EVIDENCE FOR EXTRATHYMIC CHANGES IN THE T CELL RECEPTOR γ/δ REPERTOIRE

By CHRISTINA M. PARKER,*§ VERONIKA GROH,‖ HAMID BAND,*§ STEVEN A. PORCELLI,*§ CRAIG MORITA,*§ MARINA FABBI,‖ DAVID GLASS,¶ JACK L. STROMINGER,‡‖ AND MICHAEL B. BRENNER*§

*From the *Laboratory of Immunochemistry and the ‡Division of Tumor Virology, Dana-Farber Cancer Institute; the §Department of Rheumatology and Immunology, Harvard Medical School, Boston, Massachusetts, 02115; the ‖Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138; and the ¶Division of Rheumatology, Children's Hospital Medical Center, Cincinnati, Ohio 45229*

Lymphocytes express two types of TCRs, either α/β (1-3) or γ/δ (4). The genes encoding the TCR γ and δ chains are composed of V, D, J, and C regions that undergo somatic rearrangement during development (5, 6). There are only eight functional Vγ gene segments and perhaps 5-10 functional Vδ gene segments identified in humans (6). While the specificity of the TCR-γ/δ is poorly understood, the limited germline diversity suggested that TCR-γ/δ-bearing lymphocytes might recognize antigen in the context of relatively nonpolymorphic antigen-presenting molecules (6). Recently, alloreactive TCR-γ/δ cells specific for MHC class I and class II gene products, as well as class I-like gene products (Tla and Qa), have been described (7-9). The distinct nature of the TCR-γ/δ, the fact that lymphocytes that bear it frequently lack CD4 and CD8, and the non-MHC-restricted recognition of tumor targets by some TCR-γ/δ cells (10), led us to suggest that these TCRs might recognize cell surface molecules not encoded in the MHC (10). It was recently demonstrated that a human TCR-γ/δ clone can recognize non-MHC-encoded CD1 antigens that might serve as novel antigen-presenting molecules for $CD4^-8^-$ T cells (11). In addition, some of the specific antigens that γ/δ T cells can recognize have been identified, such as mycobacterial antigens (including heat-shock proteins) (12-16) and tetanus toxoid (17). TCR-γ/δ^+ lymphocytes expand markedly in number in lymphoid organs and in tissue lesions in response to the mycobacterial antigens (13, 15).

TCR-γ/δ cells comprise ~5% (0.5-20%) of the $CD3^+$ cells in human peripheral blood and in lymphoid tissues (18-20). However, in mice, TCR-γ/δ cells preferen-

This work was supported by grants from the National Institutes of Health to M. B. Brenner and J. L Strominger. C. Parker is supported by an NIH Physician Scientist Training Award; V. Groh is supported by the Cancer Research Institute/Rudolph M. Montgelas Fellowship; H. Band is supported by a Leukemia Society of America Special Fellowship, an Investigator Award from the Arthritis Foundation, and NIH First Award; S. Porcelli is supported by an NIH Postdoctoral Fellowship; C. Morita is supported by an NIH Institutional Training Grant to the Brigham and Women's Hospital's Department of Rheumatology and Immunology, and M. Fabbi is on leave of absence from the Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

Address correspondence to Christina M. Parker, Laboratory of Immunochemistry, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

tially localize and constitute the predominant subset at epithelial surfaces, such as in the skin (dendritic epidermal cells [dEC][1]) (21) and in the intestine (intraepithelial lymphocytes [IEL]) (22, 23). dEC express a single Vγ/Vδ pair (Vγ3/Vδ1) and have very limited diversity at their V(D)J junctions, resulting in a population of T cells that express virtually identical receptors in this location (24). In contrast, gut IEL express predominantly two different Vγ/Vδ pairs (Vγ5/Vδ4 or Vγ5/Vδ6) and have much greater junctional diversity (23). In the mouse thymus, Vγ and Vδ gene rearrangements appear in a sequential order (25). This ordered pattern in development may account for the nonrandom expression of specific pairs of Vγ and Vδ gene segments observed at different anatomical sites. Alternatively, or in addition, various types of thymic selection, differential homing, or peripheral expansion might influence the V gene pairs utilized and their peripheral locations. The site-specific localization of γ/δ T cells bearing certain V gene pairs and the differences in diversity of the junctional regions has led to the hypothesis that each cell population might perform distinct functions related to their anatomical site.

In humans, the marked epithelial localization seen in the mouse has not been observed (18). However, the relatively small percentage of TCR-γ/δ cells in gut were noted preferentially to localize to the epithelium rather than the lamina propria in one study (26). Yet, selective Vγ and Vδ gene pairing and differences in V gene usage at various anatomical sites have been observed in man. The Vγ2/Vδ2 chain pair occurs in most individuals on >70% of the circulating γ/δ T cells (27, 28). In contrast, Vδ1$^+$ cells are prevalent in late fetal and postnatal thymus and in the red pulp of spleen (28, 29). To gain insight into the differential TCR-γ/δ variable chain usage in thymus compared with peripheral blood, in this report, both thymic and extrathymic influences on the TCR-γ/δ repertoire were studied. The results provide evidence that a marked extrathymic expansion of γ/δ T cells bearing certain V gene segments occurs and is an important feature in determining the peripheral TCR-γ/δ repertoire in man.

Materials and Methods

mAbs. mAbs used were SPV-T3b (anti-CD3) (30), anti-TCR-δ1 (pan-reactive anti-TCR-δ) (31), δTCS1 (anti-Vδ1-Jδ1, which appears to represent the most frequent Vδ1 rearrangement expressed in peripheral blood) (29, 32), BB3 (anti-Vδ2) (33, 34), anti-Tiγa (anti-Vγ2 [also called Vγ9, for nomenclature see reference 6]) (35), anti-CγM1 (pan-reactive anti-TCR-γ) (36), BMA 031 (pan-reactive anti-TCR-α/β; kind gift of Dr. Kurrle at Behringwerke AG, Marburg, FRG), and UCHL1 (anti-CD45RO) (37). Isotype-matched mAbs that do not react with human leukocytes were used as controls.

In Vitro Transcription/Translation. A truncated TCR-δ insert was prepared from the IDP2 TCR-δ cDNA clone (38) containing sequences downstream of the FnuD II site at the codon encoding amino acid 106 in the D region. The truncated Cδ insert was ligated into the vector pSP73 downstream from the SP6 promoter to yield the pSP73.Cδ. In this construct, the first methionine codon is at amino acid residue 138 within the constant region of the TCR-δ chain. Therefore, in vitro transcription and translation of this construct should yield a protein that only includes amino acids encoded by the Cδ gene segment. Constructs encoding the full-length IDP2-δ protein (pGEM3-0240/38) and the IDP2 γ protein (pSP65.IDP2γ) have been

[1] *Abbreviations used in this paper:* CBL, umbilical cord blood lymphocytes; CBMC, cord blood mononuclear cells; dEC, dendritic epidermal cells; IEL, intraepithelial lymphocytes.

described previously (31, 39). These three cDNA constructs were linearized with appropriate restriction endonucleases, transcribed, and translated in vitro. The resulting protein products were then immunoprecipitated utilizing mAb anti-CγM1, anti-TCR-δ1, and an IgG1 isotype-matched control antibody.

Immunofluorescence Analysis of Cell Suspensions. Heparinized blood was obtained from umbilical cords of uncomplicated deliveries at the Brigham and Women's Hospital. Cord blood mononuclear cells (CBMC) were isolated by centrifugation at 1,340 *g* with 45% sepracell-MN (Sepratech, Oklahoma City, OK), 55% cord blood. When necessary, residual RBC were lysed with ammonium chloride (40). PBMC from peripheral blood were isolated using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). Thymocyte suspensions were prepared by gently teasing thymic tissue into single cell suspensions using the entire lobule to ensure that cortical and medullary thymocytes were accurately represented. Tonsil-derived lymphocytes were prepared by gently teasing entire tonsil tissue into a single cell suspension. For single-color analysis thymocytes, PBMC, CBMC, or tonsillar lymphocytes were resuspended at 2–4 × 10^6 cells/ml in staining buffer (PBS/5% human serum/1% BSA/0.02% NaN_3) containing saturating amounts of mAbs and incubated for 1 h at 4°C. After three washes with staining buffer, cells were incubated with FITC-labeled $F(ab')_2$ goat anti-mouse Ig (Tago Inc., Burlingame, CA). Two-color staining was performed using unconjugated mAbs followed by FITC-conjugated goat anti-mouse Ig, then followed by an incubation with saturating amounts of normal mouse serum. Biotin-conjugated mAbs followed by PE-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA, or Tago Inc.) were used as the second-step reagents. Labeled cells were analyzed with either a Facscan flow cytometer (Becton Dickinson & Co.) or Epics C (Coulter Electronics Inc., Hialeah, FL).

T Cell Lines. PBMC from a normal donor were isolated by density gradient centrifugation with Ficoll-Paque (See above). The PBMC were depleted of α/β T cells by incubation with anti-TCR-α/β (BMA 031) followed by goat anti-mouse Ig-conjugated magnetic beads (M450; Dynal, Oslo, Norway). Cells with attached beads were removed by adherence to a magnet. TCR-α/β-depleted lymphocytes were then enriched for TCR-γ/δ cells by incubation with the panreactive anti-TCR-γ/δ antibody (anti-TCR-δ1) followed by incubation with the goat anti-mouse Ig-conjugated magnetic beads and magnet selection. The γ/δ T cell-enriched line was cultured with irradiated PBL feeder cells and maintained in culture by periodic restimulation with PHA as previously described (4). After 4 wk of culture, the cells in this line stained homogeneously with the pan-reactive TCR-γ/δ mAb; 7.6% of the cells in this line stained with the Vδ1-specific mAb and 93.5% stained with the Vδ2-specific mAb. Positive selection with an anti-CD8 mAb (OKT8) and magnetic beads produced a homogeneously $V\delta1^+$ cell line. In long-term culture, >99% of the lymphocytes in the OKT8-selected line were positive with the anti-Vδ1 mAb, and >99% of the lymphocytes in the original TCR-γ/δ-enriched line were reactive with the anti-Vδ2 mAb.

Results

Anti-TCRδ1 mAb Recognizes a Cδ-encoded Determinant. Four mAbs directed against distinct TCR-γ/δ determinants were utilized. mAb δTCS1 detects a Vδ1-Jδ1-encoded determinant (called anti-Vδ1 in this study) (29, 32), mAb BB3 (detects a Vδ2-encoded determinant (anti-Vδ2) (33, 34), and anti-TiγA detects a Vγ2-encoded determinant (anti-Vγ2) (35). In addition, mAb anti-TCR-δ1 has broad reactivity against γ/δ T cells and is thought to be panreactive. Here, a cDNA construct was transcribed and translated in vitro to produce a protein product including only the C region of the TCR-δ protein, and this protein product was recognized by the anti-TCR-δ1 mAb (Fig. 1). This demonstrates directly that the anti-TCR-δ1 mAb recognizes a determinant in the Cδ-encoded portion of the TCR δ chain and thus should recognize all TCR-δ-bearing lymphocytes (See Materials and Methods, and Fig. 1).

Age-related Changes in TCR-γ/δ V Gene Usage in the Periphery. Seven umbilical cord blood samples and 27 thymi were analyzed for γ/δ T cell number and variable chain

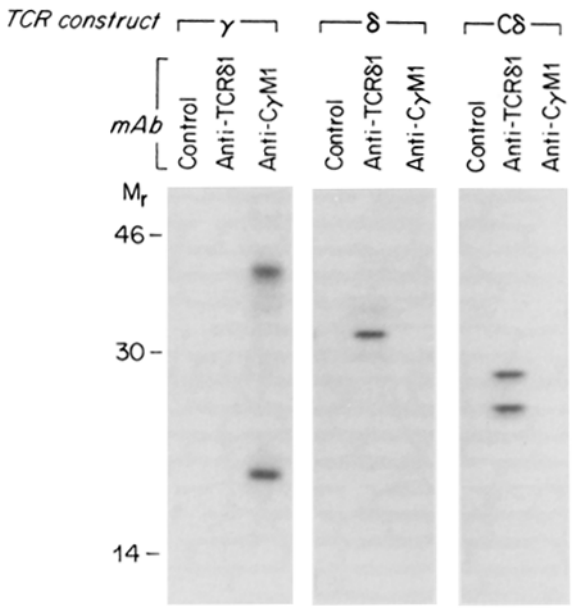

FIGURE 1. Anti-TCR-δ1 recognizes a Cδ-encoded epitope. Anti-TCR-δ1 mAb immunoprecipitated the in vitro translated product whose synthesis was directed by a full-length TCR-δ construct (see TCR-δ construct panel). To determine if this mAb reacted with a determinant in the constant region of the δ chain, in vitro transcription and translation of a truncated Cδ construct was performed to produce a C region peptide. The anti-TCR-δ1 mAb immunoprecipitated this C region peptide (see Cδ construct panel). Isotype-matched control mAb and anti-CγM1 (anti-TCR-γ) mAb failed to recognize these δ chain–encoded products. The anti-TCR-γ mAb did immunoprecipitate the protein product of the TCR-γ construct (see TCR-γ construct panel), while anti-TCR-δ1 did not immunoprecipitate the TCR-γ protein product. Thus, anti-TCR-δ1 reacts specifically with a determinant encoded in the Cδ region of the TCR-δ protein, indicating that it should be reactive with all TCR-δ-bearing T cells (see Materials and Methods for experimental details).

usage. The TCR-γ/δ V gene usage in cord blood and in thymus was quite similar, but differed from adult peripheral blood. Cord blood TCR-γ/δ cells used predominantly Vδ1-encoded products (50% of γ/δ T cells) and less frequently expressed Vδ2-encoded products (25% of γ/δ T cells). Similarly, in the thymus, Vδ1 expression was predominant as it was found on 65% of the γ/δ T cells, while Vδ2 was expressed on only 10–15% of thymic γ/δ T cells. In contrast to thymus and cord blood, adult (>21 yr old) peripheral blood V gene usage was characterized by predominant expression of Vδ2-encoded subunits (>70% of γ/δ T cells), while a smaller number of cells bore Vδ1-encoded receptors (on <30% of γ/δ T cells) in most individuals. In addition, the proportion of T cells expressing TCR-γ/δ in cord blood was quite similar in all the individuals tested (mean of 1.73% [SEM = 0.18] of $CD3^+$ cells), and was on average threefold lower than the proportion of T cells expressing TCR-γ/δ in adult peripheral blood (5.7% [SEM = 1.4] of $CD3^+$ cells). These preliminary studies thus confirmed earlier reports (19, 20, 29) showing that a difference existed between γ/δ V gene usage in thymic and peripheral locations. Furthermore, the observation that peripheral umbilical cord blood at birth was similar to thymus, but distinct from adult peripheral blood suggested the existence of a significant age-related extrathymic expansion of circulating γ/δ T cells associated with a change in the predominant V genes they expressed. This observation was examined in detail here.

The proportion of T cells expressing γ/δ TCRs (anti-TCR-$δ1^+$) gradually rose from a mean of 1.73% of the T cells at birth, to a mean of 10% of the T cells at 6 yr of age, after which the mean fell to a lower level, with a wide range of individual values in adulthood (Fig. 2 *A*, *top*). Variable gene segment usage was analyzed in

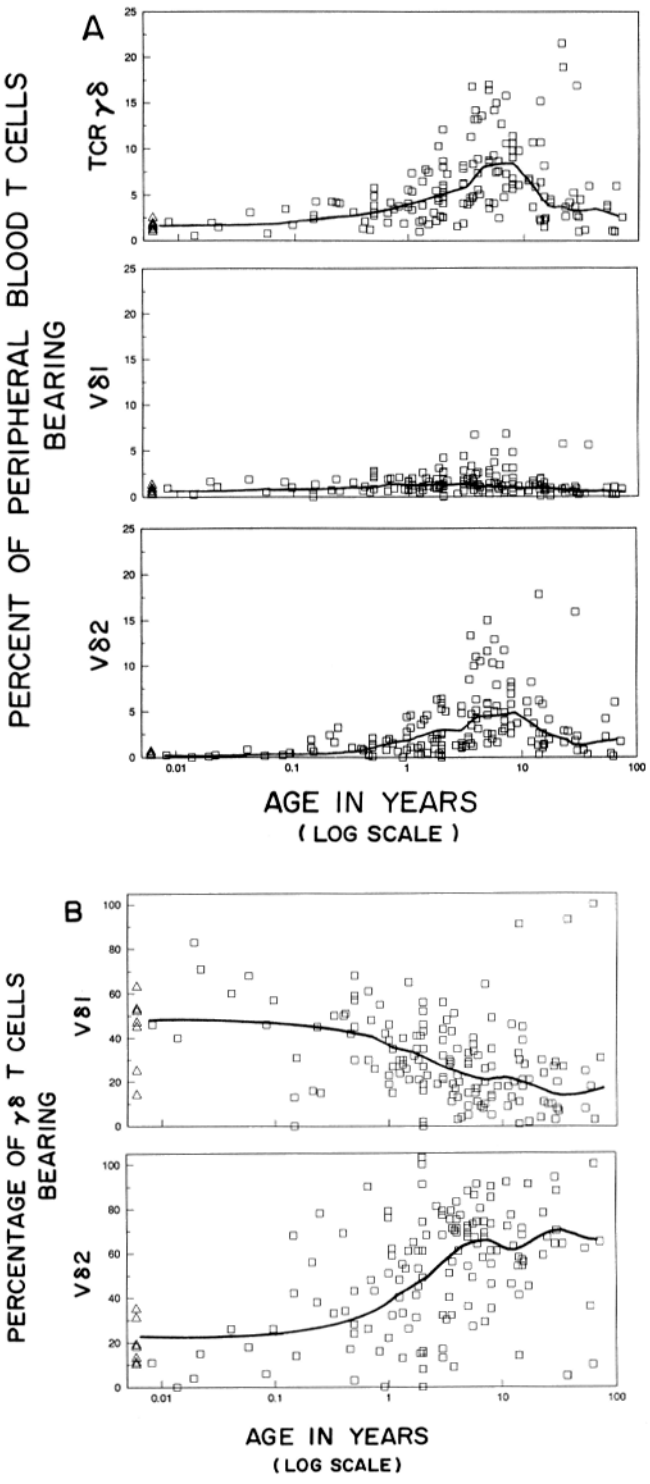

FIGURE 2. (*A*) Age-related variation in the percentage of T cells expressing TCR-γ/δ, Vδ1, or Vδ2 in human peripheral blood. The percentage of peripheral blood $CD3^+$ cells expressing TCR-γ/δ (anti-TCR-$\delta1^+$), Vδ1 (δTCS-1^+), and Vδ2 ($BB3^+$) was determined by single-color FACS analysis (see Materials and Methods), and plotted versus age. Umbilical cord blood is represented by open triangles adjacent to the *y*-axis (these values were tightly grouped so that many open triangles are overlaying one another), and samples from children and adults are represented by open squares. The lines on the plots were generated using a lowess smoother computer analysis ($f = 0.2$) (51). The percentage of cells positive for a given mAb was calculated using the formula: 100 × (percent of PBL positive with mAb shown on the *y* axis/percent of PBL positive with mAb SPV-T3b). (*B*) Age-related variation in the percentage of γ/δ T cells that bear Vδ1- or Vδ2-encoded receptors in peripheral blood. The percentage of peripheral blood anti-TCR-$\delta1^+$ cells expressing Vδ1 or Vδ2 was determined by single-color FACS analysis and plotted versus age. Umbilical cord blood determinations are represented by open triangles adjacent to the *y*-axis, child and adult samples by open squares. The lines on the plots were generated using a lowess smoother computer analysis ($f = 0.5$) (51). Percentage of cells positive for a given mAb was calculated using the formula: 100× (percent of PBL positive with mAb shown on the *y*-axis/percent of PBL positive with anti-TCR-δ1).

these individuals by staining with anti-Vδ1 and anti-Vδ2 mAbs. During this time period, the proportion of T cells expressing Vδ1 remained nearly constant while the percentage of T cells expressing Vδ2 increased strikingly (Fig. 2 *A*, *middle* and *bottom*). In addition to the age-related increase in the number of γ/δ T cells, a shift from a Vδ1 predominance to a Vδ2 predominance over the first 6 yr of life was also observed (Fig. 2 *B*, *top* and *bottom*). Thus, both the increase in cell number (Fig. 2 *A*) and the change in V gene predominance (Fig. 2 *B*) could be accounted for by an age-related expansion of the Vδ2-bearing T cell subset.

Vδ2 Expansion in the Periphery Occurs in the Absence of a Parallel Thymic Wave. To address whether a postnatal thymic wave of TCR Vδ2-expressing cells could explain the rising number of Vδ2-bearing cells in the periphery with advancing age, 29 thymi between 3 wk and 8 yr of age were examined by FACS staining for TCR-γ/δ cell number and V gene segment expression. In addition, paired samples of peripheral blood were obtained at the same point in time from 18 of these individuals and analyzed for TCR-γ/δ cell number and V gene segment expression. In contrast to the peripheral blood where an age-related increase in the proportion of T cells expressing the TCR-γ/δ was observed (Fig. 2 *A*, *top*), in the thymus, the proportion of T cells expressing the TCR-γ/δ remained constant with increasing age, up to the age of 8 yr. This resulted in a rise in the ratio of T cells expressing TCR-γ/δ in the peripheral blood compared with the thymus in the paired thymus/peripheral blood samples over time (Fig. 3 *A*). In addition, thymus samples showed no change in the percentage of TCR-γ/δ cells bearing either Vδ1- or Vδ2-encoded receptors over time. In none of the thymi did we observe >30% $V\delta2^+$ cells (mean = 12%), including thymi from individuals in whom the Vδ2 expansion (often to >85% of the γ/δ cells) had already occurred in the paired peripheral blood sample (Fig. 3 *B*). Since the percentage of γ/δ T cells and usage of V gene segments was unchanged over time for all thymus samples examined, no evidence to support the occurrence of a postnatal thymic wave of Vδ2-bearing cells was found. Instead, it seemed more likely that the expansion of TCR-γ/δ cells bearing Vδ2 in the periphery may have resulted from a postnatal extrathymic expansion of this subset, while the Vδ1-bearing cells remained constant in number.

Age-related Expression of CD45RO on Vδ2-bearing Cells. The CD45 transmembrane protein is found on all T cells and occurs in distinct isoforms as a result of alternatively spliced exons (41). The smallest isoform, CD45RO, lacks all of the differentially spliced exons and is recognized by mAb UCHL1 (42). Nearly all T cells (>95%) from neonatal peripheral blood are negative or express only low levels of CD45RO (43, 44). With increasing age, the percentage of T cells that express high levels of CD45RO rises until ~60% of all of the circulating T cells are $UCHL1^{hi}$ in adulthood (43). A similar rise has also been demonstrated for γ/δ T cells in peripheral blood (43). To confirm that both $V\delta1^+$ and $V\delta2^+$ γ/δ T cells were capable of expressing CD45RO after activation, in vitro cultured cell lines expressing these V genes were examined by FACS analysis; which revealed that both expressed increased levels of CD45RO. The $V\delta2^+$ line expressed CD45RO at high levels, while $V\delta1^+$ cell lines expressed CD45RO at moderate levels. In addition, both Vδ1- and Vδ2-expressing thymocytes and tonsil-derived lymphocytes expressed moderate or high levels of CD45RO (data not shown).

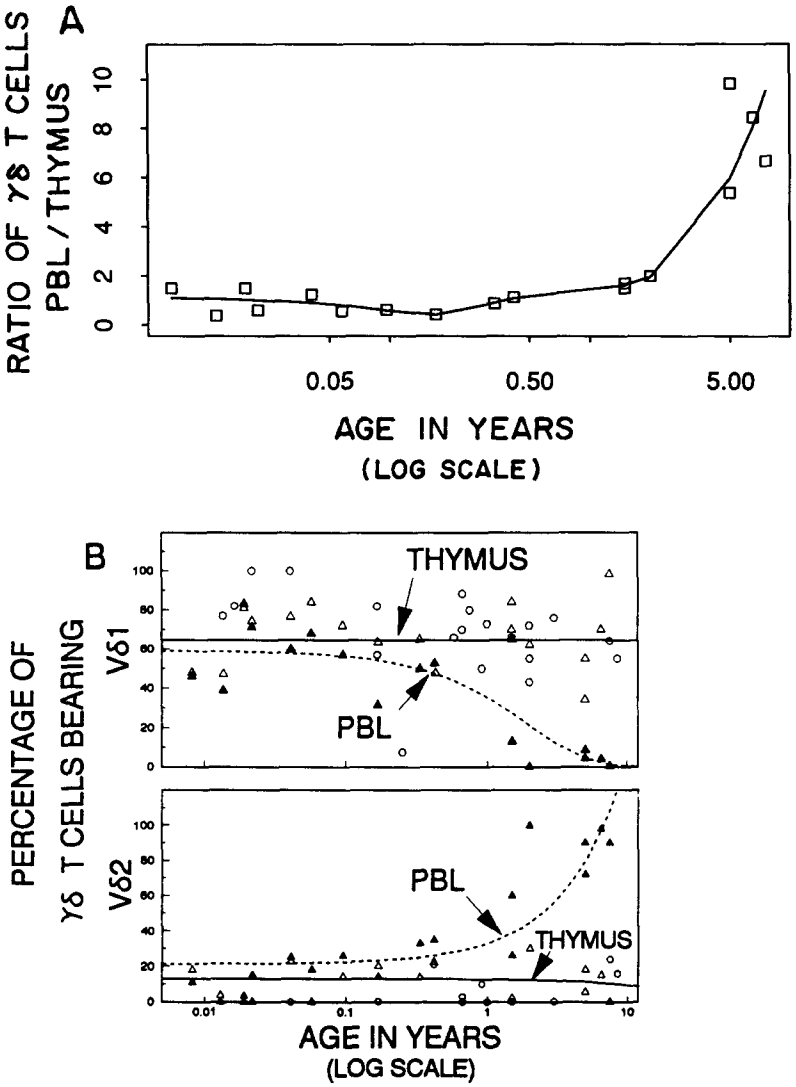

FIGURE 3. (*A*) Age-related change in the TCR-γ/δ percentage in thymus compared with peripheral blood. The percentage of $CD3^+$ ($SPV\text{-}T3b^+$) cells expressing TCR-γ/δ (anti-TCR-δ1$^+$) in the thymus and in peripheral blood in the same individual at the same point in time was determined. The ratio of the TCR-γ/δ percentage in thymus compared with peripheral blood was calculated using the formula: (percent of PBL positive with mAb anti-TCR-δ1/percent of PBL positive with mAb SPV-T3b)/(percent of thymocytes positive with anti-TCR-δ1/percent of thymocytes positive with SPV-T3b). The line on the plot was generated using a lowess smoother computer analysis ($f = 0.5$) (51). (*B*) Age-related change in TCR-δ variable gene segment using in thymus compared with peripheral blood. The percentage of $CD3^+$ ($SPV\text{-}T3b^+$) cells expressing Vδ1 (δTCS-1$^+$, *top*) or Vδ2 (BB3$^+$, *bottom*) in the thymus and in peripheral blood was determined by single-color FACS analysis and plotted versus age. Peripheral blood determinations are represented by solid symbols while thymus determinations are displayed as open symbols. Circles represent thymus samples without a paired peripheral blood sample. Triangles represent paired samples of thymus and peripheral blood obtained from the same individual at the same point in time. The percentage of cells positive with a given mAb was calculated as in Fig. 2 *A*. Computer-generated lines reveal age-related trends.

Since an increase in expression of CD45RO occurs when naive T cells are activated, this antigen may serve as a differentiation marker for previously activated or "memory" T cells (44, 45). We therefore examined whether the expansion of Vδ2-bearing cells observed here (Fig. 2 *A*, *bottom*) correlated with expansion of naive ($CD45RO^-$ or $CD45RO^{lo}$) or of previously activated ($CD45RO^{hi}$) cells. In umbilical cord blood lymphocytes (CBL), two populations of lymphocytes were observed, one that was unreactive ($CD45RO^-$) and one that was weakly reactive ($CD45RO^{lo}$) with UCHL1 mAb (Fig. 4 *A*). Very few (<5%) of neonatal T cells stained brightly with UCHL1. On PBL from children of increasing age, a third population of cells was observed that stained brightly ($CD45RO^{hi}$). The $CD45RO^{lo}$ population was small in adult PBL, while the $CD45RO^{hi}$ population predominated (Fig. 4 *A*). In two-color FACS analysis, both $V\delta1^+$ and $V\delta2^+$ T cells in umbilical cord blood lacked the $CD45RO^{hi}$ phenotype. By 1 yr of life, $CD45RO^{hi}$ was expressed on ~30% of the TCR-γ/δ^+ cells. This percentage continued to rise between 1 and 18 yr of life to ~60% of the $V\delta2^+$ cells, suggesting that a significant proportion of these cells were previously activated (Fig. 4 *B*). In contrast to $V\delta2^+$ lymphocytes, $V\delta1^+$ lymphocytes remained <20% $CD45RO^{hi}$ even in adulthood (Fig. 4 *B*). Moreover, no shift from $CD45RO^-$ to $CD45RO^{lo}$ could be identified on Vδ1-bearing T cells from children of increasing age (through age 18) (data not shown). Thus, a striking differ-

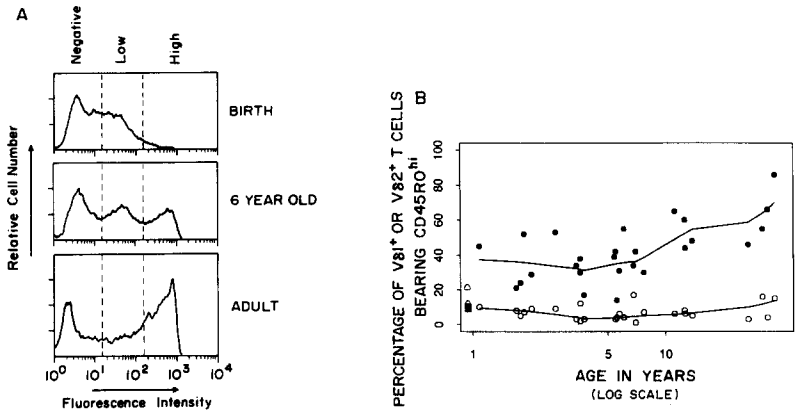

FIGURE 4. (*A*) CD45RO expression on PBL. CD45RO expression was analyzed on PBL at birth (cord blood), from a 6-yr-old child, and from an adult. The 6-yr-old child's profile was utilized to set cursors dividing the PBL into populations with negative ($CD45RO^-$), low ($CD45RO^{lo}$), or high ($CD45RO^{hi}$) expression of CD45RO determined by staining with mAb UCHL1 and one-color FACS analysis. (*B*) $CD45RO^{hi}$ expression of Vδ1- or Vδ2-bearing T cells in peripheral blood. The percentage of $V\delta1^+$ or $V\delta2^+$ γ/δ T cells that express $CD45RO^{hi}$ was determined by two-color FACS analysis on CBL (*triangles adjacent to the y-axis*) and on PBL (*circles*) and plotted versus age. $V\delta1^+$ γ/δ cells (δTCS1$^+$, *open symbols*) rarely express $CD45RO^{hi}$, while $V\delta2^+$ γ/δ T cells (BB3$^+$, *closed symbols*) showed an age-related increase in the percentage that express $CD45RO^{hi}$. The percentage of $V\delta1^+$ or $V\delta2^+$ cells that express $CD45RO^{hi}$ was determined in two-color FACS analysis by gating on the cells positive with the Vδ-specific mAb and then determining the percentage of the gated cells that were in each of the three regions of UCHL1 staining determined in Fig. 4 *A*. The percentage of cells expressing $CD45RO^{hi}$ was calculated using the formula: number of $V\delta1^+$ or $V\delta2^+$ cells in the $CD45RO^{hi}$ region/total number of $V\delta1^+$ or $V\delta2^+$ cells. The lines on the plot were generated using a lowess smoother computer analysis ($f = 0.5$) (51).

ence existed in peripheral blood between the Vδ2$^+$ TCR-γ/δ cells, which expanded in an age-related fashion and expressed a phenotype characteristic of "memory T cells", and the Vδ1$^+$ cells, which failed to expand in number or to express this memory phenotype. Acquisition of the memory phenotype on Vδ2$^+$ γ/δ T cells occurred during the age period in which the proportion of TCR-γ/δ cells bearing Vδ2 in peripheral blood was increasing (compare Fig. 2 *B*, *bottom*, with Fig. 4 *B*), suggesting that the increase in cell number might be related to activation and proliferation of these cells.

Individual Variation in TCR-γ/δ V Gene Repertoire. The observations noted above apply to the population of individuals in general. It has been reported that in most adults, >70% of the TCR-γ/δ repertoire in peripheral blood can be accounted for by a single Vγ2/Vδ2 chain pair (46). The analysis here confirmed this generalization, but also identified a number of subjects who displayed a different pattern of expression of Vγ and Vδ genes in their peripheral blood. Four representative examples of healthy adults with differing TCR-δ V gene-defined phenotypes are shown (Fig. 5). In peripheral blood from subject 1, Vδ2 was expressed on 94% of the γ/δ cells, with Vδ1 expressed on nearly all the remaining TCR-γ/δ cells. In this individual, Vδ2 was paired predominantly with Vγ2, while Vδ1 was almost never paired with Vγ2. This corresponds to the predominant phenotype previously reported by others (46). In subject 2, Vδ2 was expressed on 77% of the γ/δ T cells; the majority of the remaining γ/δ cells expressed Vδ1. Like subject 1, Vδ2 paired predominantly with Vγ2; in contrast to subject 1, Vδ1 paired with Vγ2 on ~60% of Vδ1$^+$ T cells. Subjects 3 and 4 differed strikingly from subjects 1 and 2 since Vδ2 expression was found on only 40 and 7% of the peripheral blood γ/δ T cells, respectively.

Correspondingly, in these latter subjects Vδ1 expression predominated and was present on 41 and 82% of peripheral blood γ/δ T cells, respectively. In the majority

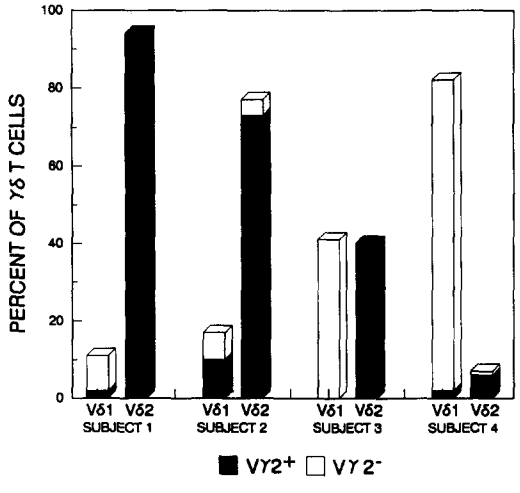

FIGURE 5. Vγ and Vδ gene segment usage differs in four adult individuals. TCR-γ/δ variable chain usage was determined on the peripheral blood of four individuals by two-color FACS analysis. For each individual, the percentage of γ/δ T cells expressing Vδ1 or Vδ2 is represented by the height of the bar. Of the cells that express either Vδ1 or Vδ2, the proportion that pairs with Vγ2 is represented by the part of the bar that is shaded. Note that both the proportion of cells that express each Vδ gene segment and the frequency of pairing of Vγ2 with Vδ1 vary strikingly from subject to subject. Vδ1, Vδ2, and Vγ2 were detected with mAbs δTCS1, BB3, and Tiγa, respectively. The percentage of CD3$^+$ cells positive for the Vδ chains was calculated as in Fig. 2 *A*. The percentage of Vδ1$^+$ or Vδ2$^+$ cells positive with anti-Vγ2 was calculated from two-color FACS by gating on the cells stained with either anti-Vδ1 (δTCS1) or anti-Vδ2 (BB3) mAbs and then determining the percentage of the gated cells that were positive with the anti-Vγ2 (Tiγa) mAb. The percentage of Vδ1$^+$ or Vδ2$^+$ cells expressing Vγ2 was calculated using the formula: number of Vδ1$^+$ or Vδ2$^+$ cells in the Vγ2$^+$ region/total number of Vδ1$^+$ or Vδ2$^+$ cells.

of individuals, Vδ2 usage predominated (subjects 1 and 2), while in the other cases, Vδ1 and Vδ2 were used about equally or Vδ1 predominated (the least frequent occurrence) (subjects 3 and 4, respectively). Thus, while the majority of TCR-γ/δ cells in all subjects expressed either Vδ1 or Vδ2, the relative proportion of cells that expressed each of these TCR δ chains varied. To confirm that an individual measurement in a person reflected a stable phenotype, four adults were examined on at least four occasions over a period of 18 mo. These analyses revealed a modest variation over time in the percentage of T cells expressing TCR-γ/δ, but no significant variation in the relative expression and pairing of Vδ1, Vδ2, and Vγ2. This suggested that the TCR-γ/δ repertoire in adults is relatively stable over a period of months to years.

The γ/δ Gene Repertoire Is Not Solely Determined by Inherited Factors. To determine if background genetic differences could account for the distinct γ/δ V gene repertoires observed among individuals, family and twin studies were performed. In family 1 (Fig. 6 *A*), both parents expressed a less common phenotype, in which Vδ1 gene segment usage predominates over Vδ2 gene segment expression (>50% Vδ1, <30% Vδ2). However, all of the children displayed the common phenotype, expressing Vδ2 on >70% of their TCR-γ/δ cells. Thus, none of the progeny displayed the parental γ/δ phenotype, despite the fact that each MHC haplotype carried by the parents was inherited by at least one of the children. In family 2 (Fig. 6 *B*), one parent ex-

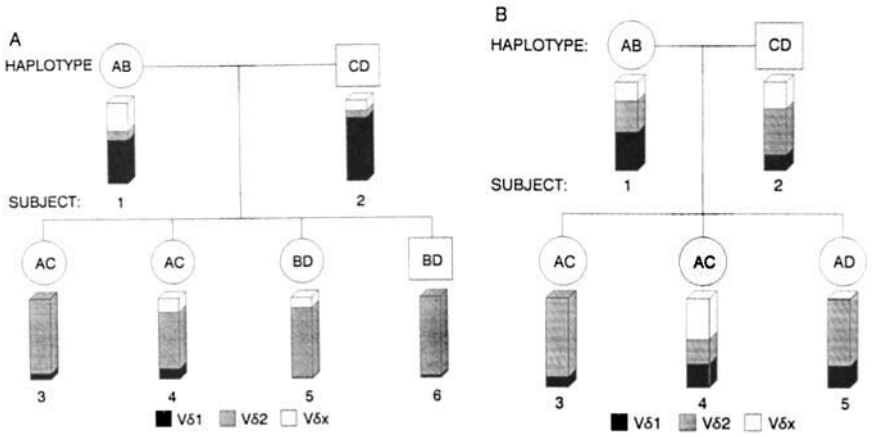

FIGURE 6. Vδ gene usage in families. Vδ gene usage was determined for the members of two families, as described in Fig. 4. The extended MHC haplotypes are represented within the circles or squares by A, B, C, and D. The histogram represents TCR-γ/δ cells, the lowest (*solid*) section represents the proportion of TCR-γ/δ cells expressing Vδ1 gene segments, the middle (*shaded*) section represents the proportion of TCR-γ/δ cells expressing the Vδ2 gene segment, and the top (*open*) section represents the proportion of TCR-γ/δ cells expressing neither Vδ1 nor Vδ2 gene segments (VδX). The ages of family 1 (*A*) members 1, 2, 3, 4, 5, and 6 are 62, 68, 34, 36, 42, and 33 yr, respectively, and the percents of $CD3^+$ cells expressing TCR-γ/δ are 1.13, 4.12, 2.78, 3.69, 8.48, and 10.64%, respectively. MHC haplotypes in family 1 are (A) A2, B5, DR5; (B) Aw24, B39, DR7; (C) Aw31, B7, DR4; and (D) A1, B41. Individual 6 has juvenile rheumatoid arthritis. The ages of family 2 (*B*) members 1, 2, 3, 4, and 5 are 69, 69, 39, 43, and 24 yr, respectively, and the percents of $CD3^+$ cells expressing TCR-γ/δ are 17.64, 0.60, 4.80, 2.51, and 2.71%, respectively. MHC haplotypes in family 2 are (A) A3, B14, DR6, DQw1; (B) A2, B60, DR4; (C) A32, B60, C3, DR4; and (D) A29, B44, DR7, DQw2. Individual 2 has rheumatoid arthritis.

pressed Vδ1 on 40% of the γ/δ cells while the other parent expressed Vδ1 on 11% of the γ/δ T cells. Interestingly, two of the progeny of this pair (subjects 3 and 4) inherited identical MHC haplotypes, yet were markedly different from each other in their expression of TCR-γ/δ variable gene segments. Individual 3 expressed Vδ2 on >90% and Vδ1 on very few of the γ/δ T cells. In contrast, individual 4 expressed Vδ2 on only 27% and Vδ1 on ~25% of the TCR-γ/δ cells. These representative family studies reveal no simple or direct correlation between inheritance of a MHC haplotype and TCR-γ/δ phenotype in PBL. This suggested that factors other than the MHC type must influence TCR-γ/δ V gene usage by peripheral blood T cells. These may include other genetic factors not encoded in the MHC, or environmental influences.

To address whether other genetic elements besides those encoded in the MHC might determine the TCR-γ/δ V gene repertoire, seven sets of identical twins were evaluated (Fig. 7). In four sets, both twins showed nearly identical γ/δ V gene repertoires, expressing the common phenotype with Vδ2 on a majority of their γ/δ T cells. However, several sets revealed divergent V gene usage. In both twin sets A and B, one individual utilized Vδ1 on ~25% of the γ/δ T cells in peripheral blood, while the other individual utilized Vδ1 on <10% of the γ/δ T cells. The twins also differed in Vδ2 usage, since one member of twin set A utilized Vδ2 on 85% of the γ/δ T cells, while the other individual utilized Vδ2 on 60% of the γ/δ T cells. Set B differed more dramatically in Vδ2 usage; one individual expressed Vδ2 on 30% of the γ/δ T cells, while the other individual expressed Vδ2 on 93% of the γ/δ T cells. In each twin set, individual 1 has diabetes mellitus and individual 2 is healthy. However, it does not appear that the disease accounts for these differences in the expression of Vδ gene segments, as the Vδ1 usage is high in the diabetic individual of one twin set, but in the nondiabetic individual of the other twin set. The different TCR-γ/δ V gene repertoire in the peripheral blood of such identical twins supports the hypothesis that there are nongenetic influences that affect TCR-γ/δ variable chain expression on peripheral blood T cells.

Discussion

The repertoire of TCR-γ/δ variable gene segments expressed on lymphocytes in the thymus at birth was very similar to that expressed in peripheral blood (umbilical

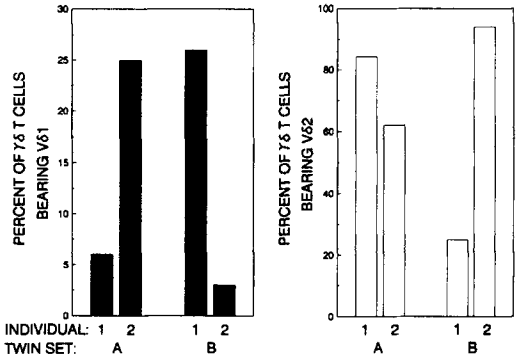

FIGURE 7. Vδ gene segment usage in identical twins. Histograms for individuals 1 or 2 of each set of twins (A and B) show the percentage of γ/δ T cells that use Vδ1 (*left, solid*) or Vδ2 (*right, open*). Note that the scale of the left panel and the right panel are different. Individual 1 has diabetes mellitus, while individual 2 is healthy in both twin sets A and B.

cord) at birth. However, with increasing age, the number of TCR-γ/δ-bearing T cells in peripheral blood increased during the first 6 yr of life and then fell slightly. This rise was accounted for by an increase in the number of Vδ2 expressing TCR-γ/δ cells in peripheral blood, resulting in the previously described predominance of TCR-γ/δ cells bearing Vδ2 in adult PBL (27, 28). However, the number of Vδ1-bearing cells in peripheral blood did not change with age. Therefore, the ratio of Vδ2/Vδ1-bearing cells rose dramatically after birth until about age 6. During the period when the Vδ2 number in PBL increased, no change in Vδ-encoded gene segment expression was observed in thymus samples, including determinations performed on paired human thymus and peripheral blood samples from the same donor. The average ratio of Vδ2/Vδ1 as well as the proportion of T cells expressing TCR-γ/δ remained stable in the thymus at all ages studied, making it unlikely that a distinct thymic wave of cells bearing Vδ2-encoded receptors occurred at the time of the observed increase in peripheral $V\delta2^+$ cells. On the other hand, a correlation existed between the rising number of $V\delta2^+$ cells and their high levels of expression of CD45RO. Taken together, the absence of a distinct $V\delta2^+$ postnatal thymic wave and the CD45RO phenotype supports the hypothesis that the age-related changes in peripheral blood TCR-γ/δ V gene repertoire are accounted for by extrathymic events, possibly antigen exposure. The peripheral population of $V\delta2^+$ T cells is polyclonal, since all of the genes analyzed from peripheral blood PCR (18/18 $V\delta2^+$ VJC junctions) (47) or Southern blot analysis (four of four clones from each of two individuals) (29) showed distinct sequences or gene rearrangements. The fact that the Vδ2 expansion (and preferential chain pairing) did not occur equally in individuals and that these differences could not be accounted for in an inherited fashion in families or identical twins suggested further that these individual variations might result from nongenetic environmental antigen or superantigen challenges. In other studies, expansion of T cells bearing specific Vβ gene segments have been noted in vitro after stimulation with staphylococcal toxins (48). In addition, T cells expressing specific Vδ gene segments have been shown to proliferate in response to mycobacterial antigens (12). Thus, antigens like these might account for the expansion of Vδ2-expressing γ/δ T cells and their high levels of expression of CD45RO in peripheral blood.

However, it is likely that a number of factors may play a role in determining the Vδ repertoire. For example, selective homing could influence the V gene repertoire at each anatomical site as recent studies in both mice and man suggest that V gene usage varies at distinct anatomical sites. Similarly, TCR Vγ- and Vδ-encoded chain pairs may interact with distinct ligands in different tissues and be expanded on that basis. In this study, $V\delta1^+$ T cells in the periphery expressed a naive phenotype. It is possible that these $V\delta1^+$ cells may then migrate to localized sites where they are activated, as is thought to occur for lymphocytes in general (49). Interestingly, in previous studies, $V\delta1^+$ lymphocytes have been shown to be present at twofold greater frequency than $V\delta2^+$ T cells in the human intestinal epithelium (50). In contrast to normal individuals, in patients with coeliac disease, the proportion of IEL T cells expressing Vδ1 is increased by more than fivefold, while the proportion of T cells expressing Vδ2 is unchanged (50). This suggests that $V\delta1^+$ T cells might be stimulated preferentially in this location in contrast to the reciprocal expansion of $V\delta2^+$ cells that occurs in the peripheral blood pool as shown in this report. Information emerging from other studies has suggested an important role for develop-

mentally regulated TCR gene rearrangements in determining the TCR-γ/δ repertoire (25). The data presented here point to the importance of extrathymic peripheral expansion of selected γ/δ subsets in generating the adult TCR-γ/δ repertoire.

Summary

The germline repertoire of variable genes for the TCR-γ/δ is limited. This, together with the availability of several Vδ-specific and a Cδ-specific mAbs, has made it possible to assess differences in the TCR-γ/δ repertoire in man. TCR-γ/δ cells expressing particular V gene segments have been previously shown to be localized in different anatomical sites. In this study, analysis of TCR-γ/δ V gene segment usage performed on subjects from the time of birth through adulthood revealed striking age-related changes in the TCR-γ/δ repertoire in peripheral blood. V$\delta1^+$ γ/δ T cells predominated in thymus as well as in peripheral blood at birth and then persisted as a relatively constant proportion of CD3$^+$ PBL. However, V$\delta2^+$ γ/δ T cells that constitute a small proportion of the CD3$^+$ cells in thymus and in peripheral blood at birth, then expand and account for the major population of γ/δ T cells in PBL in adults. No parallel postnatal expansion of V$\delta2^+$ cells in the thymus was observed, even when paired thymus-peripheral blood specimens were obtained on subjects between the ages of 3 d and 8 yr. The subset of V$\delta2^+$ lymphocytes that was expanded in peripheral blood expressed high levels of CD45RO suggesting prior activation of these cells, consistent with the possibility that their expansion might have resulted from exposure to foreign antigens or superantigens. In contrast, V$\delta1^+$ T cells in PBL showed no comparable increase in relative numbers and were either negative or expressed only low levels of CD45RO. Consistent with evidence for extrathymic peripheral expansion of selective TCR-γ/δ subsets, no link between MHC haplotype and differences in the TCR-γ/δ V gene usage between individuals was apparent, and identical twins displayed TCR-γ/δ variable gene segment phenotypes that were strikingly different from one another.

The elements that determine the TCR-γ/δ repertoire in individuals are not known. It is possible that both thymic selection and extrathymic factors may influence the peripheral repertoire. Recently, TCR-γ/δ^+ lymphocytes have been shown to expand markedly in peripheral lymphoid tissues and infectious lesions in response to mycobacterial antigens (13, 15), and a correlation between mycobacterial responses and TCR-γ/δ V gene usage has been shown in mice (12). The data presented here demonstrated peripheral age-related changes in the γ/δ repertoire and point to the importance of extrathymic expansion of specific γ/δ subsets in generating the human TCR-γ/δ repertoire.

We thank Dr. A. Castaneda (Division of Cardiac Surgery, Children's Hospital, Boston, MA) for providing postnatal thymic tissue and paired peripheral blood samples; Dr. G. Eisenbarth and T. Smith (Joslin Clinic) for providing peripheral blood samples from identical twins; the Hematology Laboratory of Children's Hospital in Boston for providing peripheral blood samples from children; and Mara St. Cyr of the Brigham and Women's Hospital for providing umbilical cord blood samples. We thank Drs. A. Moretta, T. Hercend, P. Beverly, Kurrle, and H. Spits for the kind gift of mAbs; and Janet Anderson (Division of Biostatistics and Epidemiology, Dana-Farber Cancer Institute) for statistical advice and for curve fitting analyses.

Received for publication 28 December 1989.

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