The Life Span of Naive α/β T Cells in Secondary Lymphoid Organs

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Summary

We have determined the life span of naive $CD4^-8^+$ T cells in T cell receptor transgenic mice. We find that such cells do not divide in secondary lymphoid organs in both normal euthymic mice and T cell–deficient mice. By both continuous labeling and by chasing pulse-labeled cells, we find that the minimum life span of the naive T cells is in the order of 8 wk.

The life span of a cell is the time it lives from its formation until it enters mitosis or dies. Our information on the life span of naive lymphocytes is scarce because it has been difficult in the past to distinguish naive from antigen-experienced T cells. It has been argued that the various forms of the CD45 molecule can be used to distinguish memory from naive T cells (1–5), but the fact that some of the supposed memory cells can convert back into the naive phenotype is somewhat perplexing and demands a more cautious interpretation of the results (6–9). The CD44 molecule has also been considered as a memory marker, but its expression may reflect T cell activation rather than T cell memory (10, 11).

A second reason for our limited knowledge has been the apparent different conclusions reached from experiments using different methods to estimate the life span of lymphocytes. While labeling with [^{3}H]thymidine and some experiments using bromodeoxyuridine (BrdU)[1] incorporation have yielded results indicating that a major fraction of T and B lymphocytes has a relatively long life span (12–14), experiments using hydroxyurea, which destroys dividing cells, have reached conflicting conclusions (15, 16). Both approaches have not really been used to distinguish naive from memory T cells.

Our recent experiments with T cells from TCR transgenic mice have indicated that naive T cells could have a considerable life span (17). These experiments showed that in the absence of antigen, naive T cells do not numerically increase in secondary lymphoid tissue even in T cell–deficient animals, i.e., the number of naive T cells with known specificity (HY antigen presented by D^b MHC molecules) did not increase after transfer into female nude hosts but increased dramatically in male recipients. Nevertheless, it was still possible to recover a significant portion of the injected cells in female hosts 4–8 wk after transfer. This protocol, like others (18), could not, however, reveal information on the life span of T cells as it could not be excluded that some cells were dying while others divided periodically, and because it is not clear whether the transfer of T cells in T cell–deficient mice as carried out in experiments by us and others (18) mimics anything of physiological significance. We therefore studied cell division not only in this transfer model but also in euthymic and athymic TCR transgenic mice. The conclusion from all the experiments is that naive $CD4^-8^+$ α/β T cells, which leave the thymus after positive selection, have a minimum life span of ~8 wk and thus challenge the idea that naive T cells are short lived (19).

Materials and Methods

Mice. TCR α/β transgenic mice used in this study were described previously (20). C57 Bl/6 mice were obtained from IFFA Credo (L'Arbresle, France). C57 Bl/6 (B6) nude mice were obtained from Bomholtgard (Copenhagen, Denmark).

Adult Thymectomy. Adult thymectomy was performed under anesthesia with Avertine (Fluka, Switzerland). The successful extraction of the thymus was carefully checked in all animals once they were killed for the analysis of BrdU labeling.

Continuous Labeling with BrdU. 5-bromo-2-deoxyuridine (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS (4 mg/ml). 200 μl was injected intraperitoneally at 8 a.m. and 8 p.m. for up to 25 d (21).

Antibodies. For cell surface staining we used PE-coupled CD4 (1447; Becton Dickinson & Co., Mountain View, CA) fluorescein-conjugated CD8 (1353; Becton Dickinson & Co.), and T3.70 TCR α chain antibody (22) conjugated with biotin.

Surface Staining and Sorting of Lymphocytes. 10^6 lymphocytes were pelleted in round-bottomed wells of 96-well microtiter plates. Cells were resuspended in 100 μl PBS containing the desired antibodies in optimal concentration and incubated for 15 min on ice. After incubation cells were washed twice in PBS containing 2% FCS and resuspended either in 100 μl of the same solution (in the case of direct staining with fluorochrome-marked antibodies) or in 100 μl of optimally diluted PE-streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL) or allophycocyanin-streptavidin (Molecular Probes, Inc., Eugene, OR), and incubated for another 15 min on ice. After washing cells were analyzed on

[1] *Abbreviation used in this paper:* BrdU, bromodeoxyuridine.

a FACScan® or sorted on a FACStar Plus® instrument (Becton Dickinson & Co.).

BrdU Staining. After sorting, cells were pelleted in plastic tubes. To the pellet, 500 μl of 70% ethanol was added at room temperature. After 20 min cells were washed twice in PBS without FCS (2,000 rpm). Then, 500 μl of 3 N HCl containing 0.5% Tween (BDH Chemicals, Poole, England) was added. After 20 min, cells were pelleted and 500 μl of 0.1 M sodiumtetraborate (37F 002F; Sigma Chemical Co.) was added. After an additional 3 min, cells were washed twice in PBS containing 0.5% Tween. To the pellet, 20 μl of fluorescein-conjugated BrdU antibody (7583; Becton Dickinson & Co.) was added, incubated for 20 min, and cells were washed and prepared for analysis as described for other staining procedures. Cells were analyzed on the FACScan® without gates normally excluding dead cells.

Results

Conditions of Continuous Labeling. Previous results have shown that labeling with BrdU at the indicated conditions does not have any obvious toxic effect during a 1-wk labeling period, as the number of cells in secondary and primary lymphorgans as well as the composition of various cell types in the thymus remained constant (21). Since some of the experiments to be reported required continuous labeling for up to 25 d, we conducted additional controls by comparing cell numbers and lymphocyte subset composition of BrdU-injected TCR transgenic mice or mice injected at the same time intervals with saline only. The results showed that continuous BrdU labeling did not affect cell numbers (Table 1) or subset composition in various lymphoid organs, i.e., the ratio of CD4 and CD8 cells remained constant as did the subsets expressing the transgenic receptor and cells expressing endogenous TCR α chains (not shown).

Male-specific CD4⁻8⁺ T Cells Divide in Male but Not Female Nude Hosts. In initial experiments we evaluated the efficiency of our labeling method by studying BrdU incorporation in CD4⁻8⁺ T cells expressing a male-specific transgenic TCR α/β after their transfer into male C57Bl/6 (B6) nude hosts. Previously we had reported a rapid increase in numbers of cells with that receptor in male recipients, and it can be seen in Fig. 1 that all of these cells incorporated BrdU quickly, as do bone marrow cells that we use as an internal control of a rapidly turning over population of cells in most of the subsequent experiments. In contrast, CD4⁻8⁺ $\alpha_T\beta_T$ cells do not incorporate any label during a 2-wk period of continuous labeling after injection into female B6 nude mice (Fig. 1). Because the number of cells recovered 2 wk after injection corresponds to the number of cells expected to home to the spleen immediately after injection, these data suggest that some naive CD4⁻8⁺ T cells can persist for at least 2 wk without any cell division in T cell–deficient hosts. Contrary to CD4⁻8⁺ $\alpha_T\beta_T$ cells, both CD4⁻8⁺ as well as CD4⁺8⁻ cells with endogenous TCR chains divide to some extent in the adoptive hosts. These cells have receptors of unknown specificity and may be stimulated by various unknown antigens (17).

Table 1. *Number of Lymphocytes in BrdU-injected Mice*

		Cell counts					
		Thymus		LN		Spleen	
BrdU labeling	*wk*			$\times 10^{-6}$			
	1	56	24	30	36	64	32
	2	28	50	32	42	40	56
	3	20	48	36	38	44	76
Saline only							
	–		–		–		–
	2	26	58	24	32	82	70
	–		–		–		–

Thymus, lymph node, and spleen were removed, and single cell suspension was prepared as described (Materials and Methods).

The Life Span of Naive CD4⁻8⁺ T Cells in Euthymic Hosts. To study the turnover of naive CD4⁻8⁺ T cells in euthymic hosts, we conducted continuous labeling in TCR α/β transgenic B6 female mice. As shown in Fig. 2, thymus and bone marrow cells label quickly within a few days, while there is no significant labeling of CD4⁻8⁺ $\alpha_T\beta_T$ T cells during the first 7 d. This would be the expected result if naive T cells were not dividing in peripheral lymph tissue and if their turnover would be entirely dependent on the export of newly formed cells from the thymus (21). It can be seen that after 1 wk there was a linear increase in the number of labeled cells that could be expected to reach 50% of all CD4⁻8⁺ $\alpha_T\beta_T$ cells after ~4 wk. By extrapolating and assuming linear labeling kinetics, one can calculate that the average life span of a naive CD4⁻8⁺ cell in euthymic mice is in the order of 8 wk (Fig. 2).

Continuous Labeling in Thymectomized Mice. The experiments of the preceding section indicate that T cells did not divide once they left the thymus unless they were antigenically stimulated. This was directly tested by labeling of cells in thymectomized female TCR α/β transgenic B6 mice. After thymectomy as well as sham-thymectomy, we regularly observed a rapid fall of lymphocyte numbers down to ~50% of normal and a recovery to normal numbers 2 wk thereafter (Fig. 3). At the same time interval the proportion of CD4⁻8⁺ $\alpha_T\beta_T$ cells decreased among total lymphocytes as well as among CD4⁻8⁺ T cells (Fig. 3). Again, this is consistent with our earlier observations in nude mice that naive T cells do not divide unless antigenically stimulated. According to Fig. 4, this is also true for thymectomized mice that contain a much larger number of T cells than nude mice: over a labeling period of 3 wk, starting 4 wk after thymectomy, there is no BrdU uptake by CD4⁻8⁺ $\alpha_T\beta_T$ cells.

Decay of Labeled Cells in Euthymic Mice. The previous conclusions were supported by monitoring the decay of labeled CD4⁻8⁺ $\alpha_T\beta_T$ cells in TCR α/β transgenic B6 mice after a labeling period of 2 wk. At that point in time, ~20% of these cells are labeled and their decay among the total CD4⁻8⁺ $\beta_T\alpha_T$ population can be easily determined. It can be seen that these cells are relatively long lived, as no or only

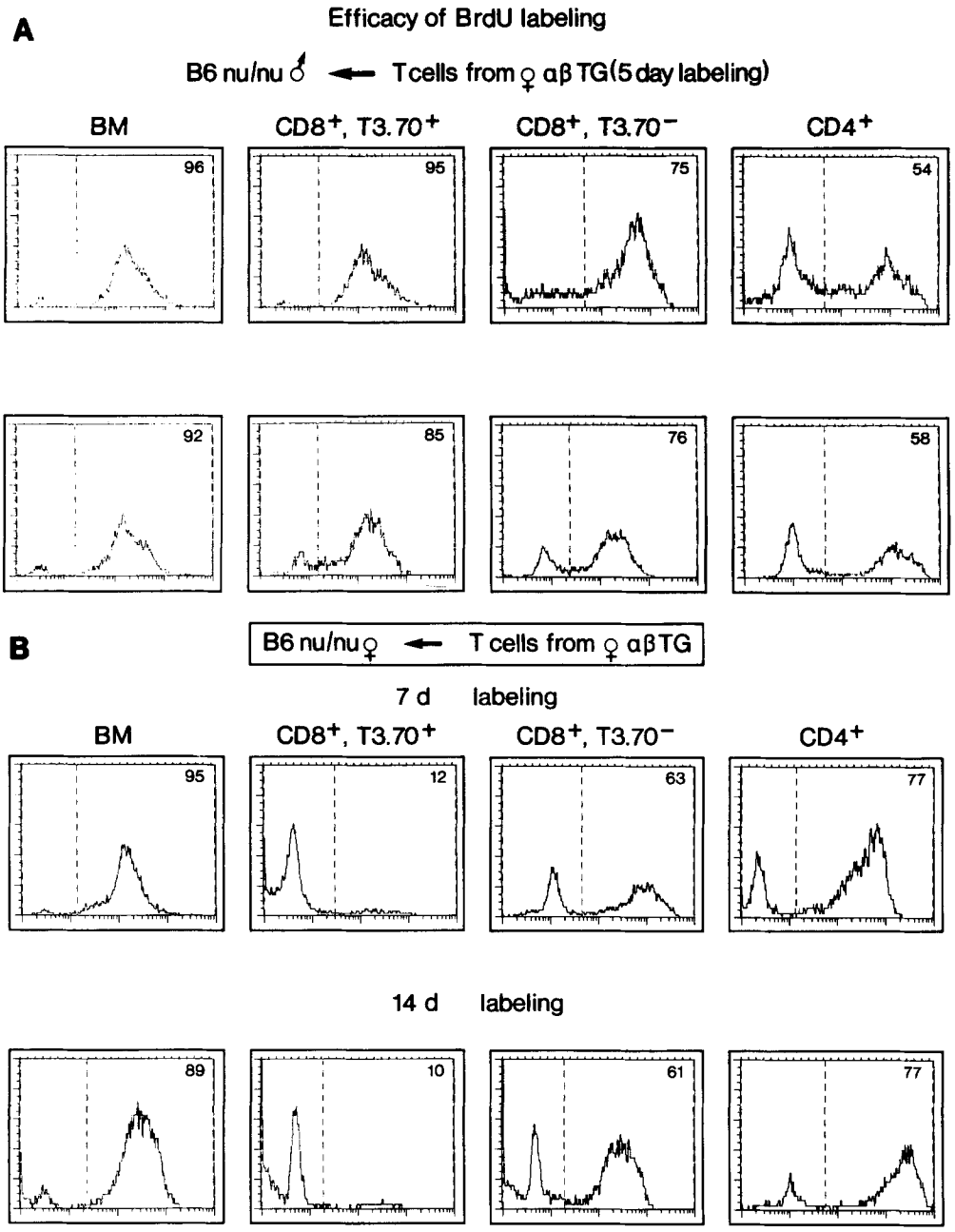

Figure 1. (*A*) The labeling of various T cell subsets transfer of B cell–depleted (anti-Ig column) T cells from female TCR α/β transgenic mice into nude male recipient mice. Labeling was assessed in bone marrow (*BM*) cells, in male-specific $CD4^-8^+$ $T3.70^+$ cells (22), in $CD4^-8^+$ $T3.70^-$ cells, which have other unknown specificities because they express endogenous TCR α chains, as well as $CD4^+$ cells, which all express endogenous TCR α chains. Cells were analyzed 5 d after the transfer and after 5 d of continuous BrdU labeling. Spleen (*top*) and lymph node (*bottom*) cells were analyzed (*BM*, bone marrow cells). (*B*) The labeling of various T cell subsets after transfer of B cell–depleted T cells from TCR α/β transgenic mice into nude female recipient mice after 7 and 14 d of transfer and continuous labeling with BrdU. Cell subsets are as in *A*. Only spleen cells were analyzed. The few labeled $CD8^+$, $T3.70^+$ cells may be cells with endogenous TCR α chains in addition to transgenic TCR α chains.

a slow decay is observed over a 3-wk period (Fig. 5). Since the previous studies have indicated that $CD4^-8^+$ $\alpha_T\beta_T$ cells are not dividing at all during that time period, the results indicate again that naive $CD4^-8^+$ T cells have a relatively long life span. It is unlikely that this result is significantly influenced by export of labeled cells from the thymus as most of the label has disappeared from the thymus after 1 wk (Fig. 5), and since in continuous labeling experiments the increase in the number of labeled cells is <20% per week. Nevertheless, to completely rule out this possibility we determined also the decay of label in thymectomized mice.

Decay of Labeled Cells in Thymectomized Mice. Mice were continuously injected with BrdU for 3.5 wk, then thymectomized, and the decay of labeled $CD4^-8^+$ $\alpha_T\beta_T$ T cells was investigated as above. Whereas the labeled cells disappeared rapidly from the bone marrow, the proportion of labeled $CD4^-8^+$ $\alpha_T\beta_T$ cells decayed very slowly during the subsequent 3-wk period of observation (Fig. 6). In fact, the decay was slower than in the euthymic mice. This may be due to better survival of cells in thymusless mice, which is expected because there is no de novo production of unlabeled cells.

Discussion

Before the advent of TCR transgenic mice, it was difficult to establish data on the life span of naive T lymphocytes be-

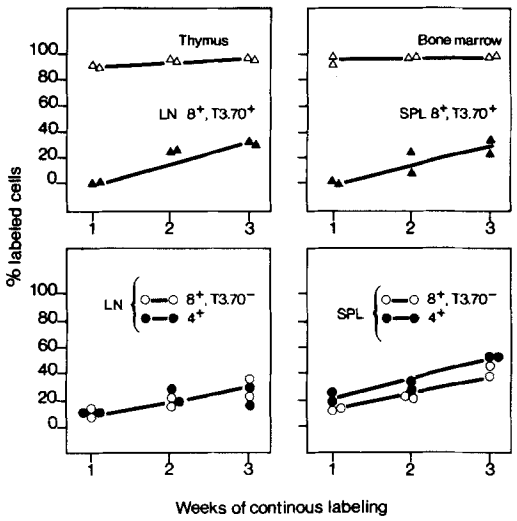

Figure 2. Continuous labeling of thymus, bone marrow, splenic (*SPL*) T, and lymph node (*LN*) T cells with BrdU in TCR α/β transgenic female mice. T cell subsets were as described in Fig. 1 *A*.

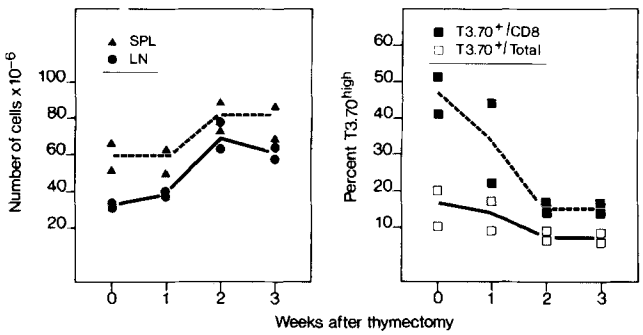

Figure 3. Increase in the number of total lymphocytes in lymph node and spleen (*left*) and decrease of the proportion of $CD4^-8^+$ $T3.70^+$ male-specific lymphocytes in spleen during weeks after adult thymectomy of female TCR α/β transgenic mice. The total number of $CD4^-8^+$ $T3.70^+$ cells that can be calculated from the total cell number and the percent of $CD4^-8^+$ $T3.70^+$ cells remained constant during the observation period.

cause one could not be certain that one was dealing with such cells. These exist no surface markers that definitely distinguish between naive and antigen-experienced T cells because most of the markers used to date are markers that correlate with cell activation rather than still ill-defined memory (23). Male-specific cells in female TCR transgenic mice fulfill the criteria of naive T cells, as these cells are never found to be CD44 positive, while they rapidly acquire CD44 after deliberate antigenic stimulation (24). The number of naive T cells with the transgenic TCR as well as the total number of lymphocytes decline initially after thymectomy performed in TCR transgenic female mice. The recovery of T cell numbers during the weeks after thymectomy is achieved exclusively by cells that can be antigenically stimulated, i.e., cells that express endogenous TCR α chains. The reduced proportion of cells with apparently naive phenotype in thymectomized mice may have previously led to the speculation that naive T cells are very short lived (19). Our results suggest that this interpretation is not correct and that it is the dilution of naive T cells in the pool of antigenically stimulated cells expanding after adult thymectomy that creates this illusion. The fact that there is no significant incorporation of DNA precursors into these cells once they leave the thymus supports the argument that these cells persist as naive T cells in peripheral lymph tissue.

By extrapolating the curve obtained during a 3-wk period of continuous labeling, one arrives at an estimate of a minimum life span of naive $CD4^-8^+$ T cells of 8 wk. This minimum estimate is supported by experiments in which the decay of labeling was monitored after a labeling period of 14 or 25 d in normal mice or mice that were thymectomized at the end

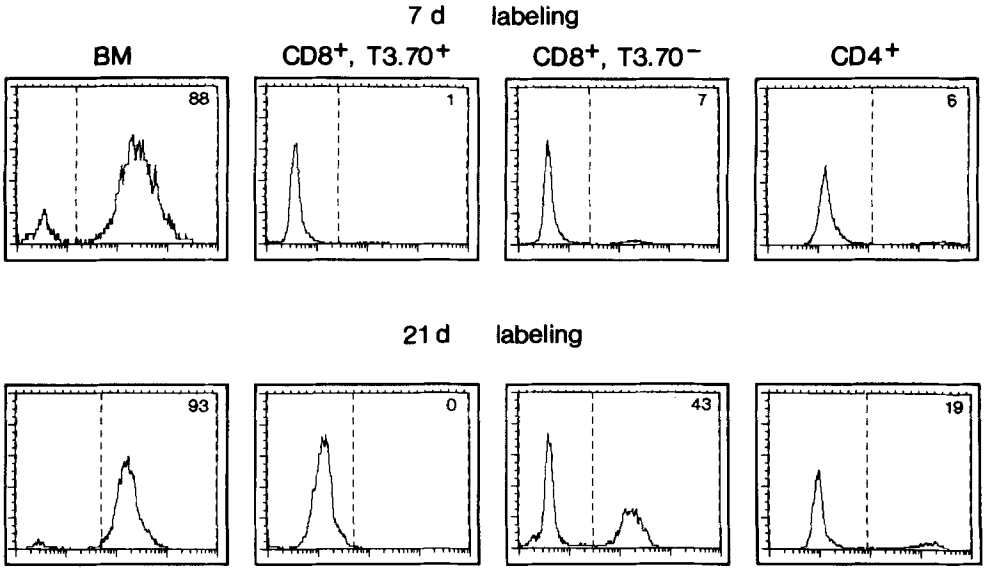

Figure 4. Continuous labeling of T cell subsets with BrdU in thymectomized female TCR α/β transgenic mice 4 wk after adult thymectomy. T cell subsets were as described in Fig. 1 *A*.

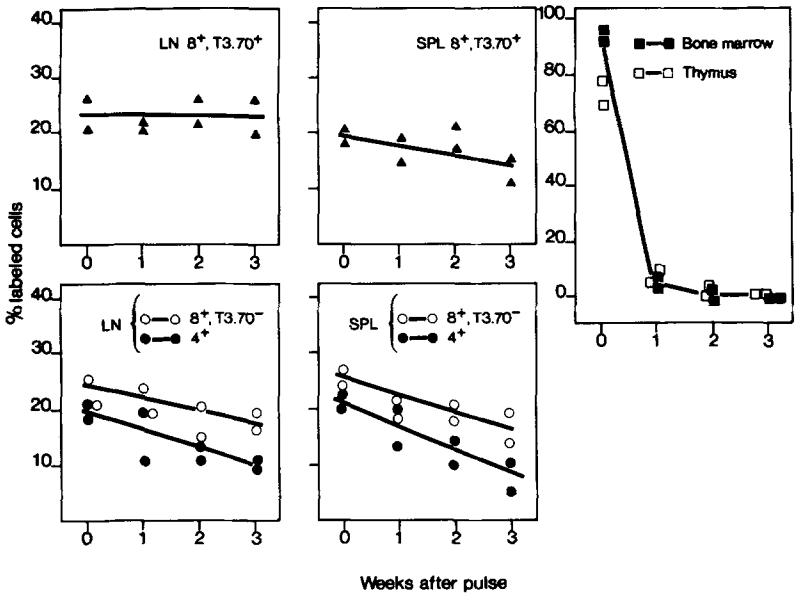

Figure 5. Decay of label in bone marrow, thymus, lymph node (*LN*) T, and splenic (*SPL*) T cells after a labeling pulse of 2 wk in female TCR α/β transgenic mice. Lymph node and splenic T cells were as described in Fig. 1 *A*.

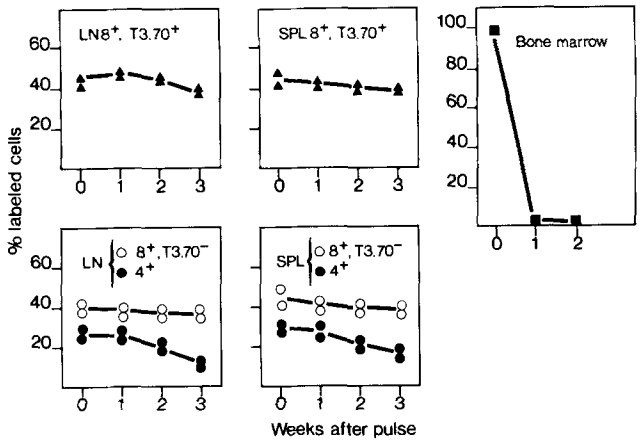

Figure 6. Decay of label in bone marrow, lymph node (*LN*) T, and splenic (*SPL*) T cells after a labeling pulse of 25 d of TCR α/β transgenic female mice. Thymectomy was performed at day 0 of the observation period. T cell subsets were as described in Fig. 1 *A*.

of the labeling period. Previous experiments have indicated that some 75% of T cells of peripheral T cells have a life span of 7 d or less (16). Assuming that this method gives a correct estimate of life span, we would have to argue that these cells do not include naive $CD4^-8^+$ T cells. It should be pointed out that not all labeling methods give the same estimate of life spans and that, for instance, labeling with [^{3}H]thymidine has lead investigators to conclude that the proportion of rapidly turning over T cells is <20% (12). While there exist continuing debates on the utility of the various labeling procedures, we feel that the BrdU procedure as used here represents an appropriate means to study life span as it does not affect cell numbers or subset composition in various lymphoid organs. At present we can also not be certain whether the results obtained with our method disagree with any other methods because the life span of naive $CD4^-8^+$ T cells has not been determined previously and the proportion of antigenically stimulated cells may vary considerably in various experimental animals.

For several reasons it is likely that the calculated life span for naive $CD4^-8^+$ $\alpha_T\beta_T$ cells of 8 wk represents a lower estimate. In female TCR α/β transgenic mice there is a slightly increased export of male-specific naive T cells from the thymus (our unpublished data), and it could be that an increased export of naive T cells from the thymus has an impact on the turnover of peripheral naive T cells. This may in fact be the reason why we still detect $CD4^-8^+$ $\alpha_T\beta_T$ cells 3 mo after adult thymectomy (unpublished results). It is possible that naive T cells have a longer life span in T cell–deficient mice or mice that export only low numbers of T cells from the thymus and thus provide more "space" for naive T cells. Whatever influence T cell export may have on the life span of naive T cells, our data indicate that the minimum life span of naive $CD4^-8^+$ T cells in a euthymic mouse is 8 wk.

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