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# Diversity in T-cell receptor γ gene usage in intestinal epithelium

**(intraepithelial lymphocytes/gene sequences/splicing/antigen)**

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**ABSTRACT** **The intraepithelial cells of the murine small intestine include a significant number of CD3$^+$ T cells that use T-cell receptor γ genes rather than T-cell receptor β genes. As with other sites of T-cell receptor γ expression, combinatorial diversity is limited, but there is junctional diversity, and this, together with the specific variable region γ gene segments used, distinguishes γ gene expression in the gut epithelium from that in cells derived from the dermal epithelium. The restriction of productive γ gene expression largely to one V–J–C (V, variable; J, joining; C, constant) gene combination may result from nonproductive joining of other V–J combinations and from productively rearranged genes rendered nonfunctional by incorrect splicing.**

The rearranging T-cell receptor (TCR) γ genes of mice and of humans are contained in relatively small families of variable (V) and constant (C) gene segments that are juxtaposed in T cells by recombination through a limited number of joining (J) gene segments (1–6). Hence, there is a real but limited capacity for combinatorial diversity. In adult humans and mice, lymphocytes isolated from the thymus, spleen, or lymph nodes predominantly express the αβ TCR. However, the γδ TCR is used by a small fraction of peripheral T cells that are mostly double-negative (DN) cells (cells lacking both CD4 and CD8 cell-surface markers) (7–9). There is increased expression of productively rearranged γ genes in murine splenic DN T cells responding to haplotype differences in mixed lymphocyte reactions (MLRs) (10, 11) and splenic DN T cells productively expressing γ genes to encode part of a complete γδ TCR have been cloned *in vitro* from athymic mice after MLR stimulation (12).

In the mouse, the γδ receptor appears at day 14, some 3 days before TCR αβ-bearing DN cells are evident (13–15). Because of this ordered appearance of TCRs in thymic ontogeny, and because utilization of γδ seems to increase as phylogeny is back-tracked (16), the γδ receptor has been termed TCR1 (16, 17).

Five observations, (*i*) the rarity of γδ cells in the circulating immune system, (*ii*) the modulation of γ expression in MLR, (*iii*) the capacity to derive major histocompatibility complex (MHC) class I product-specific TCR1-bearing cells, (*iv*) the low capacity for combinatorial diversity in γ, and (*v*) the discovery of TCR1$^+$ cells in murine epidermis (18–20), led to a hypothesis that TCR1-bearing cells might be the predominant lymphocytes of various epithelia, their role being to recognize common alterations in wounded epithelial cells that may be manifest by the expression in those cells of MHC class I related proteins or "stress" molecules (17). A limited capacity for circulation and cell–cell interaction of intraepithelial lymphocytes (IELs) may be incompatible with extremely high TCR diversity, since it would be difficult to get the appropriate receptor to its ligand. The hypothesis makes several predictions, two of which are (*i*) that various epithelial surfaces contain γδ cells and (*ii*) that there will be restricted diversity in the receptors used. Data presented here bear out both predictions. It is shown that lymphocytes of the murine small intestinal epithelium include CD3$^+$ cells that use a very restricted γ gene repertoire. There are striking differences between γ gene usage in the gut as compared with that in the epidermis.

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## MATERIALS AND METHODS

**Gut IEL Extraction.** BALB/c or C57BL/6 (8–16 weeks old) (Charles River Breeding Laboratories and The Jackson Laboratory) gut IELs were prepared by either of two methods (21, 22). Gradients of Percoll (Pharmacia LKB) [73%–47%–30% in 1× phosphate-buffered saline (PBS) (140 mM sodium chloride/10 mM phosphate)] were spun 20 min (600 × *g*, 4°C). Cells from the 73%–47% interface appeared as small resting lymphocytes, and results of their analysis are presented here.

**Fluorescence-Activated Cell Sorting (FACS) Analysis.** One million cells underwent reaction with monoclonal antibody (mAb) 536 (hamster anti-mouse V,5) from J. Allison (University of California, Berkeley) (23), mAb 2C11 (hamster anti-mouse CD3) from J. Bluestone (24), biotinylated mAb F23.1 (murine anti-$V_\beta$) from M. Bevan (25), or staining buffer, and the incubation continued for 30 min on ice. Cells were washed twice, resuspended in a 1:150 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-hamster IgG (Cappel Laboratories), or a 1:50 dilution of FITC avidin (for F23.1), incubated 30 min on ice, washed three times in cold 1× PBS/0.1% sodium azide, and fixed in 1% paraformaldehyde. Cells were analyzed on a Becton Dickinson FACS IV, using excitation wavelength of 488 nm. Emission was collected between 515 and 545 nm, with $2 \times 10^5$ cells observed for each sample. Cells were gated so that the larger epithelial cells were not sorted.

**Hybridizations *in Situ* and RNA Probes.** Cells were analyzed as described by Jones *et al.* (11). $^{35}$S-labeled RNA probes were synthesized by transcription *in vitro* of linearized pGEMR plasmids (Promega) containing γ gene fragments (Fig. 1). The $C_\beta$ gene probe was a 650-base-pair (bp) *Eco*RV/*Sca* I fragment from cDNA pHDS11 (26).

**cDNA Preparation.** Total RNA (28) was electrophoresed (29) to check integrity by ethidium bromide staining and by Northern hybridization. Ten micrograms was converted to cDNA as described (29) using 120 pmol of $C_\gamma 1$ oligonucleotide (27).

Abbreviations: IEL, intraepithelial lymphocyte; PCR, polymerase chain reaction; TCR, T-cell receptor; V, variable; C, constant; J, joining; DN, double negative; MLR, mixed lymphocyte reaction; MHC, major histocompatibility complex; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.

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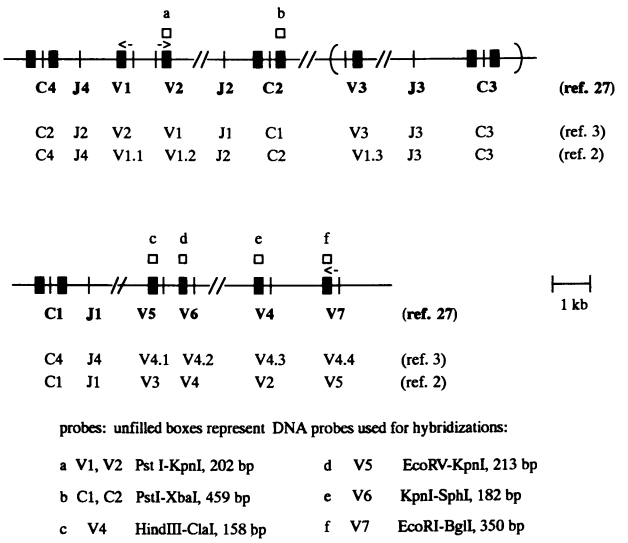


FIG. 1. The murine $\gamma$ gene family (1–3, 27). The nomenclature based on ref. 27 is used in this paper, but alternative nomenclatures are provided. Probes used to detect $\gamma$ gene expression are indicated and were designed based on DNA sequence data (1–3). Solid boxes represent exons. kb, Kilobase.

**Polymerase Chain Reaction (PCR).** cDNA was used in PCRs, according to Roth *et al.* (30). PCR products were blunt-end ligated into pGEM4Z *Sma* I site. Miniprep DNA was sequenced (31), and analyzed on 6–8% acrylamide/7 M urea gels.

## RESULTS

**TCR Usage in IELs.** The epithelium was stripped from the small intestines of adult mice (21, 22), and IELs were separated from epithelial cells according to density. Examination of 5-$\mu$m sections showed that the extraction procedure stripped epithelium from only parts of the gut, generally leaving the underlying lamina propria intact (data not shown).

The IELs underwent reaction with several antisera and were analyzed by FACS. Over 50% of IELs are T cells by the criterion of being $CD3^+$ (32) (Fig. 2). A large percentage were $CD8^+$ (data not shown). Interestingly, there was negligible reactivity to an antiserum that detects the $V_\gamma 5$ gene product (Fig. 2), expressed by T cells of murine epidermis (23, 33). A small portion clearly reacted with antibody F23.1 that detects a subset of $TCR2^+$ cells (data not shown). Therefore, TCR2-bearing cells are present in IEL preparations.

TCR usage in freshly derived IELs was tested by hybridization *in situ* of TCR gene probes (11, 34). TCR gene expression was detected in a significant percentage of IELs

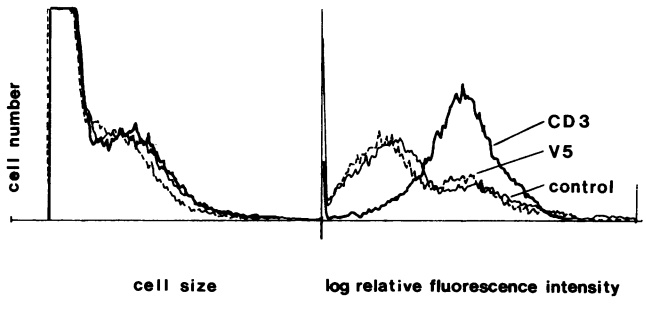


FIG. 2. FACS analysis of BALB/c gut IELs. IELs were incubated with anti-$V_\gamma 5$, anti-CD3 or staining buffer, followed by incubation with FITC rabbit anti-hamster IgG. Size plot of entire 47%/73% fraction is shown on the left; only the small cells were sorted and are represented in the fluorescence plot. The $x$ axis for fluorescence represents 3.8 log units.

Table 1. Analysis *in situ* of $\gamma$ gene segment usage

| Gene segment | % lymphocytes positive | |
|---|---|---|
| | Antisense probe | Sense probe |
| V2, V1 | <0.5 | 0 |
| V4 | 2.6 | 0 |
| V5 | 1 | <0.5 |
| V6 | 2.5 | 0 |
| $C_\gamma 1$ and 2 | 17.2 | <0.5 |
| $C_\beta 1$ and 2 | 0.3 | 0 |

C57BL/6 IEL cytospins were hybridized with antisense RNA probes, and estimates of percent lymphocytes positive for each probe were determined by microscopic examination. Parallel samples were hybridized with sense probes as controls. Percentages represent averages of two independent IEL preparations, with three cytospin slides per probe per preparation. Each slide contained $10^4$–$10^5$ cells.

(Table 1; Fig. 3). More cells reacted with antisense probes to $C_\gamma$ (detects $C_\gamma 1$ and -2) than with antisense probes to TCR $C_\beta$ (probe detects $C_\beta 1$ and -2). Cells did not react with numerous sense probes (Table 1). The percentage of $\gamma^+$ cells increased after sorting for CD3, indicating them to be TCR-expressing T cells. Therefore, of the IELs for which TCR gene usage can be deduced by hybridization *in situ*, most are TCR1-expressing T cells, by virtue of being $CD3^+$, $\gamma RNA^+$, $\beta RNA^-$. Similar results were obtained in both BALB/c and C57.BL/6 mice.

**Restricted $\gamma$ Gene Expression.** In sites of $\gamma$ gene expression in adult mice, highly restricted gene segment usage has been apparent (11, 33). To examine the situation in murine intestinal IELs, probes individually reactive to V1 and V2, V4, V5, and V6 (Fig. 1) were hybridized *in situ* to lymphocytes derived from several gut preparations (Fig. 3; Table 1). There was little hybridization to either V2 or V5, the latter being consistent with the failure of IELs to react with anti-V5 antiserum (Fig. 2). By contrast, there was consistent hybridization to V4 and to V6 probes. However, the combined utilization of all of Vs tested accounts for less than one half of the cells expressing $C_\gamma$ RNA. Hence, it seemed possible that use was being made of the recently described V7 gene, for which a probe was not at the time available. Therefore, a unique sequence oligonucleotide to V7 was made that might prime amplification of $V_\gamma 7$ RNA by PCR (35, 36).

IEL $C_\gamma 1$ RNA was converted to cDNA by reverse transcription primed with a $C_\gamma 1$ antisense oligonucleotide, and amplification of the cDNA was attempted by using sense V4, V6, and V7 oligonucleotides (Table 2). The results using V7 and V6 primers are shown in Fig. 4. The amplification using V6 independently corroborates the data from hybridization *in situ*. However, the amplified fragment of expected size is only seen after Southern blotting and hybridization to a further V6 probe. By contrast, PCR products of expected sizes directed by V7 and V4 were visible after ethidium bromide staining of the gel (e.g., see Fig. 4*a*). The identity of these fragments was confirmed by cloning and sequencing: hence, V7 is abundantly used in IELs.

**Productive $V_\gamma$ Gene Expression Includes Junctional Diversity.** The sequences (Fig. 5) show entirely productive V7–J1 joining. Productively rearranged V7 genes have not been reported before. The molecular generation of diversity follows exactly the pattern seen in V2–J2 $\gamma$ joins from alloactivated T cells (11), and it is similar to that seen in analyses of other TCR genes (37): there is high variability in the site of breakage in the V gene segment, the J gene segment breakage site being more conserved, and there is variable template-independent nucleotide insertion. Despite the different sequences used, there is frequently (6/9) incorporation of an aromatic amino acid residue at the join.

**$V_\gamma 4$ Expression.** PCR indicates a substantial amount of V4 RNA in the IEL preparation. V4 sequences were diverse but

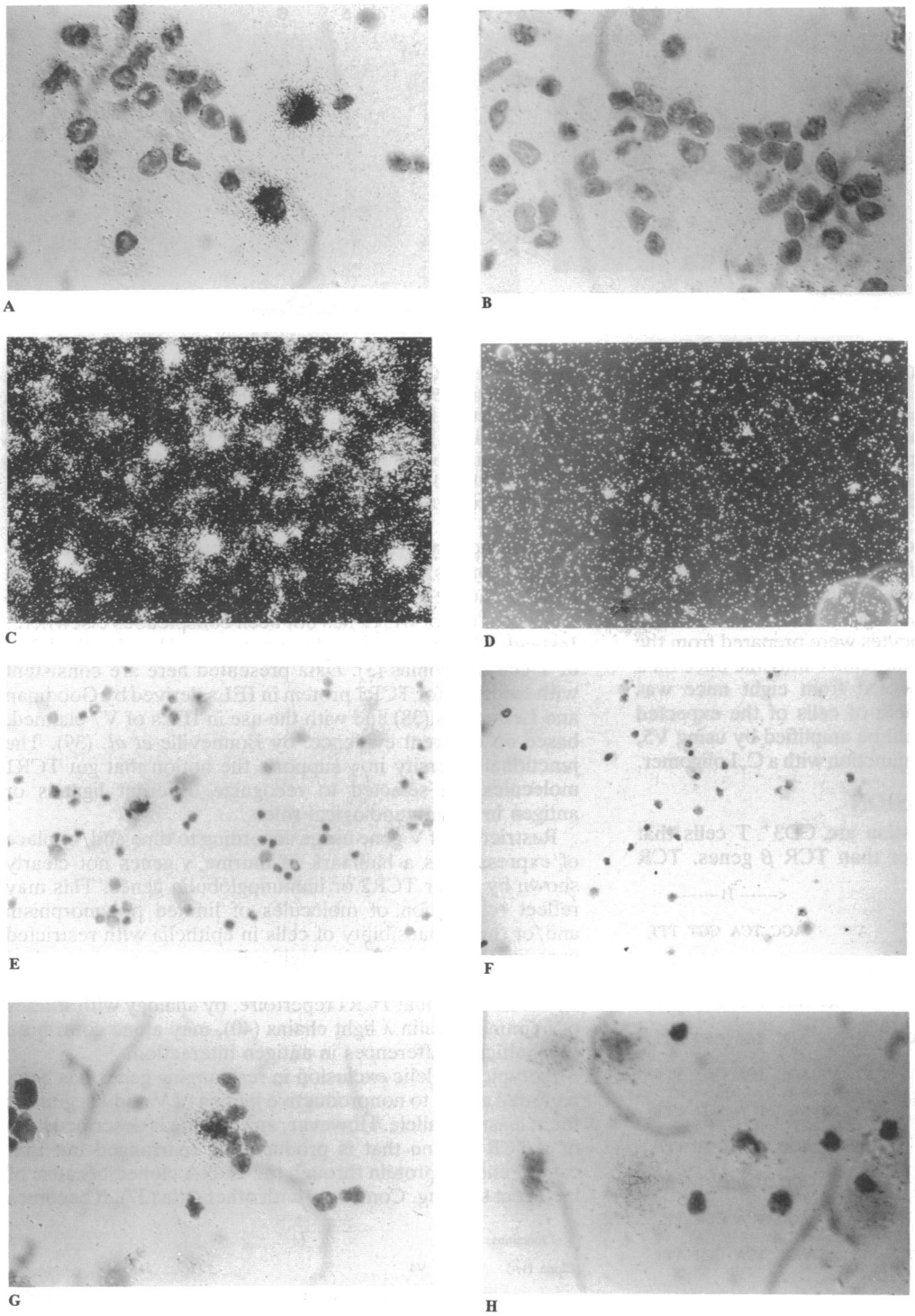


FIG. 3. Micrographs of hybridizations *in situ* of BALB/c IEL cytospins. (*A* and *B*) Bright field. (*A*) $C_\gamma 1$ and -2, antisense. (*B*) $C_\gamma 1$ and -2, sense. (*C* and *D*) Dark field. (*C*) $C_\gamma 1$ and -2, antisense. (*D*) $C_\gamma 1$ and -2 sense. (*E–H*) Bright field. (*E*) $C_\beta 1$ and -2, antisense. (*F*) $C_\beta$, sense. (*G*) $V_\gamma 6$, antisense. (*H*) $V_\gamma 6$, sense. (*A, B, G,* and *H*, ×240; *C–F*, ×60.)

quite different from V7 sequences. First, among four independently derived clones, the same out-of-frame join occurred twice (clones 5 and 10; Fig. 6), formed by ligation of cleaved V and J segments, without N nucleotides. A similar situation occurred in a previously reported nonproductive V4–J1 join (2). The J segment is cleaved 10 nucleotides downstream of its 5′ end, in striking contrast to the conservation of the 5′ end of J characteristic of in-frame γ genes. Two other V4 clones sequenced were both joined productively. One clone (clone 3, Fig. 6) has an orthodox V–J join and can presumably encode protein. Hence, in the gut, there may be some use of $V_\gamma 4$-based TCR1 molecules. By contrast, the other productively rearranged clone (clone 1) could not encode protein, because it is derived from improperly spliced RNA. Thirteen nucleotides of extra exon are present between the 3′ J gene segment splice donor and the 5′ C gene segment splice acceptor. This splice was previously reported in two of seven cases of abortively rearranged γ mRNA from fetal thymus (27); in the case described here, it may represent an alternatively spliced form of an otherwise productively utilized gene. It is generally rare

Table 2. Oligonucleotides used to drive PCR amplification of TCR γ RNA

| | |
|---|---|
| $C_\gamma 1$ | 5′-AAATGTCTGCATCAAGTCT-3′ |
| V4 | 5′-CTTGCAACCCCTACCCATAT-3′ |
| V5 | 5′-CCGCTTGGAAATTGATGAGA-3′ |
| V6 | 5′-AGAGGAAAGGAAATACGGC-3′ |
| V7 | 5′-AACTTCGTCAGTTCCACAAC-3′ |

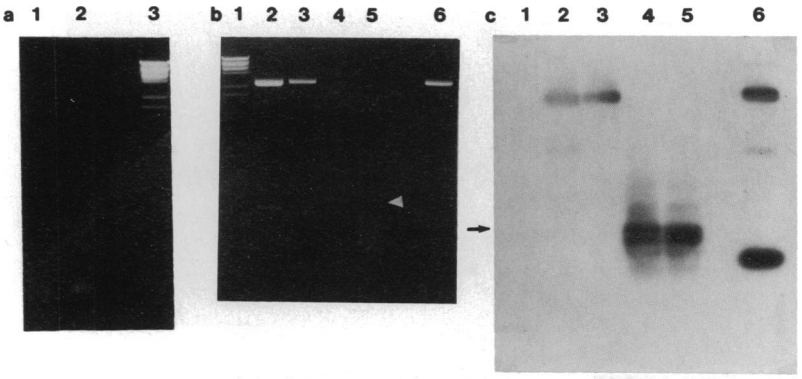


FIG. 4. PCR product analysis. (*a*) Ethidium bromide-stained 2% agarose gel. Lane 1, 20 μl of V5–C1-primed BALB/c *nu/nu* IEL cDNA; no visible 180-bp product. Lane 2: 20 μl of V7–C1-primed C57BL/6 IEL cDNA; visible 250-bp band. Lane 3, λ *Bst*EII, 800 ng. (*b*) Ethidium bromide-stained 2% agarose gel. Lane 1, λ *Bst*EII, 800 ng. Lane 2, V7 *Eco*RI/Bgl I 330 bp from cDNA, cloned into pGEM3Z (*Eco*RI/*Hin*dIII digest). Lane 3, V7–C1 PCR product, 250 bp, cloned into pGEM4Z (*Eco*RI/*Hin*dIII digest). Lanes 4 and 5, 20 μl of V6–C1-primed C57BL/6 cDNA (two cDNA preparations); no visible bands; white arrowhead indicates approximate expected size, 350 bp. Lane 6, V6 *Sph* I/*Kpn* I 182-bp cDNA, cloned into pGEM2 (*Eco*RI digest). (*c*) Southern blot of gel *b*, probed with $^{32}$P-labeled oligonucleotide primed V6 182-bp *Eco*RI fragment. A doublet of V6-specific PCR product of approximately the correct size is evident (black arrow).

for improperly spliced mRNA to be cloned at high frequency and striking that the same aberrant J1–C1 splice was never detected in the V7–J1–C1 clones. Hence, incorrect splicing may contribute to restricted $V_\gamma$ usage.

**Thymic Dependence.** Lymphocytes were prepared from the small intestinal epithelium of adult female athymic mice on a BALB/c background. RNA pooled from eight mice was examined. There was a high yield of cells of the expected density, but no γ sequences could be amplified by using V5, V6, or V7 oligonucleotides in conjunction with a $C_\gamma$1 oligomer.

## DISCUSSION

IELs of the murine small intestine are CD3$^+$ T cells that frequently express TCR γ rather than TCR β genes. TCR usage is not equivalent in different epithelia. The predominant receptor of dermal epithelia using $V_\gamma$5 (33) is not used in the gut, and the predominantly used γ gene in IELs is V7, the functional use of which has not been conspicuous elsewhere. Instead, V7 was first cloned as a rearranged band in the DNA of T-cell thymomas (3). Data presented here are consistent with evidence for TCR1 protein in IELs derived by Goodman and Lefrançois (38) and with the use in IELs of V7 claimed, based on different evidence, by Bonneville *et al.* (39). The junctional diversity in γ supports the notion that gut TCR1 molecules are selected to recognize different ligands or antigen in an immunological role.

| | ------V7--------> | | <--------J1-------- |
|---|---|---|---|
| clone | | | |
| 255 | TCC TGG GCT GGT | | AGC TCA GGT TTT |
| 36 | TCC TGG GCT GGT | T | AT AGC TCA GGT TTT |
| 67 | TCC TGG GCT GG | AT | AT AGC TCA GGT TTT |
| 2-28 | TCC TGG GCT GG | CTGG | AGC TCA GGT TTT |
| 3-36 | TCC TGG GCT | T | AT AGC TCA GGT TTT |
| 7 | TCC TGG GC | AT | AT AGC TCA GGT TTT |
| 129 | TCC TGG G | | AT AGC TCA GGT TTT |
| 239 | TCC TGG GCT | T | GC TCA GGT TTT |
| 4-54 | TCC TGG GCT GG | GG | AT AGC TCA GGT TTT |
| 4-68 | TCC TGG GCT GG | CTTAT | AT AGC TCA GGT TTT |
| amino acid sequences | | | |
| (germline) | Ser Trp Ala Gly | | Ser Ser Gly |
| (2) | Ser Trp Ala Gly | Tyr | Ser Ser Gly |
| (1) | Ser Trp Ala Gly | Trp | Ser Ser Gly |
| (2) | Ser Trp Ala | Tyr | Ser Ser Gly |
| (1) | Ser Trp | Asp | Ser Ser Gly |
| (1) | Ser Trp Ala Gly | Asp | Ser Ser Gly |
| (1) | Ser Trp Ala Gly | Leu Tyr | Ser Ser Gly |
| (1) | Ser Trp Ala | Cys | Ser Gly |

FIG. 5. V7–J1 joining. Frequencies of occurrence of amino acid sequences are in parentheses.

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Restriction of V gene usage according to time and/or place of expression is a hallmark of murine γ genes not clearly shown by either TCR2 or immunoglobulin genes. This may reflect recognition of molecules of limited polymorphism and/or the implausibility of cells in epithelia with restricted capacity to circulate having highly diverse receptors matched with highly diverse antigens. However, the junctional diversity in the intestinal TCR1 repertoire, by analogy with studies of immunoglobulin λ light chains (40), may alone contribute to significant differences in antigen interaction.

Isotypic or allelic exclusion in rearranging genes has been regarded as due to nonproductive joining of V and J segments for at least one allele. However, an example is described here of a TCR γ gene that is productively rearranged but that cannot encode protein through the cDNA cloned because of incorrect splicing. Combined with other data (27), it becomes

| V -J Junctions | | | |
|---|---|---|---|
| Clone | (+/-) | V4 | J1 |
| g | + | ACT TAC TAC TGT TCC TAC GGC | AT AGC TCA GGT TTT CAC AAG |
| 5,10 | - | ACT TAC TAC TGT TCC TAC GGC | T TTT CAC AAG |
| 1 | + | ACT TAC TAC TGT TCC TAC GGC CAA T | AT AGC TCA GGT TTT CAC AAG |
| 3 | + | ACT TAC TAC TGT TCC TAC GGC GTA T | AT AGC TCA GGT TTT CAC AAG |

| J-C splices | | | |
|---|---|---|---|
| 5, 10 | ATT CCC TCT G | gtaagt | tcttacag AC AAA AGA CTT GAT GC |
| 1 | ATT CCC TCT G | gtaagt ctttccagTT TTC TCT TAC AGA CAA AAG ACT TGA TGC | |
| 3 | ATT CCC TCT G | gtaagt | AC AAA AGA CTT GAT GC |

FIG. 6. Sequences of portions of V4–J1–C1 cDNA clones derived from IEL populations. The germ-line sequences (g) were derived from refs. 1, 2, and 25; the cDNA sequences were determined completely in both directions. Lowercase letters indicate intron sequences that flank the relevant splice donors and acceptors, and N nucleotides are underlined.

clear that γ RNA is prone to alternative splicing. It is striking that the alternative splicing occurs in an intron common to all $V_\gamma$–J1–C1 genes and yet is not found in any of the productive V7 clones. It may be that the IELs from which incorrectly spliced RNA was amplified represent a particular stage of differentiation (e.g., see ref. 41).

There is no evidence for somatic mutation of V7. However, junctional diversity that includes somatic contribution to the putative ligand combining site is potentially dangerous in its capability to create autoreactive receptors. Therefore, some form of thymic selection might be expected for intestinal IELs. That may not be so for dermal T cells that show no junctional diversity (33) and may constitute another difference between $TCR1^+$ cells of different epithelia. Athymic mice did not show expression of V7 in the gut.

Both TCR1 and TCR2 T cells are present in IEL preparations. However, $TCR1^+$ cells represent a significant proportion of IELs, whereas they comprise only 1–5% of splenic T cells. The selective accumulation of $TCR1^+$ cells in epithelia strengthens the notion (17) that they recognize particular restricted ligand systems as part of an immune response.

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