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Oligoclonality of Rat Intestinal Intraepithelial T Lymphocytes: Overlapping TCR β-Chain Repertoires in the CD4 Single-Positive and CD4/CD8 Double-Positive Subsets

Lars Helgeland, Finn-Eirik Johansen, Jon O. Utgaard, John T. Vaage, and Per Brandtzaeg

Previous studies in humans and mice have shown that gut intraepithelial lymphocytes (IELs) express oligoclonal TCR β-chain repertoires. These studies have either employed unseparated IEL preparations or focused on the CD8\(^+\) subsets. Here, we have analyzed the TCR β-chain repertoire of small intestinal IELs in PVG rats, in sorted CD4\(^+\) as well as CD8\(^+\) subpopulations, and important differences were noted. CD8\(α\) and CD8\(β\) single-positive (SP) IELs used most V\(β\) genes, but relative V\(β\) usage as determined by quantitative PCR analysis differed markedly between the two subsets and among individual rats. By contrast, CD4\(^+\) IELs showed consistent skewing toward V\(β17\) and V\(β19\); these two genes accounted collectively for more than half the V\(β\) repertoire in the CD4/CD8 double-positive (DP) subset and were likewise predominant in CD4 SP IELs. Complementarity-determining region 3 length displays and TCR sequencing demonstrated oligoclonal expansions in both the CD4\(^+\) and CD8\(^+\) IEL subpopulations. These studies also revealed that the CD4 SP and CD4/CD8 DP IEL subsets expressed overlapping β-chain repertoires. In conclusion, our results show that rat TCR-αβ\(^+\) IELs of both the CD8\(^+\) and CD4\(^+\) subpopulations are oligoclonal. The limited V\(β\) usage and overlapping TCR repertoire expressed by CD4 SP and CD4/CD8 DP cells suggest that these two IEL populations recognize restricted intestinal ligands and are developmentally and functionally related. *The Journal of Immunology, 1999, 162: 2683–2692.

A large population of lymphocytes is located between the villous epithelial cells in the small intestinal mucosa. These intraepithelial lymphocytes (IELs)\(^1\) consist of several different T cell subsets, some of which are found almost exclusively in this compartment (1–4). The presence of unique CD8\(α\) (TCR-γδ or TCR-αβ) single-positive (SP) and CD4/CD8 double-positive (DP) subpopulations has been proposed to reflect distinct origin and maturation pathways for IELs. Experimental studies in mice have suggested that such unique intraepithelial T cells as well as those expressing conventional CD8\(α\) and CD4 SP phenotypes are extrathymically derived (2, 5), but the thymic impact and the developmental relationship between the different subpopulations remain unresolved (1, 6). In the rat we have recently shown that CD8\(α\) and CD8\(α\) SP IELs populate the intestinal epithelium sequentially during the neonatal period in both the γδ and αβ T lineages, and that these CD8 populations dominate in young adult rats (7). CD4 SP and CD4/CD8 DP IELs arrive later and accumulate with age, constituting substantial numbers in aged rats (8–10). These IELs probably depend on the gut flora, as they are not found in germfree animals (10, 11). The thymus appears to be critical for normal development and maturation of all CD3\(^+\) IEL subpopulations, because athymic nude rats contain few T cells with variably distorted phenotypes that are induced by the gut microflora only late in life (7).

The role of intraepithelial T cells in mucosal immunity is poorly understood. In contrast to T cells in peripheral lymphoid organs, the functions of IELs appear to be conducted by a limited number of clones. Human TCR-αβ\(^+\) IELs have been shown to express an oligoclonal receptor repertoire that differs among individuals (12–14). Moreover, a murine study reported that the β-chain repertoire differs even among genetically identical mice from the same litter. This finding was taken as evidence for a random and individually imprinted repertoire selection (15). Most of these studies have focused on CD8\(^+\) IELs, and little is known about the CD4\(^+\) subsets. Indirect evidence in humans has suggested that CD4\(^+\) IELs are oligoclonal, as deduced from observations in unseparated colonic IELs that are relatively enriched in this subset (14, 16).

Rat IELs have not been subjected to detailed repertoire analysis, but previously we investigated V region usage of TCR-αβ IELs in AGUS rats with a small panel of V\(β\) specific mAbs (11). V\(β\) expression was found to be relatively unbiased and did not provide an indication of oligoclonality. However, with the same mAbs we recently obtained results compatible with oligoclonal expansions in PVB rats (unpublished observations), and the present report explores the β-chain repertoire of sorted IEL subpopulations in this strain. Here, we show that although both the CD4\(^+\) and CD8\(^+\) subpopulations are oligoclonal, there are important differences between them. While CD8\(α\) and CD8\(α\) SP IELs displayed variable β-chain repertoires in different animals, both CD4 SP and CD4/CD8 DP IELs showed a consistent overexpression of V\(β17\) and V\(β19\). Furthermore, CD4 SP and CD4/CD8 DP IELs expressed overlapping β-chain repertoires. These findings have significant implications for our understanding of the development and function of IELs.
Materials and Methods

Animals

Rats of the PVG strain were bred at the animal facility of the Department of Anatomy, University of Oslo (Oslo, Norway). They were reared under conventional conditions and routinely screened for common rat pathogens. Female rats were used at 5–6 mo of age.

Cell preparation and subpopulation separation

IELs were isolated as previously described in detail (7, 11). Briefly, the small intestine was flushed free of fecal content, inventoried, and cut into 3–4 cm long pieces without disruption of the Peyer’s patches. After incubation twice for 15 min each time at 37°C in PBS with 0.1% EDTA, 0.3 mg/ml DTT, 5% FCS, and 1% antibiotics, cell suspensions were further enriched for IELs by centrifugation (30 min, 600 χ g) on a one-step density gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway). With this procedure, contamination with lamina propria lymphocytes that have a high content of B lymphocytes was estimated to be <2% (7, 11). Also, the integrity of the remaining lamina propria was verified histologically (data not shown). In some experiments lamina propria lymphocytes (LPLs) were isolated. IELs were first removed, as described above, except for the exclusion of Peyer’s patches before EDTA/DTT incubation and the inclusion of an additional 15-min incubation at 37°C. LPLs were subsequently released by mechanical teasing as originally described by Lysom and Bruton (17), followed by Lymphoprep centrifugation. Lymph node lymphocytes (LNLs) were obtained from the mesenteric lymph nodes.

IEL subpopulations were prepared by FACS-sorting (FACS Vantage, BD Biosciences, Mountain View, CA). For preparation of CD8 SP IEL subsets, cells were stained with phycoerythrin-conjugated anti-CD8a (Serotec, Oxford, U.K.) followed by streptavidin-phycoerythrin (Dako) and were subsequently sorted into CD4 SP (CD4a), TCRβ1 and TCRβ2 (23), in reaction buffer dNTP (0.2 mM), MgCl2 (100 mM), and rRNasin (1 U/ml). Aliquots of 15 χ l were first heated to 95°C for 5 min and then subjected to 26, 30, or 34 cycles in a thermal cycler (Hybaid, Middlesex, U.K.: each cycle was 1 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C). The PCR products, ranging from about 290–410 bp, were electrophoresed in 1% agarose gels and vacuum-blotted onto nylon membranes (Schleicher and Schuell, Dassel, Germany). Membranes were prehybridized in hybridization solution (5 χ SSPE, 2 χ Denhardt’s solution, 0.1% SDS, and 0.2 mg/ml salmon sperm DNA) in a hybridization oven for 3 h at 42°C. Hybridization was performed at 42°C overnight with an internal antisense Cβ probe (5’-TGGGTGGAGTACCGGTTTCCAG-3’) that had been end labeled with 106 χ P (1 ϊ 106 cpn/ml of hybridization solution), followed by washing in 1 χ SSPE/1% SDS for 30 min at room temperature and finally in 0.2 χ SSPE/1% SDS for 30 min at 35°C. Quantitation was performed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Initial analyses in LNLs showed that under these conditions, PCR amplification was in the linear phase between 26–32 cycles and reached a plateau at 35–37 cycles. Representation of individual Vβ families was expressed as a percentage of the total signal obtained with all the Vβ primers and was determined at the lowest number of cycles yielding a clear signal above background (usually 26 cycles).

Analysis of TCR Vβ usage by semiquantitative PCR

To determine Vβ usage, cDNA was PCR amplified in 22 reactions corresponding to all known rat TCRBV subfamilies (Table I). Each 50-μl reaction contained approximately 1000 cell equivalents of cDNA, one of the Vβ primers (0.6 μM) and a Cβ primer (0.6 μM; 5’-TCCTGCTTCTGGTGGCAATG-3’), in reaction buffer dNTP (0.2 mM), MgCl2 (1.5 mM), Taq DNA polymerase (0.6 U), and enzyme buffer (Roche Molecular Systems, Branchburg, NJ). Aliquots of 15 μl were first heated to 95°C for 5 min and then subjected to 26, 30, or 34 cycles in a thermal cycler (Hybaid, Middlesex, U.K.: each cycle was 1 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C). The PCR products, ranging from about 290–410 bp, were electrophoresed in 1% agarose gels and vacuum-blotted onto nylon membranes (Schleicher and Schuell, Dassel, Germany). Membranes were prehybridized in hybridization solution (5 χ SSPE, 2 χ Denhardt’s solution, 0.1% SDS, and 0.2 mg/ml salmon sperm DNA) in a hybridization oven for 3 h at 42°C. Hybridization was performed at 42°C overnight with an internal antisense Cβ probe (5’-TGGGTGGAGTACCGGTTTCCAG-3’) that had been end labeled with 106 χ P (1 ϊ 106 cpn/ml of hybridization solution), followed by washing in 1 χ SSPE/1% SDS for 30 min at room temperature and finally in 0.2 χ SSPE/1% SDS for 30 min at 35°C. Quantitation was performed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Initial analyses in LNLs showed that under these conditions, PCR amplification was in the linear phase between 26–32 cycles and reached a plateau at 35–37 cycles. Representation of individual Vβ families was expressed as a percentage of the total signal obtained with all the Vβ primers and was determined at the lowest number of cycles yielding a clear signal above background (usually 26 cycles).

Analysis of CDR3 lengths

TCRs were analyzed for distribution of V region lengths, resulting from the variable deletion and addition of nongermline-encoded nucleotides in the complementarity-determining region 3 (CDR3) during V-(D)-J joining. First, expressed Vβ genes were PCR-amplified from ≈1000 cell equivalents of cDNA in 25-μl reactions for 34 cycles, using Vβ- and Cβ-specific primers under the conditions described above. PCR products were subsequently diluted 1:40 in 10-μl aliquots containing reaction buffer as described above and subjected to a 30-cycle run-off reaction with a 32P end-labeled antisense Cβ primer
FIGURE 1. Vβ gene expression in CD8 SP IELs and LNLs. Southern blots of RT-PCR-amplified Vβ genes from CD8αα⁺ IELs (A), CD8αβ⁺ IELs (B) and CD8⁺ LNLs (C) in rat 1. cDNA was amplified with Vβ- and Cβ-specific primers (26 cycles for A and C, 30 cycles for B), blotted onto nylon membranes, and hybridized with a 3²P end-labeled internal Cβ probe. Molecular size markers are indicated in base pairs.

(0.13 μM, 5’-ATGGCTCAAACAAAGAGAC-3’). Each cycle was 1 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C. Labeled primer extension products were heat denatured and separated on 5% denaturing polyacrylamide gels.

To visualize the distribution of CDR3 lengths in individual Vβ-Jβ combinations, antisense primers specific for rat TCRBJ gene segments were designed, avoiding the region upstream of the third residue 5’ to the conserved phenylalanine (Table 1). A similar approach has previously been described in the mouse (27). Amplified PCR products were diluted 1/400 (to minimize intra-PCR recombinations) in 10-μl reactions containing 0.13 μM of a 3²P end-labeled antisense Jβ primer and reaction buffer as described above and were subjected to 40 cycles of primer extension (each cycle was 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C).

Cloning and sequencing of PCR products
To determine the DNA sequence of expressed Vβ-chains, PCR products amplified with a set of Vβ- and Cβ-specific primers were either subcloned directly or after a nested PCR reaction where the Cβ primer was substituted with a Jβ-specific primer. PCR products were blunt ended with Pfu DNA polymerase, ligated into the SrfI site of pcR-Script (Stratagene, La Jolla, CA), and transformed into Epicurian coli cells according to the manufacturer’s instructions. Color-selected colonies were screened for insert of the correct size by PCR followed by agarose gel electrophoresis. Miniprep DNA was prepared by standard methods and was sequenced at Medigenomix (Martinsried, Germany) with the plasmid-specific oligonucleotide M13.

Results
Variable skewing of the Vβ repertoire in CD8αα and CD8αβ SP IELs
IELs from 6-mo-old PVG rats were isolated and analyzed for β-chain repertoire, since at this age most IELs express the TCR-αβ (28). In accordance with previous data (7–10, 28, 29), 55–65% of IELs were CD8 SP, with the ratio between CD8αβ⁺ and CD8αα⁺ cells being about 2:1. These two populations were sorted to approximately 99% purity while excluding CD4⁺ IELs. They were then subjected to Vβ repertoire analysis by semiquantitative PCR. Fig. 1 shows Southern blots of RT-PCR-amplified Vβ genes from rat 1. All Vβ families were expressed by both CD8 SP IEL populations as well as by CD8⁺ LNLs, with the exception of Vβ7. However, some Vβ genes were expressed at relatively much higher and others at relatively much lower levels in IELs than in LNLs. Differences in Vβ expression between CD8αα⁺ and CD8αβ⁺ IEL were also noted, and these findings were substantiated by subsequent quantitation in four individual rats.

Vβ usage in the CD8αα⁺ and CD8αβ⁺ IEL subsets was not correlated and showed a striking variation between rats, contrasting with the high degree of consistency observed for CD8⁺ LNLs (Fig. 2). For example, in rat 1, 18 and 19% of CD8αα⁺ IELs expressed Vβ13 and Vβ15, respectively, while the corresponding figures were 3 and 2% for CD8αβ⁺ IELs, and 3 and 4% for CD8⁺ LNLs. In rat 2, 18 and 14% of CD8αβ⁺ IELs expressed Vβ13 and Vβ19, respectively, while the numbers were only 2 and 4% for CD8αα⁺ IELs, and 3 and 7% for CD8⁺ LNLs. A few examples of coincident Vβ expansion in the two IEL subsets were also observed, e.g. Vβ20 in rat 1. Further, Vβ16 appeared to be generally increased in CD8αα⁺ IELs. However, taken together the data indicated that Vβ usage differed between the CD8αβ⁺ and CD8αα⁺ IEL subsets and between individual rats.

CD8αα and CD8αβ SP IELs are oligoclonal
Clonal status of CD8αα and CD8αβ SP IELs was investigated by analyses of CDR3 length distributions, which were visualized by
subjecting RT-PCR-amplified TCR templates to run-off PCR reactions with a radiolabeled Cβ primer, followed by separation on PAGE. Ten Vβ subfamilies were selected for analysis. As expected, LNLs displayed multiple CDR3 lengths, the run-off reactions yielded between 6 and 10 bands (Fig. 3A). The spacing between the bands indicated that they were separated by three bases, corresponding to in-frame transcripts. Band intensities also were distributed evenly, typical of a polyclonal repertoire. This was not the case for IELs, where only one or a few prominent bands were visible for several Vβ families, indicative of oligoclonal expansions. In a given IEL population, over-represented Vβ genes yielded the most distinct oligoclonal patterns, as exemplified by

Table II. Sequences of Vβ-Dβ-Jβ junctions from PCR-cloned Vβ-Cβ transcripts from CD8⁺ IELs and LNLs

<table>
<thead>
<tr>
<th>Population</th>
<th>Vβ</th>
<th>N</th>
<th>Dβ</th>
<th>Jβ</th>
<th>Gene Segments</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8αa IEL</td>
<td>TGGTGTGCATTGG</td>
<td>AAGGGGCGG</td>
<td>ACAGACAAGATATAT</td>
<td>Vβ15-Dβ2-Jβ2.3</td>
<td>9/9</td>
<td></td>
</tr>
<tr>
<td>CD8αβ IEL</td>
<td>TGGTCCAGTGGCCAAG</td>
<td>GAGACTACC</td>
<td>TCAAAACACCTTGTC</td>
<td>Vβ4-Dβ2-Jβ2.4</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>CD8 LNL</td>
<td>TGGTGGCAGTTAGGC</td>
<td>CTCGCCACAGAGGGACGGC</td>
<td>TATGACTACACC</td>
<td>Vβ4-Dβ1-Jβ1.6</td>
<td>1/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGGTGGCAGTGGCCAAG</td>
<td>GGGGGGGTGA</td>
<td>ACAGACAAGATATAT</td>
<td>Vβ4-Dβ1-Jβ1.2</td>
<td>1/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGGTGGCAGTGGCCAAG</td>
<td>TGGGGGGCAC</td>
<td>ACAGACAAGATATAT</td>
<td>Vβ4-Dβ1-Jβ1.6</td>
<td>1/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGGTGGCAGTGGCCAAG</td>
<td>AGGCCCGT</td>
<td>TATGACTACACC</td>
<td>Vβ4-Dβ1-Jβ1.2</td>
<td>1/9</td>
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<tr>
<td></td>
<td>TGGTGGCAGTGGCCAAG</td>
<td>TGGGGACAGTG</td>
<td>TATGACTACACC</td>
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<tr>
<td></td>
<td>TGGTGGCAGTGGCCAAG</td>
<td>TGGGGACAGTG</td>
<td>TATGACTACACC</td>
<td>Vβ4-Dβ1-Jβ1.2</td>
<td>1/9</td>
<td></td>
</tr>
</tbody>
</table>

a cDNA from the populations indicated in rat 1 was PCR-amplified with Vβ-Cβ primers and cloned into pCR Script, and multiple isolates were sequenced. All isolates represented functional in-frame transcripts. Dβ sequences are underlined; N-region nucleotides were defined as any nucleotide that could not have been accounted for by any of the germline sequences, and possible P additions are not distinguished. Analysis of CDR3 length distribution of the same PCR products is represented in Fig. 4.

b The exact 3’ end genomic sequences of rat Vβ gene segments and possible allelic variations are not known; the 3’ end was assigned based on the compilation of cDNA sequences in this study and apparent homology to murine germline sequences.
Vβ13 and Vβ14 in rat 1. In the CD8αα+ IEL population, Vβ13 accounted for 18% of the total Vβ repertoire and comprised only one dominant band in the CDR3 length display. In the CD8αβ+ IEL population, Vβ13 constituted only 3% of the total repertoire but apparently contained TCRs with different CDR3 lengths (see Figs. 2 and 3A, respectively). The reverse pattern was seen for Vβ14.

Some Vβ families, like Vβ16, yielded rather normally distributed bands in Cβ run-off reactions in both CD8αα+ and CD8αβ+ IELs (Fig. 3A). CDR3 length distribution was therefore investigated in closer detail in individual Vβ-Jβ combinations using primers specific for different Jβ segments (Table I). In Fig. 3, B and C, are shown run-off reactions with the Jβ1.2 and Jβ2.3 primers in 9 of the 10 Vβ families in rat 1. Only one or a few unevenly distributed bands were observed in CD8αα+ and CD8αβ+ IELs, or the Jβ segment was not expressed. Even when multiple bands appeared in the Cβ run-off reaction, as was the case for Vβ16, one dominant band was observed for both Jβ1.2 and Jβ2.3 (Fig. 3, B and C). It could therefore be concluded for CD8 SP IELs that the 10 Vβ families analyzed expressed oligoclonal repertoires. For CD8+ LNLs, on the other hand, multiple CDR3 lengths were generally observed, as expected for a polyclonal repertoire. However, some Jβ primers yielded patterns suggestive of oligoclonality also in CD8+, but not CD4+ LNLs (see below). The reason for this is unclear, but possibly reflect oligoclonal expansions of CD8+ T cells in the peripheral lymphoid organs of aged rats, as has previously been observed in mice (31).

To confirm that dominant bands in the CDR3 length displays corresponded to mono- or oligoclonal populations, TCR transcripts from rat 1 were PCR amplified, cloned, and sequenced (Table II). In CD8αα+ IELs, all nine randomly isolated Vβ15-Cβ clones contained the same junctional sequence paired with Jβ2.3. This indicated the presence of one dominant Vβ15-expressing T cell clone and was consistent with the finding of only one band in the Vβ15- Jβ2.3 run-off reaction (Fig. 3C). Likewise, in CD8αβ+ IELs 7/7 sequenced Vβ4-Cβ transcripts expressed Jβ2.4 and showed identical junctional sequences (Table II). In CD8+ LNLs, on the other hand, the same primer pair yielded different junctional sequences in all isolates, as expected for a polyclonal population.

Marked skewing toward Vβ17 and Vβ19 in CD4 SP and CD4/CD8 DP IELs, but not in CD4 SP LPLs

Detailed TCR repertoire analysis in CD4 SP and CD4/CD8 DP IELs has not been reported. In accordance with previous rat data (7–10, 28), 20–30% of IELs expressed CD4, with approximately 25% being CD4 SP and 75% being CD4/CD8 DP. The latter subset expresses only the α-chain, not the β-chain, of CD8. The CD4 SP and CD4/CD8 DP IEL subsets were sorted to approximately 99% purity and subjected to Vβ repertoire analysis by semiquantitative PCR. Fig. 4 shows representative Southern blots, in rat 5. Both the Vβ17 and Vβ19 families were markedly over-represented in CD4+ IELs. However, whereas CD4 SP IELs used all Vβ genes, except for Vβ7, this was not the case for CD4/CD8 DP IELs. In these cells, usage of some Vβ genes was negligible.
Quantitation of Vβ usage in four individual rats (Fig. 5) confirmed the marked skewing toward Vβ17 and Vβ19 in both CD4 SP and CD4/CD8 DP IELs. Skewing was especially pronounced in the CD4/CD8 DP population, where Vβ17 and Vβ19 together accounted for 50–60% of the total Vβ repertoire. In the CD4 SP subset they covered 20–35%. Over-representation of Vβ17 was a consistent finding, while Vβ19 was increased in three of four rats in CD4/CD8 DP IELs. Additional Vβ usage was mainly from Vβ genes 4, 8.2, 15, and 16. These results contrasted data obtained with CD4+ LNLs as well as with CD8+ SP IELs, where Vβ17 and Vβ19 never dominated (see above). Thus, Vβ usage in CD4+ IELs was much more restricted.

The Vβ repertoire in CD4+ IELs was also determined in two young adult rats (3.5 mo of age). Previous studies had shown that this was not long after the appearance of CD4+ cells, particularly the CD4/CD8 DP subset, in the gut epithelium under the impact of the gut microflora (10, 11, 28). Even at this early stage, Vβ17 and Vβ19 were markedly over-represented, together covering 40–50% of the Vβ repertoire in CD4/CD8 DP IELs and 24–36% in CD4 SP IELs (in the former subset Vβ19 was over-represented in only one of the two rats examined; Fig. 6).

Other studies have suggested a close developmental and functional relationship between IELs and LPLs (13, 32). It was therefore of interest to determine whether the Vβ repertoire was skewed in CD4+ LPLs, as in CD4+ IELs. In agreement with a previous rat study (17), CD4+ T cells were more frequent than CD8+ T cells in the lamina propria, and many B cells were present (30–35% sIg+). CD4 SP LPLs were purified by FACS and assessed for Vβ usage. As shown in Fig. 7, there was no significant skewing of the Vβ repertoire in CD4+ LPLs. This was in line with a previous study that concluded that Vβ usage was unbiased in human CD4+ LPLs (33). Thus, it appeared that this population differed markedly from its IEL counterpart, and that Vβ skewing was a hallmark of mucosal CD4+ T cells residing in the epithelium.

CD4 SP and CD4/CD8 DP IELs show overlapping oligoclonal TCR β-chain repertoires

The results described above suggested that CD4+ IELs are oligoclonal, and this was confirmed by CDR3 length displays. In rat 5, analysis focused on the five Vβ families 4, 15, 16, 17, and 19, which were well represented in the CD4/CD8 DP IEL subpopulation (see Fig. 5). As shown in Fig. 8, fewer bands were generally present for CD4/CD8 DP than for CD4 SP IELs in both the Cβ and Jβ run-off reactions; this further confirmed that the repertoire was more restricted in the former IEL subpopulation. Importantly, the data also suggested that CD4 SP and CD4/CD8 DP IELs expressed overlapping β-chain repertoires, because the same dominant CDR3 lengths were usually present in both. This can be exemplified for the Vβ17 and Vβ19 families. Nested PCR amplifications showed that Vβ17 was mainly coexpressed with Jβ genes 1.4 and 2.3 and Vβ19 was mainly coexpressed with Jβ1.4 in CD4/CD8 DP IELs from rat 5 (data not shown). CDR3 length distribution with these two Jβ genes showed bands of the same length in CD4 SP vs CD4/CD8 DP IELs (Fig. 8, B and C). Analogous results were obtained in three other rats (no. 6–8) for Vβ17 and Vβ19 in combination with Jβ1.4 and Jβ2.7 (Fig. 9).

TCR transcripts from both CD4+ IEL subsets in rat 5 were subjected to sequence analysis (Table III). Vβ19-Jβ1.4 isolates from the CD4/CD8 DP subset contained two different junctional sequences. However, they were of the same CDR3 length, which explained why there was only one band in the CDR3 length display in the Vβ19-Jβ1.4 and Vβ19-Cβ combinations (as described above, Vβ19 was mainly paired with Jβ1.4 in this animal; see Fig. 8). To confirm that this was indeed the case, the band in the Cβ run-off reaction (Fig. 8A) was excised from the gel, reamplified with Vβ19 and Cβ primers, and sequenced. The result revealed the same two Vβ19-Jβ1.4 clones in a similar distribution as that described above (data not shown). More importantly, however, the same two sequences were obtained in CD4 SP IELs, one was predominant (5 of 10) and the other was found in 1 of 10 isolates (Table III). Identical junctional sequences were also obtained in CD4 SP and CD4/CD8 DP IELs in another Vβ-Jβ combination, namely Vβ17-Jβ2.3 (Table III). These results firmly established
that the two CD4+ IEL subsets expressed overlapping β-chain repertoires. It should be noted that no evidence for sharing of junctional sequences between CD8αβ+ and CD8αα+ SP IELs were obtained, not even in the few instances where dominant bands of the same length were observed in the run-off reactions (e.g., as for Vβ15 in Fig. 3C and data not shown).

Interestingly, repertoire bias in CD4/CD8 DP IELs not only included Vβ segments (as Vβ17 and Vβ19) but also certain Jβ segments. This was especially true for Jβ1.4, which showed preferential pairing with Vβ19 in all four rats analyzed (data not shown). The possibility therefore existed that identical TCRs were selected in different animals. Generally, however, CDR3 lengths were not preserved, except in the Vβ19-Jβ1.4 combination, in which one prominent band of the same length was observed in all four rats (see Fig. 9B). However, sequencing revealed that Vβ19-Jβ1.4 junctions were different at both nucleotide and amino acid levels (data not shown). Thus, we concluded that the overlapping β-chain repertoire expressed by the two CD4+ IEL subsets were not conserved between rats.

Discussion

This is the first detailed description of the TCR β-chain repertoire of rat IELs. It has confirmed previous observations made in mouse and man that intestinal IELs are oligoclonal. In addition, it has revealed important differences between CD4αβ+ and CD8αβ+ IELs. The CD8αα+ and CD8αβ+ SP populations expressed a repertoire that was characterized by random oligoclonal expansions in individual rats. In contrast, in the same strain the repertoire of CD4 SP and CD4/CD8 DP cells was consistently skewed toward Vβ17 and Vβ19. This, together with the finding of overlapping β-chain repertoires led us to conclude that the two CD4+ IEL populations are closely related.

Previous repertoire studies of IELs in mice and humans have mainly focused on the CD8+ subsets. The majority of human IELs are CD8αβ+ and express TCR repertoires that differ between individuals (12–14). However, little is known about the influence from genetic and environmental factors in shaping the oligoclonal repertoires in humans. This has been addressed in mice by the use...
of inbred animals kept under the same environmental conditions. Those studies analyzed separately the CD8aa- and CD8αβ- IEL populations, but without excluding CD4+ cells. Nevertheless, they indicated that TCR usage of CD8αα and CD8αβ SP IELs was highly variable. No consistent patterns were observed by CDR3 length displays either between the two subsets or among individual animals (15, 34). These data were taken as evidence that the gut epithelium expresses a polyclonal, rather than oligoclonal, TCR repertoire (35), and this may be the case in the rat as well. In keeping with this, reconstitution experiments performed in mice have shown that the first T cells to populate the gut epithelium express complex β-chain repertoires (36). Therefore, it would be interesting to know whether the oligoclonal TCR repertoires observed here in adult rats reflect clonal expansions over a diverse polyclonal background or the presence of only a limited number of clones in the epithelium. However, these alternatives are not easily distinguishable by PCR technology. At any rate, the nature of the Ags and/or restriction elements selecting the CD8 SP IELs subsets is difficult to deduce on the basis of repertoire analysis.

In this study CD4+ IELs were also subjected to separate TCR repertoire analysis. One striking finding was the predominance of Vβ17 and Vβ19 families in both CD4 SP and CD4/CD8 DP IELs. It is tempting to speculate that such Vβ-specific expansions result from interactions with conserved microbial Ag, especially when considering that CD4+ IELs are induced by the microbial flora. Induction is especially pronounced for the CD4/CD8 DP population (10, 11, 37). In line with this, CD4/CD8 DP IELs reportedly respond to heat shock proteins from both mycobacteria (38) and E. coli (39), but only when these bacteria are present in the intestinal lumen. Bacterial superantigens might also conceivably stimulate IELs, e.g., when complexed.

Table III. TCR β-chain junctional sequences from CD4+ IELs and LNLe

<table>
<thead>
<tr>
<th>Population</th>
<th>Vβb</th>
<th>NDβN</th>
<th>Jβ</th>
<th>Frequency</th>
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<tr>
<td>CD4/CD8 DP IEL</td>
<td>Vβ17-TGTGCCACGAGCTCTGT</td>
<td>CCGGCTTA</td>
<td>ACAAGATATAT-3β2.3</td>
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<tr>
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<td>CCGGCTTA</td>
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<tr>
<td>CD4 LNL</td>
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<tr>
<td></td>
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<td>TGGGCTTG</td>
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<td>1/9</td>
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<tr>
<td></td>
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<td>CCAAGACAGGTTG</td>
<td>ACTACAGAAAGATATAT-</td>
<td>1/9</td>
</tr>
<tr>
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<td>TAGA</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>GCCAGCAAT</td>
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<td>1/9</td>
</tr>
<tr>
<td>CD4/CD8 DP IEL</td>
<td>Vβ19-TGTGCACAGCTCTGA</td>
<td>TGA</td>
<td>TTTCCATGAAGAATGTTTT-3β1.4</td>
<td>4/10</td>
</tr>
<tr>
<td></td>
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<td>TTTCCATGAAGAATGTTTT-</td>
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<td>AAAGAATGTTTT-</td>
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<tr>
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<tr>
<td>CD4 LNL</td>
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<td>1/8</td>
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a cDNA from the populations indicated in rat 5 were PCR-amplified with Vβ-Cβ primers and reamplified, substituting the Cβ primer with one of the Jβ primers. The PCR products were cloned into pCR Script and multiple isolates were sequenced. All isolates represented functional in-frame transcripts, except one in CD4/CD8 DP IEL that was out-of-frame (not included). Dβ sequences are underlined; N-nucleotides are not distinguished from possible P-nucleotides. CDR3 length distribution of the same PCR products. NDβN: Nucleotide diversity; Jβ: joined J segment; Frequency: percentage of sequences of a given type.

The exact germline 3’ ends of rat Vβ gene segments are not known; the 3’ end was assigned based on the compilation of cDNA sequences in this study and apparent homology to murine genomic sequences.
with MHC class II molecules on epithelial cells (40). However, as discussed by others (14), superantigen stimulation alone would not expectedly give rise to oligoclonality, but only when combined with Ag-specific recognition events. It is also possible that Vβ skewing reflects recognition of MHC class Ib molecules, e.g., CD1 or the thymus leukemia Ag that are expressed in the gut (41–43). Some evidence to this end has been provided by employing TAP-deficient mice, which contained many CD4/CD8 DP IELs (44). In any case, genetic background apparently influences Vβ skewing in CD4+ IELs. Although preliminary experiments in another strain (AGUS) confirmed that the repertoire was skewed toward certain Vβ genes, particularly in the CD4/CD8 DP subset, these animals did not show overexpression of Vβ17 or Vβ19 as did the PVG rats (unpublished data).

The origin and maturation pathways of IELs remain controversial. According to one theory, CD4/CD8 DP IELs arise from conventional thymus-derived CD4 SP T cells that migrate into the epithelium where they acquire CD8 expression. This possibility is supported by the observation that transfer of CD4 SP peripheral T cells into SCID mice reconstitutes CD4/CD8 DP IELs (45, 46). Here, we have provided strong evidence for such a close relationship between the two CD4-expressing IEL subsets. Analysis of CD3 length distribution as well as sequencing of TCR junctional regions demonstrated that CD4 SP and CD4/CD8 DP IELs express overlapping β-chain repertoires. Our results also showed that the CD4/CD8 DP subset express a considerably more restricted repertoire than do CD4 SP IELs, consistent with continued intraepithelial expansion. On the contrary, our data fit poorly with the hypothesis that CD4 DP IELs are analogous to DP thymocytes that develop into mature CD4+ and CD8+ SP T cells (2). Of all the IEL subpopulations analyzed, the CD4/CD8 DP subset expressed the most restricted β-chain repertoire and was unrelated to the CD8 SP subsets. Therefore, it would be very difficult to view CD4/CD8 DP IELs as precursors for conventional positive and negative selection mechanisms.

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References


