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Rearrangement and expression of $V_\gamma1$, $V_\gamma2$ and $V_\gamma3$ TCR γ genes in C57BL/6 mice

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Abstract

We have recently described a mAb (2.11) that recognizes the $V_\gamma1$–$J_\gamma4$–$C_\gamma4$ chain. With this mAb and an anti-δ mAb we separated $\gamma\delta^+$ 2.11^+ and $\gamma\delta^+$ 2.11^- intraepithelial lymphocytes (i-IEL) by FACS. Transcripts of rearranged TCR $V_\gamma1$ and $V_\gamma2$ genes in both i-IEL populations were analyzed by PCR followed by sequence analysis of cDNA spanning the junction of the variable (V) and joining (J) genes. Roughly the same number of $V_\gamma1$ and $V_\gamma2$ transcripts were found in the 2.11^+ population, while >90% of the transcripts in the 2.11^- population contained a $V_\gamma2$ gene sequence. Furthermore, >80% of the $V_\gamma1$ transcripts in the 2.11^+ population were functional, while only 30–40% of the $V_\gamma2$ transcripts in either population contained an in-frame sequence. The observed frequency of in-frame $V_\gamma2$ transcripts is what would be expected from cell populations that have not gone through cellular selection mediated by the TCR. Expression of $V_\gamma2$ mRNA in TCR$\alpha\beta$ and TCR$\gamma\delta$ thymocytes was studied by a technique that analyzes populations of transcripts of rearranged genes. In both T cell populations similar levels of $V_\gamma2$ transcripts were found and about two out of three transcripts were out-of-frame. During the cloning and sequence analysis, we identified a clone that expresses the $V_\gamma3$ segment rearranged to the $J_\gamma3$–$C_\gamma3$ region in C57BL/6 mice. Together with the PCR cloning and sequencing of the complete $C_\gamma3$ region in C57BL/6 mice, these data demonstrate that the $J_\gamma3$–$C_\gamma3$ gene is functional in this strain. Taken together, these studies revealed that: (i) cells expressing the $V_\gamma1$ chain are an important subset of the $\gamma\delta$ i-IEL population and that they show extensive junctional diversity; (ii) there is no correlation between expression of in-frame $V_\gamma2$ transcripts and expression of $V_\gamma2$ chains at the cell surface; and (iii) cells expressing the $V_\gamma3$ chain might be a minor subset of the $\gamma\delta$ T cell population in C57BL/6 mice.

Introduction

Among TCR γ genes of mice, there are seven different variable (V) γ genes that can rearrange to four different constant (C) genes, each of which is associated with a junctional (J) element. Four V_γ genes, $V_\gamma4$, $V_\gamma5$, $V_\gamma6$ and $V_\gamma7$, rearrange mostly to the $J_\gamma1$–$C_\gamma1$ gene. The other three genes ($V_\gamma1$, $V_\gamma2$ and $V_\gamma3$) show a very high level of homology and rearrange mostly to the $J_\gamma4$–$C_\gamma4$, $J_\gamma2$–$C_\gamma2$ and $J_\gamma3$–$C_\gamma3$ genes, respectively (reviewed in 1). The region containing the $J_\gamma3$–$C_\gamma3$ gene has been deleted in most of the common laboratory strains of mice (2,3), and is believed to be non-functional in BALB/c mice due to a mutation in the splice donor at the end of exon 2, a defective putative polyadenylation signal sequence and a base pair deletion in the $J_\gamma3$ region (4,5).

Although a large amount of information is available regarding the onset of appearance during ontogeny, TCR repertoire, tissue distribution and thymus dependence of the $\gamma\delta$ T cell subsets bearing the products of V_γ genes that rearrange to the $J_\gamma1$–$C_\gamma1$ gene (i.e. $V_\gamma4$, $V_\gamma5$, $V_\gamma6$ and $V_\gamma7$ subsets), analogous information regarding other $\gamma\delta$ T cell subsets is limited (reviewed in 6). Sequence analysis of the $V_\gamma1$–$J_\gamma4$ junctions of $V_\gamma1$-expressing hybridomas obtained from thymus and spleen and of cDNA obtained from dendritic epidermal cells of nude mice has shown that $V_\gamma1$-bearing cells in these organs are highly diverse (7–9). Recently, we described a mAb (2.11) that recognizes the $V_\gamma1$–$C_\gamma4$ protein, and showed that $V_\gamma1$-bearing cells constitute a large fraction of the $\gamma\delta$ T cells not only in the thymus and peripheral lymphoid organs but also in the intestinal epithelium (32). In contrast, previous studies

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have shown a large number of functionally rearranged $V_\gamma2$ genes in intraepithelial lymphocytes (i-IEL) leading to the belief that $V_\gamma2$-bearing cells constitute a significant population of mouse i-IEL (10,11). However, $V_\gamma2$ gene rearrangement and mRNA have been found in some γδ T cells that express γ chains other than the $V_\gamma2$–$C_\gamma2$ protein and even in αβ T cells. Therefore, the role of the V2–J2–C2 gene in encoding the TCR for a major γδ T cell subset has been questioned (1). To clarify this issue and to study the extent of diversity of $V_\gamma1^+$ and/or putative $V_\gamma2^+$ i-IEL, we have sorted $\gamma\delta^+$ 2.11^+ and $\gamma\delta^+$ 2.11^- i-IEL and analyzed the junctional sequences obtained after PCR amplification of cDNA isolated from both cell populations using primers that recognize both the $V_\gamma1$ and the $V_\gamma2$ genes. Our results show that $V_\gamma1$-bearing cells constitute a large and diverse population of i-IEL and that $V_\gamma2$–$J_\gamma2$–$C_\gamma2$ mRNA is frequently expressed in many T cells without apparent expression of the protein at the cell surface. Furthermore, we show that the $J_\gamma3$–$C_\gamma3$ gene is functional in C57BL/6 mice and that cells bearing the $V_\gamma3$ chain might represent a minor subset of γδ lymphocytes in this strain.

Methods

Mice

C57BL/6 (B6) mice were obtained from Iffa-Credo (L'Arbresle, France). Males that were 8–12 weeks old were used.

Cell preparations and cultures

The preparation of i-IEL has been described in detail (12). Purified TCRαβ or TCRγδ cells were obtained by stimulation of B6 total or $CD4^-CD8^-$ thymocytes with anti-β mAb (H57; 13) or anti-δ mAb (14) coated to plates and expanded in IL-2 as described (32). Cells activated with H57 were >98% $TCR\alpha\beta^+$ with no detectable $TCR\gamma\delta^+$ cells, and cells activated with 3A10 were >95% $TCR\gamma\delta^+$ with <2% $TCR\alpha\beta^+$ T cells.

Immunofluorescence staining and cell sorting

i-IEL were incubated with FITC-labeled anti-pan γδ mAb [3A10 (14)] and biotin-labeled anti-$V_\gamma1$–$C_\gamma4$ mAb [2.11 (32)] at a concentration of 10^7 cells/ml for 30 min. on ice, washed twice and incubated for 15 min with streptavidin–phycoerythrin (Southern Biotechnology, Birmingham, AL). Cell sorting was carried out in a FACStar Plus (Becton Dickinson, Mountain View, CA).

Nucleic acids

Sorted cells were mixed with 10^7 P815 mastocytoma cells as a carrier and total RNA was prepared using the guanidinium thiocyanate–CsCl method (15) cDNA was synthesized with 5 μg of total RNA using OligodT (Pharmacia, Uppsala, Sweden), and the SuperScript reverse transcriptase (Gibco/BRL, Gaithersburg, MD) following the manufacturer's instructions. In some instances, the RNA preparations were treated with DNase prior to cDNA synthesis.

Oligonucleotide primers and PCR conditions

The following oligonucleotide primers were used: $V_\gamma1$ + $V_\gamma2$: GCTATACATTGGTACCGGCA; $V_\gamma1$ + $V_\gamma2$ labeled: AATCAACGACCCTTAGGAGG; $V_\gamma1$: CCGGCAAAAAGCAAAAAAGT; $V_\gamma2$: CGGGCAAAAAACAAATCAA; $V_\gamma3$: TTGAGTATCTAATATATGTCGAG; $J_\gamma2$: CAGAGGGAATTACTATGA; $J_\gamma3$: TAAGCTCATAGTAATTCCTTCT; $J_\gamma4$: GCAAATATCTTGACCCATGA; pan-C_γ: CTTATGGAGATTTGTTTCAGC; and $C_\gamma3$ untranslated region: CAGCTGACTTGCTGTACCAC.

PCR was performed using a GeneAmp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT). Each cycle consisted of incubations at 92°C for 20 s, followed by 55°C for 30 s and 72°C for 30 s. Before the first cycle, a 2 min 94°C denaturation step was included and after the 30th cycle the extension at 72°C was prolonged for 4 min.

Cloning and sequencing

Between 5 and 10 μl of each PCR reaction was blunted, phosphorylated and cloned into the *NotI* site of the pUC18 vector using the SureClone ligation kit (Pharmacia, Uppsala, Sweden) and following the manufacturer's instructions. Plasmid DNA isolated from ampicillin-resistant white colonies was sequenced by the dideoxy chain-termination method using the sequenase enzyme (US Biochemicals, Cleveland, OH) and the M13 forward and reverse primers. In some instances colonies were screened for the presence of the right insert by PCR.

Population analysis

PCR product (2 μl) was submitted to a run-off elongation with a fluorescent primer (16) specific for the $V_\gamma1$ and $V_\gamma2$ genes; 2 μl of the elongation product was mixed with an equal volume of 95% (v/v) formamide/10 mM EDTA and loaded on an 8% denaturing polyacrylamide–urea gel cast on an automated DNA sequencer (Applied Biosystems, Foster City, CA). Size determination of the run-off products was performed with a previously described software (16). This software provides an image of the gel by analyzing each band as a peak, the area of which is proportional to the intensity of fluorescence. The length of the fragments is determined by comparison with a set of size standards run in parallel in each experiment. This set of standards consists of five labeled fragments of known size.

Results

The junctional sequences of $V_\gamma1$-bearing i-IEL are very diverse

To ensure that the cells recognized by the 2.11 mAb express functional $V_\gamma1$–$C_\gamma4$ gene products and to analyze the extent of junctional diversity of the $V_\gamma1$-expressing i-IEL, we cloned and sequenced the PCR products obtained after amplification of cDNA with $V_\gamma1/V_\gamma2$ and pan-C_γ primers. The RNA used to prepare the cDNA was isolated from sorted $\gamma\delta^+$ 2.11^- i-IEL obtained from B6 mice. The $V_\gamma1$–$J_\gamma4$ junctional sequences obtained from this population are shown in Fig. 1 and contain several points of interest. First, >80% of the $V_\gamma1$–$J_\gamma4$ sequences are joined in-frame. Second, the majority of these sequences contain one to 12 non-germline encoded nucleotides and the ends of both the V and the J segments are often shortened, generating considerable diversity in the junctions. This shortening is much more pronounced at the end of the V segment than at the end of the J segment and, as a consequence, P nucleotides are retained much more

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	Vγ1	P	N	P	Jγ4	Functional
germline Vγ1	TGT GCA GTC TGG ATA AA	TT				
germline Jγ4				GA	TCA GGC ACA	
	TGT GCA GTC TGG		C	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		CCGTCCGCCC	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG A		A	A	TCA GGC ACA	Yes
	TGT GCA GTC TGG A			GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		G	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		GGTG	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		GT	A	TCA GGC ACA	Yes
	TGT GCA GTC TGG		CGT		TCA GGC ACA	Yes
	TGT GCA GTC TGG ATA AA		ATGGC		CA GGC ACA	Yes
	TGT GCA GTC TGG		CCGA	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		TTCCC		C ACA	Yes
	TGT GCA GTC TGG				TCA GGC ACA	Yes
	TGT GCA GTC TGG ATA A		GT		TCA GGC ACA	Yes
	TGT GCA GTC TGG A				CA GGC ACA	Yes
	TGT GCA GTC TG			A	TCA GGC ACA	No
	TGT GCA GTC TGG ATA		GAGGG[illegible]	GA	TCA GGC ACA	No
	TGT GCA GTC TGG AT		G		A GGC ACA	No

Fig. 1. V–J junctional sequences of $V_\gamma1$ transcripts from B6 i-IEL. Data represent cloned $V_\gamma1$–$C_\gamma4$ PCR products from sorted $\gamma\delta^+$ 2.11^+ i-IEL.

often in the 5′ end of the J than in the 3′ end of the V. Third, the size of the CDR3 appears quite constant with >80% of the $V_\gamma1^+$ clones having the predicted germline size plus or minus three nucleotides.

Evaluation of the predicted amino acid sequences of the in-frame $V_\gamma1$–$J_\gamma4$ genes (Fig. 2) indicated that the junctional diversity also applies at the protein level. The increased frequency of a codon for Arg in the CDR3 of the 2.11^+ population can be explained by the high frequency of clones containing P nucleotides in the 5′ end of the J region.

From this experiment we conclude that the TCRγ repertoire of the $V_\gamma1^+$ i-IEL population is as diverse as that of the $V_\gamma7^+$ i-IEL population (17–19).

Rearrangement and expression of the $V_\gamma2$ gene in different γδ i-IEL populations

Analysis with TCRγ-specific mAb has shown that ~90% of i-IEL express the $V_\gamma1$ or the $V_\gamma7$ chains (21,32). On the other hand, functionally rearranged $V_\gamma2$ gene segments are abundant in i-IEL, suggesting that $V_\gamma2$-bearing cells are not a minor i-IEL subpopulation (10,11). This apparent contradiction could be resolved if the 2.11 mAb not only recognizes the $V_\gamma1$–$C_\gamma4$ protein but also the $V_\gamma2$–$C_\gamma2$ protein or if many of the functionally rearranged $V_\gamma2$ gene segments do not give rise to cell surface expression of the $V_\gamma2$–$C_\gamma2$ protein. To distinguish between these two possibilities we compared the frequencies of functional and non-functional $V_\gamma1$–$J_\gamma4$ and $V_\gamma2$–$J_\gamma2$ rearrangements in sorted 2.11^+ and 2.11^- γδ i-IEL populations. cDNA from the sorted populations was amplified by PCR. The sense primer used in this experiment has a sequence identity to the $V_\gamma1$ and $V_\gamma2$ genes, while the sequence of the antisense primer is present in all C_γ genes. Thus, this set of primers is likely to amplify the $V_\gamma1$–$C_\gamma4$ and the $V_\gamma2$–$C_\gamma2$ cDNAs proportionally to their representation in the total cDNA population.

Vγ1	P1+N+P2	Jγ4
AVWI		SGT
AVW	R	SGT
AVW	PSAR	SGT
AVW	K	SGT
AV	R	SGT
AVW	G	SGT
AVW	GG	SGT
AVW	V	SGT
AVWI	KWA	GT
AVW	PR	SGT
AVW	FP	T
AVW		SGT
AVWI	S	SGT
AVW	T	GT
AVW	R	SGT

Fig. 2. Predicted amino acid sequences encoded by the IEL in-frame $V_\gamma1$ junctions.

The frequencies of functional and non-functional rearrangements of $V_\gamma1$ and $V_\gamma2$ genes in the 2.11^+ and 2.11^- δ i-IEL populations are presented in Table 1. Three major points are worth noting. First, most of the functionally rearranged $V_\gamma1$ genes are contained in the 2.11^+ population, showing a correlation between functionally rearranged $V_\gamma1$ gene expression and 2.11^+ phenotype. Second, the frequencies of functional $V_\gamma2$ rearrangements in the two cell populations are very similar to each other (33.3% in the 2.11^+ population and 37.5% in the 2.11^- population) and are also very close to the frequency expected by random rearrangement of TCR and/or Ig genes in the absence of cellular selection (one-third of

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Table 1. Expression of functional $V_\gamma 1$–$J_\gamma 4$ and $V_\gamma 2$–$J_\gamma 2$ rearrangements in different i-IEL populations

i-IEL population[a]	Gene rearrangement	No. of clones	No. of functional clones (% of the total)
$\gamma\delta^+$ 2.11$^+$	$V_\gamma 1$–$J_\gamma 4$	17	14 (82.3)
	$V_\gamma 2$–$J_\gamma 2$	18	6 (33.3)
$\gamma\delta^+$ 2.11$^-$	$V_\gamma 1$–$J_\gamma 4$	2	1 (50.0)
	$V_\gamma 2$–$J_\gamma 2$	24	9 (37.5)

[a]Sorted $\gamma\delta^+$ 2.11$^+$ and $\gamma\delta^+$ 2.11$^-$ i-IEL population for B6 mice.

all rearrangements are expected to be in-frame). These data suggest a lack of cellular selection for functional $V_\gamma 2$ rearrangements, supporting the notion that cells expressing the $V_\gamma 2$ gene product as a part of the TCR on the cell surface are rare, if they exist at all. Third, the fact that we amplified roughly as many $V_\gamma 2$ cDNA clones as functionally rearranged $V_\gamma 1$ cDNA clones (14 clones were $V_\gamma 1$ in-frame and 18 clones were $V_\gamma 2$) in the 2.11$^+$ population suggests that most $V_\gamma 1$-bearing cells express mRNA from one rearranged $V_\gamma 2$ gene. Assuming that most $\gamma\delta$ T cells do not express two different TCR, these data indicate that there is no correlation between the expression of functionally rearranged $V_\gamma 2$–$C_\gamma 2$ mRNA and expression of the $V_\gamma 2$–$C_\gamma 2$ protein at the cell surface. Thus, the presence of functional V_γ–C_γ rearrangements *per se* should not be considered as an indication of the presence of cells expressing this V_γ–C_γ protein as part of their TCR.

Population analysis of transcripts from rearranged TCR γ chain genes

A recently developed technique allows population analysis of TCR γ chain rearrangement or mRNA expression without nucleotide sequencing. This technique takes advantage of the fact that different rearrangements between the same gene segments often display length heterogeneity due to the random shortening and/or addition of N nucleotides at the junction. The lengths of productively rearranged genes can differ by multiples of three nucleotides, whereas non-productively rearranged genes will have lengths offset by one or two nucleotides from productively rearranged sequences. PCR amplification, with V_γ–J_γ- or V_γ–C_γ-specific primers, of DNA or cDNA isolated from a polyclonal T cell population followed by a primer extension reaction with a fluorochrome-labeled nested primer will yield a labeled set of fragments of different lengths. Such fragments, differing in length by as few as a single base pair, can be resolved on denaturing polyacrylamide gels.

A plot of the fluorescence intensity profile versus length for the $V_\gamma 1$–$J_\gamma 4$ and the $V_\gamma 2$–$J_\gamma 2$ amplifications of cDNA isolated from 2.11$^+$ i-IEL and for the $V_\gamma 2$–$J_\gamma 2$ amplifications of cDNA isolated from 2.11$^-$ i-IEL is shown in Fig. 3. In concordance with the sequence data, most of the detectable fragments obtained after amplification with $V_\gamma 1$–$J_\gamma 4$ primers in the 2.11$^+$ population show length intervals of three nucleotides (Fig. 3A), while the patterns observed after amplification of both cell populations with $V_\gamma 2$–$J_\gamma 2$-specific primers show length

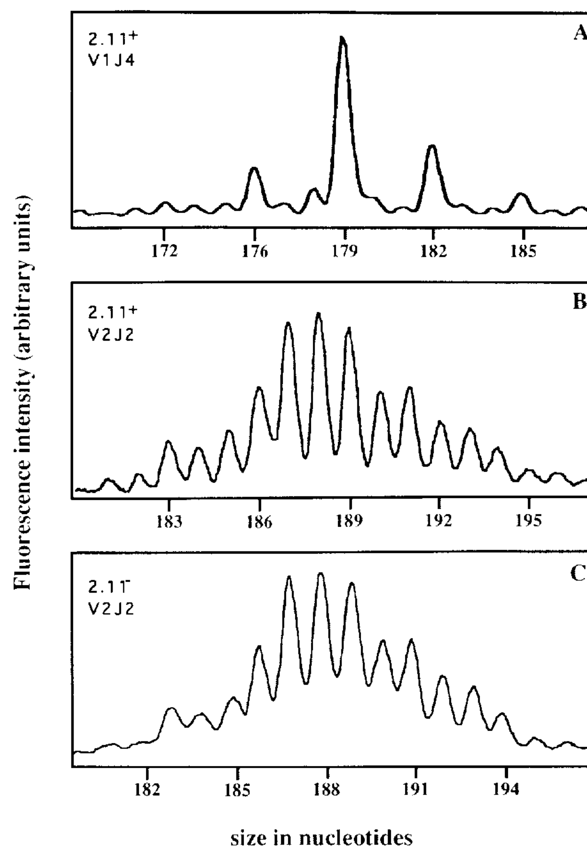

Fig. 3. TCRγ mRNA population analysis from sorted i-IEL populations. Profiles of PCRs performed on cDNA isolated from $\gamma\delta^+$ 2.11$^+$ (A and B) or from $\gamma\delta^+$ 2.11$^-$ (C) sorted i-IEL populations using $V_\gamma 1$–$J_\gamma 4$- (A) or $V_\gamma 2$–$J_\gamma 2$-specific primers (B and C).

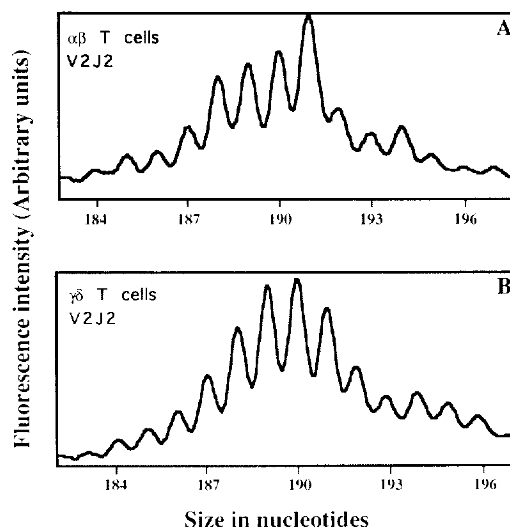

Fig. 4. Analysis of $V_\gamma 2$ mRNA expression in different T cell populations. Profiles of PCRs performed on cDNA isolated from TCRαβ$^-$ (A) and TCRγδ$^+$ thymocytes. Cells were prepared as described in Methods. The plots represents the length of the amplified fragments versus the fluorescence intensity of each band in arbitrary units.

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intervals of one nucleotide (Fig. 3B and C). Calculation of the areas of the peaks allows the quantification of the frequency of functional rearrangements for each γ gene in both cell populations. In this particular experiment, 89% of the $V_\gamma1$–$J_\gamma4$ rearrangements and 35% of the $V_\gamma2$–$J_\gamma2$ rearrangements in the 2.11$^+$ population were in-frame. In the 2.11$^-$ population, the frequency of in-frame $V_\gamma2$–$J_\gamma2$ rearrangements was 40%. These data are highly consistent with the data obtained by cDNA sequencing (see Table 1). A similar consistency was found in the modal distribution of the fragments. Thus, ~90%

```
BALB/c Jγ2     AT AGC TCG GGC TTT CAC AAG GTA TTT GCA GAA GGA ACA AAG CTC ATA GTA ATT CCC TCC G

BALB/c Jγ3     -- --T -G- -A- --- --- *-- --- --- --- --- --- --T --- --- --- --- --- --T --T -

C57BL/6 Jγ3    -- --T -G- -A- --- --- A-- --- --- --- --- --- --T --- --- --- --- --- --T --T -
```

Fig. 5. Alignment of BALB/c $J_\gamma2$, BALB/c $J_\gamma3$ and B6 $J_\gamma3$ segment nucleotide sequences. BALB/c J_γ sequences are from (5). A gap introduced to maximize homology is denoted by an asterisk. Agreement of the sequences is denoted by dashed lines

```
      V→
1  TT GGG CTT GGG CAG CTG GAG CAA ACT GAA TTA TCG GTC ACC AGA GAG ACA GAT GAG AAT GTG CAA ATA TCC TGT ATA
2  -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- CA- --- --- -G- -C- --- --- --- --- ---
3

1 GTT TAT CTT CCA TAT TTC TCC AAC ACA GCT ATA CAT TGG TAC CGG CAA AAA ACA AAT CAA CAG TTT GAG TAT CTA
2 --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- ---
3                                 --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- ---

1 ATA TAT GTC GCA ACA AAC TAC AAT CAA CGA CCC TTA GGA GGG AAG CAC AAA AAA ATT GAA GCA AGT AAA GAT TTT
2 --- --- --- -AG --- --- --- --- --- --- --- --- --- --- --- A-- --G --- --- --- --- --- --- --- ---
3 --- --- --- -AG --- --- --- --- --- -A- --- --- --- --- --- A-- --G --- --- --- --- --- --- --- ---

1 AAA AGT TCT ACC TCA ACC TTG GAA ATA AAT TAC TTG AAG AAA GAA GAT GAA GCC ACC TAC TAC TGT GCA GTC TGG
2 C-- -C- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3 C-- -C- --- --- --- --- --A --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                  J→
1 ATG AG          AT AGC TCG GGC TTT CAC AAG GTA TTT GCA GAA GGA ACA AAG CTC ATA GTA ATT CCC TCC G
2 --A -A          -- --T -G- -A- --- --- *-- --- --- --- --- --- --T --- --- --- --- --- --T --T -
3 --A -A          -- --T -G- -A- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --T --T
   CI→
1  AC AAA AGG CTT GAT GCA GAC ATT TCC CCC AAG CCC ACT ATT TTT CTT CCT TCT GTT GCT GAA ACA AAT CTC CAT AAG
2  -- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3  -- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

1 ACT GGG ACA TAC CTT TGT CTC CTT GAA AAG TTC TTT CCC GAT GTC ATA AGG GTG TAT TGG AAA GAA AAG AAT GGC ATT
2 --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- ---
3 --- --- --- --- --- --- --- --- --- --- --- --- --A --- --T --- --- --- --- --- --- --- --- G-- --- ---

1 ACT ATC CTG GAC TCC CAG GAA GGG GAT ACG CTG AAG ACT AAG GGC ACA TAC ATG AAG TTT AGC TGG CCT ACT GTG CCT
2 --C --- --- --- --- --- --- --T --- -T- --- --- --- --T -A- --- --- --- --- --- --- --- --- --- --- ---
3 --- --- --- --- --- --- --- --- --- --- --- --- --- --C -A- --- --- --- --- --- --- --- --- --A --- ---

1 GAA AGG GCA ATG GGG AAA GAG CAC AGT TGT ATT GTC AAA CAT GAG AAC AAC AAA GGA GGA GCA GAT CAA GAG ATT TTC
2 --- --- T-- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3 --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                              CII→                                  CIII→
1 TTC CCT TCA ATA AAG AAA GTT GC[                ]T ACA ACT TGC TGG CAA GAT AAA AAT GAT GTG CTG CAG TTC
2 --T --A A-- --- --- --- --- --C GTG AGT ACC AAG CC- --- --- --- --- --- --- --- --- --- --- --- --- C--
3 --T --- --- --- --- --- --- --T GTG AGT ACC AAG CC- --- --- --- --- --- --- --- --- --- --- --- --- C--

1 CAG TTC ACG AGC ACC TCT GCC TAC TAC ACC TAC CTC CTC CTG CTC CTC AAG AGT GTG ATC TAC TTG GCC ATC ATC AGC
2 --- --- --- -T- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3 --- --- --- -T- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- ---

1 TTC TCT CTG CTT AGA AGA ACA TCT GTC TGT GGC AAT GAG AAG AAG TCC     BALB/c   Vγ2-Jγ2-Cγ2
2 --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- ---     BALB/c   Vγ3-Jγ3-Cγ3
3 --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- ---     C57BL/6  Vγ3-Jγ3-Cγ3
```

Fig. 6. Comparison of B6 $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ (3), BALB/c $V_\gamma2$–$J_\gamma2$–$C_\gamma2$ (1) and $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ (2) nucleotide sequences. BALB/c sequences are from (4,5). Agreement of the sequences is denoted by dashed lines. The V region sequence shown starts at the second exon. The partial $V_\gamma3$–$J_\gamma3$ sequence was obtained from the rearranged clones shown in Fig. 7. The end of the $V_\gamma3$ and the beginning of the $J_\gamma3$ in B6 are defined by homology to the BALB/c sequences.

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of the $V_\gamma1$–$J_\gamma4$ rearrangements were distributed in three major peaks having the predicted germline length plus or minus three nucleotides.

The high level of homology between the $V_\gamma1$ and the $V_\gamma2$ genes imposed the choice of a reverse primer that would provide specificity for each J–C_γ gene. We decided to use J_γ-specific primers instead of C_γ-specific primers because the $C_\gamma4$ sequence from B6 mice is not known. To avoid amplification of rearranged DNA the RNA preparations were treated with DNase before cDNA synthesis. In addition, similar results were obtained when the amplifications were performed with a pan-C_γ primer which does not effectively amplify rearranged genomic DNA (not shown).

Analysis of $V_\gamma2$–$J_\gamma2$ mRNA was also performed on highly purified $\alpha\beta^+$ and $\gamma\delta^+$ thymocytes, and the results are shown in Fig. 4. The pattern observed in both T cell populations was very similar to the one previously found in $\gamma\delta$ i-IEL, with length intervals of one nucleotide. These data demonstrate that the unselected rearrangement and expression of the $V_\gamma2$–$J_\gamma2$ gene is not limited to the $\gamma\delta$ i-IEL population, but also takes place in other $\gamma\delta$ T cell populations and in $\alpha\beta$ T cells.

A functional $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ gene in B6 mice

During the cloning and sequencing of the PCR products presented above, we isolated one clone containing a putative J_γ segment different from all J_γ segments previously described. Comparison of its sequence with published J_γ sequences indicated a very high degree of homology with the $J_\gamma3$ segment present in BALB/c mice (5). The $J_\gamma3$ segment in BALB/c mice has a single base pair deletion compared with the $J_\gamma2$ segment. This base pair deletion causes a frame shift which, together with the fact that the $C_\gamma3$ gene lacks a proper splicing acceptor site, renders the $J_\gamma3$–$C_\gamma3$ gene non-functional (4,5). The putative J_γ gene found in B6 does not have the single base pair deletion, and with the exception of this difference, is identical in sequence to the BALB/c $J_\gamma3$ segment (Fig. 5). Thus, it is very likely that this J_γ segment corresponds to the B6 $J_\gamma3$. Analysis of the V region rearranged to the $J_\gamma3$ in this clone showed that it is the $V_\gamma3$ segment (see Fig. 6). Together with the fact that this clone was obtained from a PCR performed on cDNA, these data suggested that the $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ gene in B6 is functional.

To ascertain that this potential $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ rearrangement was genuine and not a PCR artefact, we decided to further characterize the C region associated with this J segment. We screened B6 hybridomas for the presence of a $V_\gamma3$–$J_\gamma3$ rearrangement and found one satisfying this criterion. We then prepared cDNA from this hybridoma and amplified it by PCR using primers for the $J_\gamma3$ segment and the 3′ untranslated region of the BALB/c $C_\gamma3$ gene. We then determined the nucleotide sequence of the DNA product obtained. A comparison of the putative B6 $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ with the previously described BALB/c $V_\gamma2$–$J_\gamma2$–$C_\gamma2$ and $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ sequences is shown in Fig. 6. The putative B6 $C_\gamma3$ sequence differs from the BALB/c $C_\gamma3$ sequence by only 14 of 515 nucleotides (97.3% identity). All but one of the nucleotide differences were located in the first exon. Only five of the 14 nucleotide differences between the putative B6 $C_\gamma3$ gene and the BALB/c $C_\gamma3$ gene result in amino acid changes and all of these are located in the first exon. Interestingly, four of these five different nucleotides present in the putative B6 $C_\gamma3$ sequence are found in the sequence of the BALB/c $C_\gamma2$ gene.

	Vγ3	P	N	P	Jγ3	Functional
germline Vγ3	TGT GCA GTC TGG ATA AA	TT				
germline Jγ3				AT	AT AGT TGG GAC TTT	
A) i-IEL						
	TGT GCA GTC TGG ATA		GAGAGGGG	AT	AT AGT TGG GAC TTT	Yes
	TGT GC		GAGGA		AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG			T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG		GC	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC T		TCAG	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG ATA		CCGGGACTGGA	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA		TGGGG	T	AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG A		A		AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG ATA		CG		GT TGG GAC TTT	No
	TGT GCA GTC TGG ATA AA	TT	GGA	AT	AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG		C	AT	AT AGT TGG GAC TTT	No
B) Thymus						
	TGT GCA GTC TGG			T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC T		CGG		GT TGG GAC TTT	Yes
	TGT GCA GTC TGG		GG	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG		CAT	T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG AT		CCCT		T AGT TGG GAC TTT	No
	TGT GCA GTC TGG AT		GTGGGG		AGT TGG GAC TTT	No
	TGT GCA GTC TGG		GGCCTG		T AGT TGG GAC TTT	No

Fig. 7. V–J junctional sequences of $V_\gamma3$ transcripts from B6 i-IEL and thymocytes. Data represent cloned $V_\gamma3$–$C_\gamma3$ PCR products from sorted $\gamma\delta^+$ i-IEL (A) and *in vitro* activated $\gamma\delta^+$ thymocytes (B).

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In fact, considering only the first exon, the B6 $C_\gamma3$ gene is more similar to the BALB/c $C_\gamma2$ gene than to the BALB/c $C_\gamma3$ gene, and the B6 $C_\gamma3$ and BALB/c $C_\gamma2$ genes show a higher degree of identity than do the two BALB/c C_γ regions. Thus, the first exon of the B6 $C_\gamma3$ gene differs from that of the BALB/c gene by nine nucleotides, resulting in five amino acid substitutions, while the first exons of the BALB/c $C_\gamma2$ and $C_\gamma3$ genes differ by 13 nucleotides resulting in nine amino acid changes.

The partial sequence of the $V_\gamma3$ gene in B6 differs from the $V_\gamma3$ gene of BALB/c by two nucleotides, both of which result in amino acid changes, and they are likely to represent an allelic polymorphism. Taken together, these data show that the $J_\gamma3$–$C_\gamma3$ gene is functional in B6 mice and that the $V_\gamma3$ gene rearranges to it.

Expression of the $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ gene in $\gamma\delta$ T cells from B6 mice

The fact that the $V_\gamma3$–$C_\gamma3$ gene is functional in B6 mice raises the issue of whether a subset of $\gamma\delta$ T cells expresses the $V_\gamma3$–$C_\gamma3$ protein as part of their TCR. To analyze this issue we prepared cDNAs from $\gamma\delta$ T cells isolated from the thymus and the small intestine of B6 mice, and amplified their junctional sequences with $V_\gamma3$ and C_γ-specific primers. The PCR products were then cloned and sequenced, and the $V_\gamma3$–$J_\gamma3$ junctional sequences are shown in Fig. 7. Six of the 11 clones (54.5%) isolated from the $\gamma\delta$ i-IEL and five of the nine clones (55.5%) obtained from the $\gamma\delta$ thymocytes contained functional rearrangements. These data are consistent with the possibility that a small fraction of $\gamma\delta$ T cells in those organs express the $V_\gamma3$ chain.

Discussion

$\gamma\delta$ T cell subsets that home to different epithelia are known to exhibit different degrees of diversity in the junctional sequences of their TCR. Previous analysis of $V_\gamma7$ and V_δ sequences in $\gamma\delta$ i-IEL have shown that these cells exhibit extensive junctional diversity, which is believed to confer on these cells the ability to recognize an array of different antigens (18–20). We found similar junctional diversity in the $V_\gamma1^+$ i-IEL population with no apparent selection for any particular amino acid sequence in the CDR3. The high frequency of a codon for arginine (GGA) in the $V_\gamma1$–$J_\gamma4$ junctions can be explained by the P nucleotides that are retained in the 5′ end of the joined $J_\gamma4$ segment and by the preference of the TdT enzyme for purine bases. The very high frequency of a codon for tyrosine that was previously described in $V_\gamma7$–$J_\gamma1$ junctions (18–20) (and is actually present in the junctions of other V_γ genes with $J_\gamma1$) may also be due to P nucleotides: any in-frame sequence containing at least one P nucleotide in the 5′ end of the $J_\gamma1$ segment will have a tyrosine codon, TAT.

The junctional diversity found in the TCR of $\gamma\delta$ i-IEL is in contrast with the limited diversity found in the TCR of $\alpha\beta$ i-IEL, in both mice (21) and humans (22–26). It is not clear whether this difference reflects antigenic selection or a difference in the number of precursor T cell clones that give rise to the respective i-IEL subsets. In any case, these data suggest a different function of $\alpha\beta$ and $\gamma\delta$ i-IEL.

The high expression of transcripts of rearranged $V_\gamma2$–$C_\gamma2$ genes in $\gamma\delta$ T cells known to express other V_γ chains at the cell surface and in $\alpha\beta$ T cells poses a question about the cell surface expression of the $V_\gamma2$ chain in these T cells. The ratio of functionally versus non-functionally rearranged $V_\gamma2$ genes in different $\gamma\delta$ and $\alpha\beta$ T cell populations shows that there is no selection for cells harboring in-frame rearranged $V_\gamma2$ genes. This strongly suggests that the expression of the $V_\gamma2$ chain is irrelevant for the fate of these T cells subsets. Although it is clear that a $\gamma\delta$ TCR composed of a $V_\gamma2$ chain is not expressed at detectable levels on the cell surface of $\alpha\beta$ T cells (14) one cannot exclude the possibility that the $V_\gamma2$–$J_\gamma2$–$C_\gamma2$ chain is co-expressed with other V_γ chains on the surface of some $\gamma\delta$ T cells. Lack of isotype exclusion of γ chains at the cell surface level has been reported in some $\gamma\delta$ T cell lines and hybridomas in which $V_\gamma1$–$C_\gamma4$ chains are co-expressed with either $V_\gamma2$–$J_\gamma2$ or $V_\gamma4$–$J_\gamma1$ chains (27–29). However, in these cases the expression of the $V_\gamma1$–$J_\gamma4$ chain was much higher than the expression of the other γ chains. Furthermore, double staining analysis with available γ-chain-specific antibodies in normal $\gamma\delta$ T cells shows that co-expression of detectable levels of two different γ chains in the same cell occurs rarely, if at all (unpublished results). It appears, therefore, that expression of functionally rearranged $V_\gamma2$ transcripts in $\gamma\delta$ T cells does not generally correlate with detectable levels of expression of the $V_\gamma2$ chain on the cell surface. Nevertheless, in a few instances, $\gamma\delta$ T cell lines and clones expressing the $V_\gamma2$–$J_\gamma2$ chain as part of their TCR have been reported (30,31), suggesting that a relatively small subset of $\gamma\delta$ T cells in normal mice expresses the $V_\gamma2$ chain. Likewise, according to our data a small fraction of the $\gamma\delta$ T cells in B6 mice may express the $V_\gamma3$ chain. The fact that the $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ gene has been deleted in most of the laboratory mouse strains and is non-functional in others suggests that there has been a selection against this gene. It is possible that the B6 $V_\gamma3$–$J_\gamma3$–$C_\gamma3$ gene has been subject to a different form of inactivation and that a similar case exists for the $V_\gamma2$–$J_\gamma2$–$C_\gamma2$ gene in general. A common feature of these two genes is the lack of glycosylation sites, that might interfere with the folding, transport or half-life of the molecule in the cytosol.

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