Lack of N Regions in Antigen Receptor Variable Region Genes of TdT-Deficient Lymphocytes

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During the assembly of immunoglobulin and T cell receptor variable region genes from variable (V), diversity (D), and joining (J) segments, the germline-encoded repertoire is further diversified by processes that include the template-independent addition of nucleotides (N regions) at gene segment junctions. Terminal deoxynucleotidyl transferase (TdT)–deficient lymphocytes had no N regions in their variable region genes, which shows that TdT is responsible for N region addition. In addition, certain variable region genes appeared at increased frequency in TdT-deficient thymocytes, which indicates that N region addition also influences repertoire development by alleviating sequence-specific constraints imposed on the joining of particular V, D, and J segments.

Immunoglobulin (Ig) and T cell receptor (TCR) variable region genes are created by the assembly of V, D, and J segments [V(D)J recombination] in developing lymphocytes. V(D)J recombinase activity is targeted by conserved recognition sequences (RS's) that flank each germline gene segment. The reaction involves recognition of RS's, introduction of double-strand breaks at RS-coding junctions; potential loss, addition, or both of nucleotides at coding junctions; and polymerization-ligation activities to complete joining (1, 2). The initiation of the reaction is probably mediated by one or two tissue-specific components, whereas most other events are carried out by more generally expressed activities (2).

Variable region diversity is created both by combinatorial assortment of V, D, and J segments as well as by the loss or addition of nucleotides at their junctions. Additions fall into two categories: template-dependent (P nucleotides) (3, 4) and template-independent (N regions) (5). N region addition has been hypothesized to be effected by TdT (5), which can add deoxynucleotides to available 3' ends (6). In support of this notion, TdT is found in immature lymphocytes (7) and leukemic cells (8), but not in nonlymphoid cells. Likewise, the abundance of N regions in V(D)J junctions in adult as compared to fetal lymphocytes also correlates with substantial TdT expression in precursors of the former but not the latter populations (4, 9–11). N region addition to V(D)J junctions in cell lines also has been correlated with TdT expression (12–14). However, some cell lines that lacked readily detectable TdT activity were found to add N regions (13, 14), leading to speculation that N regions also may be added by other mechanisms (15).

To unequivocally evaluate the role of TdT in V(D)J recombination and repertoire development, we used gene-targeted mutation to generate chimeric mice in which all mature lymphocytes develop from precursors lacking TdT expression. We prepared a targeting vector that eliminated the TdT promoter and first exon and allowed for both positive and negative selection (16) (Fig. 1A). We generated 43 independent TdT knockout (TdT−/−) clones of the CCE line of embryonic stem (ES) cells (Fig. 1B; representative data are shown). We then selected with increased G418 (17) and obtained independent homozygous TdT knockout clones (TdT−/−) from four different TdT−/− clones (Fig. 1B; representative clones are shown).

All mature lymphocytes in chimeras formed by injection of ES cells into RAG-2–deficient blastocysts derive from the injected ES cells [RAG-2–deficient blastocyst complementation (18)]. Complementation of RAG-2–deficient blastocysts with either TdT−/− ES cells or TdT−/− ES cells generated chimeras with substantial numbers of ES cell–derived B and T cells in primary and peripheral lymphoid organs (Fig. 1B and C). However, we did not detect either

REFERENCES AND NOTES

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TdT enzymatic activity or transcripts from the mutated TdT gene (by RNA blotting analyses) in TdT-/- thymocytes (19). Therefore, inability to express TdT has no gross effect on lymphocyte differentiation.

To characterize V(D)J junctions in TdT-/- and TdT+/+ lymphocytes, we used various specific primers (20) to amplify and sequence multiple TCR β, γ, and δ chain V-D-J or V-J junctions from RNA or genomic DNA of 2.5- to 6-week-old mouse thymuses (Fig. 2) and Ig heavy chain (lgH) V-D-J junctions from genomic DNA of 2.5-week-old mouse spleens (Fig. 3). In contrast to junctions from TdT+/+ lymphocytes, those from TdT-/- lymphocytes contained virtually no N regions (Figs. 2 and 3 and Table 1). Thus, TdT is the only major activity involved in physiologically N region addition. Other activities speculated to be involved in this process, primarily based on V(D)J recombination substrate studies and illegitimate recombination studies in lymphoid and nonlymphoid cell lines (15), are not a significant source of N regions in normal developing lymphocytes. Also, P nucleotides occurred at similar frequencies in TdT+/+ and TdT-/- junctions (Table 1), confirming that P nucleotide addition is independent of N nucleotide addition and TdT activity.

Short homologies at or near coding sequence breakpoints have been proposed to mediate V(D)J joining and lead to the appearance of certain variable regions at increased frequency in fetal repertoires (5, 10, 21). The low TdT expression in developing fetal lymphocytes has been speculated to promote this phenomenon (10). However, the overall role of such homologies in the V(D)J joining process (leading to "overlapping" junctional nucleotides) has been difficult to evaluate because N regions may have provided occult homology in junctions where none was evident (for example, in most junctions of adult repertoires). In this context, 45% (286/533) of TdT-/- junctions (V-D, D-D, D-J, or V-J) had homologies of two or more nucleotides as compared with 18% (56/306) of TdT+/+ junctions (Figs. 2 and 3 and Table 1) (19). Therefore, the occurrence of homology-mediated joins is enhanced in TdT-/- adult lymphocytes—confirming the proposed role of TdT expression on the relative occurrence of such joins in fetal as compared to adult repertoires. However, over 25% of TdT-/- junctions lacked even one base pair of overlap (Table 1), indicating the existence of a V(D)J joining pathway that is homology-independent.

The γδ T cells of epidermis mostly have a single in-frame VγJ1,1 join that also is the predominant ("canonical") VγJ1,1 join in fetal thymocytes; these repertoires also have two predominant out-of-frame VγJ1,1 joins as well (4, 22–24). The frequency of these joins in particular repertoires was suggested to result from cellular selection (25). However, experiments with transgenic TCRγ recombination substrates (23) and TCRδ-/- mice (24), coupled with observations on fetal Ig and TCR repertoires (9–11), indicated that canonical joins are favored as a result of biased joining by the recombination machinery in fetal thymic progenitors (26).

In TdT-/- thymuses, 30% of fetal VγJ1,1 joins were canonical, and 42% of 65 out-of-frame joins were one of the two joins that predominate in fetal thymocytes (Fig. 2). Taken together, these three junctions represented 70% of all VγJ1,1 junctions in TdT-/- thymocytes, a number comparable to that observed in fetal repertoires (4, 22). In contrast these same three junctions represented only 32% of the VγJ1,1 joins in the TdT+/- thymocytes, in agreement with the percentage previously observed in adult thymocytes (4, 11). Therefore, both productive and nonproductive VγJ1,1 predominant joins occurred at increased frequency in TdT-/- thymocytes. Our data strongly support the biased recombination model and indicate that lack of TdT, as opposed to either some other property of the V(D)J recombine or to cellular selection, promotes appearance of predominant junctions in fetal thymocytes. Homology-mediated joining has been proposed to promote the frequent occurrence of particular junctions (10, 26); canonical VγJ1,1 joins may be promoted by two short overlaps (AT, TAG) that appear in the majority of VγJ1,1 junctions (26) (Fig. 2). If so, the lack of use of other nearby potential overlaps (TC, CT, TA) in TdT-/- thymocytes (Fig. 2) suggests that other factors, such as
Fig. 2. Lack of N regions in V(DJ) junctions from TdT<sup>-/-</sup> T lymphocytes. Polymerase chain reaction (PCR)-amplified products (20) from thymic genomic DNA of two 4-week-old mice (a and b), two 6-week-old mice (d and e) and three 2.5-week-old mice (c, f, and g) are shown. Two independent PCR amplification reactions were analyzed from one 2.5-week-old chimera (g1 and g2). The frequency of each junction is listed to the left of the sequence. Overlapping nucleotides that could be encoded by either germline segment (including P nucleotides) are underlined. The reading frame [(+) or (−)] and canonical in-frame and predominant out-of-frame sequences (4, 22–24) are indicated by an asterisk.

Fig. 3. Lack of N regions in V(DJ) junctions from TdT<sup>-/-</sup> B cells. PCR products of genomic DNA from spleens of 2.5-week-old mice (two independent spleens each) are shown. Nucleotide sequences are aligned with the germline sequence (22, 30, 31). Overlapping nucleotides and reading frame are as in Fig. 2.
sequence context, must contribute to this process. Thus, TDML is a tissue-specific component of V(D)J recombinase. This enzyme is not required for the reaction, but if expressed it qualitatively modifies the outcome by adding N regions to V(D)J junctions. The presence or absence of TDML expression during variable region gene assembly can affect the resulting variable region repertoire in at least two ways. First, N region addition generates a substantial amount of diversity in the portion of antigen receptor variable regions that have a major role in antigen recognition and TCR-major histocompatibility complex protein interactions (27). In addition, absence of TDML leads to the appearance of particular variable region genes at increased frequency, probably because N region addition reduces the probability of the homology-mediated joining of certain variable region gene segments. In the accompanying report, another group has reached nearly identical conclusions based on the analysis of a germline TDML mutation in which a different TDML exon was targeted (28). Finally, we note that TDML−/− chimeras as old as 6 months have no marked abnormalities. However, more detailed analyses may reveal important immunological consequences of the limited variable region repertoire in TDML−/− mice.

REFERENCES AND NOTES
20. Complementary DNA was made with the use of Cc primer and cDNA, and DNA was amplified at the total volume of 100 μl by Vent polymerase (New England Biolabs). Nestled primers were as follows: 5′-AAGTGAAGGCATCTGCATCGCTCT-3′ and 5′-GCAACTCTGTTGGTATGGA-3′; Vβ8, 5′-ATGGACCGATCGCGCGAGAG-3′ and 5′-CTCCTGGAGCTCTGTGAG-3′; Vβ4, 5′-ACCTTGAGACCAGTGGGCAG-3′ and 5′-AAATCAAGGCTTTACCTAC-3′; Vγ3, 5′-CT-
Mice Lacking TdT: Mature Animals with an Immature Lymphocyte Repertoire

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In adult animals, template-independent (or N) nucleotides are frequently added during the rearrangement of variable (V), diversity (D), and joining (J) segments of lymphocyte receptor genes, greatly enhancing junctional diversity. Receptor genes from adult mice carrying a mutation in the terminal deoxynucleotidyl transferase (TdT) gene have few N nucleotides, providing proof that this enzyme is essential for creating diversity. Unlike those from normal adults, receptor genes from adult mutant mice show extensive evidence of homology-directed recombination, suggesting that TdT blocks this process. Thus, switch on of the TdT gene during the first week after birth provokes an even greater expansion of lymphocyte receptor diversity than had previously been thought.

The repertoire of B and T cell antigen receptors expressed in adult animals is more diverse than that in perinates (1). One major difference is the amount of N region diversity at the junctions of rearranged immunoglobulin (Ig) and T cell receptor (TCR) gene segments. N nucleotides are rare in V(D)J junctions from fetal or newborn animals, but constitute a major component of the diversity of IgS and TCRs from adults (2-7). This dissimilarity may be due to differential expression of TdT. Terminal deoxynucleotidyl transferase catalyzes template-independent addition of nucleotides in vitro (8), and the amount expressed in vivo correlates with the degree of N region diversity in antigen receptors (9, 10). Another difference between adult and perinatal repertoires lies in the diversity of V-J, V-D, and D-J junctional sequences. Examination of large sets of fetal and newborn Ig and γδ TCR sequences revealed overrepresentation of some junctions, coincident with short stretches of homology between abutted gene segments (3-5, 11-13). In the case of γδ TCRs, certain dominant junctions (termed "canonical") are functionally significant because they give rise to the quasi-monoclonal receptors in specific anatomical locations such as the skin. Overrepresented joints were not generally observed in adult sequences, only in some of those lacking N nucleotides. Initially, the presence of dominant junctions of γδ TCRs was attributed to cellular selection (5, 14), but a preference for rearranging at short stretches of homology is more probable (15, 16). Why such homology-directed recombination is pronounced in perinates but rare in adults is an open question.

One approach to better understanding the adult-perinate dichotomy is to artificially produce mature animals with repertoire having immature features. Thus, we generated, through homologous recombination in embryonic stem cells, a strain of mice lacking TdT (17). The mutation of TdT we obtained was an insertion of the neomycin gene into exon 4, as illustrated in Fig. 1 and confirmed by extensive Southern (DNA) blot analysis. Given the predicted location of the TdT active site and its presumed globular nature (18), exons 4 to 7 are probably critical for TdT function. No mRNA corresponding to regions 3' of the neomycin insert was detected in thymus RNA from homozygous mutant mice after polymerase chain reaction (PCR) amplification, for which we used a primer pair on the 3' side of the insertion; nor was any revealed by in situ hybridization of the appropriate probe to thymic sections (19). Abrogation of protein expression was confirmed by staining of thymocytes with a polyclonal antiserum to TdT (19).

Homozygous mutant TdT−/− mice breed well and appear healthy in a conventional animal facility, are of normal size, and do not have increased susceptibility to infection, as is common for immunodeficient animals in our colony. The mutants show no marked abnormalities in the major T or B cell compartments and are capable of mounting T and B cell responses to complex antigens like keyhole limpet hemocyanin and ovalbumin (20).

To evaluate the effect of a TdT deficiency on the lymphocyte repertoires of adult mice, we sequenced the V(D)J junctions of more than 300 rearranged Ig and TCR genes from adult animals (most from 6 to 8 weeks of age) (21). Representative sets of VJ3 DNA sequences from total thymocytes (Fig. 2), Vj8183 DNA sequences from splenocytes (Fig. 3), and Vj8 RNA sequences from CD4+CD8+CD3ε thymocytes (Fig. 3) are shown. The enzyme TdT was responsible for the bulk of N region diversity because