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Oligoclonality of Vδ1 and Vδ2 Cells in Human Peripheral Blood Mononuclear Cells: TCR Selection Is Not Altered by Stimulation with Gram-Negative Bacteria

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Despite the enormous potential repertoire of γδ T cells, there are several observations which suggest that the expressed γδ repertoire in the periphery of normal individuals is often quite restricted. To assess selective expansions among γδ T cells from both adult and newborn blood samples, PBMC from 12 normal adults and cord blood from 15 normal newborns were analyzed for TCRDV1 and TCRDV2 junctional diversity by CDR3 size spectratyping and single-strand conformational polymorphism. Although TCRBV usage showed extensive heterogeneity in adults and newborns, both populations often showed CDR3 region restriction for TCRDV1 and TCRDV2. Analysis of the CDR3 spectratype patterns of newborn twins suggested that clonal selection for TCRDV is independent of genetic background. The possible role of Gram-negative bacteria in driving selective responsiveness of γδ T cells in PBMCs from adults was examined by in vitro stimulation with *Escherichia coli* and *Pseudomonas aeruginosa.* Donors whose TCRDV repertoire was highly clonal in the unstimulated blood cells showed the same predominant clones among the bacteria-stimulated cultures. In individuals whose γδ T cells were less restricted, in vitro stimulation did not select for clonality; rather, the TCRDV repertoires were similar before and after bacterial stimulation. Together, these data indicate that γδ T cells are often clonally restricted in adults as well as in newborns and suggest that the prominent stimulatory activity of Gram-negative bacteria does not by itself account for the restriction or diversity of the γδ T cell repertoire. *The Journal of Immunology,* **1998, 160: 3048–3055.**

It has been previously noted that the natural immunity to extracellular organisms, including Gram-negative bacteria, is characterized by a disproportionate expansion of γδ T cells (1–5). The responding T cells in the peripheral blood of normal adults are almost exclusively of the $V\delta2^+$ subset, while the responding cells from newborn umbilical cord blood are predominantly $V\delta1^+$. It has been proposed that the shift from Vδ1 predominance in the blood of newborns to Vδ2-expressing cells in the blood of adults may reflect a selective response to environmental stimuli, including bacteria (5–11). Moreover, the restriction of TCRDJ junctional gene usage associated with TCRDV2 in PBMC and intraepithelial lymphocytes from normal adults (12–18) has been attributed to environmental antigenic stimulation. Additionally, specific CDR3 region usage has been demonstrated in TCR-mediated γδ T cell responses to various micro-organisms, although the results are conflicting, with data arguing both for clonal selection (19, 20) and against it (3, 21, 22). This situation is summarized in a recent review by De Libero, in which the question of whether the diversity of the TCR CDR3 contributes to the fine specificity of $V\gamma9/V\delta2^+$ T cells is addressed. This review notes that ligand recognition by these T cells is still debated, concluding that these cells are broadly cross-reactive to phosphorylated metabolites (23).

To assess the role of Gram-negative bacteria in the selective expansion of γδ T cells (1, 24), it was first necessary to verify the status of γδ TCR clonality in normal unstimulated T cells. Some of the previous reports have used TCR cloning in bacteria, followed by DNA sequencing. As a limited number of clones could be evaluated, there is a possible bias in the interpretation of results. Therefore, we have used technologies that would minimize artifacts, while providing an assessment of the CDR3 diversity among TCRDV1- and TCRDV2-expressing cells.

Single-strand conformational polymorphism (SSCP)[2] and heteroduplex conformational polymorphism have been used to assess TCR diversity based on sequence differences in the junctional region (25–28). CDR3 size spectratyping can also be used to demonstrate size heterogeneity of the CDR3 within a V gene family (25, 29). SSCP is particularly powerful for detection of unique clonal expansions, while spectratyping is more applicable for evaluating the diversity of the overall repertoire, as it provides information on the frequency of transcripts of a given size.

In this study, we combined these two technologies to assess TCR δ chain diversity in peripheral blood T cells from healthy adults and newborns, and to investigate the TCRDV diversity in relationship to the γδ T cell responsiveness to Gram-negative bacteria. By examining the selective expression of TCR among γδ T cells in cord blood, we were also able to assess possible clonal restriction before exposure to external environmental Ags. In assessing both TCRDV1 and TCRDV2 transcripts, we have addressed selective TCR usage by both subsets of γδ T cells. Finally, by analyzing TCR selection from monozygotic twins and fraternal twins from both newborn and adults we were able to demonstrate

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[2] Abbreviations used in this paper: SSCP, single-strand conformation polymorphism; TBE, TRIS-borate-EDTA.

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that clonal selection among γδ T cells is influenced by factors in addition to genetic background, since the twins selected different TCR clones among the restricted TCRDV populations detected.

Materials and Methods

Donors and PBMC isolation

Peripheral blood samples were obtained from 12 healthy adults (age range, 22–50 yr). Umbilical cord blood samples were collected from 15 newborns (nine normal deliveries of individual newborns and three pairs of twins) at Massachusetts General Hospital (Boston, MA). PBMC were isolated by Ficoll-Hypaque separation, and 5 to 10×10^6 cells were used for RNA extraction. Immunophenotypes of fresh PBMC were also examined by direct fluorescence analysis on a FACScan flow cytometer (FACScan II, Becton Dickinson, Mountain View, CA) using mouse mAbs against CD3, CD4, and CD8 (Becton Dickinson) and Vδ1 and Vδ2 (T Cell Diagnostics, Woburn, MA). PBMC from adult subjects and cord blood samples in our study showed $<5\%$ $V\delta1^+$ and $V\delta2^+$ cells before in vitro culture.

Collection of γδ T cell-enriched lymphocytes after Gram-negative bacteria stimulation from adult PBMC and cloning by limiting dilution

Escherichia coli 018K$^-$ (gift from Dr. G. Siber, Dana-Farber Cancer Institute, Boston, MA) and *Pseudomonas aeruginosa* type 6 (gift from Dr. A. Cross, Walter Reed Army Institute of Research, Washington, DC) were used for stimulation of PBMC in this study. The soluble bacteria preparations were obtained as previously described (1, 24).

PBMC from five adults (1×10^6/well) were incubated with *E. coli* and *P. aeruginosa* preparations at a dilution of 1/250. Human rIL-2 (100 IU/ml) was added on day 6 of culture to expand the reactive T cells for an additional 8 days.

After 2 wk of culture, the cells were analyzed for surface phenotype with mouse mAbs against CD3, CD4, CD8, Vδ1, and Vδ2. Cells ($5–10 \times 10^6$) from these γδ T cell-enriched cultures were collected for RNA extraction and TCR analyses.

To evaluate the γδ T cell repertoire on the basis of single cell progeny, the *E. coli*-responsive cultured lymphocytes were cloned by limiting dilution as previously described (30, 31). Although γδ T cells respond initially to *E. coli*, they do not produce IL-2 in response to restimulation with the same bacterial preparations and will be outstripped by αβ T cells (1). Therefore, limiting dilution was performed in the continued presence of 100 U of IL-2 and in response to PHA, which drives the proliferation of virtually all T cells. After 14 days in culture, the bulk culture containing approximately 60% γδ T cells, and 40% $CD4^+$ αβ T cells, was diluted to 0.5 cells/well in two 96-well plates using irradiated autologous PBL and PHA at 1 μg/ml. Every 3 days, the cultures were replenished with IL-2. As the wells became confluent (in 14–20 days), cells from positive wells were transferred to 24-well plates for additional culture. To obtain sufficient cells for assays, a second PHA and irradiated PBL restimulation was conducted in the 24-well plates. The progeny of these limiting dilution wells were phenotyped by staining with anti-CD4- and anti-Vδ2-specific Abs. Of 42 wells from which growth was accomplished (from 192 starting wells), 13 wells stained for Vδ2, 27 stained for CD4, and 2 of the wells showed a mixture of phenotypes indicative of wells that contained more than one cell. The $V\delta2^+$ cells were further analyzed for TCR usage, requiring RNA extraction, PCR amplification, and SSCP analysis as described below.

RNA extraction and cDNA synthesis

Total RNA was extracted with TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's specifications. Briefly, 5 to 10×10^6 PBMC were resuspended in 1 ml of TRIzol, with lysis facilitated by repetitive pipetting. Then, 0.2 ml of chloroform was added to the cell-TRIzol slurry. RNA was extracted from the upper (aqueous) phase of the mixture after microfuge centrifugation for 15 min at $12{,}000 \times g$. The aqueous phase was then transferred to a new tube, and 0.5 ml of isopropyl alcohol was added for 10 min at room temperature followed by a 10-min centrifugation ($12{,}000 \times g$) at 4°C. The RNA pellet was washed once with 75% ethanol and then dissolved in 50 μl of RNase-free H_2O. One microgram of total RNA was converted to first-strand cDNA using a $(dT)_{12–18}$ primer together with avian myeloblastosis virus reverse transcriptase according to the manufacturer's specifications (Promega, Madison, WI; incubation for 0.5–1 h at 42°C). Standard precautions were taken to avoid RNase contamination.

PCR and analysis of TCR by CDR3 size spectratyping and SSCP

PCR. TCR transcripts of TCRDV1, TCRDV2, and TCRBV2 were amplified using the following specific primers: TCRDV1, 5′-AGAGCTA CATGCCCACATGCT; TCRDV2, 5′-AGAGGCAGAGCTG CCCCT; TCRDC, 5′-CTTATATCCTTGGGGTA GAA; TCRBV2, 5′-GCTCC AAGGCCACATACGAGCAAGGCGTCG; and TCRBC, 5′-CTCTTGA CCATGGCCATC. The products generated by PCR were about 500 bp for TCRDV1 and TCRDV2, and 900 bp for TCRBV2. Thermocycling conditions were as follows: 5 min at 95°C (initial denaturation), followed by 35 cycles of 0.5 min at 95°C, 0.5 min at 55°C, and 1 min at 72°C. A final 5-min extension at 72°C concluded this first round of PCR.

To increase the sensitivity and specificity of PCR for SSCP and CDR3 size spectratyping analyses, a nested PCR amplification was used to generate a 200- to 300-bp product. One microliter from a 1/100 dilution of the first PCR reaction was reamplified respectively using internal primers. The same Cδ primers were used as described above: TCRDV1′, 5′-CAA GAAAGCAGCGAAATCCGT; TCRDV2′, 5′-CTATGGCCCTGGTT TCAAAGAC; TCRBV2′, 5′-CTGACCTTGTCCACTCTGACAG; and TCRBC′, 5′-GGCCTTTTGGGTGTGGGAGA TCT.

CDR3 size spectratyping. The nested PCR products were labeled with ^{32}P by incorporating 10 to 20 μCi/reaction of [^{32}P]dCTP in the amplifications. Five microliters of the final PCR reaction volume (50 μl) was added to an equivalent volume of formamide/dye loading buffer, heated at 95°C for 2 to 5 min, then applied on a prewarmed (50°C) 8% polyacrylamide sequencing gel (National Diagnostics, Atlanta, GA) for approximately 4 to 5 h. The gels were run until the 200 to 300 products reached the lower half of the gels to assure adequate separation. The gels were dried, and autoradiography was performed at room temperature for 1 to 3 days with an enhancing screen. Each specific amplification of TCRDV1, TCRDV2, and TCRBV2 resulted in a ladder of bands separated by 3-bp spacing.

Densitometry and spectratyping graphs. To assess the intensity and distribution of the amplified TCR bands, the films were scanned using a computer-linked densitometer running ImageQuant (version 3.0) software (Molecular Dynamics, Sunnyvale, CA). The data were plotted on a Macintosh computer running DeltaGraph Pro (version 3.0); DeltaPoint, Monterey, CA). A curve was generated representing the intensity of each band in the ladder, corresponding to the distribution of amplified bands representing different size classes of CDR3 regions.

SSCP. As described previously (32), the unlabeled nested PCR products of TCRDV1 and TCRDV2 were purified on a S-300 column; 10 μl of purified DNA was diluted in 2 μl of 0.5 M EDTA and 88 μl of formamide. Ten to fifteen microliters of this mixture was heated to 95°C, followed by ice-cooling immediately before loading on a 10% glycerol/8% nondenaturing polyacrylamide (0.4-mm thick) sequencing format gel, running at 15 to 20 watts for 15 to 17 h at room temperature in 1× TBE buffer. Thereafter, DNA was transferred to nylon membranes (Magnagraph, MSI, Westborough, MA) by electroblotting at 4°C in 0.5× TBE at 20 V for 3 h. The membrane was then UV cross-linked and hybridized overnight at 50°C with the following TCRDC (constant region) probe: GAACAAA TGTCGCTTGTCTGGTGAAGGAATTCTACCCCAAGGATATAAGA, which was labeled with [γ-^{32}P]ATP by T4 kinase (Promega). The membranes were washed three times at 50°C (15 min/wash step) at low stringency (0.1% SDS/1× SSC), medium stringency (1% SDS/1× SSC), and high stringency (1% SDS/0.1× SSC). Films were exposed to Kodak X-OMAT film at −80°C for 1 to 2 days with an enhancing screen.

In some experiments, directly labeled DNA was generated by incorporating [α-^{32}P]dCTP in the PCR reaction. For SSCP gel analysis, 2 μl of the PCR product was combined with 98 μl of formamide. Ten to fifteen microliters of this solution was then electrophoresed directly on the gel. Following electrophoresis, the gel was vacuum-dried to paper and autoradiographed, precluding the need for hybridization.

Sequencing. For sequencing of the CDR3 region of the TCR genes, the PCR products were purified with a Wizard Prep PCR clean up kit (Promega), using the same internal TCRDV2-specific primer and TCRDC primers. Sequencing reactions were performed by the Sanger dideoxy method, using Sequenase according to the manufacturer's specification (Sequenase version 2 kit, U.S. Biochemical Corp., Cleveland, OH), except that PCR products were denatured and quick-chilled in a dry ice/ethanol bath.

Results

Analysis of TCR junctional usage by CDR3 size spectratyping and SSCP

As shown in Figure 1, two levels of information can be obtained from CDR3 size spectratyping: band number and band intensity. The number of distinguishable bands (or peaks in the graphs) reflects the number of in-frame CDR3 sizes (size classes) present. The intensity of each band is related to the frequency of CDR3s of

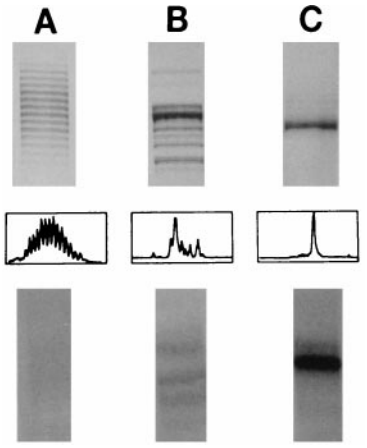

FIGURE 1. Demonstration of three different patterns representing different levels of TCR junctional diversity by CDR3 size spectratyping and SSCP. The first row shows the spectratyping gel pattern. The second row shows a graphic representation of the intensity distribution from spectratyping measured by densitometer scanning. The bottom photos show the SSCP patterns from the corresponding samples. Pattern *A* displays normally distributed intensity of size classes (peaks show a bell-shaped curve) in spectratyping, while there is a smear appearance in SSCP, indicative of polyclonal TCR usage. Pattern *B* demonstrates an abnormally distributed intensity pattern in the spectratyping and distinguishable bands with a strong smear background in SSCP, indicative of a population with limited TCR diversity. Pattern *C* shows less than five size classes in spectratyping (here a single dominant peak) and distinct bands in SSCP indicative of highly limited TCR usage.

the same length. The spectratyping graphs obtained from densitometry demonstrate the intensity distribution of the different size classes. The TCRDV1 family usually has 12 to 14 distinguishable bands (or peaks), while TCRDV2 usually shows eight to 10 bands. TCRBV2 transcripts can usually be discerned in six to eight size classes in the peripheral polyclonal populations. A typical polyclonal population usually displays a normally distributed pattern of TCR size frequency, with modal bands being the most intense; larger and smaller size classes contribute progressively less to the repertoire.

SSCP provides additional information about the clonality of the populations examined. A polyclonal population, which does not contain any predominant clones, will present a smear pattern with no distinguishable bands. A dominant clone can be detected as a distinguishable band in SSCP analysis when it is present at a level of 6 to 12% of the total population (data not shown).

As shown in Figure 1, combining the two techniques, three patterns of TCR diversity could be discerned based on differences in the junctional sequences. Pattern A (diverse, or polyclonal) shows a normal distribution of band intensity in the spectratyping analyses and a smear pattern in SSCP, suggesting a highly diverse population. Pattern B (restricted) shows contractions and gaps between bands indicative of a skewed intensity distribution pattern in spectratyping and distinguishable, but often faint, bands in the SSCP, reflecting a less diverse population. Pattern C (highly restricted) shows fewer than five bands identified by spectratyping and an SSCP pattern of distinct bands, representing a highly restricted population.

Diversity for TCRDV1 and TCRDV2 junctional usage in peripheral blood from adults

CDR3 size spectratype patterns in TCRDV1, TCRDV2, and TCRBV2 from 12 normal adults are shown graphically in Figure 2. The polyclonal nature of the TCRBV2 in all but one of the samples is indicated by the normally distributed size and intensity pattern expected of a diverse $\alpha\beta$ T cell population. The only exception was donor 10, who showed a restricted population with fewer than five peaks discernible.

In contrast, the junctional diversity patterns in TCRDV1 and TCRDV2 often showed restriction in the $\gamma\delta$ T cells in the blood of normal individuals. As shown in Figure 2, a highly restricted pattern was observed in donors 1 through 5 in TCRDV1 and in donors 1 through 6 in TCRDV2 peripheral populations, indicating oligoclonality in these donors. Donors 6 through 10 in Vδ1 and donors 7 through 10 in Vδ2 showed a restricted pattern, suggesting populations that contain relatively few clones but are more diverse than those showing the highly restricted pattern. A typical polyclonal $\gamma\delta$ T cell population in PBMC from normal donors was only observed in donors 11 and 12 with respect to both Vδ1 and Vδ2. Thus, combining the SSCP and spectratype analyses, we were able

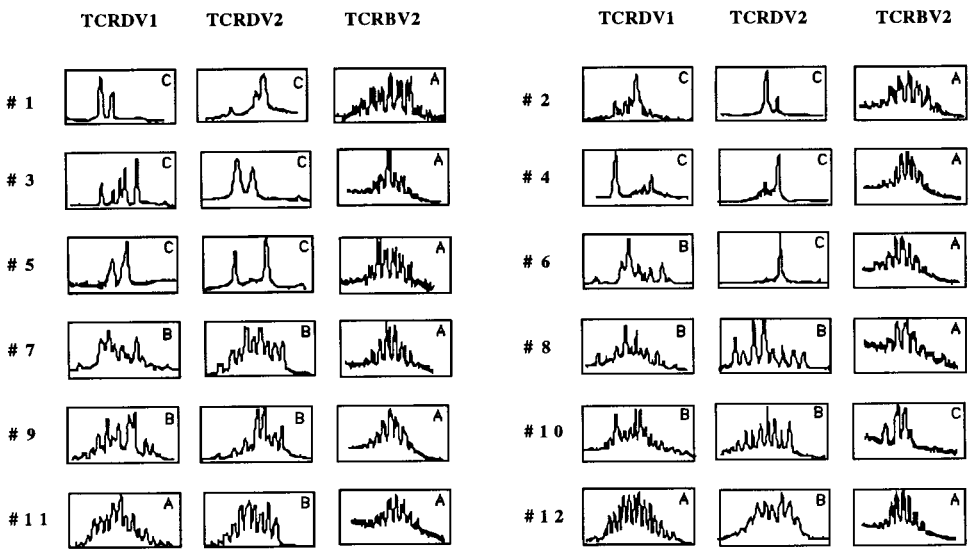

FIGURE 2. Spectratyping graphs of TCRDV1, TCRDV2, and TCRBV2 obtained from 12 normal adult donors. The *A*, *B*, and *C* designations in the *upper right* of each curve represent the extent of TCR diversity characterized by both spectratyping and SSCP as described in Figure 1 (*A*, diverse (polyclonal) TCR usage; *B*, limited TCR diversity; *C*, highly limited TCR usage).

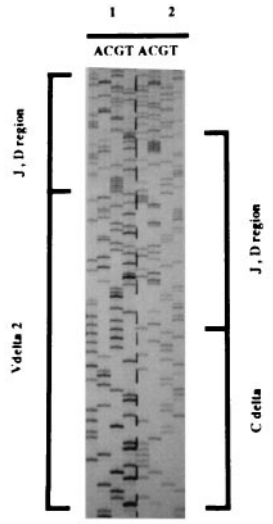

FIGURE 3. Direct sequencing of TCRDV2 in PBMC from adult donor 6. *Lanes 1* and *2* show the sequencing from the same sample beginning at the 5′ and 3′ ends, respectively. The areas for junctional (J, D), variable (V), and constant (C) regions are designated.

to demonstrate restricted TCRDV1 and TCRDV2 junctional usage in PBMC from most normal adult donors. These results for γδ T cells contrast with the diversity of the αβ cells in the same donors.

The clonal nature of the highly restricted γδ TCR repertoire was examined further in donor 6, in whom a single dominant TCRDV2-expressing clone was suggested by both spectratyping and SSCP (Fig. 1). When the TCRDV2 product was amplified by PCR and subjected to direct DNA sequence analysis, an unambiguous sequence could be determined, indicating that this clone was predominant in the starting population of γδ T cells in the PBMC (Fig. 3). As shown in the analysis of single cell progeny, this same TCR sequence could be identified in a high proportion of the *E. coli*-responsive γδ T cell clones (see below).

Proliferation of Vδ2$^+$ T cells from PBMC culture stimulated with E. coli *and* P. aeruginosa

To determine whether the restricted or polyclonal patterns of γδ T cells in the blood of normal adults were related to responses to environmental stimuli, we used the stimulation of γδ T cells by Gram-negative bacteria to assess the repertoire of γδ T cells responding to these stimuli. Adult donors 4, 6, 7, 8, and 10 were used to evaluate their TCRDV usage after stimulation with *E. coli* and *P. aeruginosa*.

Expansions of Vδ1$^+$ and Vδ2$^+$ T cells from these donors are shown in Table I. The percentage of Vδ2 cells increased in all samples stimulated with *E. coli* and *P. aeruginosa*, followed by expansion in IL-2 for 2 wk. Stimulation with *E. coli* was generally more effective at producing Vδ2$^+$ expansion (33–95%), although *P. aeruginosa* was also effective in some individuals, resulting in 15 to 84% Vδ2$^+$ cells as assessed by flow cytometric analysis. In contrast, only a few adults showed expansions of Vδ1$^+$ cells.

Adult donors 4 and 6 demonstrated highly restricted TCRDV2 populations in PBMC. Furthermore, the spectratype banding patterns of PBMC after stimulation with bacteria showed a virtually identical pattern before and after in vitro stimulation. As the spectratyping pattern was identical in the samples run on the same gel (not shown), we used SSCP to demonstrate TCR identity among the samples (Fig. 4). In donor 4 (Fig. 4*A*), in whom the Vδ2$^+$ cells accounted for 95 and 84% of the CD3$^+$ cells, respectively, after *E. coli* and *P. aeruginosa* stimulations, the demonstration of an identical TCRDV2 SSCP pattern after stimulation suggests the expansion of the clones present in peripheral blood. The identical SSCP patterns obtained at different time points during the same *E. coli* stimulation culture (*lanes 2–4*) show the consistency of the Vδ2$^+$ population during culture.

In donor 6, two stimulations performed from PBMCs drawn at two different times (at a 6-wk interval) showed the same clonal proliferation in response to *E. coli* and *P. aeruginosa* (Fig. 4*B*). Their TCRDV2 SSCP patterns also showed stability over time (data not shown). These data suggest that the clonal populations of Vδ2$^+$ cells in PBL of these donors are responsive to bacterial stimulation.

In donors 7, 8, and 10, who showed restricted patterns (but more diverse than those of donors 4 and 6) with respect to TCRDV2 junctional repertoire, their peripheral populations were also expanded by *E. coli* and *P. aeruginosa*. PBMCs drawn at up to three different times were stimulated by the same bacterial preparations to compare their Vδ2$^+$ populations. As shown in Figure 5, after *E. coli* and *P. aeruginosa* stimulations, predominant clones did not result among the cells responding to the two bacterial preparations. Samples 8 and 10 were cultured three times at, respectively, 3-wk and 1-yr intervals, and sample 7 was cultured twice at a 6-mo interval. Consistently, no predominant clones were detected from the stimulations with either bacterial preparation, and their peripheral starting TCRDV2 analyses demonstrated virtually identical CDR3 spectratype patterns (Fig. 5). These data suggest that the

Table I. *Percentage of Vδ1$^+$ and Vδ2$^+$ cells before and 2 wk after stimulations of PBMC with* E. coli *and* P. aeruginosa[a]

Donor No.	Stimulation Time	Vδ1$^+$ (%)			Vδ2$^+$ (%)		
		Before	*E. coli*-stimulated	*P. aeruginosa*-stimulated	Before	*E. coli*-stimulated	*P. aeruginosa*-stimulated
4	1	2	5	18	5	45	33
	2 (3 wk later)	2	2	12	5	65	24
	3 (1 yr later)	1	3	12	4	33	43
5	1	1	1	1	5	80	29
	2 (6 mo later)	1	2	1	2	77	61
6	1	3	2	2	5	71	37
	2 (3 wk later)	3	1	1	5	63	63
	3 (1 yr later)	1	17	1	3	43	44
7	1	1	1	1	5	38	15
	2 (6 wk later)	1	2	2	5	11	9
10	1	2	1	3	2	95	84

[a] Results are expressed as % of CD3$^+$ cells expressing Vδ1 or Vδ2.

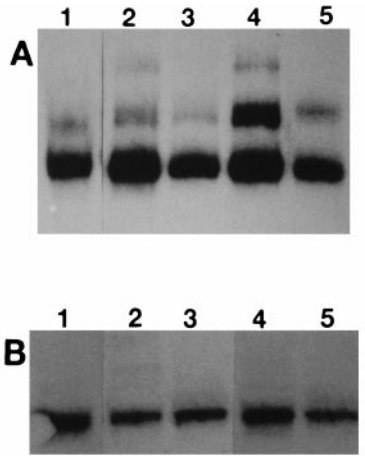

FIGURE 4. Comparison of TCRDV2 SSCP patterns from PBMCs vs those from corresponding cultures stimulated with *E. coli* and *P. aeruginosa* from donors 4 and 6. *A*, TCRDV2 SSCP pattern from adult donor 4. *Lane 1* is from PBMC before stimulation. *Lanes 2* through *4* are the TCRDV2 from populations collected, respectively, at 1, 2, and 3 wk following *E. coli* stimulation. *Lane 5* shows the TCRDV2 of PBMC 2 wk after *P. aeruginosa* stimulation. *B* shows the TCRDV2 SSCP patterns from adult donor 6. *Lane 1* shows the TCRDV2 from PBMC before bacteria stimulation. *Lanes 2* and *3* represent the TCRDV2 from PBMC from the same donor stimulated with *E. coli* on two separate occasions. (The PBMC to begin these cultures were drawn at a 6-wk interval). *Lanes 4* and *5* demonstrate the TCRDV2 of the same two PBMC populations stimulated with *P. aeruginosa* in parallel with the *E. coli* cultures in *lanes 2* and *3*).

population of γδ T cells responsive to Gram-negative bacterial stimulation is reflective of the starting population in the blood, rather than a selective, clonally restricted response toward Gram-negative bacteria.

In selected cultures, the cells were collected at different times, 1 to 2 wk apart, to analyze the consistency of TCRDV2 junctional usage during culture. The same spectratyping and/or SSCP patterns were obtained (data not shown).

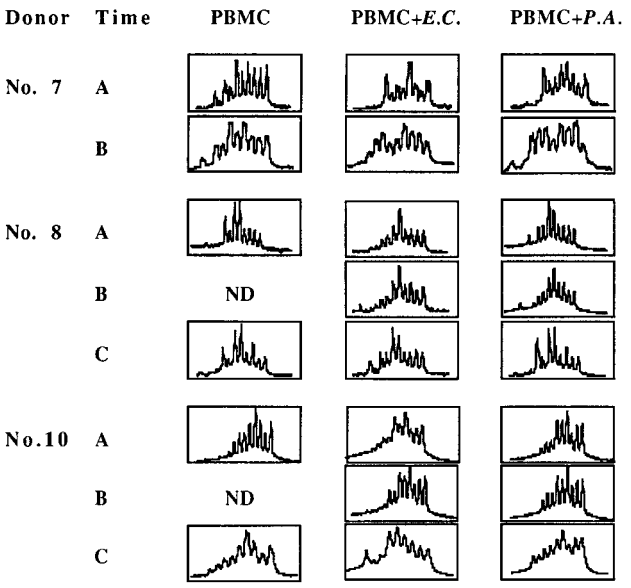

FIGURE 5. Demonstration of TCRDV2 spectratype patterns from donors 7, 8, and 10 whose PBMCs were analyzed directly (PBMC) or following stimulation with *E. coli* (PBMC + E.C.) or *P. aeruginosa* (PBMC + P.A.) on different occasions (*A*, *B*, and *C*), as described in the text.

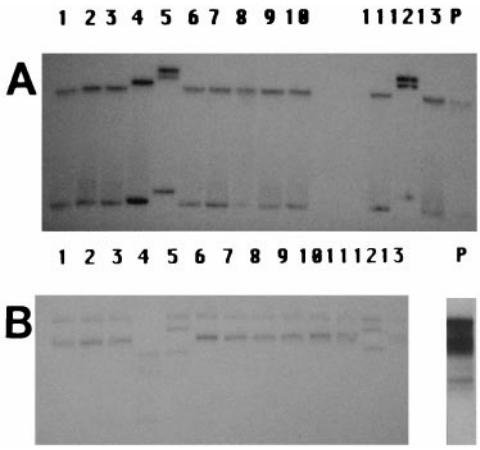

FIGURE 6. TCRDV2 and TCRGV9 SSCP patterns of clones obtained from *E. coli*-expanded culture from PBMC of adult donor 6. *A* shows TCRDV2 SSCP patterns. *B* shows TCRGV9 SSCP patterns. *Lanes 1* through *13* represent 13 different clones; the fresh PBMC is designated P.

Cloning from the E. coli*-stimulated population showed the same predominant clone as that present in PBL*

After the expansion of PBMC from adult donor 6 with *E. coli*, the cells were cloned by limiting dilution. Thirteen $V\delta2^+$ clones were obtained and grown from the culture, and they were analyzed by SSCP to evaluate the dominant clonal response. As shown in Figure 6*A*, 10 of 13 clones showed the same TCRDV2 SSCP pattern, which was identical with that of the predominant clone observed in the PBL. These γδ receptors in these $V\delta2^+$ clones are paired with Vγ9, and their TCRGV9 SSCP analysis also showed the same dominance in the same 10 clones (Fig. 6*B*). However, because there may be nonfunctional TCRGV9 transcripts in $\alpha\beta$ T cells, the same dominance is not clearly present with respect to TCRGV9 in the PBL. Instead, unlike peripheral TCRDV2 transcripts, which demonstrate one dominant TCR, TCRGV9 usage from the same sample displays a much more complex SSCP pattern (Fig. 6*B*, *lane P*). Taken together, the above results again demonstrate the clonal predominance among the $V\delta2^+$ cells in PBL from adult donor 6 and the responsiveness of this dominant clone to *E. coli* in vitro.

Diversity of TCRDV1 and TCRDV2 in umbilical cord blood samples from normal deliveries

To clarify whether a restriction in the TCRδ repertoire is also seen in newborns or represents a selection for a few clones during extrauterine life, we collected cord blood samples from nine normal deliveries. As shown in Figure 7, the TCRBV2 spectratypes showed a polyclonal feature similar to that observed in the adult donors. However, in both TCRDV1 and TCRDV2 analyses, a highly restricted pattern was observed in seven of nine cord blood samples, again indicating that oligoclonality of $V\delta1^+$ and $V\delta2^+$ cells is also present in newborns. As shown in Figure 8, in an additional three sets of twins, none of the six infants showed polyclonal spectratype patterns, indicating that of the 15 newborns analyzed, 13 showed restricted TCRDV usage.

Different TCRDV1 and TCRDV2 junctional usage in cord blood samples from twins

To determine whether the selective clonal usage of TCRδ is influenced predominantly by the genetic background of the individual or if it reflects additional regulatory control, we evaluated the TCR repertoire in twins. TCRDV1 and TCRDV2 spectratypes of six cord blood samples from three pairs of twins are shown in

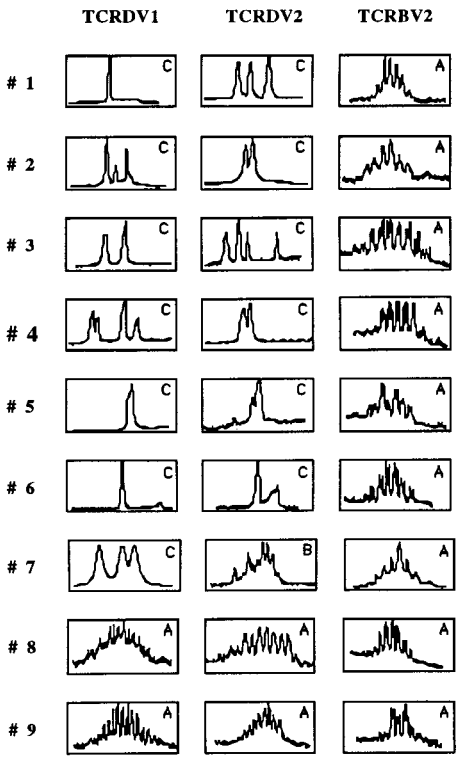

FIGURE 7. Spectratyping graphs of TCRDV1, TCRDV2, and TCRBV2 from nine cord blood samples. As in Figures 1 and 2, the designation *A, B,* or *C* reflects the extent of TCR diversity inferred from the spectratyping and SSCP patterns. (*A,* diverse (polyclonal) TCR usage; *B,* limited TCR diversity; *C,* highly limited TCR usage).

Figure 8. Samples A1/A2 and B1/B2 were collected from two pairs of identical twins, while C1 and C2 were from one pair of nonidentical twins. As shown in Figure 8, the spectratypes for both TCRDV1 and TCRDV2 junctional usage showed clear differences between twins regardless of their genetic identity. An additional pair of adult identical twins also showed different clonal predominance (donors 1 and 2 in Fig. 2).

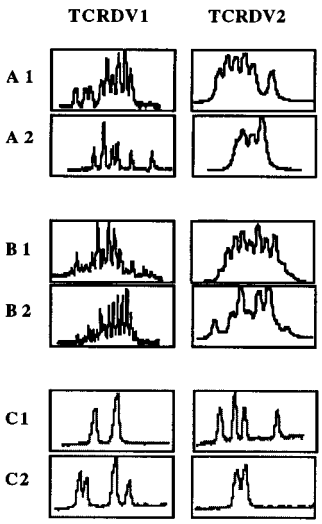

FIGURE 8. Comparison of TCRDV1, TCRDV2, and TCRBV2 from cord blood samples from three pairs of twins. *A1/A2* and *B1/B2* are from two pairs of identical twins. *C1/C2* are from a pair of nonidentical twins. (Note that adult donors 1 and 2 shown in Fig. 2 are identical twins.)

Discussion

Although there are previous studies that have suggested possible clonal selection among γδ T cells in normal PBMC, the data from several reports are somewhat contradictory and have dealt almost exclusively with the Vδ2/Vγ9 subset and not with Vδ1$^+$ T cells. In particular, the γδ T cells responding to mycobacteria have been reported to be polyclonal with respect to CDR3 (21), although unselected PBMC have been shown to be clonally restricted in some adults (12–15). It was our hope to clarify the status of the γδ T cell repertoire in adults and to determine whether the selective stimulation of γδ T cells by Gram-negative bacteria might be responsible for the clonal restriction noted in some individuals. Also, as the selection for Vδ2/Vγ9$^+$ T cells has been ascribed to environmental stimuli, we wished to determine whether the Vδ1 repertoire was similarly influenced by the bacterial stimuli or whether the clonal restriction was confined to the Vδ2/Vγ9$^+$ subset. Furthermore, we wanted to determine whether the clonal pattern seen in some adults represented a selection from a polyclonal γδ T cell repertoire at birth or, rather, if the restricted pattern was already present at birth. As the adult repertoire would be expected to be influenced by the exposure to a wide variety of environmental stimuli, the sheltered environment of the fetus coupled with immunologic immaturity would be expected to provide less opportunity for in vivo selection in the newborn. Finally, by evaluating the TCRDV repertoire in twins, we demonstrated that selective TCRDV usage is influenced by more than the genetic background of the blood donor.

The results favor the hypothesis that the γδ T cells can be clonally restricted in many normal individuals both at birth and in adult life, although there are significant differences among different individuals, some of whom show polyclonal γδ T cell populations. Both the Vδ1$^+$ and Vδ2$^+$ subsets show similar restriction patterns both at birth and in adult life.

The question of clonality of γδ T cells in newborns has been addressed in one other study (18) that reported polyclonal junctional usage in TCRDV1-DJ1 from four cord blood samples as judged by their polyacrylamide electrophoresis patterns after PCR. Thirteen of the 15 newborns we studied showed a restricted pattern in spectratyping and SSCP analyses, suggesting oligoclonality in TCRDV1 and TCRDV2 lymphocytic populations before extrauterine exposure. As two of the 15 cases we studied were polyclonal, the apparent discrepancy with the results of Beldjord's study (18) may be due to the limited number of cases they examined. By studying the TCRDV1 and TCRDV2 repertoires of a slightly larger population using two complementary techniques, our data would indicate that selection for a limited γδ repertoire is indeed a common finding among newborns. Differences between the γδ TCR repertoire in newborn twins suggests that the genetic background alone does not determine the selection for clones bearing the same γδ TCR, but, rather, like the unique attributes of fingerprints, the TCR repertoire shows phenotypic variations between individuals with genetic identity.

Although some previous studies have also noted clonal selection for γδ T cells in adults (12–15), other groups have failed to confirm this finding (20, 33–35). Our own data, derived from the combination of SSCP and spectratyping analyses of whole blood lymphocyte populations, indicate that most normal donors do show some restriction in their TCRDV repertoire, although some individuals were quite diverse in both TCRDV and TCRBV repertoires. Among the donors whose TCRDV repertoire included some different clones, but were not truly polyclonal (donors 7–10), both of the molecular approaches we used showed that there was limited diversity. In such individuals, analyses of T cell clones would

require isolating large number of cellular clones to demonstrate the selection we have found on the population level. However, nothing in any of these studies clarifies the range of TCR usage observed among normal subjects whose TCRDV repertoires varied from highly clonal to polyclonal. Although the subjects studied were of diverse racial and geographical backgrounds, the total number of subjects is still too limited to draw conclusions that would allow us to distinguish populations of individuals on the basis of γδ TCR diversity.

Although $V\delta2^+$ cells are the predominant γδ subset in PBMC in adults, while $V\delta1^+$ cells predominate in tissue or cord blood (7), the range of restriction of the TCRDV repertoires was similar in lymphocyte populations in both adults and newborns. This suggests that restriction of TCRDVJ usage exists in PBMC regardless of their relative numbers and V region usage, indicating that restriction is a general property of γδ cells. This concept is also supported by others, who found a general restriction of TCRDV gene usage in $V\delta1^+$, $V\delta2^+$, and $V\delta3^+$ T cells from PBMC and intestinal tissues (15).

Importantly, the restricted γδ repertoire seen in several of the adults could be confirmed to be stable over a period of up to 12 mo, reaffirming the findings of previous studies (13–15). Analysis of clones of bacteria-stimulated PBL of one of the donors demonstrated that 10 of 13 clones generated in response to *E. coli* had the identical TCRDV2 sequences noted to be predominant among the TCRDV2 transcripts in the fresh PBL. These data indicate that the γδ T cells that respond to *E. coli* are representative of their counterparts in the starting population.

Since the response to environmental stimuli, such as bacterial Ags, could drive clonal selection in γδ T cells, we investigated the restriction of TCR repertoire in γδ T cells derived from in vitro cultures stimulated with *E. coli* or *P. aeruginosa*. Our data show that when the unstimulated population was highly restricted, a similar pattern could be detected in the bacteria-stimulated cells. Similarly, in those individuals whose γδ T cells were polyclonal, the bacteria-stimulated cells remained polyclonal. Although it has been reported that the response to mycobacterial Ags results in polyclonal $V\delta2^+$ cells (21), we found that the response to more ubiquitous bacteria did not skew the expansion of the TCRDV repertoire. The T cell responsiveness of mycobacteria-specific clones to *Listeria monocytogenes* and *E. coli* demonstrated by Libero et al. (10) is reminiscent of our results indicating reactivity of highly clonal γδ T cells to both *E. coli* and *P. aeruginosa*.

As the response to Gram-negative bacteria does not appear to influence the γδ T cell repertoire, one might postulate that the specificity of the γδ TCR is not directly involved in the response to either *E. coli* or *P. aeruginosa*. These two Gram-negative organisms are not cross-reactive at the level of Ag recognition by $CD4^+$ αβ T cells (24, 36), although they could share antigenic structures recognizable by γδ T cells. However, the vigorous response by both clonal and polyclonal γδ T cells suggests that either all of the clones have TCRs that recognize Gram-negative bacterial Ags or that γδ T cells are stimulated by structures other than the CDR3 of the TCR. Cord blood γδ T cells, which are predominantly $V\delta1^+$, respond to the same bacteria as the predominantly $V\delta2^+$ adult T cells. This further suggests that a stimulus that is neither conventional Ag nor superantigen (1) is capable of activating γδ T cells via interaction with structures other than the CDR3 region of the γδ TCR.

Although our own previous study excluded LPS as the primary stimulus for this polyclonal activator of γδ T cells in humans (1), Leclercq et al. (3) demonstrated that LPS influenced the response of γδ T cells to Gram-negative bacteria in mice. We do not know whether LPS plays an adjuvant role in the human γδ T cell response to the proposed polyclonal response element. However, this polyclonal response of γδ T cells to Gram-negative bacteria may parallel the response of B lymphocytes to bacterial products (such as LPS from Gram-negative organisms and *Staphylococcus aureus*-derived protein A). Such polyclonal activators do not interact with the Ag-combining sites of the B cell's Ig receptors, although protein A may activate by cross-linking these Ag receptors by binding to Fc regions on the surface Igs.

Recently, it has been found that $V\delta2/V\gamma9^+$ T cell stimulation by monoethylphosphate involves the TCR complex, in that transfection of cells with such TCR confers responsiveness to such nonprotein Ags (37). It is possible that parallel to the B cell response to protein A, determinants other than the presumed Ag-combining sites encoded within the hypervariable CDR3 elements of the γδ TCR, including Cγ and Cδ structures, could interact with cross-reactive bacterial components, leading to the stimulation of both the $V\delta1^+$ and $V\delta2^+$ subsets we have noted with Gram-negative bacteria. Such a polyclonal response element on γδT cells would interact with structures on Gram-negative bacteria, resulting in upregulation of IL-2R. The addition of IL-2 then drives further γδ T cell proliferation following this bacterial stimulation (1).

In summary, PBMC from both normal adults and newborns often show restriction in the TCRDV1 and TCRDV2 repertoire. Furthermore, the primary response of γδ T cells to Gram-negative bacteria may be independent of CDR3 region specificity. The restriction of TCRDV in newborns argues against environmental antigenic selection for the γδ T cell repertoire in peripheral blood. As even identical twins show different patterns of restriction, selection for CDR3 sequences among γδ T cells seems to be independent of the genetic background. The possibility that γδ T cells are responsive as a cell lineage to some form of polyclonal activator shared among bacteria is hypothesized, but further studies are needed to elucidate the mechanisms involved.

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