Brief Definitive Report

Normal T Lymphocytes Can Express Two Different T Cell Receptor β Chains: Implications for the Mechanism of Allelic Exclusion

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Summary

We have examined the extent of allelic exclusion at the T cell receptor (TCR) β locus using monoclonal antibodies specific for Vβ products. A small proportion (~1%) of human peripheral blood T cells express two Vβ as determined by flow cytometric analysis, isolation of representative clones, and sequencing of the corresponding Vβ chains. Dual β T cells are present in both the CD45R0$^+$ and CD45R0$^-$ subset. These results indicate that dual β expression is compatible with both central and peripheral selection. They also suggest that the substantial degree of TCRβ allelic exclusion is dependent only on asynchronous rearrangements at the β locus, whereas the role of the pre-TCR is limited to signaling the presence of at least one functional β protein.

DNA rearrangements mediating assembly of β and α chain genes of the TCR are developmentally ordered (1–3). T cells developing along the α/β lineage rearrange the β locus first, and, when a functional β chain gene is generated, express its product on the cell surface in association with an invariant component constituting a pre-TCR (4). Signaling via this pre-TCR requires the tyrosine kinase lck (5) and results in: (*a*) arrest of rearrangements at the β locus, a process referred to as allelic exclusion; (*b*) thymocyte expansion and maturation to the double positive stage; and (*c*) initiation of rearrangements at the α locus (6–8). There are no reasons, a priori, to think that this mechanism may prevent maturation of T cells in which both β alleles may have productively rearranged. Therefore T cells with two β chains should be present at low frequency in the T cell repertoire, unless specific mechanisms exist to prevent simultaneous β rearrangement or to abort cells with two β-chains.

So far, all T cell clones analyzed express only one in-frame rearranged β, whereas the other chromosome carries either a partial DJ or an out-of-frame rearrangement (2). The only exception reported is that of a tetraploid clone that carries two productively rearranged Vβ (9). These findings supported the notion that a complete allelic exclusion at the β locus is achieved at the genotypic level.

We have previously documented the existence of rare human T cell clones with two in-frame β chain mRNAs expressed at apparently different levels (10 and Acuto, O., unpublished observations). Trivial explanations such as cell contamination were excluded, as only one functional α chain mRNA could be identified, suggesting that dual β expressing T cells may indeed exist.

Using mAbs that recognise Vβ gene products we have examined the surface expression of TCR-β in peripheral blood T cells. Dual β expressing cells were found at a low but significant frequency (~1%). Isolation of T cell clones and sequencing of their Vβ gene segments confirmed that both Vβ gene products are part of two functional TCRs.

The implications of these exceptions for the mechanism of allelic exclusion are discussed.

Materials and Methods

Antibodies and FACS Staining. The following mouse mAbs to human Vβ were a generous gift of Dr. A. Necker (Immunotech, Marseille, France): E2.2E7.2 (anti-Vβ2, IgM), LE89 (anti-Vβ3, IgG2a), 36213 (anti-Vβ5.2, IgG1), 3D11 (anti-Vβ5.3, IgG1), 56C5.2 (anti-Vβ8, IgG2a), JU74 (anti-Vβ13.3, IgG1), E17.5F3 (anti-Vβ17, IgG1), 417.5F3 (anti-Vβ19, IgG1), and IMMU546 (anti-Vβ22, IgG1). mAb C21 (anti-Vβ11, IgG2a) was produced in our laboratory (11). As second antibodies we used PE-labeled goat anti–mouse IgM and FITC-labeled goat anti–mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL). In three-color staining experiments, biotin-labeled WT31 (anti–human CD3, IgG1; Becton Dickinson & Co., Mountain View, CA) followed by streptavidin-allophycocyanin (SAV-APC; SBA) was used to identify T cells. In some experiments PBMC were analyzed by four-color immunofluorescence using anti-Vβ2 revealed by PE-labeled goat anti–mouse IgM, Vβ19 revealed by biotin-labeled goat anti–mouse IgG1 (SBA) followed by APC-SAV, CD3 (clone BMA031, mIgG2b FITC-conjugated; SBA) and CD45R0 (clone UCHL1, IgG2a, American Type Culture Collection, Rockville, MD) revealed by Texas red (TXRD)–conjugated goat anti–mouse IgG2a (SBA). Stained cells were analyzed by flow cytometry on a FACStar Plus® (Becton Dickinson & Co.) using the LYSYS II software. Propidium Iodide (PI) was added to gate out dead cells.

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Isolation of T Cell Clones. In sorting experiments dual Vβ cells were separated and expanded in polyclonal cell lines using 1 μg/ml PHA (Wellcome, Beckenham, UK) and allogeneic 3,000 rad irradiated mononuclear cells and subsequently cloned by limiting dilution (12). Briefly, sorted cells were cloned at 0.3 cells per well in Terasaki plates in the presence of irradiated PBMC (0.5 × 10^6/ml) in medium RPMI-5% human serum (Swiss Red Cross, Bern, Switzerland) containing 1 μg/ml PHA and IL-2 400 U/ml. T cells clones were expanded in medium supplemented with human serum and IL-2.

RT-PCR and Direct Sequencing of Vβ Chains. Total RNA was extracted from 2 × 10^6 T cells as described (13) and reverse transcribed using oligo d(T) as primer and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) in 30-μl final volume. cDNA (1.5 μl) was PCR amplified in a Perkin-Elmer 9600 Thermocycler (Cetus, Emeryville, CA) using the following oligonucleotides: Vβ2 5'-TCATCAACCATGCAAGCCTGACCT; Vβ3 5'-GTCTCTAGAGAGAAGAAGGACCGC; Vβ5 5'-TTCCCTAACTATAGCTCTGAGCTG; Vβ8 5'-ATTTACTTTAACAAGGTTCCG in association with a primer complementary to the constant region Cβ1 5'-TGCTGACCCCACTGTCGACCTCCTTCCCATT. The PCR profile was the following: 2 min at 94°C followed by 30 cycles at 94°C for 15 s, 60°C for 20 s, 72°C for 35 s, followed by a final 10-min extension. The amplification products were electrophoresed through a 0.7% NuSieve (FMC BioProducts, Rockland, ME). The bands were excised from the gel, melted at 65°C, and directly sequenced by the cycle sequencing method (Sequitherm; Epicenter Technol., Madison, WI) according to the manufacturer's instructions with an internal Cβ oligonucleotide CβII 5'-TGCTTCTGATGGCTCAA.

T Cell Proliferation. T cells (2 × 10^4) were cultured with an equal number of 6,000 rad irradiated allogeneic EBV-B cells in 200 μl RPMI-10% FCS in the presence or absence of different concentrations of bacterial toxins staphylococcal enterotoxin E (SEE) and toxic shock syndrome toxin. Thymidine incorporation was measured after 48 h.

Results

T Cells Expressing Two Vβ Are Present in Human Peripheral Blood. Using antibodies to different Vα products we have recently shown that up to 30% of human peripheral blood T cells carry two Vα as part of two functional and independent TCRs (14). To examine the extent of allelic exclusion at the β locus we performed similar experiments using a panel of anti-Vβ mAbs.

In a series of experiments, PBMC from healthy donors were stained by two-color indirect immunofluorescence with an IgM anti-Vβ2 mAb (detected by a PE-labeled goat anti–mouse IgM) and a combination of IgG antibodies specific for Vβ3, 5.2, 5.3, 8, 11, 13.3, 17, 19, and 22 (detected by FITC-labeled goat anti–mouse IgG). As shown in Fig. 1 *A*, a small proportion of cells (0.05%) was stained by both anti-Vβ2 as well as by additional anti-Vβ mAbs. The double positive cells were sorted and expanded in polyclonal cell lines using PHA and irradiated feeder cells. Analysis of these cell lines using the same antibody combination revealed a much higher frequency of cells stained by two anti-Vβ antibodies (Fig. 1 *B*). The double positive cells were sorted and cloned by limiting dilution. 252 dual β expressing clones were isolated from six donors. Among these, 85% were CD4$^+$, whereas the remaining were CD8$^+$ and only two were CD4$^-$8$^-$. Each clone was stained by the Vβ2 specific antibody and by one additional Vβ antibody, either Vβ3, 5.2, 8, 13.3, 19, or 22 (Fig. 1, *C–L* and data not shown). The relative expression of the two Vβs varied in different clones, but remained constant over extended in vitro culture. The unbalanced surface expression of the two β chains may depend on different rates of transcription, translation or, most likely, from a preferential pairing with the α chain (15).

To confirm by an independent criterion that the clones express two different Vβ products, we performed a molecular

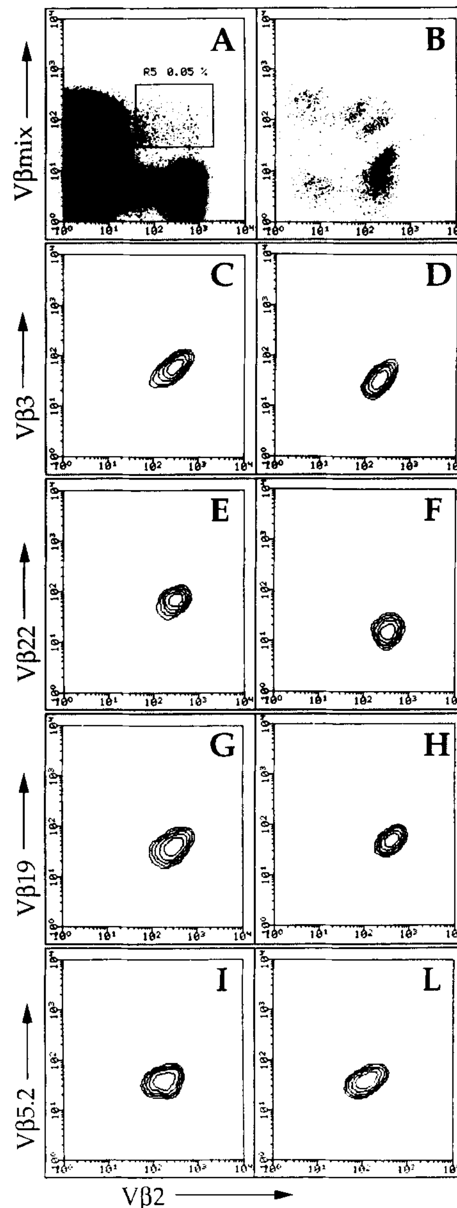

Figure 1. Identification and cloning of T cells expressing two Vβ. (*A*) PBMC stained with antibodies to Vβ2 (*x axis*) and Vβ3, 5.2, 5.3, 8, 11, 13.3, 17, 19 and 22 (*Vβ mix, y axis*) the sorting gate and the percentage of double positive T cells is shown. (*B*) Same staining on the polyclonal cell line obtained by in vitro expansion of the sorted cells. (*C–L*) Representative clones isolated from sorted cells.

Table 1. *Amino Acid Sequence of the TCR β Chains Expressed in Five Dual β T Cell Clones*

Clone Ab	Reactivity	Vβ		N D N		Jβ
2-5.2-10	Vβ2 Vβ5.2*	2‡	CSAR	HLGQV	QETQYF	2.5‡
		5.2	CA	GGVAGDL	DTQYF	2.3
2-5.2-31	Vβ2 Vβ5.2	2	CSAR	VCVWGTNFD	GYTF	1.2
		5.2	CASS	LDPGQN	SNQPQHF	1.5
2-3-33	Vβ2 Vβ3	2	CSAR	GSP	EAFF	1.1
		3	CASS	SHPGTPH	YEQYF	2.7
2-8-12	Vβ2 Vβ8	2	CSA	PGQGVALA	NYGYTF	1.2
		8	CASS	WDRL	NTEAFF	1.1
2-8-26	Vβ2 Vβ8	2	CS	SGGQTLV	TQYF	2.5
		8	CAS	ISNPQRGAGT	YEQYF	2.7

* As determined by FACS analysis using antibodies specific for Vβ2, Vβ3, Vβ5.2, and Vβ8.
‡ cDNA from the individual clones was amplified with V- and C-specific oligonucleotides and the product was directly sequenced. V, D, and J segments were assigned by comparison to the published sequences (16–18). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z48293–Z48302.

analysis of the TCRβ mRNA. cDNA was prepared from individual clones and amplified with V- and C-specific oligonucleotides. In all cases tested, a clear PCR product was obtained only with the expected oligonucleotide combination. Direct sequencing of the different Vβ products gave in all cases a single in-frame sequence (Table 1). In different clones, the N-region length varied markedly in the two alleles. These results confirm the antibody staining data and demonstrate that these clones express two different Vβs.

To rule out the unlikely possibility that dual β expressing T cells might be derived from a fusion event between two T cells we measured the total DNA content of the T cell clones by flow cytometric analysis of PI-stained permeabilized cells with standard procedures (19). In all cases a diploid DNA content was found (data not shown).

Frequency of Dual β T Cells. Due to the low frequency of dual β T cells, it was difficult to obtain an accurate estimate by direct analysis of total PBL. We therefore isolated by cell sorting T lymphocytes expressing Vβ2 or Vβ8. These cells were expanded in short-term polyclonal lines and analyzed by three-color fluorescence for the expression of CD3, the original Vβ, as well as an additional Vβ. As evident from Fig. 2 *A*, almost all $CD3^+$ cells were stained by the anti-Vβ2 antibody originally used for sorting, whereas a small percentage (0.02%) of the cells was stained by both anti Vβ2 and anti Vβ8 antibodies. If we consider that Vβ8 accounts for only 4% of the Vβs expressed in the peripheral blood T cells of this donor, we can estimate that the frequency of $Vβ2^+$ cells that express any other Vβ must be ~25-fold higher, i.e., ~0.5%. The same calculation on the $Vβ8^+$ cell line (Fig. 2 *B*) indicates a frequency of dual β T cells of ~1%.

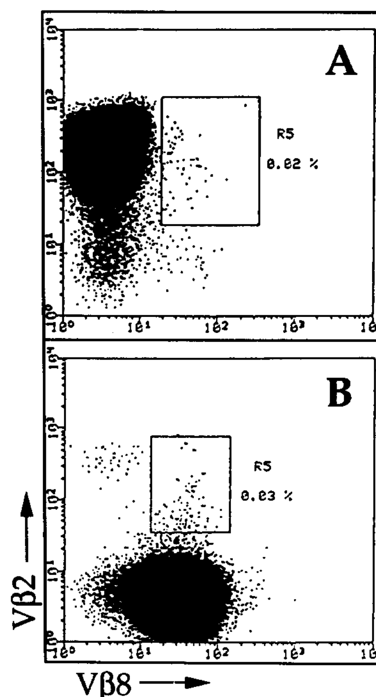

Figure 2. Frequency of dual β T cells in polyclonal T cell lines. $Vβ2^+$ and $Vβ8^+$ T cells were sorted from peripheral blood and expanded into polyclonal cell lines. After 2 wk of culture the lines were analyzed by three-color fluorescence for the expression of Vβ2, Vβ8, and CD3. Shown is the Vβ8/Vβ2 profile of CD3 gated cells. Doublets were excluded by narrow gating on both FSC and CD3. Dead cells were excluded by PI staining during acquisition. 6×10^5 T cells are displayed. (*A*) Polyclonal $Vβ2^+$ cell line. (*B*) Polyclonal $Vβ8^+$ cell line.

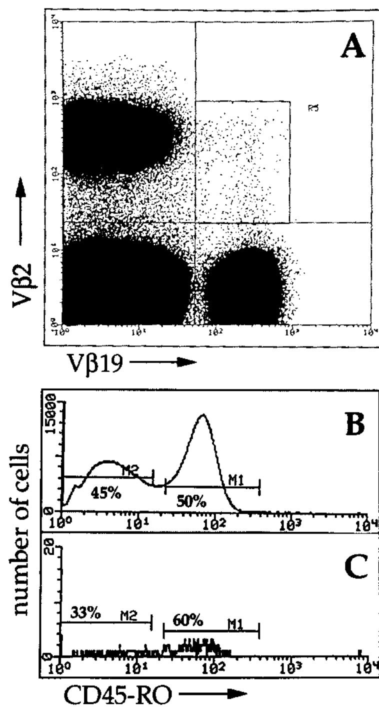

Figure 3. Distribution of dual β T cells in the CD45R0$^+$ and R0$^-$ subsets. PBMC from an adult donor were analyzed by four-color immunofluorescence with anti-Vβ2 (mIgM followed by PE-labeled goat anti-mouse IgM), Vβ19 (mIgG1 followed by biotin-labeled goat anti-mouse IgG1 and APC-SAV), CD3 (mIgG2b FITC conjugated) and CD45R0 (IgG2a followed by TXRD-conjugated goat anti-mouse IgG2a). (*A*) Vβ2 and Vβ19 profile of CD3-gated T cells. (*B*) CD45R0 staining in total CD3$^+$ cells. (*C*) CD45R0 staining of Vβ2$^+$ Vβ19$^+$ dual β T cells gated in R5 in *A*. The percentage of cells in the CD45R0$^+$ and R0$^-$ subsets is indicated.

In 10 polyclonal cell lines analyzed the frequency of dual β T cells estimated ranged between 0.5 and 3% (mean 1%).

Because dual TCR T cells express each TCR at a lower level compared to single receptor T cells, it was important to ask whether dual β T cells may undergo antigenic selection. We therefore looked at their distribution among peripheral blood CD45R0$^+$ and CD45R0$^-$ T cell subsets that correspond to memory and naive populations, respectively (20). As shown by four-color analysis (Fig. 3), the percentage of dual β T cells was actually slightly higher in the CD45R0$^+$ compartment than in the CD45R0$^-$ compartment, indicating that dual receptor T cells can participate in a normal immune response.

In Dual β T Cells the two Vβ Products Are Part of Two Functional TCR. To investigate whether dual β T cells could be activated through both TCRs, we examined the capacity of dual β T cell clones to proliferate in response to staphylococcal enterotoxins that selectively engage one or the other Vβ (21). Proliferation of Vβ2$^+$Vβ8$^+$ clones could be induced by both TSST and SEE which are Vβ2 and Vβ8 ligands, respectively, whereas clones expressing only Vβ2 or Vβ8 proliferated in response to only one of these toxins (data not shown). These results confirmed by a yet independent criterion the presence of the two Vβs as part of two functional TCRs.

Discussion

These results show that a small proportion of diploid T cells express two Vβs as part of two independent and functional TCRs. These dual β T cells are present both in naive and memory populations at a frequency of ~1%. Thus, expression of two β chains is compatible with both thymic and peripheral selection.

These findings do not question the substantial degree of allelic exclusion at the β locus, but rather the mechanism by which this exclusion is achieved. Since dual β T cells are generated, it is evident that the extent of β allelic exclusion must be determined only by the asynchrony of rearrangements at the β locus, whereas the function of the pre-TCR is limited to signaling the presence of "at least one" and not of "only one" β chain. Accordingly, the production of dual β T cells may depend on the occurrence of two simultaneous productive rearrangements on both alleles or a rearrangement on the second allele before the first β chain product had time to signal via pre-TCR. Alternatively, it is possible that insufficient signaling by the pre-TCR may allow rearrangement to proceed on the second β allele. The latter possibility is suggested by the fact that the efficiency of allelic exclusion is greatly reduced in mice that carry a dominant negative mutant lck transgene (5).

Since the α locus often escapes allelic exclusion (2, 14), it is likely that up to one third of dual β T cells might express two α chains as well. T cells with four TCR may thus exist, although the selection for preferential pairs may result in a quite unbalanced expression of the different TCRs pairs.

What could be the consequences of the incomplete allelic exclusion at the β locus? As previously discussed for T cells expressing two α chains (14), the presence of two β chains is compatible with a normal thymic censorship, since both TCRs can be simultaneously tested for reactivity to self-antigens in the thymus (22).

For positive selection, however, the situation may be different (3). Since only a small fraction of all possible TCRs is selected by a given MHC, it is expected that in a large fraction of dual receptor T cells only one TCR will have a positively selecting specificity. For this reason it could be argued that a nonpositively selecting TCR may be just an inert passenger, since it will never have the chance to recognize antigen on self-MHC. We think that this possibility cannot be excluded a priori, since we are not aware of any experimental evidence indicating that a TCR that is not positively selected cannot potentially recognize antigenic peptides bound to the nonselecting MHC molecules.

In addition, there is a low but definitive chance that the second TCR may be also self-restricted (this chance being exactly that of any random TCR to be selected by a particular MHC). Considering the very large number of T cells present in the human repertoire (~10^{11}), the existence of T cells with two receptors that fit self-MHC is unavoidable.

Dual receptor T cells may be specific for a broader range

of antigens than cells with a single receptor, which may be significant for autoimmunity and alloreactivity. The presence of two TCRs on a single T cell could pose a problem for peripheral tolerance when this is based on the inability of tissue cells to stimulate naive T cells (23, 24). Thus, once a dual receptor T cell is activated by a foreign antigen recognized by the first TCR, it may become competent to migrate to peripheral tissues and employ the second TCR to attack self. Indeed, both TCRs in dual β expressing T cells appear to be functional. This type of cross-reactivity would be based on a particular somatic combination of two distinct TCRs, rather than on structural similarity between foreign and self-antigens.

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