Selection by Two Powerful Antigens May Account for the Presence of the Major Population of Human Peripheral γ/δ T Cells

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Summary

Vγ9/Vδ2 cells represent a fraction of human γ/δ cells that is expanded after birth in the periphery, carries markers of activated cells, and becomes a major population in peripheral blood. We found that these cells do not comprise a single population but actually represent two nested sets, the smaller of which, specific for *Mycobacterium tuberculosis*-pulsed antigen-presenting cells (APC), is contained in a larger set specific for an antigen found on the Molt-4 lymphoma. The larger set, representing 40–80% of all blood γ/δ cells, is comprised of cells bearing the Vγ9/Cγ1 chain. Cells in the smaller, included set have an additional requirement for Vδ2 (and probably for certain permissive junctional regions, since a very small percentage of Vγ9/Vδ2 cells do not react against mycobacteria-pulsed APC). Optimal stimulation by mycobacteria is dependent on the presence of APC, and is not restricted by classical major histocompatibility complex molecules. Some of the Vγ9/Vδ2 mycobacteria-specific clones are also stimulated by APC pulsed with different bacteria, such as *Listeria monocytogenes* and *Escherichia coli*, indicating that the population includes several different patterns of reactivity. These data establish a relationship in humans between specificity and Vγ/Vδ gene usage, and offer an explanation for the peripheral expansion of these γ/δ cells.

In peripheral blood of normal human donors, 50–70% of γ/δ cells carry a TCR using a single set of V genes, namely Vγ9 and Vδ2, and a single γ constant region, the disulphide-linked Cγ1 (1–4). We had previously shown that such cells constitute only a minor fraction of γ/δ cells in the postnatal thymus (4) and suggested, therefore, that the overrepresentation of the single V gene pair was not due to a chemical pairing advantage but rather to selective expansion in the periphery. Suggestive evidence has recently come from Parker et al. (5), who recently showed that Vγ9/Vδ2 cells express CD45RO, a putative marker of activated cells (6), and that with age, their number increases in the blood, but not in the thymus.

There are two possibilities to explain the selective expansion of Vγ9/Vδ2 cells. The first is that these cells are expanded by stimulation with a large number of antigenic peptides in association with monomorphic restriction molecules, in accordance with the model proposed by Davis and Bjorkman (7), in which the TCR-V-encoded regions, corresponding to CDR1 and CDR2, preferentially interact with restriction molecules, while the D, J, and N regions, corresponding to CDR3, contact the antigenic peptide. In support of this view are the rare γ/δ cells that see CD1, TL, or Qa, which are nonpolymorphic class I-like molecules (8–11).

The second possibility is that the Vγ9/Vδ2 cells may be expanded by a limited but powerful set of ligands. These could be of two sorts: an environmentally recurring antigen to which the Vγ9/Vδ2 pair has a particular affinity, or a "superantigen" of the type known to bind to particular Vs regardless of the specificities encoded by the V-(D)-J junctional sequences, such as some bacterial enterotoxins and the cellular antigen Mls (12, 13).

To discriminate between the various possibilities, we analyzed the specificities expressed by a large panel of Vγ9/Vδ2 cells from adult peripheral blood. Here, we show that the selective expansion is not due to recognition of a common restriction element but rather by the recognition of two powerful ligands. The first is an antigen expressed by the tumor

cell line Molt-4, which behaves like a superantigen in that it is recognized by γ/δ cells expressing the TCR Vγ9-Cγ1 chain. The second is found on APC pulsed with various bacteria, and is recognized by most Vγ9/Vδ2 cells.

Materials and Methods

T Cell Cloning. T cell clones were established from peripheral blood of normal donors as reported (14). γ/δ cells were isolated using the FACS® (440; Becton Dickinson & Co., Mountain View, CA) and pan anti-δ mAb δ1. In some cases, $CD4^-CD8^-$ cells were sorted and later identified as γ/δ^+. Cells were cloned by limiting dilution using PHA (1 μg/ml) (Wellcome Laboratories, Dartford, UK), human rIL-2 (100 U/ml; (Hoffmann-La Roche, Nutley, NJ), and irradiated PBMC (5×10^5/ml). T cell clones were restimulated periodically following the same protocol.

Monoclonal Antibodies and Tumor Cell Lines. The following mAbs were used: δ1 (pan anti-δ) (15), BMA032 (anti-TCR-αβ) (16), TR66 (anti-CD3ε) (17), δTCS1 (anti-Vδ1-J1/3) (18), BB3 (anti-Vδ2) (3), TiγA (anti-Vγ9) (1), 4A11 (anti-Vγ4, generated in our laboratory), Leu-3a (anti-CD4) and Leu 2a (anti-CD8) (Becton Dickinson & Co.) Na 1/34 (anti-CD1a) (19), WM25 (anti-CD1b) (20), 10C3 (anti-CD1c) (21), W6/32 (anti-MHC class I) (American Type Culture Collection [ATCC], Rockville, MD), anti-β2-microglobulin antiserum (kindly provided by Dr. Jim Kaufman, Basel Institute for Immunology, Basel), L243 (anti-DR) (ATCC), B7.21 (anti-DP) (kindly provided by Dr. John Trowsdale, ICRF, London). The following tumor cell lines were obtained from ATCC: Molt-4, Jurkat, K562, U937, and Raji. OVCA 432 was kindly provided by Dr. R. Knapp, Dana Farber Cancer Institute, Boston, MA. Molt-4 variants that express high and low levels of CD1a or CD1c were kindly provided by Dr. Cesar Milstein, Cambridge, UK.

T Cell Assays. Proliferation assays were performed using 5×10^4 responder cells/well and 6,000 rad irradiated EBV-B cells (3×10^4/well) or 3,000 rad irradiated PBMC (10^5/well) as APC. RhIL-2 was added in culture at 5 U/ml. After 48 h, 1 μCi of [^{3}H]thymidine (Amersham International, Amersham, UK) was added and the cultures were harvested after an additional 18 h. T cell killing of ^{51}Cr-labeled target cells was tested as described (22).

Antigens. The following antigens were used: *Mycobacterium tuberculosis* (MT)[1] H37RA (batch No. 722075; Difco Laboratories, Detroit, MI); heat-killed *Listeria monocytogenes* strain EGD obtained from ATCC, prepared as described (23); *Escherichia coli* JM109, kindly provided by Dr. Lucia Mori, grown overnight, then heat killed 1 h at 60°C; purified protein derivative (PPD) (Statens Serum Insitut, Copenhagen); tetanus toxin (provided by Dr. G. Corradin, University of Lausanne).

Fluorescence Analysis. Cell surface marker analysis was performed using a FACScan® (Becton Dickinson & Co.). Proliferating cells were identified using forward and side scatter parameters. Dead cells were excluded with propidium iodide staining.

Southern Analysis, cDNA Amplification, and Direct Sequencing. Clones mentioned in Tables 1 and 2 were analyzed for Vγ and Vδ gene rearrangements by Southern blot as already reported (4). In $Ti\gamma A^+$ clones, the junctional segments of the γ and δ genes were amplified either from genomic DNA (clones B.1, B.3, C.4) or from cDNA and directly sequenced. 11 out of these 18 clones (C.9–17. I.6, I.7) had not previously been analyzed by Southern blot (4).

Total RNA was extracted from 5×10^6 cells as described (4). Single-stranded cDNA was synthesized from 1–5 μg total RNA using an oligo(dT) primer and MMLV reverse transcriptase. 1/20 of each cDNA sample was amplified using the following primers: Vγ9, 5′-TCATACAGTTCCTGGTGTCC-3′ (173 bases upstream to the Vγ9 3′ end); Cγ, 5′-GTGTTTGTGAGCTGCAGCAGT-3′ (28 bases downstream to the 5′ end of the C-III exon, sharing an identical sequence with Cγ1 and the three major Cγ2 alleles); Vδ2, 5′-AGGAAGACCCAAGGTAACACAA-3′ (173 bases upstream to the Vδ2 3′ end); Cδ, 5′-CTTCACCAGACAAGCGACAT-3′ (71 bases from the 5′ end of the C-I exon); Jγ1, 5′-CTTGGAAATGTTGTATTCTTCC-3′; Jδ3, 5′-AAGATCAACTCACGGGGCTC-3′. The latter two primers were used for genomic DNA samples only. The amplification of the Vδ expressed in clone F_1 was obtained by means of the "anchored" PCR, as recently described by Loh et al. (24). The amplifications were done for 40 cycles (94°C, 1 min; 55°C, 1.5 min; and 72°C, 1.5 min) with 25 pmol of each primer in 50 μl reactions. PCR products were purified from low melting agarose gel, and one-third of the final volume was used directly for sequencing. Dideoxynucleotide termination sequencing reactions were done with a modified T7 DNA polymerase (Sequenase 2.0; United States Biochemical Corp., Cleveland, OH). The primers used for sequencing were either "internal" Vδ2, JP, Jδ1 primers (Vδ2, 5′-AAGATACTTGCACCATCAGAG-3′; JP, 5′-AAGCTTTGTTCCGGGACCAA-3′; Jδ1, 5′-ACTTGGTTCCACAGTCACAC-3′), or the same Vγ9 and Vδ2 primers used in the amplifications; in some cases both strands were sequenced. 3–5 μl of the DNA template was mixed with 5 pmol of primer, 2 μl of 5× polymerase buffer, 1 μl DMSO, and denatured at 95°C for 5 min. The samples (10 μl final volume) were then transferred on ice. Labeling reactions were done with 2 μl of labeling mix (1.5 μM dGTP, dCTP, dTTP), 1 μl of 0.1M DTT, 0.6 μl of DMSO, 1 μl of α-[^{35}S]dATP, and 2 μl (2–3 U) of Sequenase. Termination reactions were according to manufacturer's instructions. The sequencing reactions were then analyzed by standard electrophoresis and autoradiography.

Results

A Large Fraction of Peripheral Blood γ/δ Cells Recognize Molt-4 Lymphoma Cells. We generated a large panel of γ/δ T cell clones (78) from peripheral blood by using PHA as a polyclonal stimulator and tested the ability of these clones to lyse different tumor target cells. This random set of clones expressed many different sorts of specificities. A few were alloreactive, recognizing specific human class II alleles (as tested on various EBV cells), and a very small set specifically saw other antigens. For example, one clone recognizes all mouse T cells. In addition, as reported by other authors (25, 26), we found that many of the γ/δ clones, when tested soon after in vitro restimulation, exert a non-MHC-restricted killing against a variety of tumor targets, including K562, U937, OVCA 432, Raji, Jurkat, and Molt-4 (Fig. 1 *A*). When the same clones were rested for 4–8 wk, they progressively lost this nonspecific reactivity, however, most of them retained the ability to lyse Molt-4 (Fig. 1 *B*).

To investigate whether the γ/δ receptor is involved in Molt-4 recognition, we performed antibody inhibition experiments with δ1 and TiγA mAbs, which recognize Cδ- and Vγ9-encoded epitopes (1, 5). Fig. 1 *C* shows that these mAbs completely abolished Molt-4 killing by five Vγ9-positive

[1] *Abbreviations used in this paper:* MT, *Mycobacterium tuberculosis*; PPD, purified protein derivative; SEA, staphylococcal enterotoxin A.

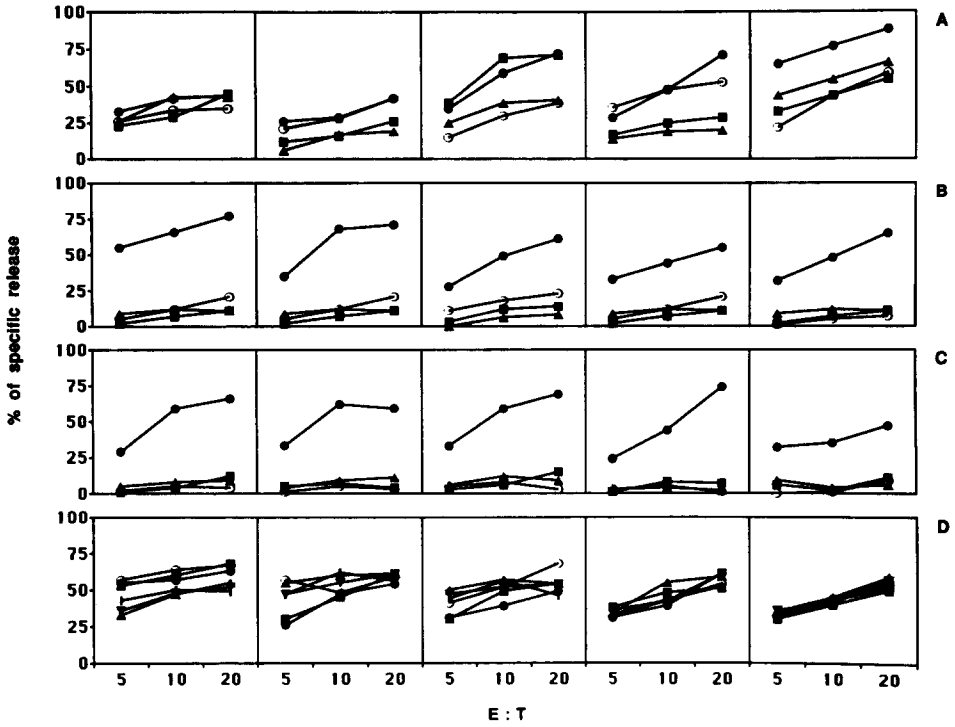

Figure 1. Killing of Molt-4 cells by rested γ/δ clones is efficient and is inhibited by anti-TCR-γ/δ mAbs. Molt-4 cells are killed with high efficiency by both freshly restimulated (*A*) and rested clones (*B*). Targets: Molt 4 (●), Jurkat (○), K562 (■), Raji (▲). (*C*) Addition of anti-CD3 (TR66) (○), anti-δ (δ1) (■), and anti-Vγ9 (TiγA) (▲) mAbs completely abolished killing of Molt-4 (●). (*D*) Addition of anti-β_2-microglobulin antiserum (○), anti-DR (L243) (■), anti-DP (B7.21) (▲), anti-MHC class I mAb (W6/32) (▼), and anti-CD1c (▮) mAbs do not inhibit killing of Molt-4 (●).

clones. Similar results were obtained with all the Vγ9$^+$ clones tested. In some experiments, we also measured the release of serine esterases, which appears to be specific for the killing mediated by the TCR and not by a putative NK receptor (27). Serine esterases activity was detected in the supernatant after triggering of Vγ9/Vδ2 cells with Molt-4 (data not shown). Thus, both mAb inhibition and serine esterases release indicate that TCR is involved in Molt-4 recognition.

In contrast to the anti-receptor antibodies, mAbs directed to various structures on the target were ineffective (Fig. 1 *D*). Neither anti-MHC antibodies (W6/32, L243, B7.21 mAbs), nor anti-β2-microglobulin antiserum, nor anti-CD1a, -b, and -c mAbs were able to block Molt-4 killing. In addition, Molt-4 variants that express low levels of CD1a and CD1c were recognized and killed by rested clones (data not shown). These results suggest that the TCRs of γ/δ cells recognize a Molt-4 surface structure that is neither a classical MHC molecule nor CD1.

Recognition of Molt-4 Is a Property of Cells Expressing the Vγ9-Cγ1 Chain. To assess the relationship between the large set of cells recognizing Molt-4 and the set of cells expressing Vγ9/Vδ2, we isolated a new panel of 189 random γ/δ clones from peripheral blood using PHA and tested them for the reactivity with the Vγ9-specific antibody TiγA, and for their capacity to kill Molt-4. None of the TiγA$^-$ clones tested (60/60) and all but one TiγA$^+$ clones (128/129) killed Molt-4. This finding indicates that the two characteristics define one set of cells. In peripheral blood, almost all Vγ9$^+$ cells recognize Molt-4, a specificity not shared by Vγ9$^-$ cells.

Since most peripheral Vγ9$^+$ cells also express Vδ2, we asked whether recognition of Molt-4 required both V genes. We selected from our collection of γ/δ clones (4) those expressing "unusual" pairings of Vγ and Vδ chains, and measured their reactivity to Molt-4. Table 1 shows 41 γ/δ clones, classified according to the expression of Vγ9, Vδ2, and the Cγ1 vs. the Cγ2 isotypes (i.e., the disulphide- or non-disulphide-linked constant region heterodimer). Although the number of clones is small, and two out of the possible eight combinations are missing, the following conclusions can be made: (*a*) killing of Molt-4 requires both Vγ9 and Cγ1, but does not require Vδ2; and (*b*) expression of Vγ9/Cγ1, though necessary, is not sufficient since one Vγ9$^+$ clone (E13) kills K562 (data not shown) but does not kill Molt-4.

Thus, Molt-4, like the well-known superantigens, is recognized by nearly all cells expressing a single particular V gene, though in the case of γ/δ cells, an additional requirement exists for the disulphide-linked form of the constant region.

Stimulation of PBMC with M. tuberculosis Leads to the Selective Expansion of Vγ9/Vδ2 Cells. It has recently been reported that a large number of human γ/δ cells proliferate in response to mycobacterial lysates (29). To characterize the TCR of these cells, we stimulated PBMC with heat-killed MT. Cells from six different donors proliferated vigorously to MT. The proliferating cells were identified by FACS® as large cells with forward and side scatter parameters and analyzed with a panel of mAbs specific for human Vγ or Vδ chains (Fig. 2 *A*). In five of six donors, there was a striking increase of γ/δ cells, identified as δ^+ (with the mAb δ1) and α/β^- (with BMA032), from 1–5% at day 0, to 40–90% at day 14. The proliferating γ/δ cells also stained with anti-Vδ2 (BB3) and anti-Vγ9 (TiγA) mAbs, while they did not react with anti-Vδ1 (δTCS1), nor with anti-Vγ4 (4A11) mAbs, indicating

Table 1. *Correlation of TCR-γ/δ, MT, and Molt-4 Recognition*

A. PHA-derived clones			mAb reactivity					Reactivity	
Name	Vγ	Vδ	δ1	δTCS1	BB3	TiγA	4A11	MT	Molt-4
Vγ9⁺Cγ1/Vδ2⁺									
A.1	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
A.2	Vγ9JP	Vδ2J1	+	−	+	+	−		+
B.1	Vγ9J1	Vδ2J1	+	−	+	+	−	+	+
B.2	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
B.3	Vγ9JP	Vδ2J3	+	−	+	+	−	+	+
B.4	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.1	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.2	Vγ9JP	Vδ2J1	+	−	+	+	−		+
C.3	Vγ9JP	Vδ2J1	+	−	+	+	−	−	+
C.4	Vγ9JP	Vδ2J3	+	−	+	+	−	+	+
E.1	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
E.3	Vγ9JP	Vδ2J3	+	−	+	+	−	+	+
E.5	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
I.3	Vγ9JP	Vδ2J3	+	−	+	+	−	+	
I.6	Vγ9JP	Vδ2J1	+	−	+	+	−	−	
I.7	Vγ9JP	Vδ2J3	+	−	+	+	−	−	
Vγ9⁺Cγ1/Vδ2⁻									
E.13	Vγ9JP	Vδ5J1	+	−	−	+	−		−
F.3	Vγ9JP1	Vδ1J1	+	+	−	+	−	−	+
G.1	Vγ9J1	Vδ1J1	+	+	−	+	−		+
Vγ9⁺/Vδ2⁻									
D.2	Vγ9J2	Vδ1J1	+	+	−	+	−	−	+
E.2	Vγ9J2	Vδ7J1	+	−	−	+	−	−	+
E.6	Vγ9J2	Vδ8J1	+	−	−	+	−	−	−
E.9	Vγ9J2	Vδ1J1	+	+	−	+	−	−	+
F.1	Vγ9J2	Vδ5J1	+	−	−	+	−	−	−
F.7	Vγ9J2	Vδ1J1	+	+	−	+	−	−	−
G.2	Vγ9JP2	Vδ1J1	+	+	−	+	−		−
E.4	Vγ9J2	Vδ1J1	+	+	−	+	−	−	
E.19			+	−	−	+	−	−	
Vγ9⁻Cγ1/Vδ2⁻									
A.4	Vγ5J1	Vδ1J1	+	+	−	−	−		−
A.10	Vγ8JP1	Vδ1J1	+	+	−	−	−		−
Vγ9⁻Cγ2/Vδ2⁺									
E.11	Vγ2J1/2	Vδ2J3	+	−	+	−	−	−	
E.15	Vγ4J2	Vδ2J3	+	−	+	−	+	−	−
Vγ9⁻Cγ2/Vδ2⁻									
A.11	Vγ4J2	Vδ3J1	+	−	−	−	+		−
B.7	Vγ2/8J2	Vδ1J2	+	−	−	−	−		−
C.5	VγxJP2	Vδ1J1	+	+	−	−	−		−
C.6	Vγ3/10J2	Vδ1J1	+	+	−	−	−		−

continued

Table 1. *(continued)*

A. PHA-derived clones			mAb reactivity					Reactivity	
Name	Vγ	Vδ	δ1	δTCS1	BB3	TiγA	4A11	MT	Molt-4
C.7	Vγ8JP2	Vδ1J1	+	+	−	−	−		−
C.8	Vγ4J2	Vδ3J1	+	−	−	−	+		−
D.1	Vγ2/8J2	Vδ1J1	+	+	−	−	−	−	−
A.5	Vγ4J2	Vδ1J1	+	+	−	−	+	−	
E.16	Vγ4J2	Vδ3J3	+	−	−	−	+	−	
B. Clones derived from MT-specific cell lines									
C.10	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.11	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.12	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.13	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.15	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.16	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.17	Vγ9JP	Vδ2J1	+	−	+	+	−	+	+
C.18			+	−	+	+	−	+	+
C.19			+	−	+	+	−	+	+
C.20			+	−	+	+	−	+	
C.21			+	−	+	+	−	+	
C.22			+	−	+	+	−	+	
C.23			+	−	+	+	−	+	
Q.1			+	−	+	+	−	+	
Q.2			+	−	+	+	−	+	
Q.3			+	−	+	+	−	+	
C.9	Vγ9JP	Vδ2J1	+	−	+	+	−	−	+
C.24			+	−	+	+	−	−	+
Q.4			+	−	+	+	−	−	

The clones were derived from peripheral blood or postnatal thymus as specified in reference 4. The Vγ and Vδ genes are named according to references 4 and 28 except for Vδ7, which is a new V gene, and Vδ8, which has already been reported in a TCR α chain (N. Migone, et al., manuscript in preparation). Blanks are not done.

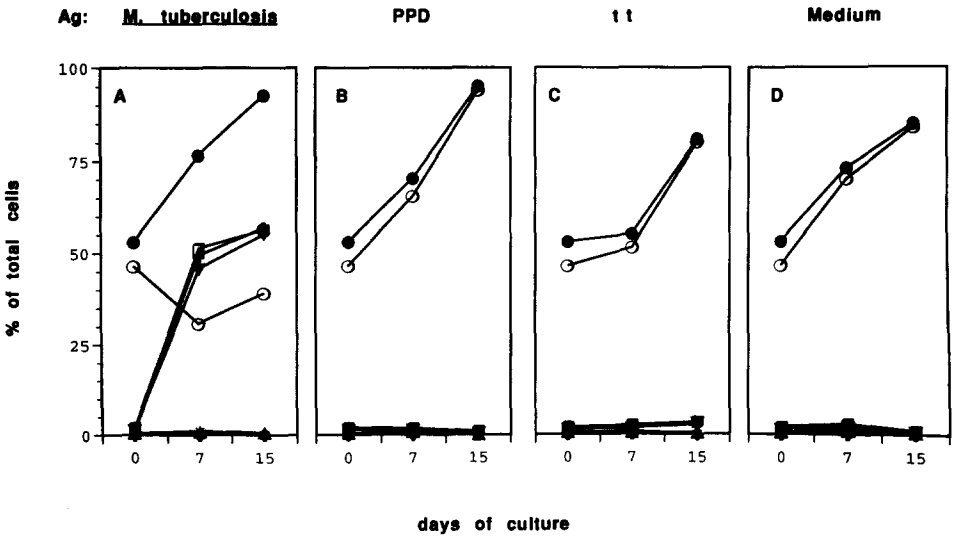

Figure 2. Stimulation of PBMC with MT induces proliferation of Vγ9/Vδ2 cells. PBMC were stimulated with 50 μg/ml of MT strain H37RA, PPD (10 μg/ml), tetanus toxoid (20 μg/ml), or 30 U/ml of IL-2. After 4 d, 10 U/ml of IL-2 was added to the cultures, and at days 7 and 15 of culture, proliferating cells were analyzed for reactivity with CD3 (●), TCR-α/β (○), Cδ (□), Vγ9 (✚), Vγ4 (*), Vδ2 (▼), and Vδ1 (▲)-specific mAbs. (■) γ/δ cells with Vγ regions different from Vγ4 and Vγ9, and (▮) cells with Vδs other than Vδ1 and Vδ2. All the donors were tuberculin skin test positive.

that virtually all the γ/δ cells carried a Vγ9/Vδ2 receptor (data not shown).

PBMC from the same donors did not show a preferential expansion of γ/δ cells when stimulated with PPD, tetanus toxin, or 30 U/ml of IL-2 (Fig. 2, *B–D*). These results indicate that only stimulation with mycobacteria and not with PPD or other protein antigens specifically activates Vγ9/Vδ2 cells in PBMC.

The proliferating T cells from MT cultures were expanded in IL-2 and tested for their response to MT. The cell lines proliferated in response to MT but not to PPD or tetanus toxoid (data not shown). They also killed mycobacteria-pulsed APC (data not shown). Thus, the response to MT is selectively comprised of γ/δ cells bearing Vγ9/Vδ2.

Recognition of Molt-4 and M. tuberculosis Are Properties of Overlapping Sets of γ/δ T Cells. Since Vγ9 is associated with recognition of Molt-4, the finding that Vγ9/Vδ2 characterizes γ/δ cultures specific for MT led us to ascertain whether the two specificities are associated with separate or overlapping sets of T cells. We therefore generated a set of clones from cultures responding to MT and analyzed their TCR usage and their specificity to various antigens. Table 1 shows a comparison of these clones with a set of randomly derived clones from peripheral blood.

Every clone derived from MT-specific cell lines reacts with BB3 and TiγA mAbs and therefore expresses a Vγ9/Vδ2 TCR (Table 1 *B*). Furthermore, all the clones that were tested express the disulphide-linked Vγ9-JP-Cγ1/Vδ2-J1 receptor, and lyse Molt-4 targets. The molecular analysis thus confirmed that the receptor of MT-reactive clones is Vγ9/Vδ2.

In the sample of randomly derived clones (Table 1 *A*), 11 of 23 of the Vγ9$^+$, and 11 of 14 of the Vγ9/Vδ2-tested clones proliferated to MT-pulsed APC, whereas none of the clones bearing other TCR combinations showed any proliferative responses to this bacterium.

Thus, the clones specific for MT appear to comprise a major proportion of the set specific for Molt-4. However, unlike the Molt-4 specificity, which appears to be dictated solely by the Vγ9-Cγ1 chain, reactivity to MT requires the additional component of Vδ2.

Because ~20% of Vγ9/Vδ2 tested cells do not react with MT, we asked whether any particular N, D, or J segment correlated with this specificity by sequencing the junctional regions of Vγ9/Vδ2 clones belonging both to MT-reactive and nonreactive groups. The results showed that the examined TCRs have different γ (Table 2) and δ (Table 3) junctional sequences, with no obvious consensus amino acid sequences or lengths (Table 4) that might distinguish between the two groups of cells. Two clones (I.7, which does not respond to MT, and C.15, which does respond to this stimulation) have an identical Vγ9 chain but different Vδs, suggesting that the γ chain alone does not confer the specificity and also that the δ chain is important. Thus, while Molt-4 behaves like a cellular superantigen, specificity for MT has features more closely resembling specificity to normal antigens. The characteristics summarized in Tables 2–4 evoke comparisons

Table 2. *TCR-γ Sequences*

Clone	MT reactivity	Germ line Vγ9	TTG	TGG	GAG	GTG	N	T	GGG AAT	CAA TAT	GAG TAT	TTG AAG	GGC AAA	JP J1/2
F.1	–		TTG	TGG	GAG	GTG	*CAGGC*					G	AAA	2
I.7	–		TTG	TGG	GAG	G	*CT*			CAA	GAG	TTG	GGC	P
C.3	–		TTG	TGG	G		*GTCTGATG*				GAG	TTG	GGC	P
I.6	–		TTG	TGG	GAG	GTG	*CCTGGGGGG*				GAG	TTG	GGC	P
C.9	–		TTG	TTG	GAG	GTG	*CACGATTTGGG*				G	TTG	GGC	P
C.10	+		TTG	TGG	GAG	G	*ACAA*		G	CAA	GAG	TTG	GGC	P
C.11	+		TTG	TGG	G		*GGC*		GG	CAA	GAG	TTG	GGC	P
C.12	+		TTG	TGG	GAG	GTG	*CGC*				GAG	TTG	GGC	P
C.13	+		TTG	TGG	GAG	GTG	*CTCCG*			A	GAG	TTG	GGC	P
C.14	+		TTG	TGG			*ATTGTC*			CAA	GAG	TTG	GGC	P
C.15	+		TTG	TGG	GAG	G	*C*		G	CAA	GAG	TTG	GGC	P
C.16	+		TTG	TGG	GAG	GTG	*CTT*			CAA	GAG	TTG	GGC	P
C.17	+		TTG	TGG	GAG	GTG	*GTG*				GAG	TTG	GGC	P
C.1	+		TTG	TGG	GAG	G	*CCCCC*		GGG	CAA	GAG	TTG	GGC	P
A.1	+		TTG	TGG	GAG	G	*ACCTCCC*				G	TTG	GGC	P
C.4	+		TTG	TGG	GAG	GTG	*CGGC*			AA	GAG	TTG	GGC	P
B.3	+		TTG	TGG	G		*TT*		GGG	CAA	GAG	TTG	GGC	P
B.1	+		TTG	TGG	GAG	GT	*A*					AAG	AAA	1

Table 3. *TCR-δ Sequences*

Clone	MT reactivity	Vδ 2 / 5	Germ line TGT GAC ACC / GCA GCA AG	N	Dδ1* GAAATAGT	N	Dδ2 CCTTCCTAC	N	Dδ3 ACTGGGGGATACG	N	AC ACC GAT AAA CTC Jδ1 / C TCC TGG GAC ACC CGA Jδ3
F.1	–	5	GCA GCA A	*CCACC*	AAT	TGCCGGT			CTGGGGGA	*GCCT*	AC ACC GAT AAA CTC 1
C.3	–	2	TGT GAC ACC			*GTCGGG*	TCCT	*T*	ACTGGGGGATAC	*CG*	AA CTC 1
I.6	–	2	TGT GAC ACC			*C*	TCC	*CG*	CTGGGGGATA	*GGG*	AC ACC GAT AAA CTC 1
C.9	–	2	TGT GAC ACC					*GTCAGT*	ACTGGGGGATACG	*GTTCCTCACCACCTAGGGG*	C GAT AAA CTC 1
I.7	–	2	TGT GAC AC					*GCCCGGGATA*	GGATAC	*A*	CC TGG GAC ACC CGA 3
C.10	+	2	TGT GAC A					*TCGTCG*	GGGGGATACG	*CTGGGGGC*	CC GAT AAA CTC 1
C.11	+	2	TGT GAC					*CCCGT*	ACTGGGGGATACG	*CTGGCTG*	CC GAT AAA CTC 1
C.12	+	2	TGT GAC ACC			*T*	TAC	*AAG*	CTGGGGG	*TCC*	C ACC GAT AAA CTC 1
C.13	+	2	TGT GAC			GA	CCTT	*ACGTT*	ACTGGGGG	*GTAAGAACG*	AC ACC GAT AAA CTC 1
C.14	+	2	TGT GAC					*TT*	ACTGGGGG	*TCCTTC*	AC ACC GAT AAA CTC 1
C.15	+	2	TGT GAC ACC					*TTGGGCAG*	TGGGGGAT	*CCGCCGAACGA*	ACC GAT AAA CTC 1
C.16	+	2	TGT GAC					*GCCG*	TGGGG	*ACTA*	AC ACC GAT AAA CTC 1
C.17	+	2	TGT GAC A	*TCGT*	GAAA				ACTGGGGG	*TCTCGAGT*	AC ACC GAT AAA CTC 1
C.1	+	2	TGT GAC ACC			*CTGA*	TCC				AC ACC GAT AAA CTC 1
A.1	+	2	TGT GAC ACC			*GT*	TTCC	*CTT*	ACTGGGGGAT	*TG*	ACC GAT AAA CTC 1
B.1	+	2	TGT GAC				TCC			*GTAGACAAACCTCGG*	ACC GAT AAA CTC 1
C.4	+	2	TGT GAC					*CCGGCGAT*	ACTGGGGGA	*CGAACT*	C TCC TGG GAC ACC CGA 3
B.3	+	2	TGT GAC			*CCAG*		T	ACTGGGGGATAC	*CCCCAATCCT*	TCC TGG GAC ACC CGA 3

* D elements were assigned with the requirement for a minimum of three contiguous matches to the germline sequence.

Table 4. *Deduced Amino Acid Sequences Encoded by V-J γ and V-D-J δ Junctions*

Clone	MT reactivity	Vγ	N	Jγ	Vδ	N-(D)-N	Jδ
F.1	–	ALWEV	*OA*	KL	CAA	*TTNCRSGGAY*	TDKL
I.7	–	ALWE	*A*	QELG	CDT	*PGIGYT*	WDTR
C.3	–	ALW	*GLM*	ELG	CDT	*VGSLLGDTE*	L
I.6	–	ALWEV	*PGG*	ELG	CDT	*LPLGDRDT*	DKL
C.9	–	ALWEV	*HDLG*	LG	CDT	*VSTGGYGSSPPRG*	DKL
C.10	+	ALWE	*DK*	QELG	CD	*IVGGIRWGP*	DKL
C.11	+	ALW	*GR*	QELG	CD	*PVLGDTLAA*	DKL
C.12	+	ALWEV	*R*	ELG	CDT	*LOAGGP*	TDKL
C.13	+	ALWEV	*LR*	ELG	CD	*DLTLLGGKND*	TDKL
C.14	+	ALW	*IV*	QELG	CD	*LLGVLH*	TDKL
C.15	+	ALWE	*A*	QELG	CDT	*LGSGGSAER*	TDKL
C.16	+	ALWEV	*L*	QELG	CD	*AVGTN*	TDKL
C.17	+	ALWEV	*VO*	LG	CD	*IVKTGGLEY*	TDKL
C.1	+	ALWE	*AP*	GQELG	CDT	*LIH*	TDKL
A.1	+	ALWE	*DLP*	LG	CDT	*VSLTGGL*	TDKL
C.4	+	ALWEV	*R*	QELG	CD	*PAILGDEL*	SWDTR
B.3	+	ALW	*V*	GQELG	CD	*PVLGDTPNP*	SWDTR
B.1	+	ALWEV		KKL	CD	*SVDKPR*	TDKL

The V and J γ and δ segments used are specified in Tables 2 and 3.

with, for example, the response to cytochrome c in mice, where T cell clones of one particular functional phenotype always express the identical Vα and Vβ combination, but vary at junctional regions (30).

Characteristics of the Response to M. tuberculosis. To analyze the biology of the response to MT, we began with the question of MHC restriction. We first asked whether the γ/δ clones showed any preference for antigen in the context of polymorphic MHC molecules using EBV-B cells bearing different HLA haplotypes as APC. Fig. 3 shows that all tested

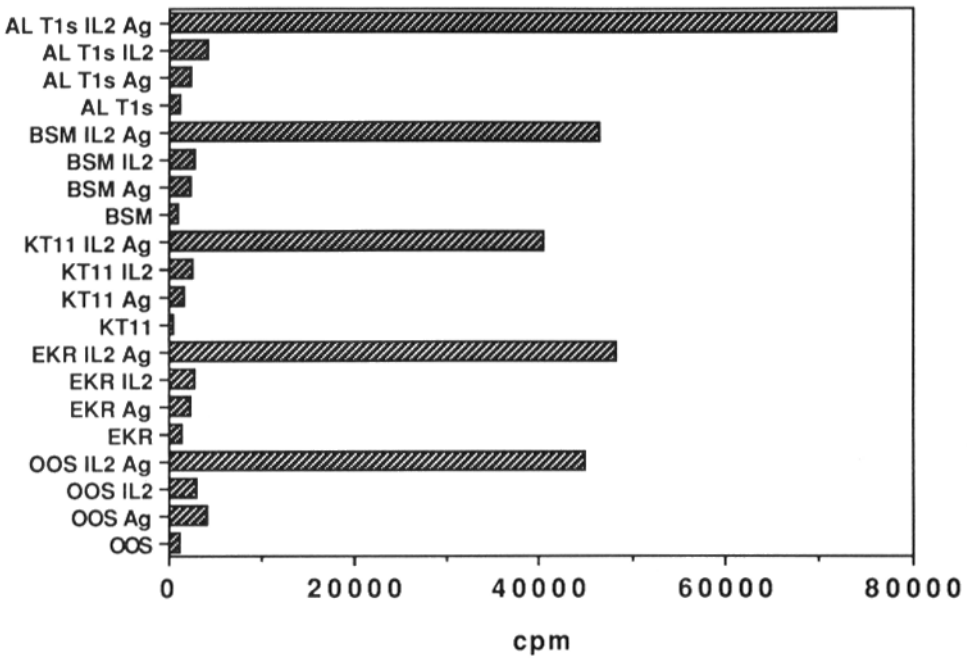

Figure 3. Vγ9/Vδ2 cells are not restricted by classical MHC molecules. γ/δ clones were stimulated with MT (50 μg/ml) and EBV-B cell lines as APC displaying different HLA class I and class II haplotypes. AL T1s: A2,26; Bw49,58; DR6, DP1; BSM: A2, Bw62, Cw3, DR4, DQw3, DP2; KT11: Aw33, Bw44, Cw3, DR3, 6, DQw1, DP2, 4; EKR: A3, B7, DR7, DQw2; OOS: A26, Bw56, Cw2, DR1, DQw1, DP2. Similar results were obtained with 19 other Vγ9/Vδ2 clones from different donors.

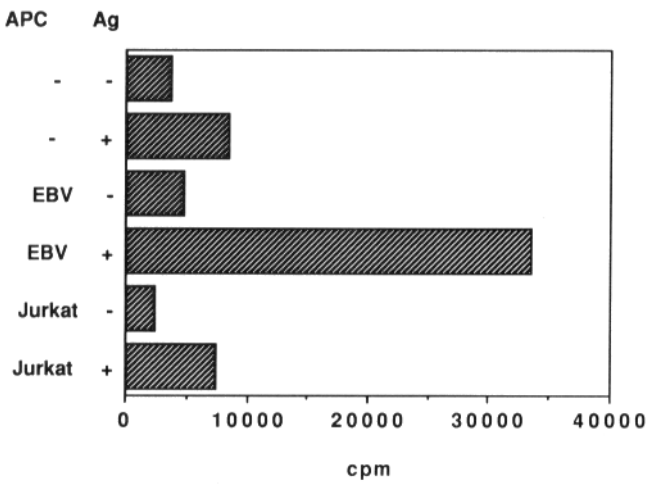

Figure 4. APC are required for optimal stimulation of Vγ9/Vδ2 clones by MT. γ/δ clones were stimulated with MT (50 μg/ml) in the absence or in the presence of various APC and tested in a proliferation assay. Similar results were obtained with 19 other Vγ9/Vδ2 clones from different donors.

EBV-B cells, irrespective of MHC type, functioned as APC for MT-reactive γ/δ clones, suggesting that polymorphic MHC determinants are not involved in the stimulation of the γ/δ clones. We also attempted to identify any nonpolymorphic restriction molecules by mAb inhibition using mAbs specific for framework regions of class I molecules (W6/32), as well as anti-β_2-microglobulin antisera, anti-DR, and anti-DP mAbs. Although these antibodies consistently block antigen recognition by TCR-α/β^+ cells (data not shown), we failed to detect consistent inhibition of γ/δ cell proliferative responses or lysis of mycobacteria-pulsed target cells (data not shown).

Having found no special role for APC of any particular haplotype, we asked whether APC are needed at all. Fig. 4 shows that T cells give a low response to MT alone, while they proliferate well when both EBV-B cells and antigen are present. These findings indicate that APC are indeed required for maximum stimulation of γ/δ cells, although self presentation by the T cells themselves may occur. Both PBMC (data not shown) and EBV-B cells functioned as APC, while a T cell line (Jurkat) did not. Thus, the bacterial antigens, like conventional antigens presented to CD4 or CD8 T cells, require the presence of APC for optimal activation of γ/δ T cells. Whether this is due to a more efficient processing, or presentation by APC, is under investigation.

To study the antigen specificity of γ/δ clones, we used APC pulsed with different bacteria such as MT, *L. monocytogenes*, or *E. coli*. Interestingly, (Table 5) some γ/δ clones, originally raised against MT, were also activated by APC pulsed with different bacteria, suggesting that the different bacteria might share common antigens or might induce the expression of similar determinants on the APC surface.

In summary, these results indicate that: (*a*) optimal stimulation of γ/δ cells requires the presence of APC; (*b*) γ/δ clones are not restricted by classical (class I or II) or nonclassical (class I–like) MHC molecules; and (*c*) some γ/δ clones recognize APC pulsed with different bacteria, suggesting that bacteria-pulsed APC display common structures.

Table 5. *Vγ9/Vδ2 Clones Recognize APC Pulsed with Different Bacteria*

		Bacteria			
Clone	APC	–	MT	*L. monocytogenes*	*E. coli*
A.1	–	0.4*	0.7	0.4	0.5
	+	14	36.6	28.4	19.8
R.4	–	12.2	15.5	7.2	7.3
	+	21.5	85.2	48.1	25.8
R.13	–	6.7	5.9	5.1	8.9
	+	14.6	72.8	32.6	12.9
R.16	–	6.6	18.2	4.1	8.2
	+	16.8	70.3	47.8	47.4
R.23	–	5	16.5	2.7	9.5
	+	15.5	87.1	54.5	23.4
R.61	–	1.9	34.3	10.3	10.8
	+	16	104.2	35.2	26.5
R.64	–	1.2	29.3	8.2	17.7
	+	17.9	120.6	37.5	50.5
R.65	–	1.1	16.8	5.6	9.3
	+	23.6	82.3	45.7	23.9
R.67	–	2.7	14.4	10.4	10.1
	+	16.8	67.9	42.5	22.8
R.74	–	5.1	42.2	6.1	11.7
	+	18.9	113.5	43.4	47.5

Cell proliferation was assessed in the presence or absence of APC (3×10^4 EBV-B/well) with the addition of 5 U/ml of IL-2.
* The results are expressed as mean cpm $\times 10^{-3}$of triplicates. Irradiated APC gave a [^{3}H]Thy incorporation of 15.4×10^3 cpm.

Discussion

Our results indicate that a large fraction of human peripheral blood γ/δ cells is able to recognize Molt-4 as well as bacteria-pulsed APC. While these two "superspecificities" are both found within the Vγ9/Vδ2 population, which is the most abundant γ/δ subset in peripheral blood, the two sets are not precisely overlapping and can be defined as follows.

The major set of peripheral γ/δ T cells, those that recognize Molt-4, is comprised of clones bearing a disulphide-linked Vγ9-bearing receptor. The Vδ chain seems to be less critical, since at least two different Vδs can pair with Vγ9-Cγ1 chains in Molt-4-reactive clones. Thus, the recognition of Molt-4 by human Vγ9$^+$ cells resembles the recognition of Mls ligands by mouse T cells expressing particular Vβ gene products (13). In both cases, the structure of the second TCR chain and of N, D, or J regions seems to be largely irrelevant.

The molecular nature of the Molt-4 associated and Mls

gene–controlled superantigens remains to be elucidated. Recently, Vγ9-related ligands have also been found to be expressed by Daudi cells (31) and by staphylococcal enterotoxin A (SEA) (32). It is possible that cellular superantigens identical or related to the Molt-4 and Daudi superantigens, or microbial superantigens like SEA, might play a role in the expansion of a subset of $V\gamma9^+$ cells in the periphery. However, this mechanism cannot explain the preferential use of Vδ2 by the vast majority of circulating $V\gamma9^+$ cells.

The second superspecificity, directed against mycobacteria-pulsed APC, is found in 80% of peripheral blood Vγ9/Vδ2 cells. This reactivity is dependent on the expression of Vγ9 and Vδ2, though the expression of both these gene segments is not completely sufficient, since ~20% of $V\gamma9^+/V\delta2^+$–tested clones do not recognize mycobacteria-pulsed APC. It is not yet clear whether the lack of reactivity is due to the presence of inappropriate TCR junctional regions or to other reasons.

The Vγ9/Vδ2 TCR of the mycobacteria-reactive cells exhibit extensive junctional diversity. In the context of the model proposed by Davis and Bjorkman (7), we might interpret our data to indicate that the mycobacteria-reactive γ/δ TCRs contact a monomorphic restriction molecule with Vγ9- and Vδ2-encoded regions and an antigenic determinant using regions encoded by the junctional sequences. However, we found no evidence that any known MHC class II, nor any known or unknown β_2-microglobulin-associated class I, molecule acted as restriction elements for either of the two major specificities.

In spite of the lack of MHC restriction, we found that APC were required for optimal proliferation of γ/δ cells. We also found that some γ/δ clones, originally raised with MT, also recognize APC pulsed with different bacteria, such as *L. monocytogenes* and *E. coli*.

These findings indicate that APC play an active role in antigen presentation but do not establish the origin of the stimulating ligand. There are two possibilities. First, this ligand may be derived from the bacteria themselves. Several reports have shown that some γ/δ cells recognize heat shock proteins (HSPs) (33–35), and different bacteria express related HSPs that may provide crossreactive epitopes (36). A recent analysis of a set of mouse γ/δ T cell hybridomas that respond to PPD and that recognize a HSP-derived peptide (35, 37) shows that these cells, like the clones described here, display considerable junctional diversity and lack classical MHC restriction (37, 38).

The second possibility is that pulsing with bacteria might induce the appearance of new surface proteins from the APC themselves. It is known, for example, that LPS can induce the expression of the MT9-defective mammary tumor virus genome in normal B cells (39). If this is the case with the ligand recognized by Vγ9/Vδ2 cells, then the APC would display a comparable set of ligands irrespective of the bacteria used.

An apparent paradox is the finding that Vγ9/Vδ2 cells are a minor population of γ/δ cells in tissues (40–43, G. De Libero unpublished results), where immune responses and cell proliferation occur (44), while they represent a major subset in peripheral blood. Vγ9/Vδ2 cells might localize in tissues during inflammatory responses (45), become activated, and then recirculate in the blood. Studies on the recirculation patterns of γ/δ cells in sheep show that they do not circulate through the T or B cell areas of lymph nodes, suggesting that they may have nothing to do with normal T-B responses (46). Studies on the recirculation and activation patterns of these cells in patients with different diseases may help to clarify their function.

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