

HUMAN LYMPHOCYTES BEARING T CELL RECEPTOR γ/δ
ARE PHENOTYPICALLY DIVERSE AND EVENLY
DISTRIBUTED THROUGHOUT THE LYMPHOID SYSTEM

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Two structurally distinct types of CD3-associated TCR exist. The majority of peripheral T lymphocytes and a sizeable portion of thymocytes express a 90-kD disulfide-linked heterodimer composed of TCR- α and - β glycoprotein subunits (1-3). TCR- α/β -expressing cells use this receptor to recognize antigens in the context of self MHC molecules (4). Recently, two novel rearranging TCR genes and their cell surface products (termed TCR- γ/δ) have been identified on a small subset of thymic and peripheral lymphocytes (reviewed in reference 5). In man, this new TCR occurs in three structurally distinct forms, depending on the usage of different TCR- γ constant region genes (6). The biological role and repertoire of TCR- γ/δ are not fully defined. While evidence exists that TCR- γ/δ is part of a cell surface structure capable of transducing activation signals resulting in proliferation (7), lymphokine production (7), and cell-mediated cytotoxicity (8, 9), the nature of both the antigens and the restriction elements recognized by this TCR is still a matter of speculation. Despite this enigma, it is likely that TCR- γ/δ^+ lymphocytes comprise a newly defined and separate sublineage of T cells, distinct from TCR- α/β^+ cells.

In man, TCR- γ/δ was initially identified on T cell lines that shared two main phenotypic characteristics, namely, lack of reactivity with mAbs against TCR- α/β and in most cases a "double-negative" (CD4⁻CD8⁻) phenotype (7, 10-12). Using indirect

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analyses, cells bearing a phenotype consistent with that of cultured TCR- γ/δ -expressing lymphocytes (CD3⁺, TCR- α/β ⁻, CD4⁻8⁻) have been found in peripheral blood in proportions ranging between 0.5 and 10% of CD3⁺ lymphocytes and at even lower frequencies (0.2-0.9%) in postnatal thymus (7, 10, 11, 13). However, WT31, the mAb generally used to demonstrate lack of TCR- α/β expression, does not exclusively stain TCR- α/β ⁺ lymphocytes; rather, it also weakly reacts with TCR- γ/δ ⁺ cells (14, and see below). Moreover the quantitative assessment of TCR- γ/δ ⁺ cells based on lack of CD4 and CD8 expression is not accurate, since the double-negative phenotype can be associated with either TCR- γ/δ or TCR- α/β expression and since CD8⁺ TCR- γ/δ ⁺ PBL have been described (10, 15, 16, and unpublished observations). A precise quantitative evaluation of the TCR- γ/δ -expressing cell population thus depends on the use of mAbs specifically directed against TCR- γ/δ . Most of the available mAbs against TCR- γ/δ (e.g., anti-NKF1 [12], anti-Ti γ A [16], BB3 [17], δ TCS-1 [18]) appear to react with only subsets of TCR- γ/δ ⁺ cells, while anti-TCR- δ 1 (19), anti-C γ M1 (6), and anti-TCR- γ/δ -1 (20) may have panreactivity with all γ/δ T cells. To more fully describe the TCR- γ/δ ⁺ T cell population, we have determined both quantitative and phenotypic features of this cell system and analyzed its tissue distribution and microanatomy within individual organs using two mAbs that are specific for the TCR- γ/δ complex and appear to have framework reactivity (anti-TCR- δ 1 and anti-C γ M1 [6, 19]). As described below such an analysis contributes to understanding the physiologic potential of TCR- γ/δ ⁺ lymphocytes in vivo.

Materials and Methods

Tissue Sampling. Fetal tissue (thymus, spleen, small and large intestine, liver, lung, kidney, and adrenal gland) was obtained at the time of postmortem examination from electively aborted fetuses ($n = 9$, gestational ages, as determined by crown rump length, menstrual records, and fetal morphology were 10, 14, 17, 18, 20, and 21 wk). Consent forms and collecting practices were approved by the Committee for the Protection of Human Subjects from Research Risks (Boston, MA). Full-term neonatal ($n = 3$) and postnatal thymi ($n = 18$, age range, 6 wk to 6 yr) were obtained from normal children undergoing corrective cardiac surgery. Adult human peripheral blood was donated by randomly selected healthy volunteers ($n = 29$, age range 23-54 yr). Specimens of adult tissue (bone marrow, $n = 1$; tonsil, $n = 9$; lymph node, $n = 8$; spleen, $n = 7$; small (duodenum, $n = 5$; jejunum, $n = 2$; ileum, $n = 6$) and large (colon, $n = 5$) intestine; liver, $n = 5$; lung, $n = 4$; skin, $n = 10$; kidney, $n = 1$; brain, $n = 4$; heart, $n = 1$; ovary, $n = 1$; adrenal gland, $n = 1$) were obtained from specimens removed for diagnostic or therapeutic purposes or at the time of autopsy.

mAbs. mAbs used were anti-Leu-9 (CD7; 21), anti-Leu-5b (CD2; 22), anti-Leu-1 (CD5; 23), anti-Leu-6 (CD1; 24), anti-Leu-3a (CD4; 25), OKT4 (CD4; 26), anti-Leu-2a (CD8; 25), OKT8 (CD8; 27), anti-Leu-4 (CD3; 25), T3b (CD3; 28), 64.1 (CD3; 29), WT31 (TCR- $\alpha/\beta > \gamma/\delta$; 30), β F1 (TCR- β ; 31), anti-TCR- δ 1 (TCR- δ ; 20), anti-C γ M1 (TCR- γ ; 6), 9.3 (CD28; 32), anti-Leu-11 (CD16; 33), anti-Leu-15 (CD11b; 34), anti-Leu-7 (35), anti-Leu-M3 (CD14; 36), anti-Leu-12 (CD19), anti-HLA-DR (37), and anti-IL-2-R (CD25; 38). Anti-Leu mAbs were purchased from Becton Dickinson & Co., Mountain View, CA; OKT mAbs were purchased from Ortho Diagnostic Systems Inc., Westwood, MA. Anti-TCR- δ 1 and anti-C γ M1 were derived in our laboratory. Isotype-matched control Ig that do not react with human leukocytes were used as controls.

Immunofluorescence Analysis of Cell Suspensions. Thymocyte suspensions were prepared by gently teasing thymic tissue into single cell suspensions using the entire thymic lobule to ensure that cortical and medullary thymocytes were accurately represented. For splenocyte suspensions, spleens were minced to prepare a single cell suspension. Viable mononuclear cells from thymus, spleen, and peripheral blood were isolated using Ficoll/Hypaque. For single-

color analysis thymocytes/PBMC/splenocytes were resuspended in PBS/1% FCS/0.02% NaN_3 at 2×10^6 cells/ml containing appropriately diluted mAbs and incubated for 30 min at 4°C . After two washes in PBS/1% FCS, antibody binding was visualized by using FITC-labeled F(ab)'_2 goat anti-mouse Ig (Tago Inc., Burlingame, CA). Two-color staining was performed using FITC-conjugated mAbs or unconjugated mAbs together with FITC-conjugated goat anti-mouse Ig and either phycoerythrin (PE)¹- or biotin-conjugated mAbs with PE-conjugated streptavidin (Becton Dickinson & Co.) as the second-step reagent(s). For three-color immunofluorescence cells were stained with FITC-, PE-, and biotin-conjugated mAbs together with APC-conjugated streptavidin (Becton Dickinson & Co.). Labeled cells were analyzed by a flow cytometer (Ortho Diagnostic Systems Inc.) or FACStar flow cytometer.

Immunohistologic Analysis of Cryostat Sections. Tissues were snap frozen in liquid nitrogen with quenching in 2-methylbutane or in cryostats and stored at -70°C until use. $4\text{-}\mu\text{m}$ serial cryostat sections were mounted on glass slides, air dried, and stored at -20°C until use. After 5-min acetone fixation tissue sections were immunostained using a three-stage biotin-avidin-peroxidase procedure as previously described (39, 40). Briefly, cryostat sections were first exposed to normal horse serum (2.5% in PBS) to inhibit nonspecific binding of horse Ig, followed by an overnight incubation with the appropriately diluted mAb. The subsequent step to block endogenous peroxidase activity with H_2O_2 was followed by incubation first with biotin-conjugated horse anti-mouse Ig and second with avidin-biotinylated peroxidase complex (both from Vector Laboratories, Inc., Burlingame, CA). Sections were then subjected to a 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI) reaction. After nuclear counterstaining with hematoxylin, sections were mounted in glycergel (Dako; Accurate Chemical & Scientific Corp., Westbury, NY). In kidney and liver sections endogenous biotin activity was blocked as previously described (41). In all tissues except gut epithelium and epidermis, percentages of anti-TCR- δ 1- and anti-C γ M1-reactive cells within the CD3^+ cell population were enumerated by counting all CD3^+ or TCR- γ/δ^+ cells, respectively, in five high power fields of each section using a calibrated optical grid. In cryostat sections of gut a 20-mm stretch of epithelium was screened for the relative number of CD3^+ and TCR- γ/δ^+ cells, respectively. Quantification of intraepidermal TCR- γ/δ^+ lymphocytes was performed in immunostained epidermal sheet preparations as previously described (42).

Results

Reactivity of mAb Anti-TCR- δ 1. mAb anti-TCR- δ 1 is directed against an epitope on the TCR- δ chain (6, 20). It binds, without exception, to all CD3^+ TCR- α/β^- T cell clones and polyclonal cell lines examined, including cells bearing TCR- γ and - δ subunits encoded by a variety of TCR- γ and TCR- δ V, J, and C region gene segments (Kragel, M. S., A. Lanzavecchia, T. Hercend, J. Borst, H. Spits, J. E. de Vries, S. H. Ip, W.-J. Tian, personal communications and our unpublished observations). To further examine the reactivity of anti-TCR- δ 1, we determined its ability to bind to CD3^+ PBL lacking TCR- α/β expression based on their staining behavior with mAb WT31. As demonstrated earlier (14), WT31 strongly binds to the surface of TCR- α/β^+ cells but weakly stains cultured TCR- γ/δ^+ lymphocytes. Two-color staining using WT31 (along the y -axis) and anti-TCR- δ 1 (along the x -axis) revealed two distinct populations (Fig. 1 *a*). In addition, note that the profile of anti-TCR- δ 1⁺ cells shifted slightly away from the x -axis (see arrow, Fig. 1 *a*), indicating that all TCR- γ/δ^+ cells also weakly react with WT31. This result was confirmed by similar two-color cytofluorographic analysis of PBMC with anti-CD3 and WT31, which revealed two distinct CD3^+ populations: one that is weakly reactive with WT31 (see arrow pointing to cell population shifted to the right of the x -axis in Fig.

¹ Abbreviation used in this paper: PE, phycoerythrin.

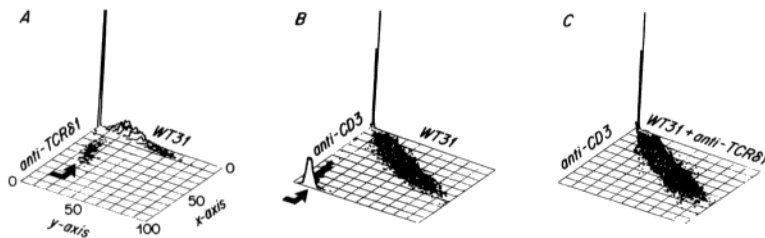


FIGURE 1. Two-color cytofluorographic analysis of TCR- α/β , TCR- γ/δ , and CD3 expression by PBL. (A) Staining with both WT31 (*y*-axis, green staining) and anti-TCR- $\delta 1$ (*x*-axis, red staining) allows visualization of two separate populations: one reactive with WT31 alone (along the *y*-axis) and a second double stained with both WT31 and anti-TCR- $\delta 1$. Note that the anti-TCR- $\delta 1$ ⁺ population has shifted away from the baseline of the *x*-axis (see *arrow*), indicating that TCR- γ/δ ⁺ cells weakly react with mAb WT31. (B) Two-color analysis using anti-CD3 (*x*-axis, red staining) and WT31 (*y*-axis, green staining) reveals that most CD3⁺ PBL unequivocally stain with WT31 and are clearly double positive (along the diagonal of the grid). In contrast, a small population of CD3⁺ PBL are only very weakly reactive with WT31, since their profile (along the *x*-axis) is shifted slightly away from the *x*-axis baseline (see *arrow*). (C) All of the CD3⁺ PBL that were weakly reactive with WT31 (B) react with anti-TCR- $\delta 1$. A combination of WT31 and anti-TCR- $\delta 1$ together stains all CD3⁺ PBL. PBMC were first incubated with WT31 (A and B) or WT31 and anti-TCR- $\delta 1$ (C) followed by FITC goat anti-mouse Ig (*y*-axis, green staining). Free binding sites of the second-step reagent were blocked with normal mouse serum and cells were then incubated with biotinylated anti-TCR- $\delta 1$ (A) or biotinylated 64.1 (anti-CD3) (B and C) followed by PE-streptavidin (*x*-axis, red staining). Using forward angle and side scatter a gate was set to include all lymphocytes.

1 *b*) and another more strongly stained with WT31 and clearly double positive for WT31 and CD3 (Fig. 1 *b*). Concomitant staining of PBMC with anti-CD3 in red (*x*-axis) and with both WT31 and anti-TCR- $\delta 1$ in green (*y*-axis) revealed one double-stained population, indicating that WT31 and anti-TCR- $\delta 1$ together react with all detectable CD3⁺ lymphocytes (Fig. 1 *c*). This type of analysis was carried out on PBL samples from 16 different individuals with similar results, indicating that WT31 and anti-TCR- $\delta 1$ together reacted with all CD3⁺ cells in peripheral blood. Taken together, the analysis of individual T cell lines and clones known to use different TCR- δ gene segments and the mAb reactivity with all WT31⁻ or WT31^{dull} cells in peripheral blood argue strongly that the anti-TCR- $\delta 1$ mAb reacts with a framework determinant present on all TCR- γ/δ -bearing lymphocytes.

The Number and Phenotype of TCR- γ/δ ⁺ Lymphocytes in Peripheral Blood and Spleen. The proportion of anti-TCR- $\delta 1$ ⁺ cells ranged between >0.5 and 16.3% (mean, 4.9%) of CD3⁺ PBL in 26 of 29 donors studied (Fig. 2). Serial measurements performed on some individuals revealed that the percentages of anti-TCR- $\delta 1$ ⁺ PBL remained nearly constant for each individual over a period of at least 6 mo. In 3 of 29 samples, anti-TCR- $\delta 1$ reactivity was below the limit of detection by cytofluorography (i.e., they contained $\leq 0.5\%$ reactive cells). However, in at least some of these individuals, TCR- γ/δ ⁺ cells were successfully cloned from peripheral blood, suggesting that even when undetectable by cytofluorographic staining, TCR- γ/δ -bearing cells appeared to be present in all individuals.

Anti-TCR- $\delta 1$ ⁺ PBL have been further examined by two- and three-color cytofluorographic analysis for the expression of other cell surface molecules relevant to T cell function and differentiation (Table I). For technical reasons this analysis was performed on PBMC samples obtained from 12 different subjects containing >2% TCR-

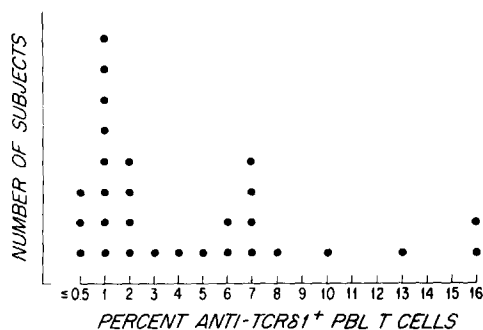


FIGURE 2. Numbers of TCR- γ/δ ⁺ lymphocytes in peripheral blood. The number of CD3⁺ PBL expressing TCR- γ/δ was determined by staining with a specific mAb (anti-TCR- δ 1) and flow cytometry. Percent anti-TCR- δ 1⁺ PBL T cells were calculated by $100 \times (\text{numbers anti-TCR-}\delta$ 1⁺ cells/numbers anti-CD3⁺ cells). Results were rounded to nearest whole percentile. 29 normal subjects (23–54 yr of age) were examined. Each closed circle represents one individual.

γ/δ ⁺ cells among their CD3⁺ PBL. In five of five samples all anti-TCR- δ 1⁺ PBL were CD7⁺, CD5⁺, and CD2⁺, although CD5 expression by these cells was about fivefold less than on TCR- α/β ⁺ lymphocytes. CD1 was consistently absent from TCR- γ/δ ⁺ PBL. Although not emphasized previously, in three of seven individuals examined, ~1–4% of the anti-TCR- δ 1⁺ cells expressed CD4 (Fig. 3, *a* and *b*). In contrast, in seven of seven individuals, a sizable portion (~10–70%) of anti-TCR- δ 1⁺ lympho-

TABLE I
Phenotype of Anti-TCR- δ 1⁺ Cells in Peripheral Blood of Normal Donors

CD antigen	Percent positive	
T cell-related antigens		
CD7	~100%	(5:5)
CD2	~100%	(5:5)
CD5	~100%	(5:5)
CD1	Not detectable	(5:5)
CD4	~1–4%	(3:7)
	Not detectable	(4:7)
CD8	~10–70%	(7:7)
NK cell-related antigens		
CD16	~10%	(1:5)
	Not detectable	(4:5)
CD11b	~50%	(5:5)
Leu 7	*	(3:5)
Non-T cell-related antigens		
CD14	Not detectable	(5:5)
CD19	Not detectable	(5:5)
T cell activation-related antigens		
CD25	Not detectable	(5:5)
Class II	Not detectable	(5:5)
CD28	~50%	(5:5)

Percent positive indicates the percent of anti-TCR- δ 1⁺ cells that express the CD antigen noted. Numbers in parentheses refer to numbers of samples containing anti-TCR- δ 1⁺ cells of the CD phenotype noted vs. the total number of individuals analyzed.

* Due to the low amounts of Leu-7 antigen expressed, accurate quantification was not possible.

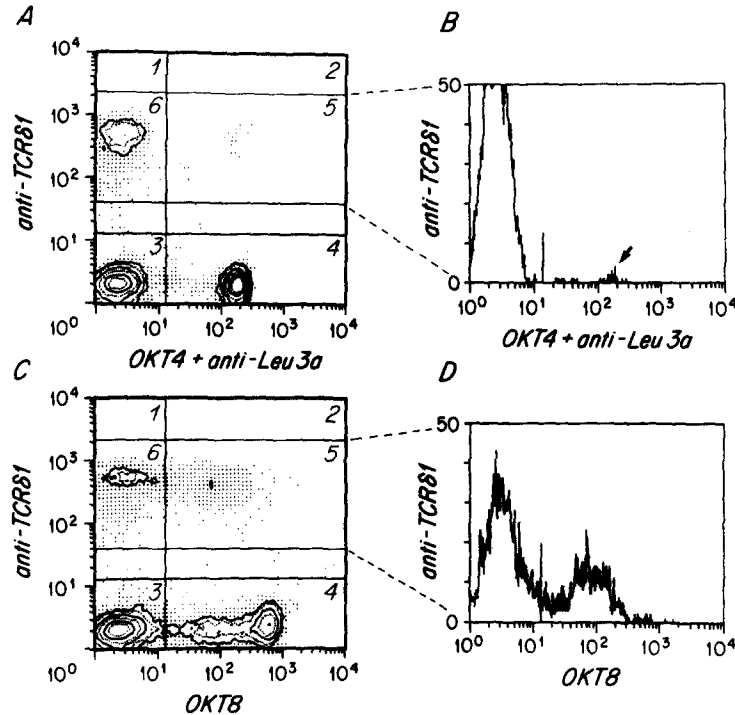


FIGURE 3. Two-color cytofluorographic analysis of CD4 and CD8 expression by TCR- γ/δ ⁺ PBL. Contour maps (A and C) show two-channel fluorescence of PBMC gated by forward and side light scatter for lymphocytes. A second gate was set to include all anti-TCR- $\delta 1$ ⁺ cells and 1,000 cells falling within this gate were analyzed for CD4 and CD8 expression respectively (B and D). (A and B) A small but distinct number of anti-TCR- $\delta 1$ ⁺ cells also reacts with anti-CD4 mAb (see dots in quadrant 5, A; as well as arrow pointing to small population of gated anti-TCR- $\delta 1$ ⁺ cells reactive with anti-CD4, B). The majority of PBL reactive with anti-CD4 mAb, however, are unreactive with anti-TCR- $\delta 1$ and presumably express TCR- α/β (quadrant 4, A). (C and D) A sizeable number of anti-TCR- $\delta 1$ ⁺ cells also reacts with anti-CD8 (see dots in quadrant 5, C; as well as the profile located to the right of vertical marker line representing gated anti-TCR- $\delta 1$ ⁺ cells that also react with anti-CD8, D). The majority of CD8⁺ PBL are unreactive with anti-TCR- $\delta 1$ and presumably express TCR- α/β (quadrant 4, C). CD8 expression by anti-TCR- $\delta 1$ ⁺ cells is slightly weaker than that observed on anti-CD8⁺ anti-TCR- $\delta 1$ ⁻ PBL. PBMC were first incubated with FITC-OKT4 together with FITC-anti-Leu-3a (A and B) or FITC-OKT8 (C and D) and second with biotinylated anti-TCR- $\delta 1$ followed by PE-streptavidin.

cytes expressed CD8, however, at lower levels than on TCR- α/β ⁺ cells (Fig. 3, c and d). In one of five subjects, ~10% of anti-TCR- $\delta 1$ ⁺ cells coexpressed low levels of CD16 (Fc-R), and in five of five subjects, CD11b (CR3) was present in low amounts on ~50% of anti-TCR- $\delta 1$ ⁺ cells. However, precise quantitation was difficult due to the low amounts of CD16 and CD11b antigen expression. The same was true for the expression of the Leu-7 antigen by some anti-TCR- $\delta 1$ -reactive lymphocytes in three of five individuals. Anti-Leu-M3 (CD14, monocyte/macrophage antigen) and anti-Leu-12 (CD19, B cell antigen) did not stain any peripheral TCR- γ/δ ⁺ cells. Furthermore, these cells did not express the p55 subunit of the human IL-2-R or HLA-DR while the T cell antigen CD28, which is expressed by virtually all TCR- α/β ⁺ cells

and is involved in T cell activation, was expressed on ~50% of anti-TCR- $\delta 1^+$ cells in five of five individuals studied.

TCR- γ/δ^+ splenocytes comprised between >0.5 and 15% of CD3⁺ cells in this organ and their phenotype was similar to TCR- γ/δ^+ PBL.

The Number and Phenotype of TCR- γ/δ^+ Lymphocytes in Thymus. Immunohistologic studies on the ontogeny of T cell antigen expression in human thymus suggested that CD3 expression occurs as early as 10 wk of gestation (43). However, at that gestational age, the fetal thymus consists of two 1 × 3-mm lobes such that an analysis of cell suspensions is not possible. The earliest fetal thymic tissue that could be subjected to cytofluorographic analysis came from a fetus of 14 wk of gestation. Results from this and several other fetal thymocyte suspensions as well as a total of 21 newborn and young children's thymi revealed that anti-TCR- $\delta 1^+$ cells constitute >0.5–16% (mean, 3.4%) of CD3⁺ thymocytes in 21 of 29 samples. In 8 of 29 samples we failed to detect anti-TCR- $\delta 1^+$ cells in cytofluorographic analysis (i.e., ≤0.5% positive cells). However, in each of these thymi, small numbers of TCR- γ/δ -bearing cells could be unequivocally detected by immunohistologic means using anti-TCR- $\delta 1$. The numbers of anti-TCR- $\delta 1^+$ cells did not correlate with either the pre- or postnatal age of a given fetus/child from the range of ages studied. More extensive studies on fetal tissue, however, are necessary to determine whether, as shown for the murine thymus (44), human TCR- γ/δ T cells are more abundant early in thymic ontogeny. In contrast to the homogeneously strong anti-TCR- $\delta 1$ staining pattern of peripheral TCR- γ/δ^+ lymphocytes, in 16 thymus samples two separate anti-TCR- $\delta 1^+$ cell populations were distinguished consisting of dull and brightly stained subsets. As in peripheral blood, anti-TCR- $\delta 1$ and WT31 together reacted with virtually all CD3⁺ thymocytes.

In general, anti-TCR- $\delta 1^+$ thymocytes were similar in phenotype to their circulating peripheral counterparts as determined by two- and three-color cytofluorographic analysis of a total of seven samples containing >2% TCR- γ/δ^+ thymocytes. Anti-TCR- $\delta 1^+$ thymocytes expressed CD7, CD2, and CD5 in amounts comparable with TCR- α/β^+ thymocytes. Occasionally (two of seven samples) a subpopulation of anti-TCR- $\delta 1^+$ cells (~10–20%) was weakly CD8⁺, although in most cases, TCR- γ/δ^+ thymocytes were CD4⁻8⁻. In contrast to anti-TCR- $\delta 1^+$ PBL, however, a sizeable portion (~50%) of each anti-TCR- $\delta 1^+$ thymocyte population analyzed expressed low levels of CD1, while CD16, CD11b, Leu-7, and CD28 could not be detected. TCR- γ/δ^+ thymocytes, furthermore, lacked IL-2-R p55 subunit and HLA-DR expression and were negative for CD14 and CD19.

Immunohistologic Analysis of TCR- γ/δ^+ Lymphocytes. Immunohistologic screening of various lymphoid and nonlymphoid organs was performed in order to identify the organs populated by TCR- γ/δ^+ cells and to characterize their specific location within each tissue. To further assure that anti-TCR- $\delta 1$ staining in tissues accurately reflected the number and location of TCR- γ/δ^+ lymphocytes, we used an additional mAb against the TCR- γ/δ complex, anti-C γ M1. This mAb is directed against a sequence encoded by the TCR- γ C1 exon that is shared in C γ 1 and C γ 2 gene segments (6). Immunoprecipitation analysis showed that the mAb reacts with a constant region determinant on both C γ 1- and C γ 2-encoded TCR- γ chains, predicting that anti-C γ M1 has framework reactivity (6). It reacts with the TCR- γ protein of

cells in tissue sections and is thus useful for immunohistologic analysis. Immunolabeling with both mAbs, anti-TCR- $\delta 1$ and anti-C γ M1, gave quantitatively and qualitatively similar staining results in all tissues examined.

Tissue samples from all fetal thymi analyzed by flow cytometry, one additional specimen from a 10-wk-old fetus, three neonatal, and 12 young children's thymi were analyzed. Cells reactive with both anti-TCR- $\delta 1$ and anti-C γ M1 were present in each sample (Fig. 4). Numbers of immunohistologically visualized TCR- γ/δ^+ thymocytes closely paralleled those obtained by cell surface staining except that a few positive cells were found to be present in all those thymi that gave negative results by cytofluorographic analysis on thymocyte suspensions. At 10 wk of gestation, while most of the thymocytes stained CD3⁺, only a small population (~5%) of fetal thymocytes reacted with both of the TCR- γ/δ -specific mAb. Larger tissue sampling, however, is necessary to study whether TCR- γ/δ^+ cells are the first TCR-expressing cells in ontogeny comparable with the murine system (45). In all tissue specimens studied, most TCR- γ/δ^+ thymocytes were present within the medulla and the corticomedullary junction while fewer TCR- γ/δ^+ cells were distributed throughout the cortex (Fig. 4 *b*).

In postnatal tonsils (Fig. 5) and lymph nodes TCR- γ/δ^+ cells were primarily localized within interfollicular and paracortical areas with occasional positive cells within mantle zones and germinal centers of secondary follicles. This distribution corresponds to the location of TCR- α/β^+ lymphocytes in these same tissues, assessed by staining with mAb β F1 directed against the TCR- β chain (31, and data not shown). In both fetal (14–21 wk) and adult spleen, TCR- γ/δ^+ cells were found in the periarteriolar lymphocyte sheath. Rare positive cells were localized within marginal zones and the red pulp. At these latter sites in some samples the proportion of TCR- γ/δ^+ to TCR- α/β^+ cells was higher than in T zones. Numbers of TCR- γ/δ^+ lymphocytes detected in tonsil, lymph node, and spleen were comparable with those observed in thymus and peripheral blood, accounting for between ~1 and 15% of CD3⁺ cells.

As within all other lymphoid tissues, in fetal (14–21 wk) and adult small and large intestine numbers of TCR- γ/δ^+ lymphocytes varied considerably between individual samples ranging from ~1–15% of total CD3⁺ gut lymphocytes (Fig. 6). In general, TCR- γ/δ^+ cells were found at similar frequencies in both compartments of gut-associated lymphoid tissue, i.e., within epithelium close to the basal membrane and within the lamina propria. The distribution and numbers of TCR- γ/δ^+ lymphocytes in Peyer's patches were similar to those found in lymph nodes and tonsils. In some sections anti-TCR- $\delta 1$, and less frequently anti-C γ M1, weakly stained the cytoplasmic content of large CD3⁻ epithelial cells. This reactivity, however, was interpreted as reflecting nonspecific binding of these mAbs to crossreactive determinants present in epithelial cells.

In cryostat sections of normal adult skin from various body areas, we found TCR- γ/δ^+ cells both intraepidermally irregularly scattered within the basal keratinocyte layer as well as in the dermis (Fig. 7). These cells were rare, but were detectable in each skin biopsy analyzed. In clinically normal skin, T lymphocytes, mainly of the TCR- α/β type, constitute discrete perivascular infiltrates within the dermis and occur in rare irregular clusters, interspersed between the most basal keratinocyte layers, predominantly along the rete ridges (46, Foster, C. A., H. Yokozeki, F. Koning, et al., manuscript in preparation and our unpublished observation) (Fig. 7 *a*). Their

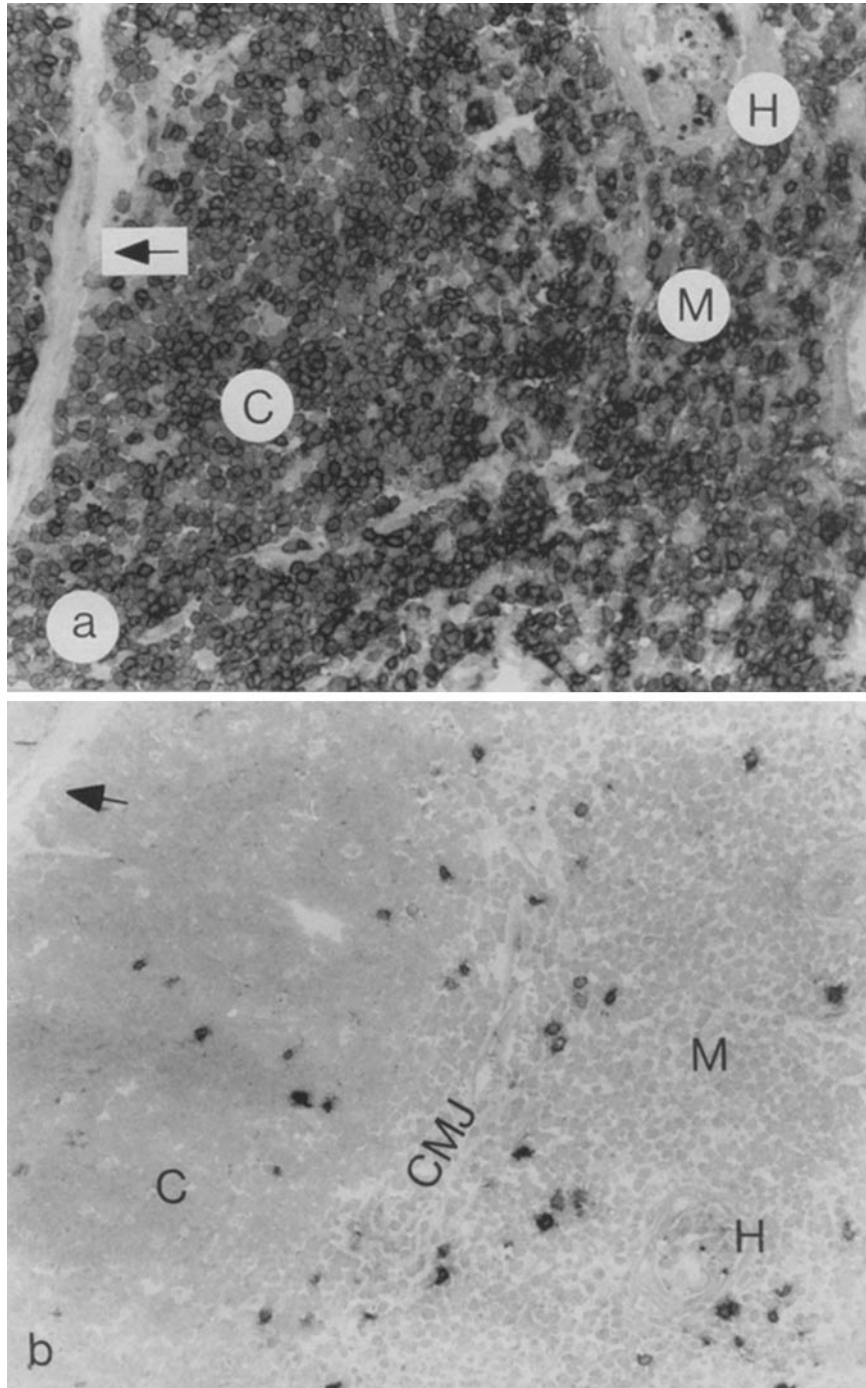


FIGURE 4. Immunohistologic visualization of TCR- $\gamma\delta^+$ thymocytes. Serial cryostat sections of normal postnatal thymus were stained by a biotin-avidin-peroxidase procedure using anti-Leu-4 (a) and anti-TCR- $\delta 1$ (b) as first-step mAb. (a) The majority of both cortical (C) and medullary (M) thymocytes stain with anti-Leu-4. (b) Anti-TCR- $\delta 1$ -reactive cells are scattered throughout the tissue. These cells are more frequent within the medulla (M) and cortico-medullary junction (CMJ) than within the cortex (C). In the photomicrographs, the arrow points to capsule of lobe and H identifies a Hassall's body within the medulla.

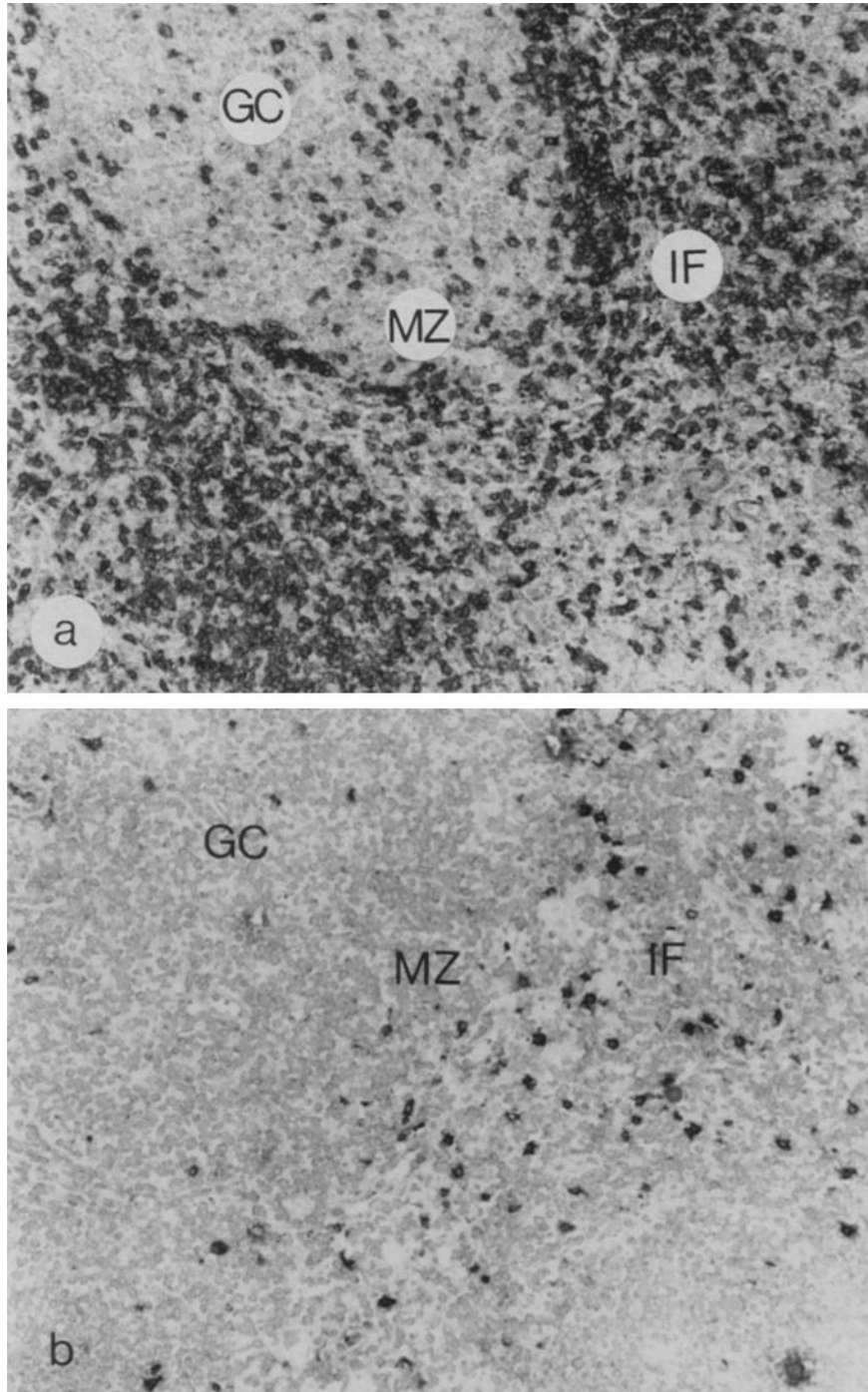


FIGURE 5. Immunohistologic visualization of TCR- γ/δ ⁺ lymphocytes in tonsil. Serial cryostat sections of tonsil were stained by a biotin-avidin-peroxidase procedure using anti-Leu-4 (a) and anti-TCR- δ 1 (b) as first-step mAb. (a) Numerous anti-Leu-4⁺ cells constitute the interfollicular (IF) T cell area, while a smaller but substantial number of anti-Leu-4⁺ cells is also present within germinal centers (GC) and mantle zones (MZ) of secondary follicles (B cell area). (b) Anti-TCR- δ 1⁺ cells are localized mainly in the interfollicular area (IF), while a few TCR- γ/δ ⁺ cells are present within mantle zone (MZ) and germinal center (GC) of a secondary follicle.

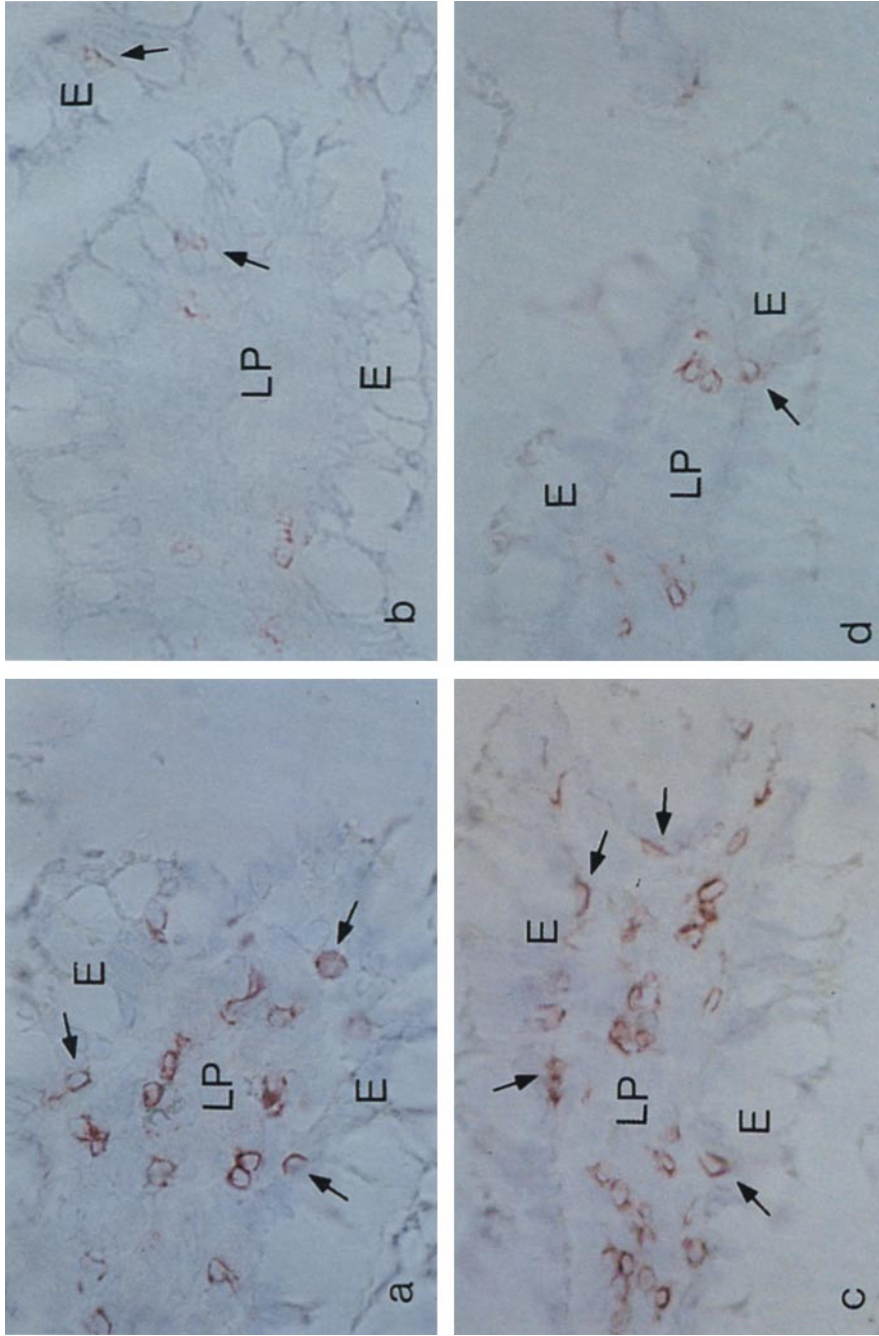


FIGURE 6. Immunohistologic visualization of TCR- γ/δ^+ lymphocytes in serial cryostat sections of normal small intestine were stained by a biotin-avidin-peroxidase procedure using anti-Leu-4 (a and c) and anti-TCR- $\delta 1$ (b and d). Numerous anti-Leu-4 $^+$ cells are present within the lamina propria (LP) and the epithelium (E) (arrows). (b and d). Compared with the total number of CD3 $^+$ gut lymphocytes, only a small number of lymphocytes present within the lamina propria (LP) or the epithelium (E) (arrows) react with anti-TCR- $\delta 1$.

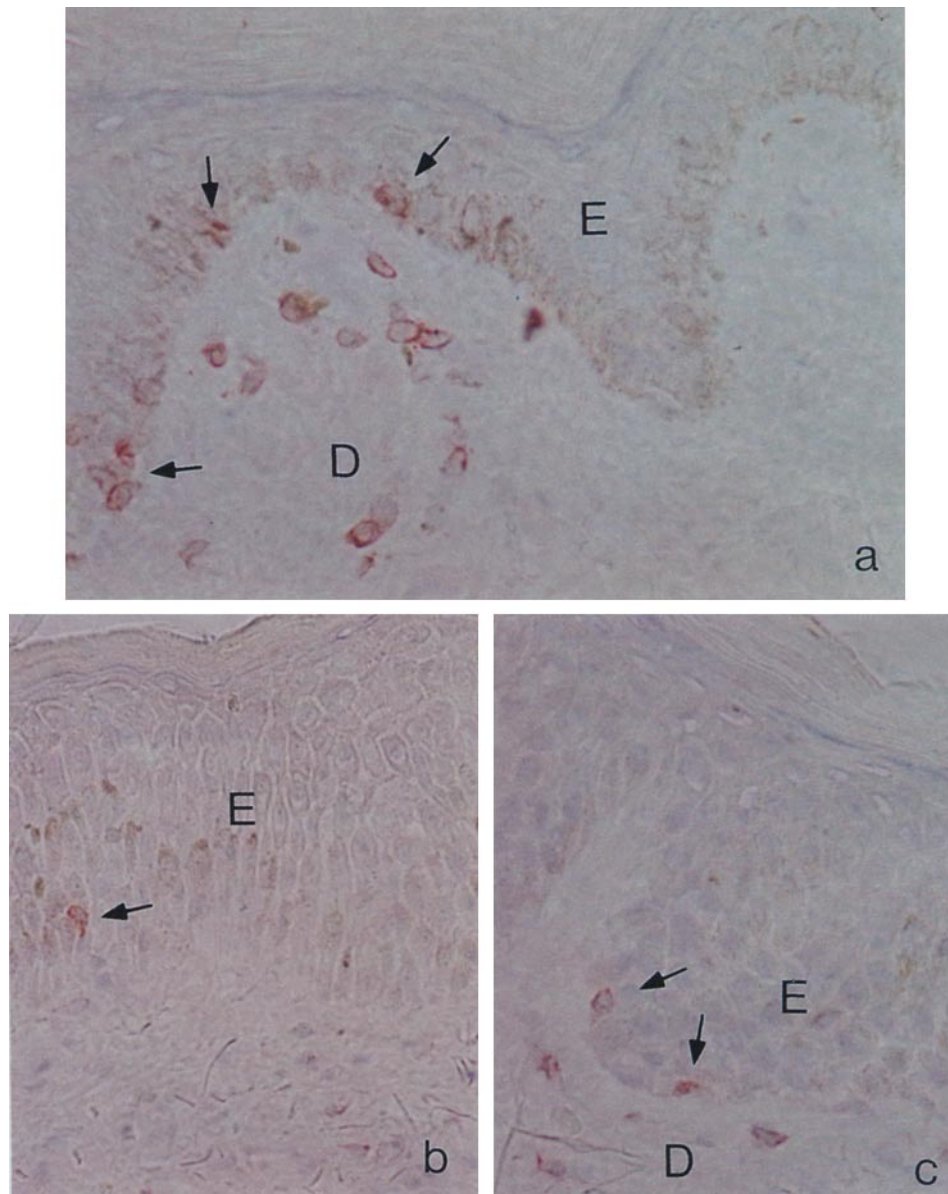


FIGURE 7. Immunohistologic visualization of TCR- γ/δ^+ lymphocytes in skin. Cryostat sections of normal skin were stained by a biotin-avidin-peroxidase procedure using anti-Leu-4 (a) and anti-TCR- $\delta 1$ (b and c) as first-step mAb. (a) Intraepidermal anti-Leu-4 $^+$ cells (arrows) are interspersed throughout the upper dermis (D), some anti-Leu-4 $^+$ cells are also scattered throughout the epidermis (E). (b) One single intraepidermal anti-TCR- $\delta 1^+$ cell (arrow) is located within the most basal keratinocyte layer of the epidermis (E). In another section anti-TCR- $\delta 1^+$ cells are noted within the basal epidermis (E) (arrows) and the dermis (D). (a-c) In normal skin T lymphocytes are not evenly distributed throughout E and D, rather they form clusters at both these sites. Therefore, in order to immunohistologically demonstrate the presence of T cells within skin, numerous serial sections of each skin specimen were screened to obtain the examples shown. The majority of skin sections contained no lymphocytes.

irregular intraepidermal distribution pattern can be examined reliably only in whole epidermal mounts. Therefore, we analyzed epidermal sheets, which showed that TCR- γ/δ^+ cells comprise between ~ 1 and 15% of CD3 $^+$ lymphocytes within the basal epidermis. Thus, the relative proportion of intraepidermal CD3 $^+$ lymphocytes that were TCR- γ/δ^+ was comparable with the other lymphoid tissues. The same was true for perivascular TCR- γ/δ^+ cells.

TCR- γ/δ^+ lymphocytes in all tissues harboring these cells did not occur in large clusters and were observed only rarely in small groups of two to five cells. Instead, TCR- γ/δ^+ lymphocytes appeared to be dispersed as single cells in the respective tissue. In the few samples of fetal (14–21 wk) and adult lung analyzed, we were unable to detect TCR- γ/δ^+ cells. More extensive studies are therefore needed to reliably determine the presence or absence of TCR- γ/δ^+ lymphocytes in bronchus-associated lymphoid tissue. None of the nonlymphoid fetal or adult tissues examined showed staining with either anti-TCR- $\delta 1$ or anti-C γ M1. We occasionally observed one or two TCR- γ/δ^+ lymphocytes in very close proximity of or even within blood vessels in several organs, reflecting the ability of these cells to circulate.

Discussion

Using two specific mAbs, anti-TCR- $\delta 1$ and anti-C γ M1 we carried out a direct analysis of TCR- γ/δ^+ cells in man. Anti-TCR- $\delta 1$ stained all WT31 $^-$ or WT31 dull lymphocytes, indicating that all CD3 $^+$ TCR- α/β^- cells in adult peripheral blood and in fetal as well as postnatal thymus express a TCR- δ chain. This staining of all WT31 $^-$ or WT31 dull CD3 $^+$ cells taken together with other data, suggests that anti-TCR- $\delta 1$ and anti-C γ M1 recognize TCR- γ/δ determinants shared by all γ/δ T cells.

Based on anti-TCR- $\delta 1$ reactivity in peripheral blood, thymus, and lymphoid tissue, we conclude that γ/δ T cells range between >0.5 and 16% (mean, $\sim 4\%$) of CD3 $^+$ cells in a panel of individuals. Given the variation of TCR- γ/δ^+ T lymphocyte counts in individuals, several lymphoid tissues (thymus, spleen, intestine, and liver) were available from the same fetuses in eight of nine cases and simultaneous quantitative analysis of TCR- γ/δ^+ cells indicated that the relative percentage of TCR- γ/δ^+ cells populating these organs was consistent for a given fetus. The presence of 10–16% of the T cells as TCR- γ/δ^+ cells was occasionally found for a particular donor. Whether high TCR- γ/δ^+ lymphocyte proportions in some individuals with an otherwise normal distribution of haemopoietic cell populations reflect individual variations within a quantitatively normal resting range, or rather, indicate a state of activation of this cell pool, remains to be determined.

TCR- γ/δ -bearing lymphocytes have been generally referred to as double negatives. Although a sizeable portion of γ/δ T cells do lack CD4 and CD8 expression, nearly half express CD8 (however at lower levels than TCR- α/β^+ lymphocytes) and a very small portion express CD4. Other markers of differentiation and activation that are found on α/β T cells also occur on TCR- γ/δ -bearing cells although not always at similar frequencies (Table I). Thus, the γ/δ T cell population appears to be phenotypically diverse and may therefore itself be composed of several subsets.

Anti-TCR- $\delta 1$ and anti-C γ M1 mAbs also made it possible to analyze the tissue distribution and microanatomical location of these cells. TCR- γ/δ^+ cells regularly populate all lymphoid tissues and were usually present as ~ 1 –15% of the total CD3 $^+$ cells. In organized peripheral lymphoid tissues (tonsils, lymph nodes, Peyer's patches,

and spleen), TCR- γ/δ^+ cells were scattered throughout the classical T cell areas and thus, in general, paralleled the microanatomical location of TCR- α/β^+ lymphocytes, however, at lower densities. Some TCR- γ/δ^+ cells were present in B cell areas of lymph follicles as well as within marginal zones and red pulp in spleen. At these two latter sites, in some specimens the proportion of TCR- γ/δ^+ to TCR- α/β^+ cells appeared higher than within T zones in general. This pattern raises the possibility of a site-specific role for TCR- γ/δ in such predominantly B cell areas and in the red pulp of the spleen.

The vast majority of murine intraepithelial gut lymphocytes appear to bear TCR- γ/δ (47). Within the avian intestine, TCR- γ/δ^+ lymphocytes predominantly localize to the gut epithelium (48). This abundant presence of TCR- γ/δ^+ cells in surface epithelia has fostered speculations that these cells may recognize a specific set of antigens prevalently entering the organism via the gut or might have specific effector functions closely related to this particular microenvironment (49). In man, in addition to Peyer's patches, the gut-associated lymphoid tissue consists of CD3⁺ lymphocytes located within the epithelium and the subepithelial connective tissue (39). In human gut we found TCR- γ/δ^+ lymphocytes at both these locations accounting for ~1-15% of the total CD3⁺ cell population. Thus, unlike the results in mouse, in none of the human samples analyzed in this study were TCR- γ/δ^+ gut-associated lymphocytes found to comprise more than ~15% of total CD3⁺ lymphocytes. Furthermore, these cells did not preferentially populate the gut epithelium, but they were present in both the epithelium and within the lamina propria. Thus, while TCR- γ/δ^+ cells are present as part of the human gut-associated lymphoid system, these results differ both quantitatively and qualitatively from that reported for mice and chicken (47, 48). It remains to be determined whether the biological role of murine and avian TCR- γ/δ^+ intraepithelial gut lymphocytes in man is provided by a specific subset of TCR- α/β^+ cells or rather is adequately fulfilled by the more limited number of TCR- γ/δ^+ gut lymphocytes found in man.

Murine CD3⁺ epidermal lymphocytes are highly dendritic, located at a supra-basal position, and most, if not all of them, express TCR- γ/δ (50). In man, normal skin is regularly populated by small numbers of lymphocytes (46, Foster, C. A., H. Yokozeki, F. Koning, et al., manuscript in preparation and our unpublished observation). Within the epidermis these cells are scattered throughout the most basal keratinocyte layers, in the dermis they mainly home to perivascular areas. This study shows that ~1-15% of both these intraepidermal and dermal CD3⁺ lymphocytes expressed TCR- γ/δ . Based on immunohistologic studies of epidermal sheet preparations, preliminary evidence exists that in the human epidermis, in addition to intraepidermal basally located TCR- γ/δ^+ lymphocytes, dendritic suprabasal CD1⁺, HLA-DR⁺, Fc-R⁺, CD4⁺ epidermal cells (presumably Langerhans cells) display weak anti-CD3/TCR- γ/δ reactivity (V. Groh, unpublished observation). It remains to be determined whether these staining results indeed reflect CD3/TCR- γ/δ expression by these cells, which according to present knowledge, are thought to belong to the monocyte/macrophage lineage (51). However, both their microanatomy within the skin as well as their phenotype and function would still differ from their putative murine counterpart. Thus, at present, compared with the situation in mice, it is less clear that a prevalent tropism of TCR- γ/δ^+ cells for human epithelia occurs.

Taken together, it appears that human TCR- γ/δ^+ cells are present in similar

proportions throughout virtually all lymphoid organs, and in general, tend to be located within each organ wherever TCR- α/β^+ lymphocytes are found.

Rather than existing only as a precursor population of TCR- α/β^+ lymphocytes, the results presented in this study and data emerging regarding the effector capabilities of these cells (52) suggest that TCR- γ/δ^+ cells constitute a distinct mature subpopulation of T cells distributed along with TCR- α/β^+ cells throughout the human lymphoid system. While the possibility of a special role for TCR- γ/δ^+ cells in epithelial organs in man must be considered, it seems likely that these cells utilize their distinct TCR and possibly distinct antigen-presenting molecules to broaden the T cell repertoire. As the current study shows they may do this as a phenotypically diverse population of T cells able to participate in immune responses at virtually all immunologically relevant sites within the human organism.

Summary

A direct quantitative and phenotypic cytofluorographic analysis of TCR- γ/δ^+ lymphocytes as well as an immunohistologic study of their tissue distribution and microanatomy was made possible by the availability of two mAbs (anti-TCR- $\delta 1$ and anti-C γ M1) specific for framework determinants on human TCR γ and δ chains, respectively. TCR- γ/δ^+ lymphocytes, ranging between >0.5 and 16% of CD3 $^+$ cells, were found in fetal and postnatal thymus, fetal and adult peripheral lymphoid organs, and adult peripheral blood. While TCR- γ/δ^+ lymphocytes comprised a small subpopulation of T cells (mean, \sim 4%) occasionally >10–16% of CD3 $^+$ cells expressed TCR- γ/δ . Virtually all TCR- γ/δ^+ thymocytes/lymphocytes expressed CD7, CD2, and CD5 but were heterogeneous with respect to their expression of CD1, CD4, CD8, CD28, CD11b, CD16, and Leu-7. Human TCR- γ/δ^+ cells populate both organized lymphoid tissues (thymus, tonsil, lymphnode, and spleen) as well as the gut- and skin-associated lymphoid systems at similar frequencies without obvious tropism for epithelial microenvironments. TCR- γ/δ^+ lymphocytes tend to be located within a given organ wherever TCR- α/β^+ lymphocytes are found. This study shows that TCR- γ/δ^+ lymphocytes constitute a small but numerically important, phenotypically diverse T cell population distributed throughout the body. These results support the concept that TCR- γ/δ^+ cells comprise a distinct, functionally heterogeneous, mature T cell sublineage that may substantially broaden the T cell repertoire at all immunologically relevant sites.

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