Peripheral Selection of Vδ1+ Cells with Restricted T Cell Receptor δ Gene Junctional Repertoire in the Peripheral Blood of Healthy Donors

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Summary
To characterize the T cell receptor (TCR) repertoire expressed by the Vδ1+ γ/δ T cell population, we have studied the Vδ1-Jδ1 junctional sequences from peripheral blood samples of healthy donors. We show that, surprisingly, this repertoire is restricted in most healthy adults, with a donor-specific and relatively stable pattern, whereas this repertoire remains unrestricted in infants, and is similar to that of thymocytes. These data contrast with the general assumption that the junctional repertoire of Vδ1+ γ/δ T cells is extensive, and strongly suggest that peripheral recruitment of Vδ1+ cells bearing particular TCR occurs in humans during the postnatal stage.

Materials and Methods

Donors and Cells. Blood samples were obtained from 29 healthy adult blood donors. Cells from two pediatric thymuses (obtained from children undergoing heart surgery) and PBL from healthy infants (n = 8; 5-d-24-mo-old) and children (n = 6; 25-72-mo-old) were studied. Four umbilical cord blood samples were also used in some experiments. Immunophenotypes of fresh or frozen PBMC were determined by direct fluorescence analysis on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA) using the FITC-o’CS1 (Vδ1-Jδ1/Jδ2 specific) mAb (3) from T Cell Sciences (Cambridge, MA). The Vδ1+ cell count was found to be normal in all cases (n = 20).

PCR Analysis of Vδ1(D)Jδ1 Junctional Sequences. Genomic DNA from PBL was phenol-chloroform extracted. Total cellular RNA was isolated by the Chomczynski and Sacchi method (7) using RNAlater (Bioprobe System, Montreuil Sous Bois, France). cDNA were synthesized from 1-5 μg of total RNA using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase in the presence of RNAse (Promega Corp., Madison, WI). RNA were primed at 42°C for 30 min in a final vol of 20 μl. PCR reactions (8) were performed in a thermocycler (Perkin-Elmer Corp., Norwalk, CT) using either whole cDNA or 500 ng of genomic DNA, 2.5 U of Taq DNA Polymerase (Perkin-Elmer Corp.), and 25 pmoles of each primer. Amplifications were carried out in a total vol of 100 μl containing 67 mM Tris-HCl (pH, 8.8), 16.6 mM (NH4)2SO4, 6.7 mM MgCl2, 10% DMSO (vol/vol), and 1.5 U Taq polymerase. The products were resolved on an agarose gel and visualized with ethidium bromide. Primer pairs used were Vγ9-Jδ1 and Vγ9-Jδ1, which amplifies a 300-bp fragment representing the predominant Vγ9-Jδ1 junctional sequence found in the human thymus (4) and large intestine (5). To date, very little is known about the biology of these cells (6).

To define the TCR-δ repertoire of Vδ1+ cells, we have characterized the Vδ1-Jδ1 junctional diversity in the PB of healthy donors. We show that the junctional repertoire of normal infants displays extensive diversity, whereas this repertoire is restricted in most healthy adults with a donor-specific and relatively stable pattern. These data strongly suggest that peripheral selection occurs in the postnatal period.

1 Abbreviations used in this paper: AJO, antijunctional oligonucleotide; PB, peripheral blood; PBL, peripheral blood leukocyte; SSCP, single-strand conformation polymorphism.
Results

Restriction of the V81-J81 Junctional Diversity in Adults. To define the CDR3 repertoire of normal V81-J81 rearrangements, the junctional sequences from two pediatric thymocyte samples and 29 unselected healthy adult donor peripheral blood leukocytes (PBL) were amplified by PCR. Sizes of amplified products were analyzed by PAGE. Representative experiments are shown in Fig. 1.

As expected, in the case of postnatal thymocytes, major variations of PCR product size were observed, leading to a smearlike electrophoresis pattern due to the extensive junctional diversity of TCR-δ rearrangements (13).

In marked contrast, very different results were obtained with healthy donor PBL (Fig. 1). A smearlike pattern similar to that observed in thymus DNA was found in only 8 of 29 cases. Predominant rearrangements were present in the other cases. Each pattern appeared to be specific for a given individual and was reproducible in independent PCR performed at least twice in different laboratories (data not shown). The PAGE pattern did not correlate with the V81+ cell count (data not shown).

To assess whether the predominantly amplified band(s) correspond(s) to repeated junctional sequences and not merely to junctions of identical size, genomic DNA PCR products obtained from eight adult donors, selected to be representative of restricted and unrestricted PAGE patterns, were cloned and sequenced (Fig. 2). Diverse and nonrepeated V81-J81 sequences were found in the two samples displaying a predominantly unrestricted PAGE pattern (samples 125 and 134). Conversely, repeated junctions were observed in all six cases demonstrating a more or less discrete V81-J81 PAGE pattern (Figs. 1 and 3, and data not shown). None of these sequences were shared by different donors. The predominant rearrangement in most cases includes extensive random nucleotide deletions/additions and the use of D regions. In all but one case (sample 127), repeated sequences were in-frame and could therefore encode a functional δ chain. In one case (sample 119), cloning procedures were used on both DNA- and cDNA-derived V81-J61 PCR products. The same predominant V81-J61 junction was observed in both experiments (of 14 cDNA PCR-derived clones hybridizing to an internal V81 probe, 8 also hybridized to an antijunctional oligonucleotide (AJO)-specific for the predominant DNA junctional sequence), suggesting that the restricted sequence may be expressed at the cell surface of at least a part of the V81+ γ/δ T cells in this donor.

An alternative strategy based on SSCP was used to confirm the CDR3 restriction in selected samples, including three samples also studied by sequence analysis. PCR carried out with α-[32P]dCTP was performed on genomic DNA and the resulting products fully denatured. One aliquot was loaded as a control on a high resolution denaturing acrylamide gel and another aliquot on a nondenaturing one to analyze single-
Figure 2. Nucleotide sequences of V61-J81 junctions from healthy donors. (id) Sample identification. All but two samples (samples 649 and 655) correspond to adult donors. Samples 649 and 655 correspond to infants aged 2 and 3 mo, respectively. (IF +) In-frame; (IF -) out-of-frame. Nongermline-encoded sequences are underlined. (DEL) Deletion of nucleotides; the corresponding number of deleted bases is indicated.

Table 1. Summary of the number of clones with the corresponding sequence/total number of sequenced clones. Nongermline-encoded sequences are underlined.

| Sample | In-frame | Out-of-frame |
|--------|----------|--------------|
| 120     | 156      | 4            |
| 121     | 150      | 5            |
| 122     | 130      | 10           |
| 123     | 100      | 17           |
| 124     | 75       | 25           |
| 125     | 50       | 45           |
| 126     | 30       | 30           |

Stability of the V61-J81 Junctional Repertoire in Adults. To determine the stability of the V61-J81 junctional repertoire, we performed the analysis on a large number of healthy donors. The results showed that the observed CDR3 restriction is not due to a sampling effect. One sample (number 119) obtained from a donor with a normal V61 + cell count (i.e., lymphocytes, 1,269/mm²; V61 + cells, 9/mm²) was further studied by dilution analysis. It is to be expected that the PAGE-specific pattern would be lost or would vary in increasing dilutions if the CDR3 restriction is only due to a sampling effect. The characteristic oligonclonal-specific pattern was observed unchanged from 580 ng (roughly equivalent to 87,000 PBL and to 610 V61 + cells, as expected from the percentage of δTCS1 + cells in the PB of this particular donor) to 15 ng (equivalent to 16 V61 + cells) dilutions and lost only at 7 ng (equivalent to eight V61 + cells) (Fig. 4). Moreover, the AJO (14) recognizing the overrepresented junctional sequence was used as a probe in this experiment showing the corresponding clone-specific sequence to be detectable in experiments performed with as little as 15 ng of DNA (Fig. 4). This experiment shows that the observed CDR3 restriction is not due to a sampling effect.

Stability of the V61-J81 Junctional Repertoire in Adults. To understand the stability of the V61-J81 junctional repertoire, we performed the analysis on a large number of healthy donors. The results showed that the observed CDR3 restriction is not due to a sampling effect. One sample (number 119) obtained from a donor with a normal V61 + cell count (i.e., lymphocytes, 1,269/mm²; V61 + cells, 9/mm²) was further studied by dilution analysis. It is to be expected that the PAGE-specific pattern would be lost or would vary in increasing dilutions if the CDR3 restriction is only due to a sampling effect. The characteristic oligonclonal-specific pattern was observed unchanged from 580 ng (roughly equivalent to 87,000 PBL and to 610 V61 + cells, as expected from the percentage of δTCS1 + cells in the PB of this particular donor) to 15 ng (equivalent to 16 V61 + cells) dilutions and lost only at 7 ng (equivalent to eight V61 + cells) (Fig. 4). Moreover, the AJO (14) recognizing the overrepresented junctional sequence was used as a probe in this experiment showing the corresponding clone-specific sequence to be detectable in experiments performed with as little as 15 ng of DNA (Fig. 4). This experiment shows that the observed CDR3 restriction is not due to a sampling effect.
analyze the stability of the Vδ1-Jδ1 CDR3 repertoire, sequential studies were performed in six healthy adult donors (Fig. 5).

In one of these (sample 813), a complex unrestricted PAGE pattern was evident, that remained unchanged 15 mo later.

In the five other donors (samples 119, 120, 127, 811, and 815), a more or less restricted repertoire was documented. In three cases (samples 119, 120, and 127), the PAGE pattern was again found to be identical 4 yr later. Vδ1-Jδ1 fragments amplified from two 811 samples taken at 2-yr intervals were hybridized with the AJO specific for the predominant and formerly identified sequence. As shown in Fig. 5, the same dominant junctional sequence was found. In the sample 811, the PAGE pattern was roughly similar at 3-yr intervals, although some alterations could be observed. Contrasting with these findings, significant alterations were documented in the last case (sample 815).

**Unrestricted Vδ1-Jδ1 Junctional Repertoire in Infants.** We wondered whether the nonrandom presence of certain Vδ1 junctions might be related to the donor’s age. To answer this question, PB from 5-d–24-mo-old infants (n = 8) and from 25–72-mo-old children (n = 6) were evaluated. Four umbilical cord blood samples were also studied. Representative experiments are shown in Fig. 6 and all data are summarized in Table 1.

Samples from infants aged <2 yr and umbilical cord blood samples display a smearlike PAGE pattern, comparable with
Figure 4. Dilution analysis of the Vδ1-Jδ1 predominant sequence in the 119 healthy donor. (Top) Ethidium bromide staining. (Middle) Hybridization with a Vδ1 probe. (Bottom) Hybridization with a clone-specific AJO recognizing the predominant sequence AATCAAGTTTCCGCGGGCCC.

that found in thymic cells (Fig. 6). Vδ1-Jδ1 PCR products from two samples, 649 (2-mo-old) and 655 (3-mo-old) were sequenced. As expected from the PAGE pattern, no repeated sequences were found (Fig. 2). One sample (sample 655) was also studied by SSCP analysis. As expected, a smearlike pattern was observed (Fig. 3).

In four children (36–60-mo-old), an unrestricted pattern was found. A discrete band associated with a predominantly unrestricted pattern was documented in two donors (25- and 36-mo-old). Finally, a clearly restricted repertoire is evident in the sample from the oldest child (72-mo-old).

As is shown in Table 1, there is an evident increase in the number of individuals demonstrating a restricted repertoire with increasing age.

Collectively, these data clearly demonstrate that a restriction of the PB lymphocyte Vδ1-Jδ1 junctional repertoire is frequent among healthy donors, that this restriction is acquired during the postnatal period, and finally, that this repertoire is generally a stable feature. These results are strongly suggestive of a postnatal positive selection process involving Vδ1+ γδ T cells expressing specific CDR3 sequences.

Discussion

In this work, we have characterized the TCR-δ gene diversity in the PBL of healthy donors. We have demonstrated that the Vδ1-Jδ1 junctional repertoire is restricted in a majority

Table 1. Correlation between the Vδ1-Jδ1 Junctional Repertoire and the Donor Age

| Total number of cases | Number of cases with a restricted PAGE pattern |
|-----------------------|-----------------------------------------------|
| Umbilical cord blood  | 4                                             |
| Infants (5 d–24 mo)   | 8                                             |
| Children (25–72 mo)   | 6                                             |
| Adults                | 29                                            |

* Includes two cases with a discrete band associated with a predominantly unrestricted pattern.
of healthy adult donors, leading to a relatively stable and donor-specific pattern. Moreover, we have also shown that this repertoire is not restricted in the PBL of infants, and is therefore similar to that observed in postnatal thymus.

It is generally assumed that γ/δ T cells function as a first line of defense against infectious pathogens. The nature of presenting molecules that may be potentially involved in antigen recognition is unclear, at least for the majority of γ/δ T cells, although evidence for allore cognition of MHC class I (15) and II (16), and of other less polymorphic molecules (17-19) has been documented. A few examples of clones recognizing nominal antigens in the context of MHC molecules have also been described (20, 21).

Little is known about the function of the Vδ1 subset. It has been shown, however, that the binding to Vδ1 + cells of a Vδ1-specific mAb elicits Ca2+ signaling and cell proliferation (22), and that activated Vδ1 + expressing clones are cytolytic and can secrete IL-5, IFN-γ, and GM-CSF (23). Moreover, TCR-dependent recognition of CD1c (17) and CD48 (TCT.1) (18, 19) molecules by Vδ1 + cells has been documented. More recently, it has been shown that the Vδ1 + subset of γ/δ T cells may be triggered to proliferate by T-cell interaction with EBV-infected Burkitt's lymphoma cells or EBV-transformed B cells (24).

The new molecular data we have obtained regarding Vδ1 + cells in healthy donors are consistent with the involvement of Vδ1 + cells in the recognition of processed peptides. Peptide plus MHC recognition by α/β T cells is mainly mediated by the V(D)J junctional CDR3 (25, 26), and the role of TCR-γ/δ junctional sequences in the specificity of antigen recognition has been recently stressed (27). In the present work, we have shown that Vδ1-CD3 junctions, while displaying the extensive alterations of germline sequences that are characteristic of post fetal stages (14), are not randomly represented among PBL Vδ1 + cells in a large fraction of healthy donors. The description of such repeated sequences in the PBL of healthy donors is unprecedented since only a few Vδ1-CD3 sequences have been published to date (28-31). Two studies showed an unrestricted repertoire in PBL from patients and normal controls, but predominant sequences in acute multiple sclerosis (29) and leprosy lesions (30), respectively, suggesting in situ selection of particular Vδ1 + cells by so far undefined antigens. One further study demonstrated a restricted Vδ1 repertoire in the peripheral blood of certain patients with rheumatoid arthritis but not in four healthy controls (30). However, it is worth noting that a recent report of preferential Vδ1-Jδ1 junctional repertoires in sarcoidosis, demonstrated a repetitive sequence in cDNA from the PBL of one of the normal controls (31).

The occurrence of dominant receptors in PBL demonstrated here may be due to thymic selection, since it has been suggested that negative thymic selection of a limited part of the Vδ1 T cell population can occur in appropriate transgenic mice (32). In addition, it is generally assumed that Vδ1⁺ PBL derive from the predominant Vδ1⁺ thymic γ/δ T cell subset. On the other hand, the hypothesis of thymic selection of defined Vδ1-Jδ1 junctions is not substantially supported by our demonstration that these junctions are not restricted in infant PBL, in whom the pattern is roughly similar to the one observed in thymus. The age corresponding to the switch between an unrestricted and a restricted repertoire remain to be determined. However, it is worth noting that a clearly restricted repertoire was documented in a 72-mo-old child in our series. We therefore favor the hypothesis that the restricted repertoire we have found in the majority of healthy donors is due to a recruitment of γ/δ T cells that express particular Vδ1-Jδ1 junctions. A similar mechanism has been speculated to occur in the case of murine pulmonary γ/δ T cells with canonical invariant TCR-δ sequence (33). Since the deduced CDR3 amino acid sequences vary from one donor to another, the results suggest that these cells recognize a limited number of nominal antigens, distinct from one donor to another, and presented by nonclassical proteins like the CD1 family. Alternatively, these antigens may be similar in all donors, but could be presented by highly polymorphic molecules. Studies of identical twins should be performed to further clarify this point.

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