T Cell Receptor Gene Recombination Patterns and Mechanisms: Cell Death, Rescue, and T Cell Production

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Summary

The antigen-specific receptors of T and B lymphocytes are generated by somatic recombination between noncontiguous gene segments encoding the variable portions of these molecules. The semirandom nature of this process, while desirable for the generation of diversity, has been thought to exact a high price in terms of sterile (out-of-frame) products. Historically, the majority of T lymphocytes generated in mammals were thought to be useless, either because they generated such sterile rearrangements or because the receptors generated did not appropriately recognize self-molecules (i.e., positive and negative selection). In the studies described here, we characterize the onset of T cell receptor (TCR) α and β chain gene rearrangements and quantitate their progression throughout T cell development. The results show that T cell production efficiency is enhanced through (a) rearrangement of TCR-β chain genes early during T cell development, with selective expansion of those cells possessing in-frame rearrangements; (b) deletion of sterile rearrangements at the TCR-α chain locus through ordered (proximal to distal) sequential recombination; and (c) modification of nonselectable α/β heterodimer specificities through generation and expression of new TCR-α chains. In addition, we demonstrate strict correlations between successful TCR-β gene rearrangement, the onset of TCR-α gene rearrangement, rapid cell division, and programmed cell death, which together serve to maintain cell turnover and homeostasis during T cell development.

The lymphoid immune system consists of T and B cells, each of which expresses a heterodimeric protein cell surface receptor of (generally) clonal specificity. The potential number of antigens that an organism could expect to encounter during its lifespan is large, such that germline genes sufficient to encode proportionate numbers of specific receptors would represent a substantial biological burden. To accommodate both genetic simplicity and antigenic diversity, a system of somatic gene recombination has evolved (1), whereby families of similar but nonidentical gene clusters rearrange to generate proteins of random and diverse specificity. In developing T cells, a heterodimeric TCR pair (designated α/β, or in some cases γ/δ) is generated through recombination of V-D-J (TCR-β or -δ) or V-J (TCR-α or -γ) gene clusters. Since the final specificity of the receptor depends on both α and β chain gene segment usage, and since the murine TCR-β locus consists of at least 28 V, 2 D, and 12 J gene segments, with 70–100 V and 50 J gene segments for TCR-α, the potential diversity generated by recombination is thus quite large, while the genetic burden is minimized.

The generation of receptor diversity through semirandom recombination of related gene segments is not without liability, however. Since amino acids are encoded by triplets of DNA bases, the usefulness of any given rearrangement will depend on both the sequence and the number of DNA bases located between initiation and termination codons. Consequently, the majority of such random rearrangements are thought to result in sterile gene configurations, mainly through out-of-frame sequences or premature termination codons. Thus, most developing lymphocytes are thought to fail to generate an antigen receptor. In the case of developing T cells, the assembly of in-frame receptor genes is only the first low frequency event. Subsequently, the heterodimers must be expressed on the cell surface, and must successfully and appropriately bind to self-MHC antigens (i.e., positive selection) before the cell can fully mature. Only a small fraction of receptor-expressing cells are thought to complete this requirement (2). Therefore, the vast majority of T cells generated by the thymus have been thought to be wasted, because of lack of receptor assembly or failure to be positively selected.

We have previously demonstrated that the frequency of TCR expression on developing T cells is far in excess of that which could be predicted on the basis of conventional models of gene recombination (3). Based on this and other related observations, it has been speculated that the TCR-α locus might be capable of more than one rearrangement on each

allele (3, 4). In the studies described here, we directly demonstrate that this is the case, using simultaneous multiprobe Southern blotting to quantitate deletion of the Jα cluster. Furthermore, we show that rearrangements at the TCR-α locus are proximally (V-J) biased, as has been postulated (4, 5), and that the first rearrangements at this locus occur immediately subsequent to the generation of an in-frame rearrangement at the TCR-β locus. We also quantitate the rearrangement of the two TCR-β gene clusters, both temporally and in relation to each other, using simultaneous multiprobe Southern blotting. Finally, we demonstrate a tight correlation between the status of gene rearrangement and the onset of programmed cell death, which serves as a model for homeostasis and biological efficiency in T cell development.

Materials and Methods

Mice. The origin of the bcl-2–transgenic strain 36 has been described (6). The mice used in these studies have been back-crossed onto the C57BL/6 background for at least eight generations by the authors. Nontransgenic C57BL/6 mice were either purchased from a commercial breeder (Jackson Laboratory, Bar Harbor, ME) or were the nontransgenic littermates from heterozygous breeding of bcl-2–transgenic mice.

Isolation of Defined T Cell Subsets. The fundamentals of the cell staining and sorting procedures used here have been described in detail previously (3, 7). For the purification of $CD3^-4^+8^+$, $CD3^{lo}4^+8^+$, $CD3^+4^+8^-$, or $CD3^+4^-8^+$ thymocytes, freshly prepared single cell suspensions of thymocytes were first treated with anti-CD3 antibody (clone KT3). Cells were then washed and stained with PE-conjugated goat anti–rat immunoglobulin (Biomeda, Foster City, CA). The cells were again washed and then treated first with rat Ig blocking reagent, followed by FITC-conjugated anti-CD8 (CALTAG Laboratories, South San Francisco, CA) and allophycocyanin (APC)[1]-conjugated anti-CD4 (CALTAG Laboratories). For the purification of lymph node T or B cells, fresh single cell suspensions were prepared from pooled axillary, brachial, and superficial inguinal lymph nodes and stained with FITC-conjugated anti–TCR-β chain (H57-597, CALTAG Laboratories) and PE-conjugated anti–heat-stable antigen (HSA; PharMingen, San Diego, CA). Subsets of triple negative $CD3^-4^-8^-$ (TN) thymocytes were prepared as previously described (3, 7). Briefly, single cell suspensions of freshly isolated thymocytes were depleted of CD3-, CD4-, and CD8-expressing cells by two rounds of depletion. The first round included treatment with anti-CD4 (clone RL172.4) and anti-CD8 (clone 3.155) antibodies, followed by lysis using rabbit serum complement. The second round of depletion involved treatment with anti-CD3 (clone KT3), anti-CD4 (clone GK1.5), and anti-CD8 (clone 53-6.7) antibodies, followed by depletion of positively-stained cells using anti-Ig–coated paramagnetic beads (Dynal, Oslo, Norway). The resulting depleted cells were then stained with FITC-conjugated anti-CD44 (PharMingen), PE-conjugated anti-HSA (PharMingen), and biotin-conjugated anti-CD25 (PharMingen), followed by APC-streptavidin (Biomeda). All preparations for cell sorting contained propidium iodide (500 ng/ml) for the identifica-

tion and exclusion of dead cells. Cell sorting was performed on a FACStar^plus® cell sorter (Becton Dickinson & Co., Mountain View, CA) equipped with argon ion and rhodamine dye lasers.

Cell Culture. The culture system for analysis of gene rearrangements in vitro was essentially as previously described (3). $CD3^{lo}DP$ (double positive; $CD4^+8^+$) thymocytes or $CD3^+$ LN cells from bcl-2–transgenic mice were resuspended at 0.3–1.0 × 10^6 cells/ml in RPMI-1640 medium containing 5% fetal bovine serum, 1 mM glutamine, 10 mM Hepes buffer, and 5 × 10^{-5} M β-ME. 1–2-ml aliquots were then dispensed to the wells of 24-well tissue culture trays. Cultures were incubated for 3 d at 37°C in a humidified atmosphere containing 8% CO_2 in air.

DNA Preparation. To minimize the numbers of cells required as sources for DNA for Southern blotting, DNA was isolated in agarose plugs as described (8). Briefly, cells of interest were aliquoted at 2.5–3.0 × 10^5 cells per 1.5-ml microfuge tube and centrifuged at 400 g. All of the supernatant was removed, and the cells were washed in Dulbecco's PBS without divalent cations and with 20 mM EDTA (PBSE). Washed cell pellets were resuspended in 30 μl of 1% low melting point agarose (FMC Corp., Rockland, ME) in PBSE at 37°C and solidified on ice. Plugs were then overlaid with 200 μl of 50 mM, Tris, pH 8.0/20 mM EDTA containing 1% sodium lauryl sarcosine and 1 mg proteinase K/ml, and incubated overnight at 50°C. The supernatant was then removed, and the plugs were washed for 8–10 h in 1 ml of 10 mM Tris/1 mM EDTA (TE). Digestion and washing steps were repeated one to two additional times. Plugs were then treated for 30 min at room temperature with 0.5 mM Pefabloc (Boehringer-Mannheim Corp., Indianapolis, IN) in 50 mM Tris/20 mM EDTA and stored at 4°C in TE until use.

RNA Preparation and Northern Blotting. Total RNA was extracted from sorted cells as described (8). Formaldehyde gel electrophoresis was performed as described (3). RNA was blotted onto GeneScreen Plus (DuPont-NEN, Boston, MA) by capillary transfer overnight in 10× SSPE (1.8 M NaCl, 0.1 M Na_2HPO_4, 0.1 M EDTA). Blotted membranes were baked for 30 min at 80°C, wetted in 2× SSC, and prehybridized for 2 h at 42°C in 5× SSC/0.5% SDS/50% formamide/1× Denhardt's reagent containing 100 μg herring sperm DNA/ml. An EcoRI fragment of clone Ncm-4 (9) was labeled with [α-^{32}P]dCTP by random hexamer priming and used as a probe for bcl-2 expression. Hybridization was carried out overnight at 42°C in fresh buffer as described for prehybridization. Hybridized blots were washed with decreasing concentrations of SSC/0.5% SDS until background noise was sufficiently reduced, and they were imaged using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA). RNA from equivalent cell numbers (∼2 × 10^6) were loaded in each lane. Equal loading was confirmed by visualization of ethidium bromide staining of ribosomal RNA bands (not shown).

Probes for Southern Blotting. The complete sequence of the J region cluster of the TCR-α locus, including the 5'-flanking C-δ region and the 3' flanking C-α region, has been published (10). Using this data, it was possible to select a restriction endonuclease combination (BamHI/HindIII) that yielded DNA fragments of sufficiently different sizes to be discriminated by agarose gel electrophoresis, and that spanned the Jα cluster at approximately equal distances (Fig. 1). In addition, fragments selected for analysis were chosen to have extensive (>500 bp) noncoding regions located 5' to most Jα coding sequences within that restriction fragment, such that this noncoding region would be deleted by rearrangements to those Jα segments. Oligonucleotide primers for PCR amplification of ∼500 bp products within this 5' noncoding region were then designed and synthesized. The primer sequences (5'-3') and

[1] *Abbreviations used in this paper:* APC, allophycocyanin; DP, double positive ($CD4^+8^+$); HSA, heat-stable antigen; TD, triple dull ($CD3^{lo}4^{lo}8^{lo}$); TN, triple negative ($CD3^-4^-8^-$).
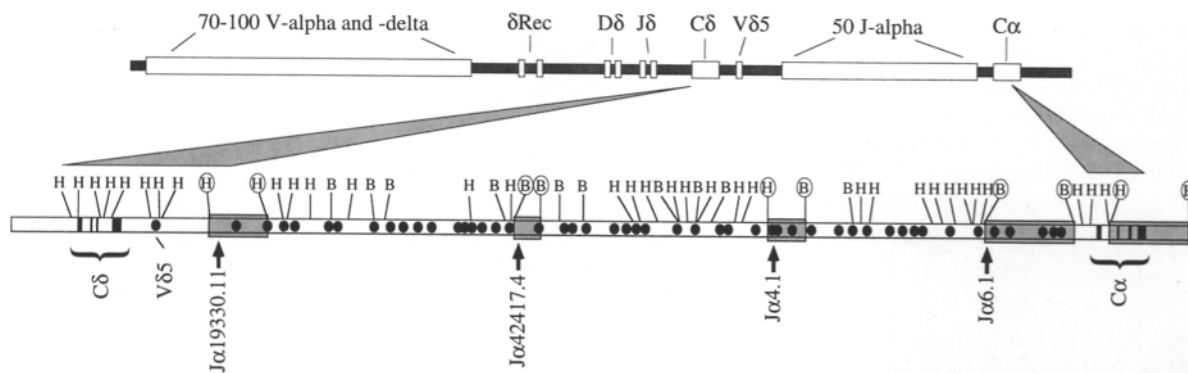
Figure 1. Strategy for simultaneous multiprobe Southern blotting of the Jα cluster. A general representation of the TCR-α locus is shown on the top strand (not to scale). The bottom strand shows an expanded view of the Jα cluster (with 5' flanking Cδ and 3' Cα regions), drawn approximately to scale. Black ovals represent the location of Jα (or in one case, Vδ) coding sequences. Black vertical bars represent the locations of Cα or Cδ introns. Restriction enzyme sites (B, BamHI; H, HindIII) are shown above the lower strand. Restriction sites flanking specific DNA fragments analyzed by Southern blotting are circled; the fragments themselves are highlighted by shaded rectangles. Approximate locations for hybridization of the Jα probes are indicated by arrows below the strand.

product sizes were as follows: Jα19330, gtcacggatgccatctctggagcag/ggaaatggcccgtggatacccagg, 520 bp; Jα42417, cagactcccaggcgtcct-cattctg/gactggccactcactgaaatgtacag, 540 bp; Jα4, gcgggatcctgggct-ctggctagg/gcggatccagtgggcatggagctgg, 700 bp; Jα6, gatcctacagcg-agcgagtagagt/gatccagtcatggcaggtagc, 430 bp. The primer sequences for Jα4 and Jα6 included artificially introduced BamHI sites; these were cloned into pBSK⁻ (Stratagene Inc., La Jolla, CA). PCR products of Jα19330 and Jα42417 were cloned directly into a T/A cloning vector (pCRII; InVitrogen, San Diego, CA). All plasmids were carried in infected *Escherichia coli*. Plasmids were purified from 200-ml *E. coli* cultures using columns (Tip-500; QIAGEN, Chatsworth, CA). Inserts were purified by digestion with the appropriate restriction enzyme, followed by agarose gel electrophoresis and extraction using Qiaquick spin columns (QIAGEN).

For TCR-β gene rearrangement analysis, a similar strategy was used (Fig. 2), as described previously (11). PCR primers for partial known sequences of the intronic regions between Dβ1 and the Jβ1 cluster (D-Jβ1) or Dβ2 and the Jβ2 cluster (D-Jβ2) were used to amplify 730- or 670-bp products, respectively. Clone D-Jβ1 included the Dβ1 coding region and 60 bp of 5' intronic DNA; clone D-Jβ2 included Dβ2 and 80 bp of upstream DNA. PCR-amplified products were ligated into pCRII, cloned, and purified as above.

Quantitative Southern Blotting. The system used for DNA digestion in agarose plugs was essentially as described (8). DNA from 2–3 × 10⁵ cells was digested using the appropriate restriction endonucleases: BamHI/HindIII for quantitation of TCR-α gene rearrangement or EcoRI for quantitation of TCR-β gene rearrangement. Digested DNA was electrophoresed in 20 × 25 cm gels

of 0.6% agarose in TBE (90 mM Tris HCl, 90 mM borate, 2 mM EDTA). Gels were depurinated in 0.25 N HCl for 17 min and soaked in 0.4 N NaOH for 30 min. DNA was then transferred onto GeneScreen Plus (DuPont-NEN) in 0.4 N NaOH using a Posi-Blotter (Stratagene Inc.). Blotted membranes were washed briefly in 2× SSC and baked for 30 min at 80°C before hybridization. For quantitation of TCR-α gene rearrangement, purified inserts from clones Jα19330, Jα42417, Jα4, and Jα6, hybridizing with 4.4-, 2.2-, 2.8-, or 6.9-kb fragments of BamHI/HindIII–digested genomic DNA, respectively, were labeled with [α-³²P]dCTP by random hexamer priming and used simultaneously as probes. Also included as a genomic probe for comparisons of lane-to-lane DNA loading was a 590-bp PstI fragment of a ~9 kb BamHI genomic clone spanning the Cα cluster of exons (10). This probe hybridizes 2,100 bp 3' of Cα exon IV and identifies a 6.1-kb fragment of BamHI/HindIII–digested genomic DNA, which is always present at genomic levels. For quantitation of TCR-β gene rearrangement, purified inserts from clones D-Jβ1 and D-Jβ2 (hybridizing with 10.5- or 2.2-kb fragments of EcoRI-digested genomic DNA, respectively) were labeled and used simultaneously as probes. The 590 bp PstI fragment hybridizing 2,100 bp 3' to Cα exon IV was again used for genomic quantitation. Hybridization was performed at 65°C in 4× SSC/1% SDS/5× Denhardt's reagent containing 100 μg of herring sperm DNA/ml. Hybridized blots were washed in decreasing concentrations of SSC/1% SDS at 68°C until background hybridization was sufficiently reduced. Blots were imaged and quantitated using a molecular imager (model GS-250; Bio-Rad Laboratories, Hercules, CA). The percentage of germline DNA
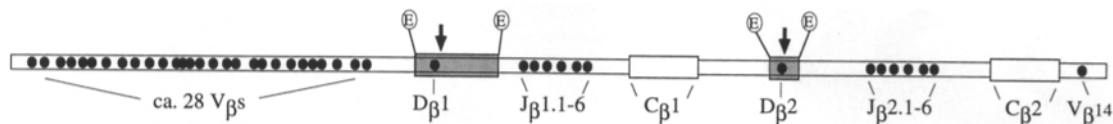

Figure 2. Strategy for simultaneous multiprobe Southern blotting of D-Jβ intronic regions. A general representation of the TCR-β locus is shown (not to scale). The location of V, D, and J coding sequences are represented by black ovals; the two Cβ clusters of exons are represented by white rectangles. The approximate locations of the two EcoRI genomic DNA fragments analyzed by Southern blotting are indicated by shaded rectangles; the locations of cloned probe sites are shown by arrows above the strand.

remaining for any given locus was calculated using the following equation: *percent of germline remaining* = {[X^t (C^g/C^t)]/X^g} × 100, where C represents Cα band intensity, X represents Jα or D-Jβ band intensity, and g and t represent germline and T cell DNA samples, respectively.

Results

TCR-α Chain Gene Rearrangement during T Cell Development. The progression of TCR-α gene rearrangement, as assessed by quantitative Southern blotting across the Jα cluster, is depicted in Fig. 3; actual values are shown in Table 1. No rearrangements are detectable before the CD25$^-$44^{lo}TD (triple dull; CD3^{lo}4^{lo}8^{lo}) stage, which correlates with the transition from TN to DP cells (7) and an in-frame TCR-β gene rearrangement (11). Immediately upon completing this transition, the first (proximal) V-J rearrangements are seen. By the next stage of development (i.e., CD3$^-$DP), substantial rearrangements (representing a deletion of 75% of germline sequences) are seen at the 5' end of the Jα cluster. Deletions of germline sequences are also seen one and two thirds of the way across the Jα cluster by this stage. However, the number of rearrangements located 3' of these probe sites is proportionately less than their distance across the locus. Together, these results demonstrate that recombination at the TCR-α locus is ordered and proximally (V-J) biased, suggesting that the distance between recombining elements may be a factor in the regulation of TCR-α gene rearrangement. This is consistent with the finding that both alleles of the TCR-α gene tend to rearrange simultaneously to approximately equivalent sites (4, 5).

Multiple Sequential Rearrangements at the TCR-α Locus. CD3/TCR^{lo}DP thymocytes represent a population of cells that possess functional rearrangements at both the TCR-α and -β loci (as is evidenced by the expression of the heterodimer on the cell surface), but have failed to undergo, or have not yet undergone, positive selection. The demonstration that noncycling cells of this phenotype expressed high levels of RAG-1 and -2 (3) suggested that such cells may be continuing to rearrange their TCR genes, possibly in an attempt to generate proteins of a new specificity. Thymocytes of this phenotype from *bcl*-2–transgenic mice also possess the capacity to survive extended periods in culture (3, 6). These characteristics allowed us to assess whether the TCR-α locus was capable of being rearranged more than once on each allele, by analyzing changes in the distribution of rearrangements in TCR-expressing immature cells; secondary rearrangements should be detectable as 3' shifts in the representation of the Jα cluster (as well as 5' shifts in Vα). Using the same quantitative Southern blotting strategy shown in Figs. 1 and 3, we found that the pattern of rearrangement after culture (i.e., the nonselecting environment) is substantially shifted toward the 3' end of the locus when compared with the same cells ex vivo (Fig. 4). Similar analyses of RAG-1$^-$ and -2$^-$ peripheral T cells did not demonstrate such a shift (not shown). These experiments directly demonstrate the widely held assumption that the TCR-α locus is capable of multiple, sequential rearrangements at each allele.

TCR-β Chain Gene Rearrangement During T Cell Development. Transition from the TN to DP stage of T cell development requires an in-frame rearrangement at the TCR-β locus (11). However, TCR-β chain gene rearrangement during T cell development has not been well characterized; in particular, the significance of duplication of the D, J, and C clusters, and their relationship to one another, is not known. Gene rearrangements between D and J segments precede those between V and D (12). Thus, it is possible to assess the initiation of TCR-β gene rearrangement by quantitative measurement of the intronic regions between D- and J-β. Using the approach depicted in Fig. 2, we have quantitated the initiation of rearrangement at each cluster of the TCR-β locus. No detectable deletions of D-J intronic regions were seen before the CD25$^+$44^{lo}TN stage of development (Fig. 5). On reaching the CD25$^+$44^{lo}TN stage, substantial deletions are seen in both D-J intronic regions. No further deletions in either D-Jβ1 or D-Jβ2 introns are detected at the next stage of development (i.e., CD25$^-$44^{lo}TD), where complete (V-DJ) and in-frame gene rearrangements are required (11). Since the D-Jβ1 intronic region should also be deleted during complete recombination of V to D-Jβ2, this suggests that not only does all D-Jβ recombination occur rapidly and wholly at the CD25$^+$44^{lo}TN stage, as has been suggested (13), but that D-J and V-DJ recombination probably occur very rapidly in succession. In addition, the D$_1$-J$_1$ intron is always present at approximately half the level of the D$_2$-J$_2$ intron. Since V-DJβ2 recombination deletes the D-Jβ1 site, this pattern suggests that cluster usage in TCR-β gene rearrange-
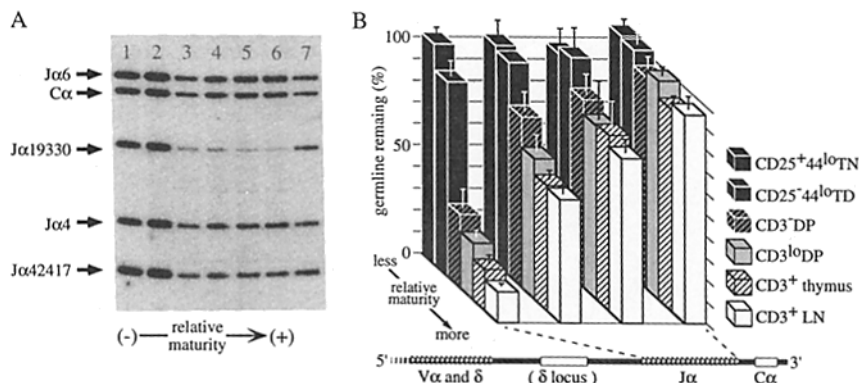

Figure 3. Quantitative assessment of TCR-α gene rearrangement among developing T cells. (*A*) A representative Southern blot showing the relative intensities and locations of the DNA fragment analyzed. DNA samples are from C57BL/6 mice as follows: (lane *1*) CD25$^+$44^{lo}TN; (lane *2*) CD25$^-$44^{lo}TD; (lane *3*) CD3$^-$DP; (lane *4*) CD3^{lo}DP; (lane *5*) CD3$^+$ thymocytes; (lane *6*) CD3$^+$ LN; (lane *7*) LN B cells (genomic control). (*B*) quantitation of Jα gene rearrangement; bars represent mean ± SE for at least three Southern blots, each containing DNA from between one and five mice per sample of sorted cells.

	CD25$^+$44^{lo}TN	CD25$^-$44^{lo}TD	CD3$^-$DP	CD3^{lo}DP	CD3$^+$ thymocytes	CD3$^+$LN
Jα19330	102 ± 5*	90 ± 7	34 ± 9	26 ± 3	19 ± 4	14 ± 1
Jα42417	101 ± 9	99 ± 2	79 ± 9	65 ± 8	58 ± 4	57 ± 8
Jα6	98 ± 8	101 ± 11	87 ± 9	80 ± 18	81 ± 13	76 ± 6
Jα4	106 ± 5	105 ± 5	98 ± 7	101 ± 4	93 ± 3	96 ± 6

* Values represent means ± SE for at least three separate Southern blots, as described in the legend to Fig. 3.

ments is nonpreferential, and that both D-J clusters on a given allele rearrange independently and simultaneously.

Temporal Relationship between TCR Gene Rearrangement and Programmed Cell Death. Successful and productive rearrangement of the TCR-β locus marks the onset of a number of important developmental functions (see Discussion), among which is the characteristic of limited lifespan, and ultimately (in the absence of rescue by positive selection) programmed cell death. Since bcl-2 gene expression is thought to be an important regulator of programmed cell death, it was logical to evaluate the pattern of endogenous bcl-2 gene expression during T cell development, particularly in relation to the known stages of TCR-α and -β gene rearrangement. The results of these studies are shown in Fig. 6. We find that bcl-2 is expressed at high levels during the stage at which TCR-β chain genes are being rearranged. Earlier stages of development, while difficult to analyze by Northern blotting, are likely to express bcl-2 as well, since they are relatively long-lived in culture (Petrie, H., unpublished observations). However, upon generation of an in-frame VDJ rearrangement at the TCR-β locus (11), and concurrent with the onset of TCR-α gene rearrangement (Fig. 3), bcl-2 is downregulated to approximately fivefold lower levels. During the subsequent DP stages of development, while TCR-α chains continue to rearrange, bcl-2 mRNA is completely undetectable, but is reexpressed upon positive selection. These findings

are generally in agreement with the related findings of others (14, 15), except that Andelejic et al. (15) did not find any bcl-2 gene expression in CD25$^-$44^{lo}TD cells by PCR using RNA as template. The reasons for these differences are not clear, but may be related to differences in cell purification techniques.

Discussion

The historical view of T cell development suggests that, either through failure to successfully rearrange antigen receptors, or though inappropriate receptor specificity, the vast majority (>97%) of T cells generated by the thymus die without maturing fully (2). Such a concept is unattractive from an evolutionary standpoint, where efficient systems confer a survival advantage and thus are preferentially
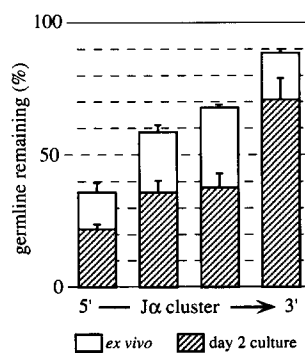

Figure 4. Secondary TCR-α gene rearrangements in T cells already possessing an in-frame rearrangement. Using the quantitative multiprobe Southern blotting procedure described in Figs. 1 and 3, and bcl-2–transgenic mice, the ability of TCR- and RAG-expressing (3) immature cells to make subsequent rearrangements at the TCR-α locus was examined. A substantial shift toward the 3' end of the Jα cluster was noted, compared with the same cells ex vivo, demonstrating that more distal V-J gene rearrangements were occurring. Bars represent mean ± SE for two individual Southern blots.
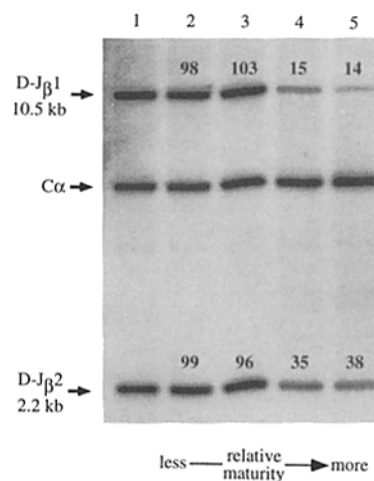

Figure 5. Quantitative assessment of TCR-β gene rearrangement during T cell development. A representative Southern blot indicating the locations of the EcoRI-digested genomic DNA fragments analyzed is shown. The pooled values for percentage of germline genes remaining in two experiments are shown above the respective bands of hybridization. DNA samples are from C57BL/6 mice as follows: (lane 1) LN B cells (genomic control); (lane 2) CD25$^-$44$^+$TN; (lane 3) CD25$^+$44$^+$TN; (lane 4) CD25$^+$44^{lo}TN; (lane 5) CD25$^-$44^{lo}TD; a complete and in-frame V-D-J recombination is required before transition to the latter stage (11). These results show that D-J recombination is both initiated and completed at the CD25$^+$44^{lo}TN stage.
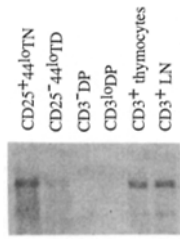
Figure 6. The relationship between TCR gene rearrangement and the onset of programmed cell death. A Northern blot for *bcl*-2 expression in RNA from progressively more mature T cells (L → R) is shown. RNA samples are from C57BL/6 mice, purified as indicated. The arrow indicates the location of the primary (7.9-kb) form of *bcl*-2 mRNA (9). *bcl*-2 expression is rapidly downregulated at the transition from out-of-frame to in-frame TCR-β gene rearrangements; expression remains depressed thoughout the time of TCR-α gene rearrangement, but is upregulated after positive selection. Similar results were obtained in at least three experiments for each sample indicated.

propagated. However, random gene recombination and the selection of antigen receptor specificities also confer biological advantages, as discussed above. Based on current knowledge, it is now possible to reconcile these apparently contradictory concepts based on the following model. Early T cell precursors migrate to the thymus, where they divide to generate a small pool of cells (∼2% of all thymocytes; 2) that undergo TCR-β gene rearrangements (Fig. 5). Since the rules for allelic exclusion at the TCR-β locus are fairly strict and conventional, ∼56% of these cells can be expected to make in-frame rearrangements at the β locus (for review see reference 4). Successful cells then undergo a series of divisions that generate the remainder of all thymocytes (2); thus, only 1% of all thymocytes are wasted because of failure to successfully rearrange TCR-β chain genes. Immediately pursuant to the generation of an in-frame TCR-β chain gene, and concurrent with most cell division, the TCR-α chain gene begins to rearrange (Fig. 3). Rearrangement at this locus is unconventional in several respects. First, both alleles rearrange simultaneously to relatively coincident loci (4, 5); allelic exclusion, therefore, operates at neither the recombination nor the gene expression levels at this locus (3, 16–18). Second, through multiple sequential rearrangements, out-of-frame configurations on one or both alleles can be replaced with new ones (Fig. 4), resulting in a substantial increase in the frequency of in-frame rearrangements. Since all cells rearranging TCR-α chain genes already possess in-frame TCR-β chain genes (reference 11; Fig. 3), this mechanism

would account for the overabundant frequency of TCR-acquisition described previously (3). Finally, an increased frequency of in-frame TCR-α gene rearrangements (and thus TCR acquisition) is not the only advantage of multiple rearrangements at this locus. Immature cells that already express TCR (and thus have in-frame rearrangements at both α and β loci) continue to rearrange TCR-α chain genes until positive selection (or programmed cell death) occurs (reference 3; Fig. 4). The newly formed TCR-α chains can then pair with the preexisting TCR-β chain to generate a heterodimer of entirely new specificity; this serves to increase the frequency of positive (and, ostensibly, negative) selection as well. Thus, the overall efficiency of T cell production is maximized by a combination of selective expansion of cells with in-frame TCR-β chain genes, together with multiple rearrangements at the TCR-α locus.

Based on the phenotypic characteristics of these cells in culture, we have previously predicted that the transition from CD25+TN to CD25-TD cells represents an important control point in T cell development (7). It is now clear that a variety of critical developmental events intersect at this point. First, this transition marks the generation of a productive TCR-β chain gene (11). Second, low-level surface expression of CD4, CD8, and CD3 (hence the TD designation) as well as TCR-β (probably in association with gp33/pTα; 19) occurs at this point (20, 21). Subsequently, these TD cells spontaneously acquire high levels of CD4 and CD8 and typical immature levels of CD3/TCR-α/β (3). Third, this transition marks the onset of an enormous burst of cell division, which accounts for the majority (∼98%) of cells in the thymus (2). Finally, initiation of TCR-α gene rearrangement (Fig. 3) and the down-regulation of *bcl*-2 gene expression (Fig. 6) both occur at this transition; in fact, an inverse correlation between TCR-α gene rearrangement and *bcl*-2 gene expression can be seen throughout T cell development. This suggests that successful TCR-β gene rearrangement may be the factor that initiates a "biological clock." Subsequently, rearrangement of TCR-α chain genes and downregulation of *bcl*-2 expression work antagonistically, the former to maximize the efficiency of selection and the latter to limit the time frame available to do so. Together, these functions would serve to maintain cell turnover and homeostasis in the thymus while maximizing thymic output.

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