of memory-phenotype and naive-phenotype cells. With regard to this point, for CD4<sup>+</sup> cells, there was a definite switch from Pgp-1<sup>hi</sup> to Pgp-1<sup>lo/int</sup> cells during the chase period; this was much less apparent for CD8<sup>+</sup> cells.

## Discussion

The main conclusion arising from the data in this paper is that naive- and memory-phenotype T cells turn over at different rates: most memory cells divide rapidly whereas naive T cells do not. It is notable, however, that a sizeable proportion of memory-phenotype T cells resemble naive cells in remaining in interphase for prolonged periods. It is also striking that some T cells appear to undergo cell division (incorporate BrdU) without losing their naive phenotype, even after many weeks. The surface phenotype of T cells thus correlates poorly with their prior life history.

FACS<sup>®</sup> analysis of BrdU-labeled cells is now the method of choice for studying lymphocyte turnover. Some workers have reported that BrdU is toxic for mice and causes heavy mortality (40). Other workers, however, have failed to see evidence of toxicity (12, 27). This has been our experience. Thus, in our hands, placing mice on drinking water con-

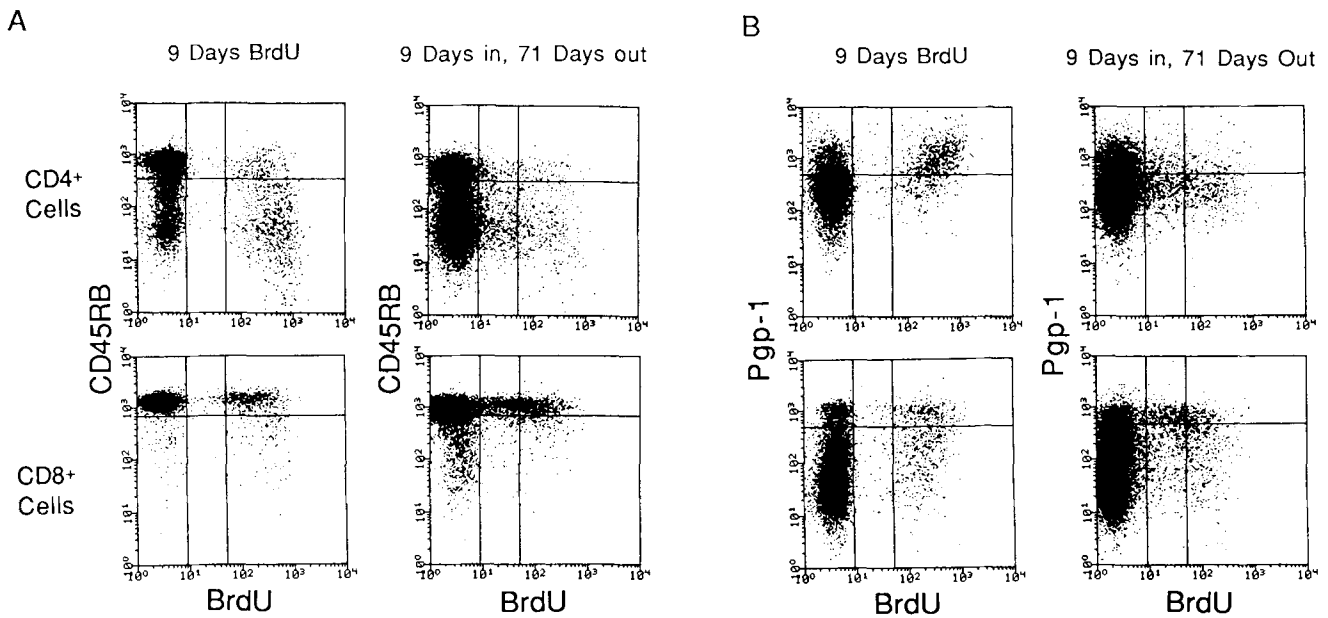


**Figure 9.** Decline in percent BrdU labeling of T cells after transferring mice to normal water. ATx mice were placed on BrdU water for 9 d (arrow) and then transferred to normal water. The data show percent BrdU labeling (BrdU<sup>lo+hi</sup>) of CD4<sup>+</sup> (top) and CD8<sup>+</sup> (bottom) cells expressing lo, int, and hi levels of the markers shown in Fig. 6 A. Data represent two to three mice per point.

taining low doses of fresh BrdU (made daily) causes no signs of ill health. In particular, we find no evidence of thymic atrophy, even after 1 mo on BrdU water. In accordance with the findings of others (32, 33), we observed that virtually all CD4<sup>+</sup>8<sup>+</sup> thymocytes incorporated BrdU within 6 d. Since CD4<sup>+</sup>8<sup>+</sup> thymocytes are the progeny of rapidly cycling CD4<sup>+</sup>8<sup>-</sup> stem cells, it would appear that BrdU incorporation by thymocytes is not antimitotic and allows precursor cells to undergo multiple rounds of cell division.

Although thymocytes showed rapid incorporation of BrdU, it is of interest that the intensity of BrdU labeling in thymocytes was quite low, probably because of local cold target competition from dying thymocytes. By contrast, in the secondary lymphoid organs (spleen/LN) two discrete peaks of BrdU<sup>lo</sup> and BrdU<sup>hi</sup> cells were evident. Since the mean fluorescence intensity of the BrdU<sup>lo</sup> subset in spleen/LN corresponded closely with the homogeneous population of BrdU<sup>lo</sup> single-

positive cells in thymus, it seemed highly likely that the BrdU<sup>lo</sup> cells in the secondary lymphoid organs represent recent thymic emigrants. The finding that the BrdU<sup>lo</sup> subset of cells was absent in ATx mice provided strong support for this prediction. By counting total numbers of BrdU<sup>lo</sup> cells in the spleen and pooled LN of ATx vs. STx mice, we were able to estimate the rate at which newly formed T cells are released from the normal thymus. In the 8-wk-old mice studied, the release of T cells from the thymus was calculated to be about 10<sup>6</sup> cells/day. This figure is in surprisingly close agreement with the estimates of Scollay and co-workers (35–37) on the release of labeled cells from the thymus injected with FITC under anesthesia. It is of interest that placing mice on BrdU water led to a 2-d lag period before labeled cells (BrdU<sup>lo</sup> cells) appeared in the secondary lymphoid organs. This finding implies that differentiation of cycling CD4<sup>+</sup>8<sup>+</sup> precursor cells into mature single-positive cells



**Figure 10.** Intensity of BrdU labeling in T cells from ATx mice placed on BrdU water for 9 d and then on normal water for a further 71 d (see Fig. 9). The data show the level of CD45RB (A) and Pgp-1 (B) expression on BrdU-labeled CD4<sup>+</sup> cells (top) and CD8<sup>+</sup> cells (bottom).

(positive selection), and movement of these cells from the cortex to the site of the cell exit from the medulla requires a minimum of 2 d.

In accordance with peripheral BrdU<sup>lo</sup> cells being recent thymic emigrants, most of the BrdU<sup>lo</sup> cells in spleen and LN expressed the phenotype of naive T cells. It is interesting to note, however, that a substantial proportion of the BrdU<sup>lo</sup> cells, especially CD8<sup>+</sup> cells, were MEL-14<sup>lo</sup> (but Pgp-1<sup>lo</sup> CD45RB<sup>hi</sup>). In agreement with this finding, we have found that MEL-14 expression is much lower on mature (HSA<sup>-</sup>) CD8<sup>+</sup> thymocytes than on typical extrathymic T cells (Ernst, B., C. D. Surh, and J. Sprent, unpublished data). The implication therefore is that, as for certain other markers (36–39), upregulation of MEL-14 expression on CD8<sup>+</sup> thymic emigrants takes place predominantly in the extrathymic environment.

Because of the continuous release of newly formed T cells from the thymus, studying the turnover of mature naive T cells in the postthymic environment necessitates prior removal of the thymus. As expected, BrdU incorporation by naive-phenotype T cells in ATx mice was found to be very slow, >80% of the cells remaining unlabeled after 5 wk on BrdU water. The simplest explanation for this finding is that, after leaving the thymus, thymic emigrants join the recirculating lymphocyte pool as long-lived resting cells (20). The longevity of these virgin T cells allows the immune system to continue to mount primary responses to new antigens even in advanced age, i.e., long after the onset of thymic atrophy. This notion rests on the assumption that naive-phenotype T cells in aged animals have remained in interphase from the time of their initial release from the thymus. This assumption may be invalid because in the present study, BrdU labeling

of naive-phenotype T cells was clearly significant. Thus, in young ATx mice, 10–20% of naive-phenotype T cells were labeled after 5 wk on BrdU water. This finding indicates that some mature T cells can divide without losing their naive phenotype. The implication therefore is that a sizeable proportion of naive-phenotype T cells in aged animals might not be true virgin cells but the progeny of cells stimulated by various environmental antigens.

The BrdU pulse–chase experiments provided further evidence that T cells can divide without losing their naive phenotype. In these experiments, placing mice on normal water after a 9-d pulse on BrdU water failed to cause a decline in percent labeling of naive-phenotype cells. In fact, the proportion of these cells tended to increase during the chase period. At the end of this period, the intensity of BrdU labeling in the naive-phenotype cells varied from quite high to very low, implying that some of the cells remained in interphase whereas others underwent extensive division. The retention of a naive-phenotype by BrdU-labeled cells was especially striking for CD8<sup>+</sup> cells. Thus, nearly all BrdU-labeled CD8<sup>+</sup> cells were CD45RB<sup>hi</sup> MEL-14<sup>hi</sup>, even after extensive dilution of label (indicative of cell division) at the end of the chase period (Fig. 10 A and data not shown); some of the labeled CD8<sup>+</sup> cells were Pgp-1<sup>hi</sup>, but many were Pgp-1<sup>lo/int</sup> (Fig. 10 B). These findings are surprising because CD8<sup>+</sup> cells proliferating in response to defined antigens (H-2 antigens) *in vivo* display the typical CD45RB<sup>lo</sup> MEL-14<sup>lo</sup> Pgp-1<sup>hi</sup> phenotype of activated/memory cells (30). As discussed elsewhere (17, 41, 42), the expression of these three markers on CD8<sup>+</sup> cells appears to be highly complex.

The slight increase in labeled naive-phenotype T cells during the BrdU chase period (most noticeable for CD45RB and

Pgp-1 expression on CD4<sup>+</sup> cells) (Fig. 9) raises the question of whether some of these cells were revertants from memory-phenotype cells. A precedent for this idea is provided by the report that purified CD45RC<sup>-</sup> rat T cells can revert to CD45RC<sup>+</sup> cells after adoptive transfer (5). In the case of Pgp-1 expression on CD4<sup>+</sup> cells, we observed a clear shift from labeled Pgp-1<sup>hi</sup> cells to Pgp-1<sup>lo</sup> cells during the chase period (Fig. 10 B). Although this finding is consistent with a reversion in phenotype, the alternative possibility is that the Pgp-1<sup>lo</sup> cells were not descended from Pgp-1<sup>hi</sup> cells: the Pgp-1<sup>hi</sup> cells died rapidly whereas the Pgp-1<sup>lo</sup> cells underwent several divisions without changing their phenotype and gave rise to an expanded population of Pgp-1<sup>lo</sup> cells. These two possibilities are not mutually exclusive.

In agreement with the data of others on CD44 expression on sheep T cells (24), most T cells with a memory phenotype showed a rapid rate of turnover. Thus, a large proportion of these cells incorporated BrdU within 3 wk and then lost this label during the chase period, presumably because of extensive cell division. Loss of labeled cells was less evident for CD8<sup>+</sup> cells, perhaps signifying that cell division during the chase period was less extensive for CD8<sup>+</sup> cells than CD4<sup>+</sup> cells. These data on the turnover of memory cells thus support the idea that most memory-phenotype cells in unimmunized animals are activated cells engaged in chronic responses to various environmental antigens. It is notable, however, that a sizeable proportion of memory-phenotype cells failed to incorporate BrdU over a 5-wk period. This ap-

plied to 20–30% of CD4<sup>+</sup> cells and 40–60% of CD8<sup>+</sup> cells (depending on the marker concerned). It is clear, therefore, that many T cells with a memory-phenotype are resting cells that can remain in interphase for prolonged periods. This notion is in line with the evidence that T (and B) cells carrying long-term memory for defined antigens are not cycling cells (43, 44). Why typical memory cells retain the expression of “memory” markers is unclear. In view of the evidence that maintenance of memory requires persistence of antigen, it is conceivable that retention of memory markers on T cells is a reflection of continuous low-level TCR stimulation. Such covert stimulation maintains the expression of molecules such as Pgp-1 but is insufficient to lead to cell division. This notion deserves serious consideration because many Pgp-1<sup>hi</sup> cells are reported to be in G<sub>1</sub> rather than G<sub>0</sub> (9).

In considering the prolonged maintenance of T cell numbers after the onset of thymic atrophy at puberty, the present data suggest that there are at least four types of potentially long-lived T cells: (a) virgin T cells released from the thymus; (b) primed T cells that retain or reacquire the phenotype of typical resting T cells; (c) primed T cells that revert to resting T cells but maintain expression of memory markers; and (d) overtly activated T cells engaged in chronic proliferative responses. These latter three populations are all candidates for carrying long-lived immunological memory to defined antigens. Direct evidence on this question will hinge on studying memory cell generation in antigen-specific lines of TCR transgenic mice.

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