Assembly of Productive T Cell Receptor $\delta$ Variable Region Genes Exhibits Allelic Inclusion

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Summary

The generation of a productive “in-frame” T cell receptor $\beta$ (TCR $\beta$), immunoglobulin (Ig) heavy (H) or Ig light (L) chain variable region gene can result in the cessation of rearrangement of the alternate allele, a process referred to as allelic exclusion. This process ensures that most $\alpha\beta$ T cells express a single TCR $\beta$ chain and most B cells express single IgH and IgL chains. Assembly of TCR $\alpha$ and TCR $\gamma$ chain variable region genes exhibit allelic inclusion and $\alpha\beta$ and $\gamma\delta$ T cells can express two TCR $\alpha$ or TCR $\gamma$ chains, respectively. However, it was not known whether assembly of TCR $\delta$ variable region genes is regulated in the context of allelic exclusion. To address this issue, we have analyzed TCR $\delta$ rearrangements in a panel of mouse splenic $\gamma\delta$ T cell hybridomas. We find that, similar to TCR $\alpha$ and $\gamma$ variable region genes, assembly of TCR $\delta$ variable region genes exhibits properties of allelic inclusion. These findings are discussed in the context of $\gamma\delta$ T cell development and regulation of rearrangement of TCR $\delta$ genes.

Key words: T cells • $\gamma\delta$ T cells • T cell receptor $\delta$

Lymphocyte antigen receptor variable region genes are assembled during development from component variable (V), diversity (D), and joining (J) gene segments in the case of the TCR $\beta$ and $\delta$ chain genes and the Ig heavy (H) chain gene or from V and J gene segments in the case of TCR $\alpha$ and $\gamma$ chain genes and Ig light (L) chain genes (1, 2). Productive rearrangement of TCR $\beta$ or IgH chain variable region genes results in cessation of further V to DJ rearrangements on the alternate allele, a process referred to as allelic exclusion (2, 3). “Functional” rearrangement of IgL $\kappa$ or $\lambda$ L chain genes (i.e., rearrangements which generate an IgL chain that can pair with a pre-existing IgH chain) also lead to cessation of further IgL chain rearrangements resulting in both allelic and IgL chain isotype exclusion (4). In contrast, TCR $\alpha$ and TCR $\gamma$ chain variable region gene assembly does not exhibit properties of allelic exclusion (3, 5–7). Consequently $\alpha\beta$ and $\gamma\delta$ T cells can express two TCR $\alpha$ or $\gamma$ chains, respectively (6, 7).

Several models have been proposed to account for allelic exclusion. One model proposed that the probability of a productive rearrangement is low making it unlikely that an individual cell could have two productive rearrangements (8). However, it is now known that the probability of a productive rearrangement can be as high as 33% (9). Another model proposed that the probability of two complete V(D)J rearrangements in any one cell was low. However, a significant percentage of peripheral B and T cells have two IgH or TCR $\beta$ V(D)J rearrangements, respectively, arguing against this model (3, 10). It has been proposed that IgH chain allelic exclusion occurs due to a toxic effect of expressing two IgH chains (11). However, the recent demonstration that B cell development proceeds normally in mice that express two IgH transgenes essentially rules out this model (12). An early model, based on analyses of rearrangement patterns in cell lines, proposed that allelic exclusion is regulated and that expression of a productively rearranged IgH or IgL chain prevents further rearrangements at the IgH and IgL chain loci, respectively (4, 13, 14). This regulated model was supported by studies demonstrating that expression of IgH or IgL transgenes resulted in a block in endogenous IgH or IgL chain gene rearrangement, respectively (15–18). Studies of TCR $\beta$ transgenic mice have supported an analogous model by which the TCR $\beta$ transgene feeds...
back to block endogenous TCR β rearrangements (19). In addition, it has recently been demonstrated that expression of IgH or TCR β chains as pre-B or pre-T cell receptors, respectively, is required for allelic exclusion (20–23).

T cells can be divided into two distinct lineages based on expression of either αβ or γδ TCRs. The genes that encode the TCR β and TCR γ chains lie in distinct loci, whereas the genes that encode the TCR δ and TCR α chains lie in a single locus (TCR α/δ locus, Fig. 1; references 24, 25). In the adult thymus TCR β rearrangements are initiated at the CD4⁺/CD8⁺ (double negative, DN) stage of thymocyte development and are ordered with DJβ 1 to DJβ rearrangements occurring on both alleles before Vβ to DJβ rearrangement (3, 26, 27). Once a productive V(D)Jβ rearrangement is made and a TCR β chain expressed, cells proceed to the CD4⁻/CD8⁻ (double positive, DP) stage of development and further Vβ to DJβ rearrangements cease (3, 26, 27). As a result, many αβ T cells have DJβ rearrangements on a single allele (28). Vα to Jα rearrangements are initiated at the DP stage. However, unlike the TCR β locus, expression of a TCR α chain does not result in cessation of Vα to Jα rearrangements (3, 26, 27). This process continues on both alleles, and Vα to Jα rearrangements can result in the deletion of previously assembled productive Vβ to DJβ rearrangements (29). It has been proposed that the downregulation of recombining activating gene (RAG) gene expression may ultimately be responsible for termination of Vα to Jα rearrangement (30).

Several notable differences exist between the developmental regulation of assembly of αβ and γδ TCR variable region genes. Assembly of TCR γ and TCR δ variable region genes occurs at the DN stage of thymocyte development (31). It is not known whether rearrangement of these genes is concurrent or sequential. In addition, assembly of TCR γ genes does not appear to exhibit allelic exclusion (7). Similar to the TCR β locus, assembly of TCR δ variable region genes does not proceed to completion on all alleles. However, unlike the TCR β locus, TCR δ variable region genes do not appear to be ordered, since incomplete DDδ, DJδ and VDδ rearrangements have been described (32, 33). It is unresolved whether productive TCR δ rearrangements lead to termination of further TCR δ rearrangements (allelic exclusion) or whether TCR δ rearrangements are limited by factors independent of the formation of productive rearrangements. To address this issue, we have analyzed TCR δ rearrangements in a panel of T cell hybridomas derived from splenic γδ T cells. We find the percentage of cells with two in-frame V(D)Jδ rearrangements is similar to that predicted in the absence of allelic exclusion. These findings are discussed in the context of γδ T cell development.

**Materials and Methods**

Isolation of γδ T Cells and Production of γδ T Cell Hybridomas. Whole spleen cell suspensions from C57BL/6 × CBA mice were incubated in DMEM-15 containing 40 U recombinant human IL-2/ml (PharMingen, San Diego, CA) on plates that had been coated with 10 μg/ml rat anti–hamster Ig (PharMingen) followed by 10 μg/ml of an anti-TCR δ chain mAb (GL4; PharMingen). Cultures were maintained for 6 d and the resulting cells were >90% pure γδ T cells as determined by flow cytometry (data not shown). Hybridomas were produced by fusing these γδ T cells to the thymoma BW-1100.129.237 using a fusion protocol that has been described elsewhere (34, 35).

Flow Cytometry. Single cell suspensions were prepared from thymus, spleen and lymph nodes as previously described (36). Hybridomas were stained with FITC-conjugated anti-TCR β chain (H57-597) and PE-conjugated anti-TCR-δ chain (GL3) monoclonal antibodies from PharMingen and were analyzed by a FACScan® (Becton Dickinson & Co., Sparks, MD).

Germinal DNA Analysis. Genomic DNA was isolated and Southern blotting carried out as previously described using Zetaprobe membranes (Bio-Rad Laboratories, Hercules, CA) and probes generated by random hexamer priming (Boehringer Mannheim Corp., Indianapolis, IN) using α-[32P]dCTP (34, 35). Probe 1 is a 600-bp HindIII fragment (39). Probe 3 is a 550-bp MspI to Ndel fragment and probe 4 a 1-kb Ndel-Xbal fragment from pTA-7 (40). Probe 5 is a 350-bp PCR product generated as described elsewhere (41). Probe 6 is a 1.5-kb EcoRI C δ cDNA fragment (42).

PCR and Sequence Analysis. PCR reactions were carried out using 200 ng of genomic DNA isolated from hybridomas and 2.5 U AmpliTaq polymerase (Perkin-Elmer Corp., Norwalk, CT). PCR conditions were: 92°C for 1 min 30 s, 62°C for 2 min 30 s, 72°C for 1 min 30 s cycled 30 times. PCR products were subcloned into pT7blue (Novagen Inc., Madison, WI) before sequencing on an ABI Prism 377 DNA sequencer (Perkin-Elmer Corp.). The Vδ nomenclature of Arden et al. (43) is used. The Vδ and Jδ primers used to PCR VDJδ joins were as follows: Vδ primers: ADV7 (Vδ6/Vδ6), TACACCTGACTTCTCATAT; ADV1155, TATTTTACGACCCACATGAGG; ADV1752 (Vδ9), ATGCTGATTTCAAGCCTGCT; DV258 (Vδ8), AGCAGGT-GAGACAAAGTCC; DV458, AGCATAGTGGCAGACATC- TCA; DV652 (Vδ3), ATGGAGATGTGAGTGGAA; V1057 (Vδ7), TGAAGAGGCTGCTGTGCTC; V1051(S1), AT- GCTTTTGGAGATGTCAGCT; V1025(S2), ATGAGGAT- TCCTTCAAGT; V1045(S1), CAGGTGGCAGACATAACTGA-CAA; V10551(Vδ5), ATGATTGGTGGCCGCAGC; Jδ primers: Jδ1, AGAGTCCAAAGATCCTCAG; Jδ2, CTTC-TGTGACTCTTTTTC.

Southern blotting of PCR products was carried out with internal oligos to Jδ1 (CGACAAACTCGTCTTTGG) or Jδ2 (CTCTGGGACACCCGACAGA).

Theoretical Determination of In-Frame Rearrangement Percentages. All mature γδ T cells must have at least one productive VDJδ rearrangement. If the probability that a VDJδ rearrangement will be in-frame equals P then the probability that a VDJδ rearrangement will be out of frame will be (1 – P). If there is an equal chance of a rearrangement in each of the three reading frames then P = 1/3. In the absence of allelic exclusion and in cells with two VDJδ rearrangements, the percentage of cells with two in-frame TCR δ rearrangements will be equal to the probability that the cell will have two in-frame rearrangements divided by the probability that the cell will have at least one in-frame rearrangement.

\[
p^2 \left( \frac{1}{3} \right)^2 = \frac{1}{3}
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Results

Generation of γδ T Cell Hybridomas. Splenic γδ T cell hybridomas were generated from C57BL6 × CBA F1 mice by stimulating unfraccionated spleen cells with plate-bound anti-TCR δ antibody (GL3) as described in Materials and Methods section. The resulting cell population was >90% pure γδ T cells as determined by flow cytometry (data not shown). These cells were fused to the BW-1100.129.237 (BW) thymoma which is incapable of producing TCR δ, β, or α chains (35). T cell hybridomas generated by fusion of a γδ T cell to BW were identified by flow cytometric analysis of cell surface TCR δ expression (data not shown). Only those hybridomas that expressed TCR δ were chosen for further analysis.

To ensure that both TCR δ alleles were present in the resulting panel of γδ T cell hybridomas genomic DNA isolated from these hybridomas was assayed by Southern blot analyses using TCR δ restriction fragment length polymorphisms that exist between C57BL6 and CBA mice (44). Genomic DNA isolated from γδ T cell hybridomas was digested with HindIII and subjected to Southern blot analysis (Fig. 1). The 2-kb marker is shown. T cell hybridomas that satisfied the above criteria (Fig. 1) was digested with HindIII and subjected to Southern blot analysis using probe 6 (Fig. 1). The 2-kb marker is shown.

TCR δ Allele Configurations in γδ T Cell Hybridomas. To determine whether the γδ T cell hybridomas chosen for analysis were clonal, genomic DNA was subjected to Southern blot analysis using probe 4 to detect rearrangements to Jδ1 gene segment. F1D.58 exhibited single nongermline bands with probes 4 and 5, demonstrating that it had undergone rearrangements to Jδ1 and Jδ2 (Fig. 3 b). All

Figure 1. Schematic of the mouse TCR α/δ locus. Shown are the Vα/Vδ gene segments, the Dδ and Jδ gene segments, the TCR δ enhancer (Eδ), the TCR δ constant region gene (Cδ), and the Dβ5 gene segment. This is followed by the Jα gene segments, the TCR α constant region gene (Cα), and the TCR α enhancer (Eα). Also shown are probes 1 through 6 and the approximate position of the BglII (B2) site. The schematic is not drawn to scale.

Figure 2. Analysis for the presence of the CBA and C57BL6 TCR δ alleles. Genomic DNA from the hybridoma fusion partner BW 1100.129.237 (BW), CBA kidney (CBA), C57BL6 kidney (B6), or γδ T cell hybridomas (F1D) was digested with HindIII and subjected to Southern blot analysis using probe 6 (Fig. 1). The 2-kb marker is shown.

Figure 3. Analysis of rearrangements to Jδ1 and Jδ2. Genomic DNA samples described in the legend to Fig. 1 were digested with either BglII (a) or HindIII (b) and subjected to Southern blot analysis using probes 1, 3, and 4 (a) or probe 5 (b). Shown are the 9-, 6- and 4-kb molecular mass markers.
other hybridomas exhibited germline bands from the C57BL/6 and CBA TCR δ alleles using probe 5, showing that there is minimal rearrangement to the Jδ2 gene segment in splenic γδ T cell hybridomas analyzed here (Fig. 3b, data not shown).

To assay for incomplete TCR δ rearrangements, BglII-digested hybridoma genomic DNA was probed with probes 1 and 3 (Figs. 1 and 3a, data not shown). Probe 1 hybridizing bands of similar size to probe 4 hybridizing bands would be generated by alleles that have undergone Dδ1 to Dδ2 or Dδ1 to Jδ1 rearrangements. Hybridomas F1D.17, 23, 32, and 61 all have 9-kb BglII probe 1 hybridizing band generated by the same rearrangement. Hybridoma DV105S1 to Dγ1 rearrangement and, therefore, a non-germline probe 1 hybridizing band would likely be of a different size than the probe 4 hybridizing band that hybridizes to probe 3 and was found to have a Vδ to Dδ2 rearrangement by PCR analysis (Fig. 3a, Table 1).

These Southern blot analyses revealed that, of the 27 γδ T cell hybridomas analyzed, all had complete V(D)J δ rearrangements on one allele (Fig. 3a, a and b, Tables 1 and 2). On the other allele, 19 hybridomas also had complete V(D)J δ rearrangements, one had a Dδ1Dδ2 rearrangement, one had a VDδ2 rearrangement and 6 had Dδ1Jδ1 rearrangements (Fig. 3a, Table 1). In addition, the Jδ2 gene segment was used in only one rearrangement (Fig. 3b, Table 1).

Analysis of V(D)J δ rearrangements in γδ T cell hybridomas using primers that should recognize the members of the 11 known mouse Vδ gene families in conjunction with primers that were just downstream of Jδ1 or Jδ2, PCR analysis (Table 2). Finally, Vδ to Dδ rearrangements by Vδ gene segments other than DV105S1 will result in loss of probe 1 hybridizing bands and generation of a non-germline probe 3 hybridizing band that should be similar in size to the band generated by probe 4 when probing BglII-digested DNA. In this regard F1D.68 has a 3.5-kb BglII band that hybridizes to probes 3 and 4 and was found to have a Vδ to Dδ2 rearrangement by PCR analysis (Fig. 3a, Table 1).

### Table 2. γδ T Cδ H hybridomas with two complete VDJ δ rearrangements

| Hybridoma | V(D)J δ rearrangement | Incomplete rearrangement |
|-----------|----------------------|--------------------------|
| F1D.9     | DV105S1-Jδ1          | D(D)Jδ1                  |
| F1D.17    | ADV75S-Jδ1           | Y                         |
| F1D.23    | ADV75S-Jδ1           | Y                         |
| F1D.29    | ADV75S-Jδ1           | Y                         |
| F1D.32    | DV7S-Jδ1             | Y                         |
| F1D.33    | ADV75S-Jδ1           | Y                         |
| F1D.36    | DV104S1-Jδ1          | Y                         |
| F1D.42    | DV7S-Jδ1             | Y                         |
| F1D.49    | DV7S-Jδ1             | Y                         |
| F1D.57    | DV7S-Jδ1             | Y                         |
| F1D.60    | ADV75S-Jδ1           | Y                         |
| F1D.69    | DV105S1-Jδ1          | Y                         |
| F1D.64    | N                    | Y                         |
| F1D.67    | YDV7S-Jδ1            | N                         |
| F1D.73    | YDV7S-Jδ1            | N                         |
| F1D.75    | YDV7S-Jδ1            | N                         |
| F1D.83    | YDV7S-Jδ1            | N                         |
| F1D.89    | YDV7S-Jδ1            | N                         |
| F1D.91    | YDV7S-Jδ1            | N                         |

F1D.32 has a rearrangement utilizing a DV7S Vδ gene segment that differs from other known family members by at least five nucleotides (data not shown). This Vδ gene segment may represent a novel DV7S family member or is due to strain differences. The two DV104S1 rearrangements in F1D.73 are distinct as determined by differences in junctional diversity (data not shown). The in-frame TCR δ rearrangement in F1D.64 is presumed as the cell expresses a TCR δ chain.
discussion

to determine if assembly of tcr γ variable region genes is regulated in the context of allelic exclusion, we have analyzed a panel of 27 clonal hybridomas derived from mouse splenic γδ t cells. of the 17 hybridomas with defined v(d)jδ rearrangements on both alleles, 6 (35%) have two in-frame rearrangements. this demonstrates that tcr γ variable region gene assembly does not exhibit allelic exclusion. although this percentage is higher than the 20% (see materials and methods for calculations), which would be expected in the absence of allelic exclusion, this difference is not statistically significant (p > 0.10). two human γδ t cell clones with in-frame tcr γ rearrangements on both alleles have been described previously (33, 45). however, given the number of cells analyzed in these studies, it was not possible to determine whether these clones represented rare events or a general lack of tcr γ allelic exclusion. as tcr γ rearrangements do not exhibit allelic exclusion, failure of tcr γ allelic exclusion further increases the possibility that a single γδ t cell will express two or more distinct γδ tcrs (7).

it is possible that one of the tcr δ rearrangements in each of the six cells with two in-frame rearrangements encodes for a tcr δ chain that cannot be expressed on the surface of the cell and therefore would not signal a block of further tcr δ rearrangements. this may occur, for example, if the tcr δ chain were not able to pair with a tcr γ chain or a component of a γδ pre-tcr, if such a receptor exists. in this regard, it has recently been shown that 2-4% of peripheral b cells have two in-frame igh rearrangements but that only one encodes for an igh chain that is capable of forming a pre-b cell receptor (22). our data is more consistent with the notion that assembly of tcr δ variable region genes exhibits properties of allelic inclusion as the percentage of γδ t cell hybridomas with two in-frame tcr δ rearrangements is in agreement with the percentage expected in the absence of allelic exclusion. furthermore, this percentage is similar to that of αβ t cells with two in-frame rearrangements at the tcr α locus, which also exhibits allelic inclusion (3).

it has been proposed for the igh locus (and by analogy for the tcr β locus) that the precise ordering of variable gene segment rearrangement during lymphocyte development may be important for effecting allelic exclusion (14). in both of these loci, d to j rearrangement occurs on both alleles before v to dj rearrangement. presumably v to dj rearrangement proceeds initially on one allele, at which point the rearrangement is "tested." if it encodes a protein that can be expressed, signals are generated that prevent further v to dj rearrangements on the other allele. in accordance with this model, the expected number of b and t cells have v(d)j/dj configured rearrangements of their igh and tcr β alleles, respectively (3, 10).

unlike the igh and tcr β loci, assembly of tcr δ variable gene segments is not ordered during development, and we now show that the tcr δ locus is not regulated in the context of allelic exclusion. however, the finding that many γδ t cells have incomplete tcr δ rearrangements demonstrates that rearrangement is frequently terminated before completion. the events that lead to termination of tcr δ rearrangement are not known. thymic γδ t cells do not express rag-1 or rag-2, and it is possible, as proposed for tcr α rearrangement, that down regulation of rag expression leads to termination of tcr δ rearrangement (30, 46). termination of tcr δ rearrangement, by whatever mechanism, may be part of a developmental program that is independent of tcr δ expression. alternatively, rearrangement may cease upon tcr δ expression, and failure of allelic exclusion may be due to the unordered simultaneous rearrangement of tcr δ alleles.
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