FURTHER ANALYSIS OF THE T CELL RECEPTOR γ/δ^+ PERIPHERAL LYMPHOCYTE SUBSET

The Vδ1 Gene Segment Is Expressed with either Cα or Cδ

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A subpopulation of lymphocytes expressing the CD3 proteins but not the α/β receptor has been identified recently. These cells represent ~5% of the lymphocytes in human peripheral blood; they have been found to possess an additional type of TCR that associates two chains designated γ and δ. The corresponding proteins are encoded by genes that rearrange specifically in T lymphocytes.

The γ chain locus was the first to be characterized; it includes two C regions (Cγ1 and Cγ2) whose use determines whether the receptor is expressed as a disulfide-linked (Cγ1) or a non-disulfide-linked (Cγ2) γ/δ heterodimer. Two J segments are located upstream of Cγ1 and three upstream of Cγ2 (1–3). 14 Vγ genes have been described upstream of the Cγ1 region (4–6).

The δ locus, identified more recently, is found between Vα and Cα genes (7, 8); one C region has been characterized with three D segments and three J segments located upstream. Six Vδ genes have been described. Five are located upstream of Cδ (9–11); one of them belongs to the Vα6 subfamily (12). One additional V segment lies downstream of Cδ in an inverted orientation (8, 11).

Several mAbs specific for various segments of the γ/δ receptor have been described. Anti-TCR-γ/δ-1 (13) recognizes a Cγ-encoded antigenic determinant. Anti-TCR-δ1 reacts with a Cδ-encoded epitope (14, 15). Thus, such reagents define the whole γ/δ^+ fraction in a given cell suspension. The anti-TiγA mAb (16) was found to recognize the Vγ9 gene product (17). Anti-δTCS1 (18) is likely to react with an antigenic determinant that includes Vδ1 and Jδ1 (19), or Vδ1 and Jδ2 (20). Anti-BB3 was found initially to recognize δTCS1$^-$ cells expressing disulfide-linked receptors (21, 22); additional experiments suggested that this mAb has a Vδ2 specificity (10).

The characterization of a novel antibody, designated anti-TiVδ2, is reported here. It is shown that this reagent is specific for an epitope encoded by the Vδ2 gene. In addition, we have further investigated the reactivity of an additional mAb, termed anti-A13 (23). This reagent has been described recently; it was found to recognize a fraction of circulating γ/δ^+ cells. These A13$^+$ lymphocytes did not react with the

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anti-BB3 mAb while many of them were δTCS1$^+$ (23). Our data indicate that anti-A13 is directed at an antigenic determinant encoded by the Vδ1 gene.

The organization of the γ/δ$^+$ peripheral cell fraction has been reassessed here in light of the reactivity of anti-A13 (Vδ1), anti-δTCS1 (Vδ1-Jδ1 or Vδ1-Jδ2), and anti-TiVδ2 (Vδ2) mAbs. It is shown that the expression of the δTCS1 and the TiVδ2 antigenic determinants accounts for the great majority of γ/δ$^+$ circulating cells. In certain donors, the summed reactivity of anti-Vδ1 and anti-Vδ2 was greater than that of anti-TCRδ1 mAb. Experiments designed to understand this apparent discrepancy led to the demonstration that the Vδ1 segment is expressed with Jα-Cα segments in a sizeable population of A13$^+$ TCR-α/β$^+$ peripheral lymphocytes.

Materials and Methods

Production of the Anti-TiVδ2 Antibody. 6-wk-old Biozzi mice were immunized with AB12 cells (10). The immunization schedule consisted of an intraperitoneal injection of 3×10^6 cells emulsified in CFA followed by three intraperitoneal injections of 3×10^6 cells emulsified in IFA at 2-wk intervals. 2 wk later, 3×10^6 cells were injected intravenously in PBS, followed by splenectomy 3 d later. Somatic hybridization was carried out as previously described (24).

Generation and In Vitro Culture of Cell Lines. Some of the cell lines analyzed in this study have been described previously. F6C7 and G6 clones were derived from peripheral blood of a 19-wk-old fetus (25–27). The other clones were obtained from PBL of four different adult healthy donors (27, 28). The polyclonal cell line TH6.4 was derived from the peripheral blood of a patient with a renal cell carcinoma. The A13$^+$ polyclonal cell line was obtained from peripheral blood of an adult healthy donor. These cells were further purified to generate the T1 and T2 cell lines.

For expansion, cells were plated in 96-well V-bottomed microtiter plates on a feeder layer containing an EBV-transformed B cell line (LAZ 388) plus irradiated allogenic lymphocytes. Cultures were fed every 3 d with lymphocyte-conditioned medium containing IL-2.

Immunofluorescence Assays and Cytofluometric Analysis. Studies on "fresh" lymphocytes were performed using monocyte-depleted cell fractions extracted by Ficoll-Hypaque density gradient centrifugation.

Indirect immunofluorescence assays were performed as described previously (24). Cells were incubated with predetermined saturating concentrations of antibody plus fluorescein-conjugated goat anti-mouse serum. For double-color assays, cells were stained with fluoresceinated BMA031 (IgG2b) mAb and unconjugated anti-A13 (IgG1); reactivity of the latter reagent was revealed by a phycoerythrin-conjugated goat anti-mouse serum specific for the IgG1 subclass.

For PBL and cultured cells, samples containing 5×10^4 and 5×10^3 cells, respectively, were analyzed on the Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL). In the experiments where the functional effects of the antibodies have been tested, cells were incubated overnight with the relevant reagent at the predetermined saturating concentration. Immunofluorescence assays on the treated cells were performed in parallel.

mAbs. Anti-NKTa mAb recognizes an infrequent α/β clonotypic determinant (24). Anti-TiγA mAb (16) is specific for a Vγ9-encoded epitope; it delineates a subset including ~3% of CD3$^+$ TCR-α/β$^-$ PBL (17). Anti-BMA031 mAb, kindly provided by Dr. R. Kurrle (Behring Co., Marburg, FRG), recognizes a monomorphic determinant of the TCR-α/β receptor (29). Anti-TCR-δ1 mAb (14), kindly provided by Dr. M. Brenner (Dana-Farber Cancer Institute, Boston, MA), recognizes a constant epitope of the human TCR-δ protein. Anti-TCR-γ/δ-1 (13), which reacts with a Cγ determinant, was generously given by Dr. J. Borst (Netherlands Cancer Institute, Amsterdam, Netherlands). Anti-δTCS1 (18), prepared by the T Cell Sciences Co. (Cambridge, MA), is most likely directed at a Vδ1-Jδ1- (19) and Vδ1-Jδ2- (20) encoded epitope. The XC3 mAb reacts with the CD2 molecule (unpublished observations). Anti-CD3 mAb used for immunofluorescence assays was OKT3 (Ortho Diag-

nostic Systems Inc., Westwood, MA), whereas immunoprecipitation experiments were conducted with an additional anti-CD3 mAb (CD3X3) kindly provided by Dr. A. Bernard (Institut Gustave-Roussy, Villejuif, France). Each mAb was used at a saturating concentration predetermined by titration curves on positive cloned cell lines.

Immunoprecipitation of the TCR-γ/δ Receptor. AB12 and TH6.4 cells were surface labeled with ^{125}I using a standard lactoperoxidase method and then lysed with a phosphate buffer containing either 0.1 or 1.0% Triton X-100, as previously described (16). The lysates were precleared several times with Staphylococcus A suspension as well as irrelevant antibodies; the specific immunoprecipitation was carried out overnight at 4°C with either anti-TiVδ2 mAb coupled to bromocyanogen-Sepharose beads for lysates obtained with high detergent concentration or anti-CD3X3 mAb coupled to protein A-Sepharose beads for lysates obtained with a low concentration of detergent. SDS-PAGE analysis was performed using 10% polyacrylamide gels either under nonreducing conditions or under reducing conditions after addition of 5% 2-ME.

Cytotoxicity Assays. Cytotoxic activity of the cells was measured by a conventional 3-h ^{51}Cr release assay using triplicate cultures in V-bottomed plates. The E/T ratio was 1:1 with 5,000 target cells per well. To test the functional effects of the antibodies, either effector or target cells were incubated before the assay for 2 h or for 30 min, respectively, using the reagents at the predetermined saturating concentrations. Percent specific cytotoxicity was calculated conventionally; SDs were <5%.

Southern and Northern Blot Analyses. High molecular weight genomic DNA samples (10 μg) were digested with Eco RI, Xba I, or Pvu II, subjected to electrophoresis through 0.7% agarose gels, and blotted in alkaline buffer onto Gene Screen Plus nylon membranes (Dupont Co., Wilmington, DE). The blots were hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 100 μg/ml salmon-sperm DNA, and 10% polyethylene glycol 6000 (PEG 6000) at 42°C for 16 h. They were then washed twice at 65°C for 30 min in 0.1× SSC, 0.1% SDS.

For the Northern blot analysis, total cytoplasmic RNA (4 μg) was denatured in glyoxal-dimethyl sulfoxide, separated on 1% agarose gels, transferred to nylon membranes, and hybridized using the same conditions described for the Southern blot analysis.

DNA Probes. ^{32}P-labeled DNA probes were prepared from agarose-purified DNA fragments by the hexamer-priming method as previously described (10). The Vδ1-specific probe is an Eco RI-Sac I fragment isolated from clone 0240/38.5 (9), kindly given by Dr. Krangel (Dana-Farber Cancer Institute). The Cδ probe is an Eco RI fragment obtained from the same clone (9). The Cα probe (a 420-bp Pvu II fragment) and the Cβ probe (a 400-bp Bgl II fragment) have been isolated as previously described (17).

Isolation and Characterization of cDNA Clones. Double-stranded (ds)^{1} cDNA was synthesized from poly(A)$^{+}$ RNA derived from the CD3^{+} BMA031^{+} A13^{+} TCR-δ1^{-} δTCS1^{-} T2 cell line. After size selection and ligation of Eco RI linkers, dscDNA was cloned into the Eco R1 site of the vector λ gt10, before the packaging of the λ gt10-cDNA hybrids. Screening of 7.5×10^{4} recombinant phages was carried out with the Cα probe using standard procedures.

The cDNA inserts were subcloned into the T3-T7 transcriptional promotor containing vector pBS. Nucleotide sequences were determined by the dideoxy chain termination method using the modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). cDNA sequencing was performed on both strands using either a T3 primer, a T7 primer, or a Cα-specific primer.

Results

Generation and Characterization of the Anti-TiVδ2 mAb. Mice were immunized with the AB12 cells whose phenotypic, functional, and molecular characterization have been reported previously (10, 17, 27). These TiγA^{+} cells express a γ chain encoded by a Vγ9-JγP-Cγ1-rearranged gene. In AB12 lymphocytes, the Vδ1 segment is in germline configuration on both chromosomes (10); the functional δ transcript de-

tected in these cells was found to include a novel Vδ gene designated Vδ-AB12 or Vδ2 (10), as well as the Jδ1 and the Dδ3 segments (19).

Hybridoma supernatants were screened on the basis of a differential reactivity between AB12 cells and polyclonal α/β^+ T cell lines. A selected hybridoma, now termed anti-TiVδ2, was further tested in comodulation experiments, after overnight incubation of AB12 cells with a saturating concentration of OKT3 mAb. As shown in Fig. 1, these data indicated that the TiVδ2 epitope is linked physically to the CD3 proteins.

The functional effects of the anti-TiVδ2 mAb on the "non-MHC-requiring" cytotoxic activity of AB12 cells was tested using two target cell lines: JM (a T cell leukemia highly susceptible to NK lysis) and U937 (a cell line of histiocytic origin relatively resistant to NK activity). As shown in Fig. 2 *A*, AB12 lymphocytes exhibited a strong cytotoxicity against JM cells leading to >50% lysis at a 1:1 E/T ratio in a 3-h assay. Treatment of the cells with anti-TiVδ2 virtually abrogated this cytotoxic activity. The blocking effects of anti-TiVδ2 were similar to those induced by either OKT3 or anti-TiγA mAb, which are both reactive with the receptor complex ex-

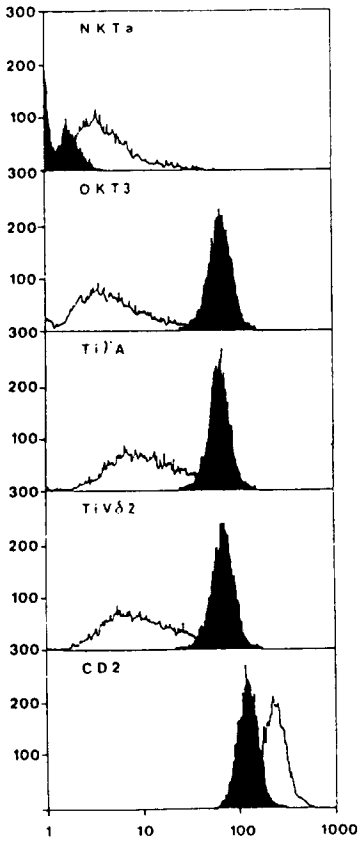

FIGURE 1. Comodulation experiment on AB12 cells. Cells were preincubated overnight with the OKT3 mAb or with control medium. Indirect immunofluorescence analyses were then performed with the indicated mAb on OKT3-treated cells (*white areas*) and on control cells (*black areas*).

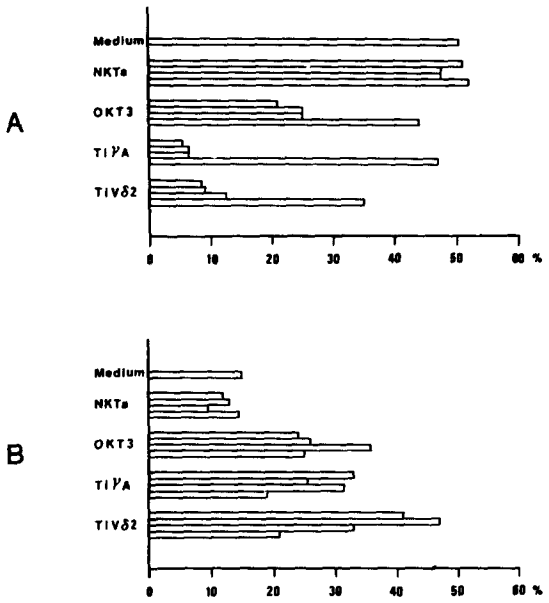

FIGURE 2. Cytotoxic activity of AB12 cells at a 1:1 E/T ratio in a 3-h experiment. (*A*) AB12 effector cells were preincubated with various mAbs before the cytotoxic assay against JM cells. (*B*) U937 target cells were preincubated with mAbs, and then AB12 cells were added. Series of mAb dilutions were tested from the saturating concentration predetermined in indirect immunofluorescence assays. (*Upper columns*) Saturating concentration (1/200 to 1/800 ascites dilutions depending upon individual mAb); (*second columns*) half of the saturating concentration; (*third columns*) 1/10; (*fourth columns*) 1/20.

pressed on the cloned cells. As compared with JM, U937 cells are relatively resistant to lysis by AB12 lymphocytes (<20% of cytotoxicity at 1:1 E/T ratio); because they express a Fc receptor (FcRγII), they are useful target cells to test the potential activity of a mAb in a "reverse ADCC" system. Again, anti-TiVδ2 was found to enhance lysis substantially as well as the other antireceptor antibodies (Fig. 2 *B*). Note that the control-irrelevant anti-NKTa mAb (of the IgG1 subclass like anti-TiVδ2) had no effect in any of the two series of experiments. Together, these data further supported the view that anti-TiVδ2 was directed at a TCR determinant.

Immunoprecipitations with Anti-TiVδ2 mAb. To characterize the molecule recognized by the anti-TiVδ2 mAb, we performed a series of immunoprecipitations with various clones and polyclonal cell lines expressing the γ/δ receptor. One representative experiment is shown in Fig. 3. The TH6.4 polyclonal cell line derives from the peripheral blood of a patient with renal cell carcinoma. More than 95% of these cells have been found to express the TiVδ2 epitope in repeated analyses over a period of several weeks in culture (data not shown). Note that the TH6.4 lymphocytes do not carry the TiγA epitope encoded by the Vγ9 gene (data not shown). TH6.4 and AB12 cells were surface labeled with ^{125}I. Immunoprecipitations from Th6.4 were performed with anti-TiVδ2; for AB12 cells, parallel experiments were undertaken using either the CD3X3 anti-CD3 mAb or the anti-TiVδ2 mAb. Before the CD3X3 precipitations, cell lysis was done in the presence of a low detergent concentration (0.1% of Triton X-100) to avoid the dissociation of the CD3-Ti complex.

Immunoprecipitations from the AB12 clone with the anti-CD3 mAb resulted in the detection, after SDS-PAGE under nonreducing conditions (Fig. 3, lane *a*), of diffuse bands between 18 and 28 kD and of a unique band at 82 kD (corresponding, respectively, to the CD3 proteins and the γ/δ heterodimer). In parallel, the material

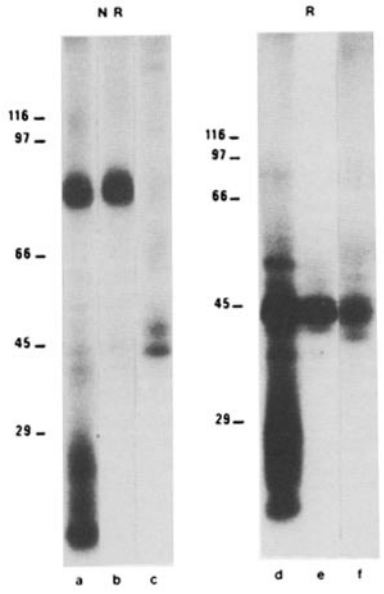

FIGURE 3. Immunoprecipitation of TCR-γ/δ molecules from CD3⁺ TCR-γ/δ⁺ cell lines by the anti-CD3 and anti-TiVδ2 mAbs. Immunoprecipitated material was analyzed by SDS-PAGE (10% polyacrylamide gel) under nonreducing (lanes *a*, *b*, and *c*) or reducing (lanes *d*, *e*, and *f*) conditions. Immunoprecipitations from the AB12 cells were performed using either the CD3X3 anti-CD3 mAb (lanes *a* and *d*) or the anti-TiVδ2 mAb (lanes *b* and *e*). Lysates from the TH6.4 cell line were immunoprecipitated by the anti-TiVδ2 mAb (lanes *c* and *e*).

precipitated with anti-TiVδ2 (Fig. 3, lane *b*) displayed the same electrophoretic mobility (82 kD), confirming that the antibody is specific for the CD3-associated receptor. Precipitations performed with anti-TiVδ2 from the TH6.4 cell line followed by migration under nonreducing conditions led to the detection of two bands at 48 and 45 kD (Fig. 3, lane *c*). These results indicated that the TiVδ2⁺ TH6.4 lymphocytes express a nondisulfide-linked form of the γ/δ receptor with a Cγ2-encoded γ chain. Note, therefore, that the anti-TiVδ2 mAb recognizes a γ/δ determinant whose expression is not dependent upon the quaternary structure of the receptor (i.e., Cγ1 vs. Cγ2). When SDS-PAGE was performed under reducing conditions, γ/δ proteins were visualized as three bands at 46, 43, and 41 kD for AB12 cells (Fig. 3, lanes *d*, *e*) and at 46, 43, and 39 kD for TH6.4 cells (lane *f*); in both cases, the 43-kD band was found to be predominant. These biochemical results are in line with previous reports on the γ/δ complex (30).

Gene Assignment of the TiVδ2 and A13 Antigenic Determinants. The anti-A13 mAb was initially found to recognize γ/δ⁺ cells that often express the δTCS1 determinant while being nonreactive with anti-BB3 mAb (23). However, the fine specificity of anti-A13 has remained unknown. Using immunofluorescence assays, we have tested here a series of γ/δ⁺ clones characterized previously (10, 15, 19, 28, and unpublished data) at both the γ and the δ loci in an attempt to define the TiVδ2 and the A13 epitopes.

The anti-TiVδ2 mAb was generated against AB12 cells that express a TiγA⁺ V9-JP-C1-encoded γ chain and a V2-D3-J1-C-encoded δ chain (10, 19). Immunoprecipitation experiments (Fig. 3) have indicated that the TiVδ2 epitope can be associated with either type of Cγ segment. The clonal analysis showed that the majority (seven of eight), but not all, of V9-JP TiγA⁺ clones react with the antibody (Tables I and II). Indeed, the TiγA⁺ F6C7 cells are TiVδ2⁻. The TiγA⁻ G6-cloned cells, which

TABLE I
Phenotypic Analysis of CD3$^+$ TCR-γ/δ$^+$ Clones

Clones	OKT3	BMA031	TCR-δ1	TiγA	TiVδ2	A13	δTCS1
G6	+	–	+	–	+	–	–
AB12	+	–	+	+	+	–	–
BD	+	–	+	+	+	–	–
BG	+	–	+	+	+	–	–
BK	+	–	+	+	+	–	–
BO	+	–	+	+	+	ND	–
BS	+	–	+	+	+	–	–
BX	+	–	+	+	+	–	–
F6C7	+	–	+	+	–	+	–
A2C12	+	–	+	–	–	+	+
J1F1	+	–	+	–	–	+	+
J2B7	+	–	+	–	–	+	+
J2D2	+	–	+	–	–	+	+
N2A11	+	–	+	–	–	+	+
A2E9	+	–	+	–	–	–	–
N1C3	+	–	+	–	–	–	–

Reactivity of cultured cell lines with specific mAbs was assessed by indirect immunofluorescence assays followed by cytofluorometric analysis. +, >95%; –, <5%.

are recognized by the anti-TiVδ2 reagent, display at the γ locus (Table II) a V4-J1 rearrangement on one chromosome, while the other one is in germline configuration. Together, these data suggested that the anti-TiVδ2 mAb does not react with a γ chain determinant. In contrast, the reagent was found to recognize all cloned cell lines displaying a rearrangement involving the Vδ2 gene (Tables I and II): among the 16 clones studied, the eight expressing the TiVδ2 epitope (G6, AB12, BD, BG, BK, BO, BS, and BX) have rearranged the Vδ2 segment while being in germ-line configuration for Vδ1. Together, there was a strict correlation between the expression of the TiVδ2 epitope and the recombination of the Vδ2 gene segment. Furthermore, an in-frame message corresponding to the 5.5-kb Eco RI Vδ2-D3-Jδ1 rearrangement has been shown previously to be transcribed in the TiVδ2$^+$ AB12 cells (10). Finally, a message corresponding to a 7-kb Eco RI Vδ2-(D)-Jδ3 rearrangement (17) has been shown to be expressed productively in the TiVδ2$^+$ G6 cells (19). Thus, it is most likely that anti-TiVδ2 is specific for a Vδ2-encoded epitope (and not a Vδ2/J determinant).

Testing the anti-A13 mAb on the present series of cloned cell lines confirmed that its reactivity was restricted to a fraction of γ/δ$^+$ cells. It was also found that the reactivity of anti-A13 is not dependent upon the use of a unique Cγ segment because both the Cγ1$^+$ F6C7 and J2D2 cells and various Cγ2$^+$ clones (A2C12, J2B7, N2A11) express the corresponding epitope (Tables I and II); furthermore, the six A13$^+$ clones tested (F6C7, A2C12, J1F1, J2B7, J2D2, and N2A11) use distinct Vγ genes. In contrast, these six cells display a Vδ1 rearrangement. One of the A13$^+$ clones (F6C7) is δTCS1$^-$; it was shown previously to surface express a Vδ1-Jδ3-encoded δ chain (19). Together, these data strongly suggest that anti-A13 mAb recognizes the

TABLE II

Rearrangements on γ and δ Loci in a Panel of CD3⁺ TCR-γ/δ⁺ Clones

	γ locus			δ locus	
Clones	Ch1*	Ch2*	Cγ‡	Vδ2§	Vδ1‖
G6	V4-J1	G	Cγ1	G/R (J3)	G/G
AB12	V9-JP	V10-JP1	Cγ1	G/R (J1)	G/G
BD	V9-JP	V10-JP1	Cγ1	D/R (J1)	G/G
BG	V9-JP	V10-JP1	ND	G/R (J1)	G/G
BK	V9-JP	V10-JP1	ND	G/R (J1)	G/G
BO	V9-JP	V8-J1	ND	G/R (J1)	G/G
BS	V9-JP	V10-JP1	Cγ1	G/R (J1)	G/G
BX	V9-JP	V10-JP1	Cγ1	D/R (J1)	G/G
F6C7	V9-JP	V3-J1	Cγ1	D/R (J1)	G/R (J3)
A2C12	V8-J2	V11-J2	Cγ2	D/G	G/R (J1)
J1F1	V4-J2	V9-J2	ND	D/G	G/R (J1)
J2B7	V2-J2	V3-J2	Cγ2	D/R (J1)	G/R (J1)
J2D2	V2-J1	G	Cγ1	D/G	G/R (J1)
N2A11	V3-J2	V3-J2	Cγ2	D/R (6.5 kb)	G/R (J1)
A2E9	V2-J2	V4-J2	Cγ2	G/G	G/G
N1C3	V10-J2	ND	Cγ2	G/G	G/G

Rearrangements were determined by Southern blot analysis on genomic DNA (10, 19, 26, and unpublished data). G, germ-line position; D, deleted.

* γ locus rearrangements on both chromosomes detected with pH60 Jγ probe.

‡ Use of Cγ segment deduced from SDS-PAGE analysis (i.e., disulfide-linked vs. non-disulfide-linked dimer.

§ δ locus rearrangements detected with the Vδ2 probe.

‖ δ locus rearrangements detected with the Vδ1 probe.

product of the Vδ1 segment exclusively, as opposed to anti-δTCS1, which reacts with cells surface expressing Vδ1⁺Jδ1⁺ chains (19) or Vδ1⁺Jδ2⁺ chains (20).

Using the Anti-Vδ1 and Anti-Vδ2 mAbs to Characterize the γ/δ⁺ Fraction in Peripheral Blood. The respective expression, in circulating lymphocytes, of the Vδ1, the Vδ1-Jδ1/Jδ2, and the Vδ2 gene segments was reassessed here using the relevant antibodies, namely anti-A13, anti-δTCS1, and anti-TiVδ2. As expected (10), a large majority (80%) of the γ/δ⁺ T cells were recognized by anti-TiVδ2, while δ chains, including a Vδ1-Jδ1 or Vδ1-Jδ2 gene product, were present on only a small (13%) γ/δ⁺ subset (Table III). The summed reactivity of the anti-A13 and the anti-TiVδ2 mAbs was 3.2%, whereas the anti-TCRδ1 mAb recognized 3.0% of the cells in this series of 41 individuals (Table III). Such an advantage (+0.2%) of the anti-(Vδ1 + Vδ2) mAb reactivities over that of anti-TCRδ1 was surprising. A detailed analysis of the results indicated that in 13 of 41 donors, the sum of Vδ1 + Vδ2 exceeded the Cδ expression in a significant fashion ranging from 0.5 to 2.8%.

Expression of the A13 Antigenic Determinant Is not Restricted to γ/δ⁺ T Lymphocytes. Several hypotheses could be made to explain this apparent discrepancy, including a crossreactivity of the anti-A13 mAb with, for example, other V-encoded TCR epitopes. In any case, it was important to assess whether the antibody recognized cells expressing the γ/δ receptor in an exclusive fashion. Thus, we performed a series of double-color immunofluorescence analyses with anti-A13 and anti-BMA031, a reagent specific for a constant epitope of the α/β receptor. A representative experi-

TABLE III
Immunofluorescence Analysis of PBL

Donors	TiVδ2	A13	δTCS1	TCR-δ1	Vδ1 + Vδ2
1	2.5	<0.1	<0.1	3.4	2.5
2	1.9	<0.1	<0.1	2.3	1.9
3	0.4	0.2	0.1	0.6	0.6
5	1.3	0.3	<0.1	1.6	1.6
6	11.0	0.4	<0.1	12.0	11.4
7	1.5	0.7	0.2	2.5	2.2
9	2.0	0.8	0.4	2.2	2.8
10	2.9	1.3	0.7	3.8	4.2
11	6.5	0.4	0.1	6.9	6.9
12	1.5	0.7	0.4	2.2	2.2
15	2.7	0.5	0.3	2.8	3.2
16	0.3	1.3	1.3	1.9	1.6
17	2.3	0.3	0.3	3.0	2.6
18	4.3	0.5	<0.1	4.6	4.8
19	1.5	0.4	<0.1	1.9	1.9
20	0.9	0.9	0.6	1.4	1.8
21	3.0	1.4	0.9	3.9	4.4
22	3.1	0.7	0.2	3.5	3.8
23	0.5	0.5	0.3	1.2	1.0
24	1.3	1.0	0.3	1.6	2.3
25	3.1	1.1	0.6	4.0	4.4
26	2.3	0.9	0.4	2.8	3.2
27	1.5	0.5	0.1	1.5	2.0
28	3.8	0.9	0.5	4.1	4.7
29	0.7	0.2	0.1	0.9	0.9
30	1.2	1.3	1.1	3.0	2.5
31	1.5	0.2	0.4	2.5	1.7
32	1.9	1.3	0.5	3.7	3.2
33	11.3	1.0	0.9	13.4	12.3
34	2.2	0.2	<0.1	3.0	2.4
35	0.6	0.5	0.3	0.9	1.1
36	0.6	0.4	0.2	0.8	1.0
37	2.5	1.3	1.0	3.3	3.8
38	3.1	1.3	0.6	3.9	4.4
39	2.8	1.1	0.5	3.1	3.9
40	1.7	0.8	0.6	1.9	2.5
41	1.0	0.8	0.2	1.4	1.8
4	0.4	1.1	0.3	0.7	1.5
8	1.2	3.4	0.3	1.8	4.6
13	1.2	1.0	0.5	1.3	2.2
14	2.0	1.2	0.7	2.1	3.2
Mean	2.4	0.8	0.4	3.0	
SD	2.3	0.6	0.3	2.5	
Percent*	80	27	13	100	

Percent of positive cells was determined by indirect immunofluorescence. Vδ1 + Vδ2 represents the summed reactivities of the anti-TiVδ2 and anti-A13 mAbs.

* The anti-TCR-δ1 reactivity is designated 100%, the other numbers are calculated as compared relative percentages.

ment (Fig. 4) shows the results obtained with donors 11, 14, and 32. These three individuals were tested on the basis of the summed (Vδ1 + Vδ2) reactivity compared with that of anti-TCRδ1 (donor 32, −0.5%; donor 11, 0%; donor 14, +1.1%). In each of these donors where expression of the Cδ segment was greater, equal, and inferior, respectively, to that of the Vδ1 plus Vδ2 segments, a minor but clearly detectable BMA031$^+$ A13$^+$ cell population was identified (Fig. 4).

To understand this phenomenon, we further studied the donor (no. 8) who displayed the largest (Vδ1 + Vδ2)/Cδ difference (TCR-δ1, 1.8%; A13, 3.4%; δTCS1, 0.3%; TiVδ2, 1.2%; [Vδ1 + Vδ2]-Cδ, 2.8%). A13$^+$ PBL from this individual were purified by cell sorting procedures after treatment with the corresponding antibody. The sorted cells were stimulated with PHA and expanded in the presence of IL-2. On day 15, cultured lymphocytes were analyzed with a series of mAbs. As shown in Fig. 5 *A*, all these cells were A13$^+$ while 85% were BMA031$^+$ WT31$^+$ (i.e., α/β^+), and 15% were TCR-δ1$^+$ TCR-γ/δ−1$^+$ (i.e., γ/δ^+).

These data suggested that the A13 epitope may be expressed on α/β receptors. To analyze this observation, the TCR-δ1$^+$ A13$^+$ and the BMA031$^+$ A13$^+$ lymphocytes were purified from the cell line with an additional round of cell sorting. After in vitro expansion, the secondary cultures (termed T1 and T2) were phenotyped on day 25. The quality of the purification was satisfactory with respective percentages of TCR-δ1$^+$ and BMA031$^+$ lymphocytes >95% in the relevant corresponding fractions (Fig. 5, *C* and *B*, respectively). T2 cells were found to be CD3$^+$ BMA031$^+$ WT31$^+$ TCR-δ1$^-$ TCR-γ/δ-1$^-$ (Fig. 5 *B*). There was a typical bright expression of the CD8 molecule on all the α/β^+ cells, while CD8 was present at low density on only 30% of the γ/δ^+ cells. Note that a substantial number (25%) of the T1 TCR-δ1$^+$ cells coexpressed the TiγA and the δTCS1 epitopes (Fig. 5 *C*). Such an association is infrequent in peripheral blood of most individuals (10, 15).

The Vδ1 Gene Segment Is Productively Transcribed with Either the Cα or the Cδ Segment. DNA and RNA were extracted from either the A13$^+$ BMA031$^+$ (T2 cell line)

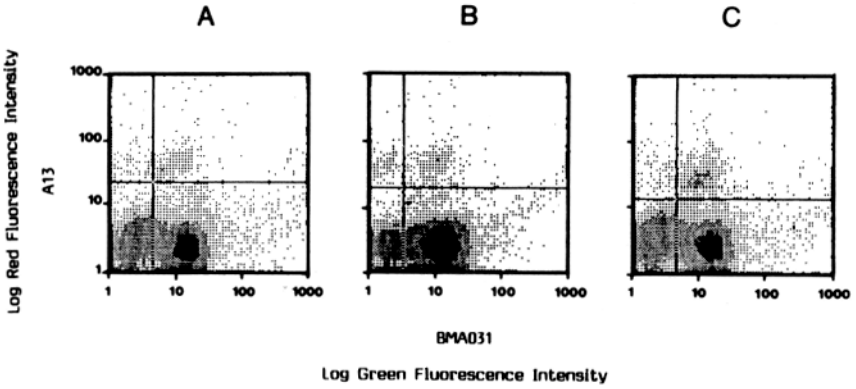

FIGURE 4. Double-color immunofluorescence analysis of PBL. The *x*-axis shows $\log_{10}$ green fluorescence intensity obtained with fluoresceinated anti-BMA031 mAb. The *y*-axis represents $\log_{10}$ red fluorescence intensity obtained with anti-A13 antibody plus phycoerythrin-conjugated goat anti-mouse IgG1 serum. *A*, *B*, and *C* show the results for PBL from donors 11, 14, and 32, respectively.

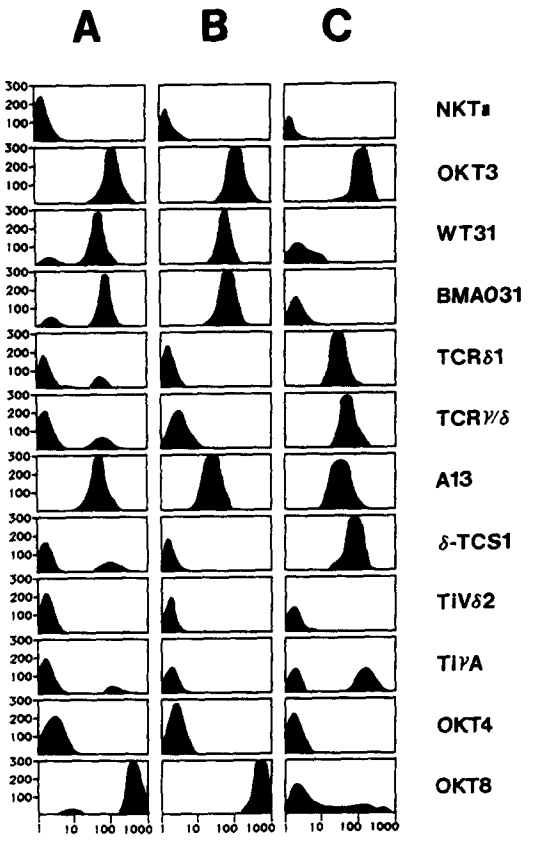

FIGURE 5. Phenotypic analyses of polyclonal cell lines from donor 8 performed by indirect immunofluorescence experiments. (*A*) The A13$^+$ cell line derived from PBL; (*B*) the BMA031$^+$ cells (T2 cell line) derived from the A13$^+$ cell line; (*C*) the TCR-δ1$^+$ cells (T1 cell line) derived from the A13$^+$ cell line.

or the A13$^+$ TCRδ1$^+$ (T1 cell line) cultured cells in order to study the genes encoding the TCR chains expressed by the respective lymphocyte fractions.

For Southern blots analyses, we used a Cδ and a Vδ1 probe (see Materials and Methods). The K562 myeloid cell line was tested to detect the germ-line positions; T1 and T2 cells were studied in parallel. The Cδ segment was found to be deleted from both Eco RI and Xba I restriction fragments in the T2 cell line, while 3.5-kb Eco RI and 9.5-kb Xba I germ-line signal were detected in both K562 and T1 cells (Fig. 6, *C* and *D*). With the Vδ1 probe, germ-line bands were found in K562 cells at 3 and 23 kb on Eco RI and Pvu II fragments, respectively. As shown in Fig. 6, *A* and *B*, one germ-line signal was detected in both T1 and T2 cells. On the second chromosome, the Vδ1 gene segment was rearranged in both cell types. For T1 cells, the Vδ1 rearrangement corresponded to the expected (given the fact that T1 cells are δTCS1$^+$) well-defined V1-J1, 3.3-kb Eco RI, and 10-kb Pvu II signals (9). For T2 cells, a rearrangement was detected as a 5.4-kb Eco RI and a 12-kb Pvu II band. Experiments were also performed with the pH60 Jγ probe (6) with DNA from T1 and T2 cells; several rearranged bands were found in each case, indicating both cell lines are polyclonal (data not shown).

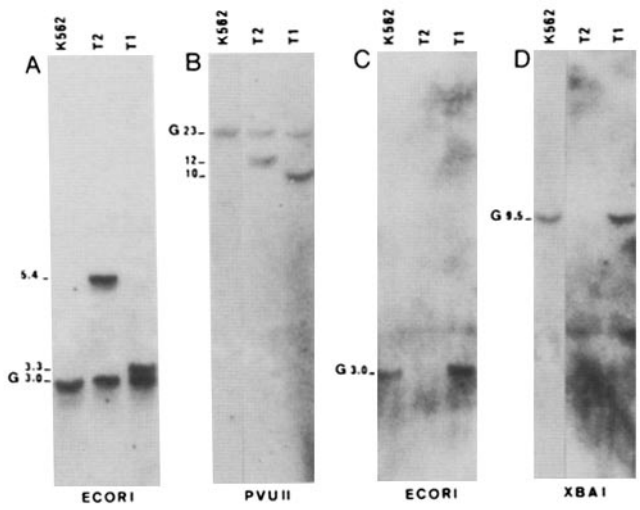

FIGURE 6. Southern blot analysis with DNA extracted from the K562, T1, and T2 cell lines. DNA samples were digested with Eco RI (*A* and *C*), Pvu II (*B*), or Xba I (*D*). (*A* and *B*) Hybridization with the Vδ1 probe; (*C* and *D*) hybridization with the Cδ probe.

For Northern blot analyses, we used a Cα and a Cβ probe in addition to the Cδ and Vδ1 probes. RNA was prepared from T1 and T2 cells. A TCR-δ1$^+$ δTCS1$^+$ cloned cell line, termed A2C12, was used as a positive control for the transcription of the Vδ1 gene segment. After Cα hybridizations, a full-length 1.6-kb message was found in T2 cells, while a 1.3-kb truncated transcript was detected in both A2C12 and T1 lymphocytes (Fig. 7 *A*). Results obtained with the Cβ probe were similar with a full-length (1.3 kb) transcript in T2 cells and a truncated signal (1.0 kb) in the others (Fig. 7 *C*). As expected from Southern blots that showed a deletion of this segment, there were no detectable transcripts hybridizing with the Cδ probe in the T2 cells (data not shown). For A2C12 and T1 cells, the Cδ signals (not shown) were identical to those obtained with the Vδ1 probe, which detected the two well-characterized 2.2- and 1.3-kb bands corresponding to distinct polyadenylations of

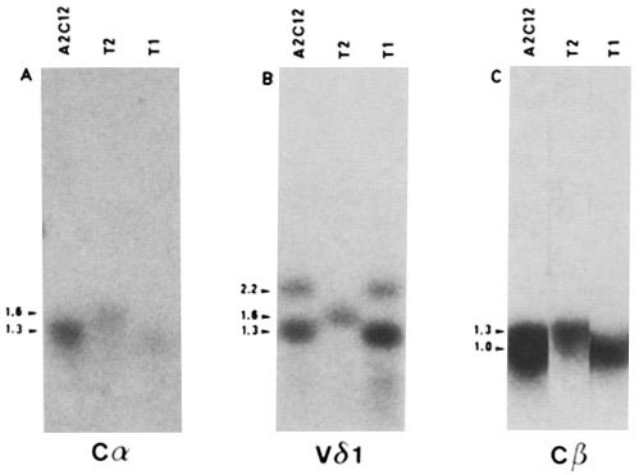

FIGURE 7. Northern blot analysis with RNA extracted from the A2C12, T1, and T2 cell lines. RNA samples were hybridized with the Cα probe (*A*), the Vδ1 probe (*B*), and the Cβ probe (*C*).

the δ chain transcripts. In T2 cells, the Vδ1 hybridization led to a distinct 1.6-kb signal (Fig. 7 *B*), which corresponds to the size of the full-length α chain transcript.

Note that Cδ is undetectable in T2 cells while a germ-line Vδ1 band is present (Fig. 6, *C* and *D*). These results suggest that, at least in some of these cells, rearrangements have occurred in the α locus on both chromosomes. It is therefore likely that an additional V segment located downstream of Vδ1 has been used in the other recombination.

To demonstrate unequivocally that the Vδ1 gene segment is transcribed with the Cα segment, we prepared an expression library from the T2 cells that were screened with the Cα probe. Three positive cDNAs, which included 85% of the V segment for the longest and 35% for the shortest, were sequenced. In each case (data not shown), 100% homology was found with the original Vδ1 sequence (9). The 3′ end of the cDNAs included a Jα segment, termed AC24 (31), and the Cα segment also with 100% homology to these previously reported sequences (data not shown).

Discussion

We report here the characterization of two mAbs directed at the TCR-γ/δ heterodimer. In earlier series of experiments, multiple $CD3^+$ TCR-α/β^- cloned cell lines derived from human peripheral blood have been analyzed for rearrangements and transcription of the genes encoding their TCR-γ and TCR-δ chains. These lymphocytes have also been tested with the available anti-TCR mAbs, such as anti-TCR-δ1, anti-TiγA, or anti-δTCS1. Furthermore, expression libraries were produced from some of these clones, which led to the molecular cloning of the respective γ and δ transcripts (19). This well-defined cell panel (10, 15, 19, 28, and unpublished data) can now be used to assess the fine specificity of novel anti-TCR antibodies by simple immunofluorescence analyses. Thus, testing the reactivity of anti-A13 and anti-TiVδ2 on the clones led to the conclusion that they recognize Vδ1 and Vδ2 gene products, respectively.

An mAb, designated anti-BB3, has been reported previously (21). Its reactivity on circulating lymphocytes correlated with the surface expression of disulfide-linked γ/δ dimers. It was therefore postulated that the reagent may recognize a Cγ1-encoded epitope (21, 22). Analyses of our clones did not support this initial working hypothesis, rather suggesting a Vδ2 reactivity (10, 19). With the present characterization of the anti-TiVδ2 mAb, this point can be concluded definitely. Indeed, we have studied here a polyclonal cell line, Th6.4, that displays virtually 100% reactivity with both anti-TiVδ2 and anti-BB3 mAbs (data not shown). Using the former reagent to immunoprecipitate the receptor complex, TH6.4 cells were found to surface express a non-disulfide-linked, i.e., Cγ2-encoded, γ chain.

The TH6.4 line, developed from blood of a patient with renal cell carcinoma, has been very useful to study the specificity of the antibodies. In addition, its characterization has shown that there are no sterical constraints prohibiting the association of a $V\delta2^+$ δ chain and a $C\gamma2^+$ γ chain in a non-disulfide-linked receptor. Nevertheless, such association is likely to be infrequent in peripheral blood because all other $V\delta2^+$ peripheral cells derived from normal donors that we and other groups (10, 21) have studied were found to carry a $C\gamma1^+$ (most often $V\gamma9^+$) disulfide-linked receptor.

In the original report on the anti-A13 mAb, it was found that this reagent recog-

nizes a BB3$^-$ TCR-γ/δ^+ peripheral blood cell fraction partially reactive with anti-δTCS1 (23). Our present findings are in line with these initial studies. In light of the gene assignment proposed here for anti-A13, i.e., Vδ1, one can explain why a minority of TCR-γ/δ^+ A13$^+$ circulating lymphocytes are δTCS1$^-$. The latter antibody reacts with either Vδ1-Jδ1 (19) or Vδ1-Jδ2 chains (20); the A13$^+$ δTCS1$^-$ fraction is likely to include infrequent cells expressing Vδ1-Jδ3 receptors such as the previously described F6C7 clone (19).

We have used the series of antibodies specific for the δ chain to reassess the distribution of the Vδ segments in the circulating TCR-γ/δ^+ fraction of adult healthy donors. In a series of 41 individuals, the summed reactivity of anti-TiVδ2 plus that of anti-δTCS1 (2.4 + 0.4 = 2.8%) almost reached that of anti-TCR-δ1 (3.0%). Evidently, such a sum does not account for the minor fractions of Vδ1$^+$ lymphocytes using Jδ3. These data further support the view that the Vδ1 and the Vδ2 gene segments are used in a virtually exclusive fashion by peripheral cells of most individuals, while Vδ2 is largely predominant.

Double-color immunofluorescence experiments showed the existence of a sizeable BMA031$^+$ A13$^+$ peripheral lymphocyte subpopulation in all individuals tested in this study. The development of the T2 polyclonal cell line has allowed us to analyze this observation. Northern blots demonstrated that Vδ1 is indeed transcribed with the Cα segment in these BMA031$^+$ A13$^+$ cells. This has been confirmed by sequencing three clones isolated from a T2 cell cDNA library with the Cα probe. The cDNA V regions showed 100% homology with the original Vδ1 sequence.

In the mouse, nine families of Vδ segments have been identified. Five of them are quite distinct from the previously described Vα genes (32–35). Four are closely related to Vα segments. Indeed, the murine cDNAs Z68, VM23, δ2.3, and KN25 isolated from distinct thymic libraries have 99, 94, 97, and 95% nucleotidic homology to the TA1, TA27, TA65, and FN1.18 Vα gene segments, respectively (33–36). It is therefore clear that murine "Vδ" and "Vα" genes are partially overlapping sets.

In the human, there has been only one similar observation. An infrequently used gene segment displaying 96% homology to Vα6.1 (HAP01 cDNA clone) was found to be used in a γ/δ receptor (12). The 11 nucleotidic differences between this Vδ (designated Vδ4 by Takihara et al. [11]) and Vα6.1 were found to be dispersed throughout the gene segment. Note that Southern blots performed with the Vδ4 probe led to the detection of two bands with common restriction enzymes (11), suggesting that this V segment is a member of a family.

Vδ1 has been the first human gene segment shown to rearrange at the δ locus (9). Further studies have indicated that it was the predominant segment used in thymic δ chains while present on a sizeable minority of peripheral cells with the γ/δ receptor (15, 30, 37, 38). Sequence comparison of Vδ1 with that of previously reported Vα segments showed that it matches a human Vα consensus in 75% of the residues (9); at the protein level, it is distantly related (57% identity) to the human Vα sequence PGA5 (9). As opposed to other examples discussed here, Vδ1 has no strong homology with other known V sequences. This correlates with the fact that the Vδ1 probe detects only one band in genomic DNA digested with a variety of restriction enzymes (9, 11). Together, it is most likely that Vδ1 defines a one-member family. Our present experiments indicate that this unique V segment, representing

one of the two major Vδ genes, is in fact used in either type of receptor structure (i.e., α/β and γ/δ).

Southern blots performed with a Jγ probe in the T2 cells showed that the line is polyclonal while the Vδ1 probe detected a unique rearranged band. These results (in line with the sequencing data where the same Jα segment was found in the three cDNA clones) support the view that BMA031$^+$ A13$^+$ cells may preferentially use certain Jα segments. Further characterization of the corresponding lymphocyte subset in series of individuals will have to address this point.

We have originally identified (10), in a cloned cell line termed AB12, a Vδ segment (Vδ2) that is used by ~80% of peripheral TCR-γ/δ$^+$ lymphocytes (Table III). The same sequence has been reported by other groups (11, 39). It does not display a significant homology with a Vα consensus (10). Using anti-TiVδ2 mAb, which recognizes the corresponding gene product, we have performed double-color immunofluorescence assays in order to potentially identify BMA031$^+$ TiVδ2$^+$ lymphocytes. Such cells were undetectable in PBL from five normal donors (data not shown). It is therefore likely that Vδ2 represents a "δ-specific" (or "highly selective") gene segment.

We and others have shown that the combinatorial diversity of the γ/δ receptors expressed in human circulating lymphocytes is very limited (15, 21). This may provide a unique opportunity to easily assess, with a relatively limited panel of specific mAbs, the molecular structure of TCR chains expressed by lymphocytes in both normal and immunopathological situations. For example, it has been shown, using anti-TiγA, that cells expressing V9$^+$ γ chains are present with high frequency in the altered joints of patients with chronic rheumatoid arthritis (40, 41). When the role of γ/δ$^+$ T lymphocytes in immune responses is better understood, potential structure/function correlations may be of clinical interest. Anti-A13 and anti-TiVδ2 will further contribute to complete a panel of useful antibodies. However, the former reagent will have to be used in association with antibodies directed at constant regions of the receptors for appropriate interpretation of the data.

Summary

In the present study, we have characterized the reactivity of two mAbs that are directed at the human TCR-γ/δ. These reagents, designated anti-A13 and anti-TiVδ2, were found to recognize antigenic determinants encoded by the TCR Vδ1 and Vδ2 gene segments, respectively. Immunofluorescence analyses performed with the antibodies confirmed that, in the TCR-γ/δ$^+$ cell subpopulation, the expression of Vδ2$^+$ δ chains is largely predominant, as compared with the Vδ1$^+$ counterparts. However, these experiments led to an apparently discrepant finding. Indeed, the total number of cells recognized by the anti-A13 plus the anti-TiVδ2 antibodies was often greater than that detected with anti-TCR-δ1, a reagent specific for a constant epitope of the human δ chain. Further investigation showed the presence of a sizeable peripheral lymphocyte subset coexpressing the BMA031 and the A13 epitopes. Because the former antibody is known to recognize an invariant antigenic determinant of the TCR-α/β dimer, these results suggested that the Vδ1 gene segment may be expressed with either Cδ or Cα. This hypothesis was confirmed using T2, an IL-2-dependent BMA031$^+$ A13$^+$ polyclonal cell line developed from peripheral blood of

a healthy adult donor. Indeed, T2 cells were found to have productively rearranged the Vδ1 gene. Together, results of Northern blot analysis and cDNA cloning indicated that Vδ1 was expressed in these cells as part of a 1.6-kb full-length message including Jα-Cα segments.

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