

## EVIDENCE FOR EXTRATHYMIC CHANGES IN THE T CELL RECEPTOR $\gamma/\delta$ REPERTOIRE

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Lymphocytes express two types of TCRs, either  $\alpha/\beta$  (1-3) or  $\gamma/\delta$  (4). The genes encoding the TCR  $\gamma$  and  $\delta$  chains are composed of V, D, J, and C regions that undergo somatic rearrangement during development (5, 6). There are only eight functional V $\gamma$  gene segments and perhaps 5-10 functional V $\delta$  gene segments identified in humans (6). While the specificity of the TCR- $\gamma/\delta$  is poorly understood, the limited germline diversity suggested that TCR- $\gamma/\delta$ -bearing lymphocytes might recognize antigen in the context of relatively nonpolymorphic antigen-presenting molecules (6). Recently, alloreactive TCR- $\gamma/\delta$  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- $\gamma/\delta$ , the fact that lymphocytes that bear it frequently lack CD4 and CD8, and the non-MHC-restricted recognition of tumor targets by some TCR- $\gamma/\delta$  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- $\gamma/\delta$  clone can recognize non-MHC-encoded CD1 antigens that might serve as novel antigen-presenting molecules for CD4<sup>-</sup>CD8<sup>-</sup> T cells (11). In addition, some of the specific antigens that  $\gamma/\delta$  T cells can recognize have been identified, such as mycobacterial antigens (including heat-shock proteins) (12-16) and tetanus toxoid (17). TCR- $\gamma/\delta$ <sup>+</sup> lymphocytes expand markedly in number in lymphoid organs and in tissue lesions in response to the mycobacterial antigens (13, 15).

TCR- $\gamma/\delta$  cells comprise ~5% (0.5-20%) of the CD3<sup>+</sup> cells in human peripheral blood and in lymphoid tissues (18-20). However, in mice, TCR- $\gamma/\delta$  cells preferen-

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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. Fabbri is on leave of absence from the Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

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tially localize and constitute the predominant subset at epithelial surfaces, such as in the skin (dendritic epidermal cells [dEC]<sup>1</sup>) (21) and in the intestine (intraepithelial lymphocytes [IEL]) (22, 23). dEC express a single V $\gamma$ /V $\delta$  pair (V $\gamma$ 3/V $\delta$ 1) 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 V $\gamma$ /V $\delta$  pairs (V $\gamma$ 5/V $\delta$ 4 or V $\gamma$ 5/V $\delta$ 6) and have much greater junctional diversity (23). In the mouse thymus, V $\gamma$  and V $\delta$  gene rearrangements appear in a sequential order (25). This ordered pattern in development may account for the nonrandom expression of specific pairs of V $\gamma$  and V $\delta$  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  $\gamma/\delta$  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- $\gamma/\delta$  cells in gut were noted preferentially to localize to the epithelium rather than the lamina propria in one study (26). Yet, selective V $\gamma$  and V $\delta$  gene pairing and differences in V gene usage at various anatomical sites have been observed in man. The V $\gamma$ 2/V $\delta$ 2 chain pair occurs in most individuals on >70% of the circulating  $\gamma/\delta$  T cells (27, 28). In contrast, V $\delta$ 1<sup>+</sup> 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- $\gamma/\delta$  variable chain usage in thymus compared with peripheral blood, in this report, both thymic and extrathymic influences on the TCR- $\gamma/\delta$  repertoire were studied. The results provide evidence that a marked extrathymic expansion of  $\gamma/\delta$  T cells bearing certain V gene segments occurs and is an important feature in determining the peripheral TCR- $\gamma/\delta$  repertoire in man.

## Materials and Methods

*mAbs.* mAbs used were SPV-T3b (anti-CD3) (30), anti-TCR- $\delta$ 1 (pan-reactive anti-TCR- $\delta$ ) (31),  $\delta$ TCS1 (anti-V $\delta$ 1-J $\delta$ 1, which appears to represent the most frequent V $\delta$ 1 rearrangement expressed in peripheral blood) (29, 32), BB3 (anti-V $\delta$ 2) (33, 34), anti-Ti $\gamma$ a (anti-V $\gamma$ 2 [also called V $\gamma$ 9, for nomenclature see reference 6]) (35), anti-C $\gamma$ M1 (pan-reactive anti-TCR- $\gamma$ ) (36), BMA 031 (pan-reactive anti-TCR- $\alpha/\beta$ ; 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- $\delta$  insert was prepared from the IDP2 TCR- $\delta$  cDNA clone (38) containing sequences downstream of the FnuD II site at the codon encoding amino acid 106 in the D region. The truncated C $\delta$  insert was ligated into the vector pSP73 downstream from the SP6 promoter to yield the pSP73.C $\delta$ . In this construct, the first methionine codon is at amino acid residue 138 within the constant region of the TCR- $\delta$  chain. Therefore, in vitro transcription and translation of this construct should yield a protein that only includes amino acids encoded by the C $\delta$  gene segment. Constructs encoding the full-length IDP2- $\delta$  protein (pGEM3-0240/38) and the IDP2  $\gamma$  protein (pSP65.IDP2 $\gamma$ ) have been

<sup>1</sup> 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-C $\gamma$ M1, anti-TCR- $\delta$ 1, 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  $2-4 \times 10^6$  cells/ml in staining buffer (PBS/5% human serum/1% BSA/0.02% NaN<sub>3</sub>) 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)<sub>2</sub> 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  $\alpha/\beta$  T cells by incubation with anti-TCR- $\alpha/\beta$  (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- $\alpha/\beta$ -depleted lymphocytes were then enriched for TCR- $\gamma/\delta$  cells by incubation with the panreactive anti-TCR- $\gamma/\delta$  antibody (anti-TCR- $\delta$ 1) followed by incubation with the goat anti-mouse Ig-conjugated magnetic beads and magnet selection. The  $\gamma/\delta$  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- $\gamma/\delta$  mAb; 7.6% of the cells in this line stained with the V $\delta$ 1-specific mAb and 93.5% stained with the V $\delta$ 2-specific mAb. Positive selection with an anti-CD8 mAb (OKT8) and magnetic beads produced a homogeneously V $\delta$ 1<sup>+</sup> cell line. In long-term culture, >99% of the lymphocytes in the OKT8-selected line were positive with the anti-V $\delta$ 1 mAb, and >99% of the lymphocytes in the original TCR- $\gamma/\delta$ -enriched line were reactive with the anti-V $\delta$ 2 mAb.

## Results

*Anti-TCR $\delta$ 1 mAb Recognizes a C $\delta$ -encoded Determinant.* Four mAbs directed against distinct TCR- $\gamma/\delta$  determinants were utilized. mAb  $\delta$ 1CS1 detects a V $\delta$ 1-J $\delta$ 1-encoded determinant (called anti-V $\delta$ 1 in this study) (29, 32), mAb BB3 (detects a V $\delta$ 2-encoded determinant (anti-V $\delta$ 2) (33, 34), and anti-Ti $\gamma$ A detects a V $\gamma$ 2-encoded determinant (anti-V $\gamma$ 2) (35). In addition, mAb anti-TCR- $\delta$ 1 has broad reactivity against  $\gamma/\delta$  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- $\delta$  protein, and this protein product was recognized by the anti-TCR- $\delta$ 1 mAb (Fig. 1). This demonstrates directly that the anti-TCR- $\delta$ 1 mAb recognizes a determinant in the C $\delta$ -encoded portion of the TCR  $\delta$  chain and thus should recognize all TCR- $\delta$ -bearing lymphocytes (See Materials and Methods, and Fig. 1).

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

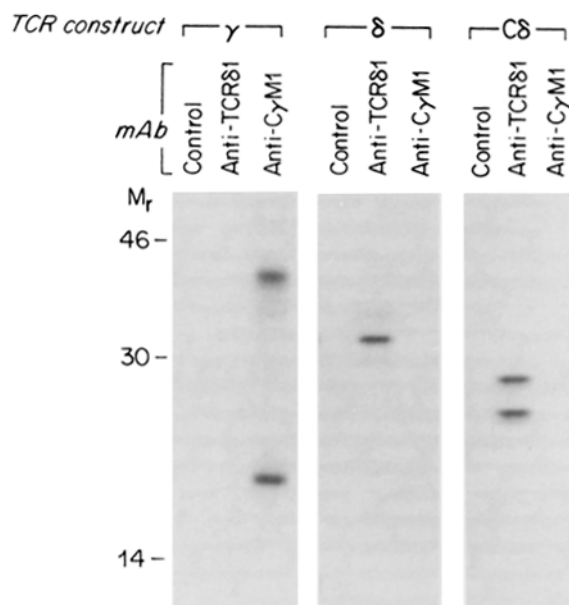


FIGURE 1. Anti-TCR- $\delta 1$  recognizes a C $\delta$ -encoded epitope. Anti-TCR- $\delta 1$  mAb immunoprecipitated the in vitro translated product whose synthesis was directed by a full-length TCR- $\delta$  construct (see TCR- $\delta$  construct panel). To determine if this mAb reacted with a determinant in the constant region of the  $\delta$  chain, in vitro transcription and translation of a truncated C $\delta$  construct was performed to produce a C region peptide. The anti-TCR- $\delta 1$  mAb immunoprecipitated this C region peptide (see C $\delta$  construct panel). Isotype-matched control mAb and anti-C $\gamma$ M1 (anti-TCR- $\gamma$ ) mAb failed to recognize these  $\delta$  chain-encoded products. The anti-TCR- $\gamma$  mAb did immunoprecipitate the protein product of the TCR- $\gamma$  construct (see TCR- $\gamma$  construct panel), while anti-TCR- $\delta 1$  did not immunoprecipitate the TCR- $\gamma$  protein product. Thus, anti-TCR- $\delta 1$  reacts specifically with a determinant encoded in the C $\delta$  region of the TCR- $\delta$  protein product, indicating that it should be reactive with all TCR- $\delta$ -bearing T cells (see Materials and Methods for experimental details).

usage. The TCR- $\gamma/\delta$  V gene usage in cord blood and in thymus was quite similar, but differed from adult peripheral blood. Cord blood TCR- $\gamma/\delta$  cells used predominantly V $\delta 1$ -encoded products (50% of  $\gamma/\delta$  T cells) and less frequently expressed V $\delta 2$ -encoded products (25% of  $\gamma/\delta$  T cells). Similarly, in the thymus, V $\delta 1$  expression was predominant as it was found on 65% of the  $\gamma/\delta$  T cells, while V $\delta 2$  was expressed on only 10–15% of thymic  $\gamma/\delta$  T cells. In contrast to thymus and cord blood, adult (>21 yr old) peripheral blood V gene usage was characterized by predominant expression of V $\delta 2$ -encoded subunits (>70% of  $\gamma/\delta$  T cells), while a smaller number of cells bore V $\delta 1$ -encoded receptors (on <30% of  $\gamma/\delta$  T cells) in most individuals. In addition, the proportion of T cells expressing TCR- $\gamma/\delta$  in cord blood was quite similar in all the individuals tested (mean of 1.73% [SEM = 0.18] of CD3<sup>+</sup> cells), and was on average threefold lower than the proportion of T cells expressing TCR- $\gamma/\delta$  in adult peripheral blood (5.7% [SEM = 1.4] of CD3<sup>+</sup> cells). These preliminary studies thus confirmed earlier reports (19, 20, 29) showing that a difference existed between  $\gamma/\delta$  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  $\gamma/\delta$  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  $\gamma/\delta$  TCRs (anti-TCR- $\delta 1$ <sup>+</sup>) 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

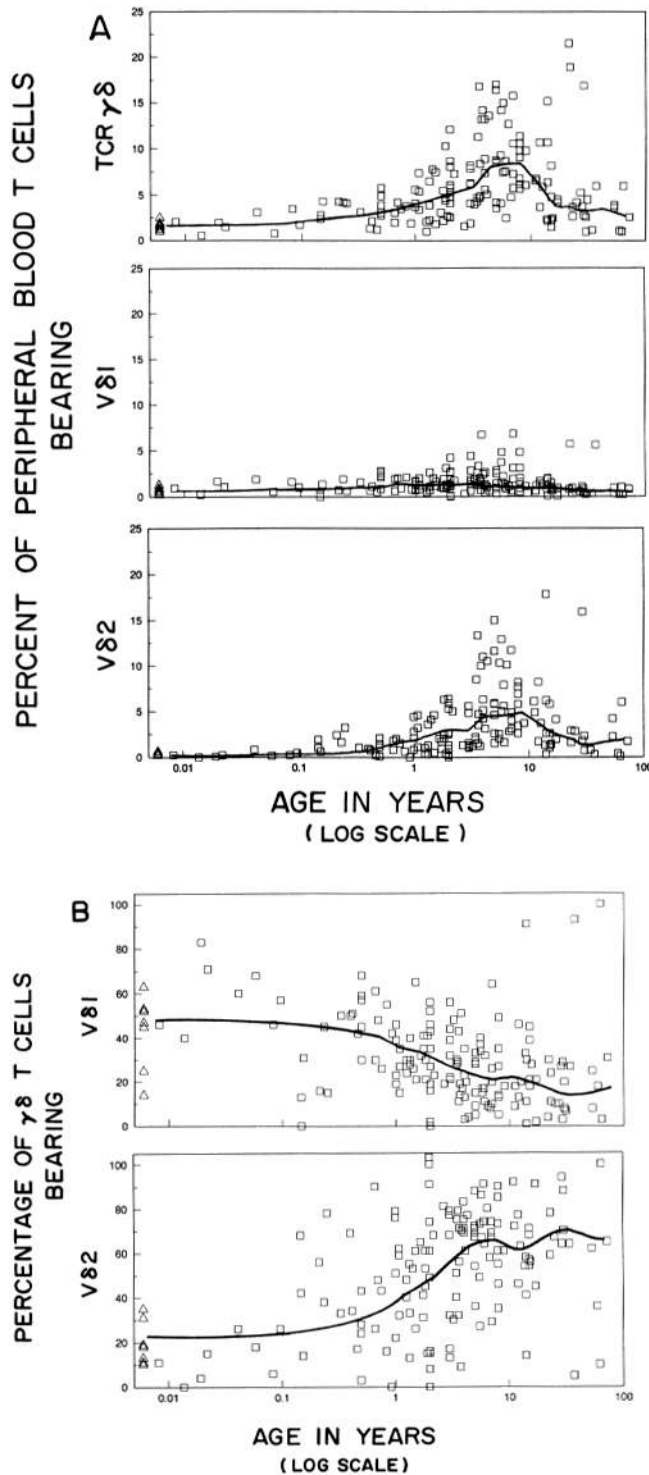


FIGURE 2. (A) Age-related variation in the percentage of T cells expressing TCR- $\gamma/\delta$ , V $\delta$ 1, or V $\delta$ 2 in human peripheral blood. The percentage of peripheral blood CD3<sup>+</sup> cells expressing TCR- $\gamma/\delta$  (anti-TCR- $\delta$ 1<sup>+</sup>), V $\delta$ 1 ( $\delta$ TCS-1<sup>+</sup>), and V $\delta$ 2 (BB3<sup>+</sup>) 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:  $100 \times (\text{percent of PBL positive with mAb shown on the y axis} / \text{percent of PBL positive with mAb SPV-T3b})$ . (B) Age-related variation in the percentage of  $\gamma/\delta$  T cells that bear V $\delta$ 1- or V $\delta$ 2-encoded receptors in peripheral blood. The percentage of peripheral blood anti-TCR- $\delta$ 1<sup>+</sup> cells expressing V $\delta$ 1 or V $\delta$ 2 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:  $100 \times (\text{percent of PBL positive with mAb shown on the y-axis} / \text{percent of PBL positive with anti-TCR-}\delta$ 1).

these individuals by staining with anti-V $\delta$ 1 and anti-V $\delta$ 2 mAbs. During this time period, the proportion of T cells expressing V $\delta$ 1 remained nearly constant while the percentage of T cells expressing V $\delta$ 2 increased strikingly (Fig. 2 A, *middle* and *bottom*). In addition to the age-related increase in the number of  $\gamma/\delta$  T cells, a shift from a V $\delta$ 1 predominance to a V $\delta$ 2 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 V $\delta$ 2-bearing T cell subset.

*V $\delta$ 2 Expansion in the Periphery Occurs in the Absence of a Parallel Thymic Wave.* To address whether a postnatal thymic wave of TCR V $\delta$ 2-expressing cells could explain the rising number of V $\delta$ 2-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- $\gamma/\delta$  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- $\gamma/\delta$  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- $\gamma/\delta$  was observed (Fig. 2 A, *top*), in the thymus, the proportion of T cells expressing the TCR- $\gamma/\delta$  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- $\gamma/\delta$  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- $\gamma/\delta$  cells bearing either V $\delta$ 1- or V $\delta$ 2-encoded receptors over time. In none of the thymi did we observe >30% V $\delta$ 2<sup>+</sup> cells (mean = 12%), including thymi from individuals in whom the V $\delta$ 2 expansion (often to >85% of the  $\gamma/\delta$  cells) had already occurred in the paired peripheral blood sample (Fig. 3 B). Since the percentage of  $\gamma/\delta$  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 V $\delta$ 2-bearing cells was found. Instead, it seemed more likely that the expansion of TCR- $\gamma/\delta$  cells bearing V $\delta$ 2 in the periphery may have resulted from a postnatal extrathymic expansion of this subset, while the V $\delta$ 1-bearing cells remained constant in number.

*Age-related Expression of CD45RO on V $\delta$ 2-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<sup>hi</sup> in adulthood (43). A similar rise has also been demonstrated for  $\gamma/\delta$  T cells in peripheral blood (43). To confirm that both V $\delta$ 1<sup>+</sup> and V $\delta$ 2<sup>+</sup>  $\gamma/\delta$  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 $\delta$ 2<sup>+</sup> line expressed CD45RO at high levels, while V $\delta$ 1<sup>+</sup> cell lines expressed CD45RO at moderate levels. In addition, both V $\delta$ 1- and V $\delta$ 2-expressing thymocytes and tonsil-derived lymphocytes expressed moderate or high levels of CD45RO (data not shown).

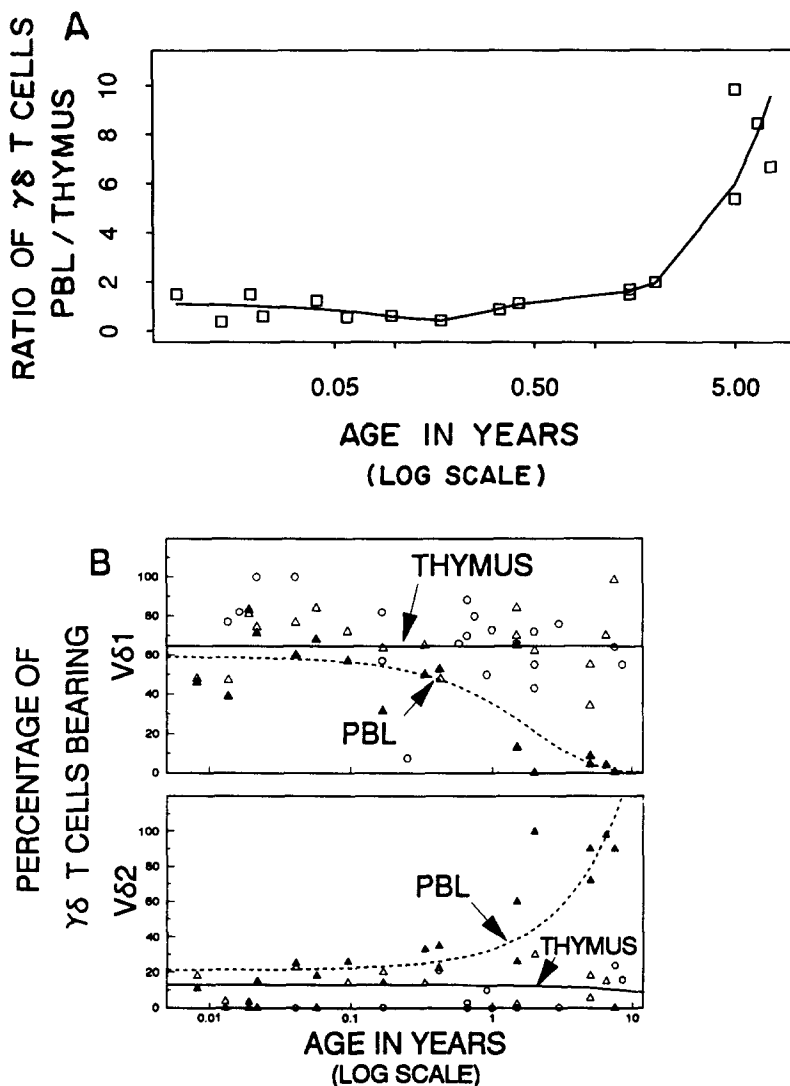


FIGURE 3. (A) Age-related change in the TCR- $\gamma/\delta$  percentage in thymus compared with peripheral blood. The percentage of CD3<sup>+</sup> (SPV-T3b<sup>+</sup>) cells expressing TCR- $\gamma/\delta$  (anti-TCR- $\delta$ 1<sup>+</sup>) in the thymus and in peripheral blood in the same individual at the same point in time was determined. The ratio of the TCR- $\gamma/\delta$  percentage in thymus compared with peripheral blood was calculated using the formula: (percent of PBL positive with mAb anti-TCR- $\delta$ 1/percent of PBL positive with mAb SPV-T3b)/(percent of thymocytes positive with anti-TCR- $\delta$ 1/percent of thymocytes positive with SPV-T3b). The line on the plot was generated using a lowest smoother computer analysis ( $f = 0.5$ ) (51). (B) Age-related change in TCR- $\delta$  variable gene segment using in thymus compared with peripheral blood. The percentage of CD3<sup>+</sup> (SPV-T3b<sup>+</sup>) cells expressing V $\delta$ 1 ( $\delta$ TCS-1<sup>+</sup>, top) or V $\delta$ 2 (BB3<sup>+</sup>, 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 V $\delta$ 2-bearing cells observed here (Fig. 2 A, *bottom*) correlated with expansion of naive (CD45RO<sup>-</sup> or CD45RO<sup>lo</sup>) or of previously activated (CD45RO<sup>hi</sup>) cells. In umbilical cord blood lymphocytes (CBL), two populations of lymphocytes were observed, one that was unreactive (CD45RO<sup>-</sup>) and one that was weakly reactive (CD45RO<sup>lo</sup>) 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<sup>hi</sup>). The CD45RO<sup>lo</sup> population was small in adult PBL, while the CD45RO<sup>hi</sup> population predominated (Fig. 4 A). In two-color FACS analysis, both V $\delta$ 1<sup>+</sup> and V $\delta$ 2<sup>+</sup> T cells in umbilical cord blood lacked the CD45RO<sup>hi</sup> phenotype. By 1 yr of life, CD45RO<sup>hi</sup> was expressed on  $\sim$ 30% of the TCR- $\gamma/\delta$ <sup>+</sup> cells. This percentage continued to rise between 1 and 18 yr of life to  $\sim$ 60% of the V $\delta$ 2<sup>+</sup> cells, suggesting that a significant proportion of these cells were previously activated (Fig. 4 B). In contrast to V $\delta$ 2<sup>+</sup> lymphocytes, V $\delta$ 1<sup>+</sup> lymphocytes remained <20% CD45RO<sup>hi</sup> even in adulthood (Fig. 4 B). Moreover, no shift from CD45RO<sup>-</sup> to CD45RO<sup>lo</sup> could be identified on V $\delta$ 1-bearing T cells from children of increasing age (through age 18) (data not shown). Thus, a striking differ-

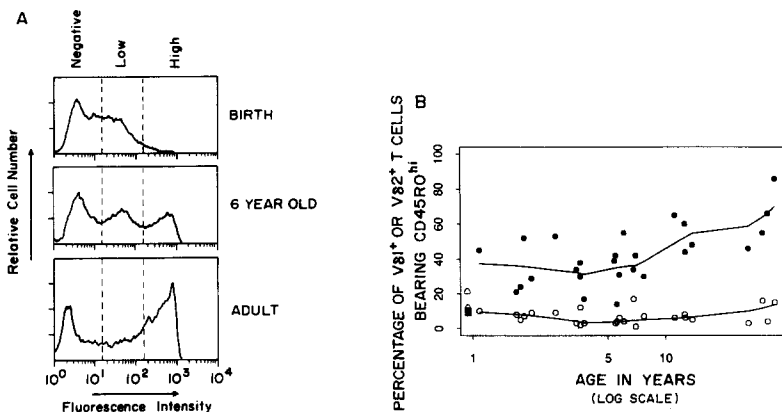


FIGURE 4. (A) CD45RO expression on PBL. CD45RO expression was analyzed on PBL at birth (cord blood), from a 6-yr-old child, and from an adult. The 6-yr-old child's profile was utilized to set cursors dividing the PBL into populations with negative (CD45RO<sup>-</sup>), low (CD45RO<sup>lo</sup>), or high (CD45RO<sup>hi</sup>) expression of CD45RO determined by staining with mAb UCHL1 and one-color FACS analysis. (B) CD45RO<sup>hi</sup> expression of V $\delta$ 1- or V $\delta$ 2-bearing T cells in peripheral blood. The percentage of V $\delta$ 1<sup>+</sup> or V $\delta$ 2<sup>+</sup>  $\gamma/\delta$  T cells that express CD45RO<sup>hi</sup> was determined by two-color FACS analysis on CBL (triangles adjacent to the y-axis) and on PBL (circles) and plotted versus age. V $\delta$ 1<sup>+</sup>  $\gamma/\delta$  cells ( $\delta$ TCS1<sup>+</sup>, open symbols) rarely express CD45RO<sup>hi</sup>, while V $\delta$ 2<sup>+</sup>  $\gamma/\delta$  T cells (BB3<sup>+</sup>, closed symbols) showed an age-related increase in the percentage that express CD45RO<sup>hi</sup>. The percentage of V $\delta$ 1<sup>+</sup> or V $\delta$ 2<sup>+</sup> cells that express CD45RO<sup>hi</sup> was determined in two-color FACS analysis by gating on the cells positive with the V $\delta$ -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<sup>hi</sup> was calculated using the formula: number of V $\delta$ 1<sup>+</sup> or V $\delta$ 2<sup>+</sup> cells in the CD45RO<sup>hi</sup> region/total number of V $\delta$ 1<sup>+</sup> or V $\delta$ 2<sup>+</sup> 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  $V\delta 2^+$  TCR- $\gamma/\delta$  cells, which expanded in an age-related fashion and expressed a phenotype characteristic of "memory T cells", and the  $V\delta 1^+$  cells, which failed to expand in number or to express this memory phenotype. Acquisition of the memory phenotype on  $V\delta 2^+$   $\gamma/\delta$  T cells occurred during the age period in which the proportion of TCR- $\gamma/\delta$  cells bearing  $V\delta 2$  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- $\gamma/\delta$  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- $\gamma/\delta$  repertoire in peripheral blood can be accounted for by a single  $V\gamma 2/V\delta 2$  chain pair (46). The analysis here confirmed this generalization, but also identified a number of subjects who displayed a different pattern of expression of  $V\gamma$  and  $V\delta$  genes in their peripheral blood. Four representative examples of healthy adults with differing TCR- $\delta$  V gene-defined phenotypes are shown (Fig. 5). In peripheral blood from subject 1,  $V\delta 2$  was expressed on 94% of the  $\gamma/\delta$  cells, with  $V\delta 1$  expressed on nearly all the remaining TCR- $\gamma/\delta$  cells. In this individual,  $V\delta 2$  was paired predominantly with  $V\gamma 2$ , while  $V\delta 1$  was almost never paired with  $V\gamma 2$ . This corresponds to the predominant phenotype previously reported by others (46). In subject 2,  $V\delta 2$  was expressed on 77% of the  $\gamma/\delta$  T cells; the majority of the remaining  $\gamma/\delta$  cells expressed  $V\delta 1$ . Like subject 1,  $V\delta 2$  paired predominantly with  $V\gamma 2$ ; in contrast to subject 1,  $V\delta 1$  paired with  $V\gamma 2$  on ~60% of  $V\delta 1^+$  T cells. Subjects 3 and 4 differed strikingly from subjects 1 and 2 since  $V\delta 2$  expression was found on only 40 and 7% of the peripheral blood  $\gamma/\delta$  T cells, respectively.

Correspondingly, in these latter subjects  $V\delta 1$  expression predominated and was present on 41 and 82% of peripheral blood  $\gamma/\delta$  T cells, respectively. In the majority

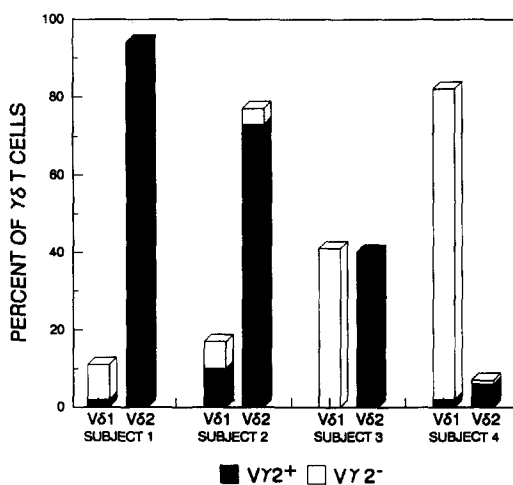


FIGURE 5.  $V\gamma$  and  $V\delta$  gene segment usage differs in four adult individuals. TCR- $\gamma/\delta$  variable chain usage was determined on the peripheral blood of four individuals by two-color FACS analysis. For each individual, the percentage of  $\gamma/\delta$  T cells expressing  $V\delta 1$  or  $V\delta 2$  is represented by the height of the bar. Of the cells that express either  $V\delta 1$  or  $V\delta 2$ , the proportion that pairs with  $V\gamma 2$  is represented by the part of the bar that is shaded. Note that both the proportion of cells that express each  $V\delta$  gene segment and the frequency of pairing of  $V\gamma 2$  with  $V\delta 1$  vary strikingly from subject to subject.  $V\delta 1$ ,  $V\delta 2$ , and  $V\gamma 2$  were detected with mAbs  $\delta$ TCS1, BB3, and T $\gamma$ a, respectively. The percentage of  $CD3^+$  cells positive for the  $V\delta$  chains was calculated as in Fig. 2 A. The percentage of  $V\delta 1^+$  or  $V\delta 2^+$  cells positive with anti- $V\gamma 2$  was calculated from two-color FACS by gating on the cells stained with either anti- $V\delta 1$  ( $\delta$ TCS1) or anti- $V\delta 2$

(BB3) mAbs and then determining the percentage of the gated cells that were positive with the anti- $V\gamma 2$  (T $\gamma$ a) mAb. The percentage of  $V\delta 1^+$  or  $V\delta 2^+$  cells expressing  $V\gamma 2$  was calculated using the formula: number of  $V\delta 1^+$  or  $V\delta 2^+$  cells in the  $V\gamma 2^+$  region/total number of  $V\delta 1^+$  or  $V\delta 2^+$  cells.

of individuals, V $\delta$ 2 usage predominated (subjects 1 and 2), while in the other cases, V $\delta$ 1 and V $\delta$ 2 were used about equally or V $\delta$ 1 predominated (the least frequent occurrence) (subjects 3 and 4, respectively). Thus, while the majority of TCR- $\gamma/\delta$  cells in all subjects expressed either V $\delta$ 1 or V $\delta$ 2, the relative proportion of cells that expressed each of these TCR  $\delta$  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- $\gamma/\delta$ , but no significant variation in the relative expression and pairing of V $\delta$ 1, V $\delta$ 2, and V $\gamma$ 2. This suggested that the TCR- $\gamma/\delta$  repertoire in adults is relatively stable over a period of months to years.

*The  $\gamma/\delta$  Gene Repertoire Is Not Solely Determined by Inherited Factors.* To determine if background genetic differences could account for the distinct  $\gamma/\delta$  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 V $\delta$ 1 gene segment usage predominates over V $\delta$ 2 gene segment expression (>50% V $\delta$ 1, <30% V $\delta$ 2). However, all of the children displayed the common phenotype, expressing V $\delta$ 2 on >70% of their TCR- $\gamma/\delta$  cells. Thus, none of the progeny displayed the parental  $\gamma/\delta$  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-

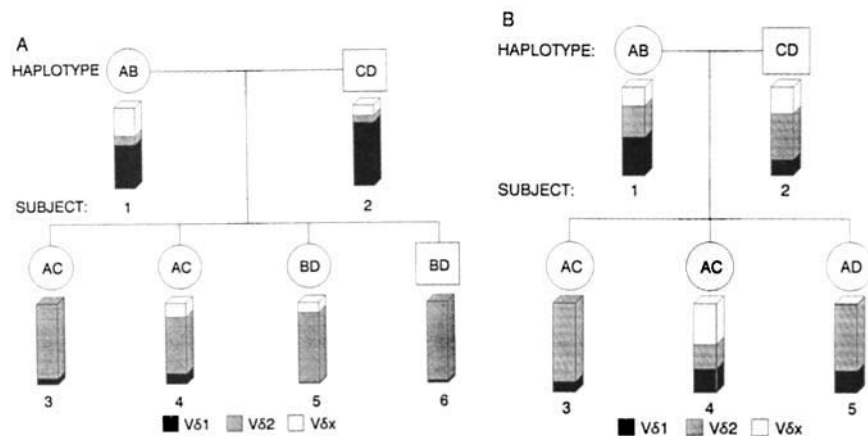


FIGURE 6. V $\delta$  gene usage in families. V $\delta$  gene usage was determined for the members of two families, as described in Fig. 4. The extended MHC haplotypes are represented within the circles or squares by A, B, C, and D. The histogram represents TCR- $\gamma/\delta$  cells, the lowest (*solid*) section represents the proportion of TCR- $\gamma/\delta$  cells expressing V $\delta$ 1 gene segments, the middle (*shaded*) section represents the proportion of TCR- $\gamma/\delta$  cells expressing the V $\delta$ 2 gene segment, and the top (*open*) section represents the proportion of TCR- $\gamma/\delta$  cells expressing neither V $\delta$ 1 nor V $\delta$ 2 gene segments (V $\delta$ X). 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<sup>+</sup> cells expressing TCR- $\gamma/\delta$  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<sup>+</sup> cells expressing TCR- $\gamma/\delta$  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 V $\delta$ 1 on 40% of the  $\gamma/\delta$  cells while the other parent expressed V $\delta$ 1 on 11% of the  $\gamma/\delta$  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- $\gamma/\delta$  variable gene segments. Individual 3 expressed V $\delta$ 2 on >90% and V $\delta$ 1 on very few of the  $\gamma/\delta$  T cells. In contrast, individual 4 expressed V $\delta$ 2 on only 27% and V $\delta$ 1 on  $\sim$ 25% of the TCR- $\gamma/\delta$  cells. These representative family studies reveal no simple or direct correlation between inheritance of a MHC haplotype and TCR- $\gamma/\delta$  phenotype in PBL. This suggested that factors other than the MHC type must influence TCR- $\gamma/\delta$  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- $\gamma/\delta$  V gene repertoire, seven sets of identical twins were evaluated (Fig. 7). In four sets, both twins showed nearly identical  $\gamma/\delta$  V gene repertoires, expressing the common phenotype with V $\delta$ 2 on a majority of their  $\gamma/\delta$  T cells. However, several sets revealed divergent V gene usage. In both twin sets A and B, one individual utilized V $\delta$ 1 on  $\sim$ 25% of the  $\gamma/\delta$  T cells in peripheral blood, while the other individual utilized V $\delta$ 1 on <10% of the  $\gamma/\delta$  T cells. The twins also differed in V $\delta$ 2 usage, since one member of twin set A utilized V $\delta$ 2 on 85% of the  $\gamma/\delta$  T cells, while the other individual utilized V $\delta$ 2 on 60% of the  $\gamma/\delta$  T cells. Set B differed more dramatically in V $\delta$ 2 usage; one individual expressed V $\delta$ 2 on 30% of the  $\gamma/\delta$  T cells, while the other individual expressed V $\delta$ 2 on 93% of the  $\gamma/\delta$  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 V $\delta$  gene segments, as the V $\delta$ 1 usage is high in the diabetic individual of one twin set, but in the nondiabetic individual of the other twin set. The different TCR- $\gamma/\delta$  V gene repertoire in the peripheral blood of such identical twins supports the hypothesis that there are nongenetic influences that affect TCR- $\gamma/\delta$  variable chain expression on peripheral blood T cells.

### Discussion

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

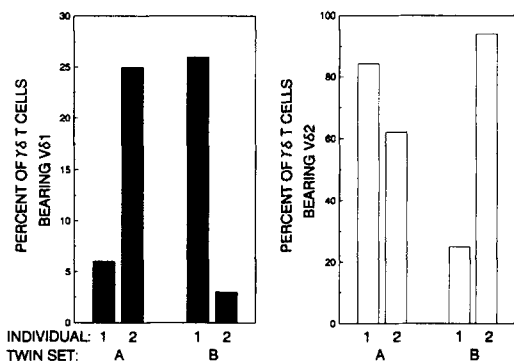


FIGURE 7. V $\delta$  gene segment usage in identical twins. Histograms for individuals 1 or 2 of each set of twins (A and B) show the percentage of  $\gamma/\delta$  T cells that use V $\delta$ 1 (left, solid) or V $\delta$ 2 (right, open). Note that the scale of the left panel and the right panel are 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- $\gamma/\delta$ -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 V $\delta$ 2 expressing TCR- $\gamma/\delta$  cells in peripheral blood, resulting in the previously described predominance of TCR- $\gamma/\delta$  cells bearing V $\delta$ 2 in adult PBL (27, 28). However, the number of V $\delta$ 1-bearing cells in peripheral blood did not change with age. Therefore, the ratio of V $\delta$ 2/V $\delta$ 1-bearing cells rose dramatically after birth until about age 6. During the period when the V $\delta$ 2 number in PBL increased, no change in V $\delta$ -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 V $\delta$ 2/V $\delta$ 1 as well as the proportion of T cells expressing TCR- $\gamma/\delta$  remained stable in the thymus at all ages studied, making it unlikely that a distinct thymic wave of cells bearing V $\delta$ 2-encoded receptors occurred at the time of the observed increase in peripheral V $\delta$ 2<sup>+</sup> cells. On the other hand, a correlation existed between the rising number of V $\delta$ 2<sup>+</sup> cells and their high levels of expression of CD45RO. Taken together, the absence of a distinct V $\delta$ 2<sup>+</sup> postnatal thymic wave and the CD45RO phenotype supports the hypothesis that the age-related changes in peripheral blood TCR- $\gamma/\delta$  V gene repertoire are accounted for by extrathymic events, possibly antigen exposure. The peripheral population of V $\delta$ 2<sup>+</sup> T cells is polyclonal, since all of the genes analyzed from peripheral blood PCR (18/18 V $\delta$ 2<sup>+</sup> 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 V $\delta$ 2 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 V $\beta$  gene segments have been noted in vitro after stimulation with staphylococcal toxins (48). In addition, T cells expressing specific V $\delta$  gene segments have been shown to proliferate in response to mycobacterial antigens (12). Thus, antigens like these might account for the expansion of V $\delta$ 2-expressing  $\gamma/\delta$  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 V $\delta$  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 V $\gamma$  and V $\delta$ -encoded chain pairs may interact with distinct ligands in different tissues and be expanded on that basis. In this study, V $\delta$ 1<sup>+</sup> T cells in the periphery expressed a naive phenotype. It is possible that these V $\delta$ 1<sup>+</sup> 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 $\delta$ 1<sup>+</sup> lymphocytes have been shown to be present at twofold greater frequency than V $\delta$ 2<sup>+</sup> 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 V $\delta$ 1 is increased by more than fivefold, while the proportion of T cells expressing V $\delta$ 2 is unchanged (50). This suggests that V $\delta$ 1<sup>+</sup> T cells might be stimulated preferentially in this location in contrast to the reciprocal expansion of V $\delta$ 2<sup>+</sup> 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- $\gamma/\delta$  repertoire (25). The data presented here point to the importance of extrathymic peripheral expansion of selected  $\gamma/\delta$  subsets in generating the adult TCR- $\gamma/\delta$  repertoire.

### Summary

The germline repertoire of variable genes for the TCR- $\gamma/\delta$  is limited. This, together with the availability of several V $\delta$ -specific and a C $\delta$ -specific mAbs, has made it possible to assess differences in the TCR- $\gamma/\delta$  repertoire in man. TCR- $\gamma/\delta$  cells expressing particular V gene segments have been previously shown to be localized in different anatomical sites. In this study, analysis of TCR- $\gamma/\delta$  V gene segment usage performed on subjects from the time of birth through adulthood revealed striking age-related changes in the TCR- $\gamma/\delta$  repertoire in peripheral blood. V $\delta 1^+$   $\gamma/\delta$  T cells predominated in thymus as well as in peripheral blood at birth and then persisted as a relatively constant proportion of CD3<sup>+</sup> PBL. However, V $\delta 2^+$   $\gamma/\delta$  T cells that constitute a small proportion of the CD3<sup>+</sup> cells in thymus and in peripheral blood at birth, then expand and account for the major population of  $\gamma/\delta$  T cells in PBL in adults. No parallel postnatal expansion of V $\delta 2^+$  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 $\delta 2^+$  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 $\delta 1^+$  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- $\gamma/\delta$  subsets, no link between MHC haplotype and differences in the TCR- $\gamma/\delta$  V gene usage between individuals was apparent, and identical twins displayed TCR- $\gamma/\delta$  variable gene segment phenotypes that were strikingly different from one another.

The elements that determine the TCR- $\gamma/\delta$  repertoire in individuals are not known. It is possible that both thymic selection and extrathymic factors may influence the peripheral repertoire. Recently, TCR- $\gamma/\delta^+$  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- $\gamma/\delta$  V gene usage has been shown in mice (12). The data presented here demonstrated peripheral age-related changes in the  $\gamma/\delta$  repertoire and point to the importance of extrathymic expansion of specific  $\gamma/\delta$  subsets in generating the human TCR- $\gamma/\delta$  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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