Ontogenic Development and Tissue Distribution of Vγ1-expressing γ/δ T Lymphocytes in Normal Mice

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Summary

A hamster monoclonal antibody (mAb) recognizing an epitope in the Vγ1-Jγ4-Cγ4 chain of the γ/δ T cell receptor has been generated. Using this mAb, we have quantitated the occurrence of Vγ1-bearing γ/δ T cells in the developing thymus and in the lymphoid organs and several epithelia of adult mice. The Vγ1-expressing cells constitute a minor γ/δ T cell subpopulation during fetal and early postnatal life, but they constitute a major population of γ/δ T cells in the thymus and in the peripheral lymphoid organs in adult mice. In addition, we found that Vγ1-bearing cells comprise a large proportion (15–60%) of the γ/δ T cells present in the intestinal epithelium (i-IEL) in all strains of mice tested. $V\gamma1^+$ i-IEL are present in athymic (nude) mice and in antigen-free mice, demonstrating that they can develop extrathymically and that their presence in the intestinal epithelium is independent of the antigenic load of the gut. Our results show that Vγ1-bearing lymphocytes account for the largest population of γ/δ T cells in the mouse. This population includes a thymus-dependent component that homes to the secondary lymphoid organs and a thymus-independent component that constitutes a major fraction of the γ/δ i-IELs.

T lymphocytes bearing the γ/δ TCR constitute a minor T cell subpopulation in the thymus and in the peripheral lymphoid organs but are often the major T cell type in epithelial tissues (1–9). γ/δ T cells comprise several different subsets as defined by such distinctive parameters as TCR repertoire, time of appearance during ontogeny, thymus dependence, and anatomical localization (for review, see reference 10). The physiological role of γ/δ T cells is still poorly understood, partially because of the lack of knowledge about γ/δ T cell ligands.

A large fraction of γ/δ T cell hybridomas expressing a TCR composed of Vγ1-Jγ4-Cγ4 and Vδ6-Cδ chains is constitutively activated to secrete IL-2 (11). The role of a specific interaction involving the TCR in this activation has been suggested by the ability of mAbs against the TCR–CD3 complex to inhibit spontaneous IL-2 production and by the fact that transfection of the Vγ1/Vδ6 cDNAs into a TCR^- murine hybridoma confers the phenotype of constitutive activation onto the recipient cells (11, 12). In some cases, the reactivity of Vγ1/Vδ6 TCR-expressing hybridomas to purified protein derivative (PPD)[1] and to the 180–196 amino acid region of the mycobacterial heat shock protein (HSP) 60 has been demonstrated (13), although failures to detect such reactivities have also been reported (14, 15). Constitutively activated hybridomas expressing the Vγ1/Vδ6 TCR have been isolated from newborn and adult thymus (11, 14, 16), adult spleen (16), liver (17), skin epidermis (18), and from the intestinal epithelium of weanling mice (15), suggesting that γ/δ T cells expressing the Vγ1 gene product are normally present in different lymphoid organs and epithelia. Nevertheless, neither quantification of Vγ1-bearing γ/δ T cells in different tissues nor studies on the origin of these cells has been performed, mainly because of the lack of mAbs specifically recognizing this population. Here we describe a hamster mAb (named 2.11) specific for the mouse Vγ1-Cγ4 protein and the studies performed to analyze these issues. Our results show that Vγ1-bearing lymphocytes account for the largest population of γ/δ T cells in the mouse, including a thymus-dependent component that localizes to the secondary lymphoid organs and a thymus-independent component that comprises a significant fraction of the γ/δ intestinal intraepithelial lymphocytes (i-IEL).

Materials and Methods

Animals. C57BL/6 (B6), BALB/c, DBA/2, and C3H/HeJ (C3H) mice were obtained from The Jackson Laboratory (Bar

[1]*Abbreviations used in this paper:* AgF, antigen free; DEC, dendritic epidermal cells; HSP, heat shock protein; i-IEL, intestinal intraepithelial lymphocytes; PPD, purified protein derivative; SPF, specific pathogen free.

Harbor, ME) or from Iffa-Credo (L'Arbresle, France). BALB.B10 and BALB.C-3H mice were purchased from Bomholtgard Breeding and Research Center Ltd. (Bomholtvej, Denmark) and bred at the MIT facilities. BALB/c specific pathogen–free (SPF) and antigen-free (AgF) animals were a kind gift of Dr. C. Heusser (Ciba-Geigy, Basel, Switzerland). Athymic (nu/nu) and (nu/+) littermates were purchased from The Jackson Laboratory, Charles River Laboratories, Inc. (Wilmington, MA), and Iffa-Credo. All other mouse strains were purchased from The Jackson Laboratory. Adult Armenian hamsters were obtained from Cytogen (West Roxbury, MA).

Abs. For complement-mediated killing, we used anti-CD4 (RL.174; reference 19) and anti-CD8 (HO 2.2; 20). Both mAbs were used as culture supernatants. Other mAbs used were H57-597, anti-Cβ (21); GK1.5, anti-CD4 (22), 53-6.7, anti-CD8 (23); RA3.6B2, anti-CD45R/B220 (24); M1/70 anti-Mac-1 (25) 3A10, anti-δ (2), and 536 anti-Vγ5 (26). All of these mAbs were purified from culture supernatant either by ion exchange chromatography on DEAE–cellulose or by affinity chromatography on protein A–Sepharose (Pharmacia, Uppsala, Sweden) and biotinylated by standard procedures. Biotin-labeled goat anti–mouse IgM was purchased from Caltag Laboratories (San Francisco, CA); biotin-labeled anti-CD5 was from Becton Dickinson & Co. (Mountain View, CA); and FITC-labeled anti-CD8β, FITC-labeled anti-Thy1, biotin-labeled anti-Vδ4, and biotin-labeled anti-Vγ4 were from PharMingen (San Diego, CA).

Immunofluorescence Staining and Flow Cytometric Analysis. Cells (10^5–10^6) were incubated in staining buffer (PBS, 3% FCS, 0.1% NaN_3) with the indicated labeled Abs for 30 min on ice. After two washes, the cells were incubated with streptavidin-PE (Southern Biotechnology Associates, Birmingham, AL) for 15 min on ice. After another two washes, 10,000 viable cells were analyzed using a FACScan® flow cytometer (Becton Dickinson & Co.). Dead cells were gated out by their staining with propidium iodide.

Cell Purifications and Cultures. The preparation of dendritic epidermal cells (DEC) and i-IEL has been previously described in detail (27, 28). The preparation of γ/δ T cell blasts used to produce γ/δ T cell hybridomas was as follows: Thymus and spleen cell suspensions were prepared according to standard procedures. Thymocytes were incubated with biotinylated anti-CD4 and anti-CD8 mAbs, and splenocytes were incubated with biotinylated anti–TCR-β, anti-CD4, anti-CD8, anti-B220, and anti-MAC-1 mAbs for 35 min on ice. After washing the unbound Abs, the cells were incubated with Tonsyl-activated magnetic beads (Dynal Inc., Great Neck, NY) that had been previously coupled to streptavidin according to the manufacturer's instructions. The incubation was performed for 60 min at 4°C with a slight rocking of the tubes. After separation of the beads, unbound cells were cultured in complete medium (DME supplemented with L-glutamine, sodium pyruvate, β-ME, nonessential amino acids, penicillin, and streptomycin, plus 10% fetal bovine serum) together with irradiated (1,000 rad) B6 spleen cells (2×10^6/ml) in the presence of 50 μg/ml of PPD and 10 U/ml of rIL-2. Alternatively, thymocytes were treated with anti-CD4 and anti-CD8 mAbs and complement for 45 min at 37°C. $CD4^-CD8^-$ live cells were purified by density gradient centrifugation using Lympholyte M (Cederlane Laboratories, Ltd., Hornby, Ontario, Canada) and cultured (5×10^5/ml) in complete medium plus 10 U/ml of rIL-2, in plates previously coated with the 2.11 mAb (20 μg/ml).

Production of T Cell Hybridomas. 3–5 d after initiation of the cultures described above, growing blasts were fused to the TCR-α/β^- variant of the BW5147 thymoma cell line (29) at a 1:1 ratio in 0.5 ml of 50% polyethylene glycol as described (30). The cells were then distributed in 96-well flat-bottomed plates with either HAT or azaserine-hypoxanthin medium. The hybridomas named T and S come from PPD-stimulated thymocytes and splenocytes, respectively. The hybridomas named BTC come from thymocytes activated with the 2.11 mAb.

Production of the 2.11 mAb. Armenian hamsters were immunized intraperitoneally three times at 3-wk intervals with 2×10^6–10^7 irradiated, TCR γ/δ^+ T3.13.1 hybridoma cells resuspended in saline. The T3.13.1 hybridoma expresses a TCR composed of Vγ1 and Vδ6.3 chains and constitutively secretes IL-2 when cultured in vitro. 3 d after the last injection, spleen cells were fused with the murine myeloma SP2/0 at a ratio of 10:1 (spleen cell/myeloma) in 1 ml of 50% polyethylene glycol as described (2). The cells were then distributed in 96-well flat-bottomed plates with HAT medium. Culture supernatants from growth-positive wells were tested for their ability to inhibit the constitutive IL-2 production of the immunizing hybridoma. IL-2 production was assayed by the growth of the HT-2 cells, scored by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) assay as originally described (31). Cells from wells inhibiting >75% of the IL-2 secretion were subcloned several times by limiting dilution until stable Ab-secreting hybrids were obtained.

Cell Surface Labeling and Immunoprecipitation. 8×10^6 Vγ1-expressing hybridoma cells were harvested by centrifugation, washed four times with PBS, and resuspended in 4 ml of PBS. Cells were split into nine glass test tubes coated with Iodogen (Pierce, Rockford, IL), and 200 μCi $Na^{125}I$ was added to each tube. After a 15-min incubation on ice with occasional mixing, cells were pooled and washed four times with PBS. The cell pellet was lysed in 1.5 ml ice cold lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF), vortexed for 20 s, and incubated on ice for 30 min. The lysate was clarified by centrifugation at 13,000 rpm at 4°C in a microfuge. 0.1 vol of normal hamster serum was added to the supernatant, followed by two rounds of preclearing with 0.1 vol protein A–Sepharose beads (Pharmacia). One quarter of the precleared lysate was immunoprecipitated with 10 μl of either 2.11- or 3A10-coated protein A–Sepharose beads. Immunoprecipitates were washed, resuspended in reducing sample buffer, and subjected to SDS-PAGE on a 10% gel, according to standard procedures. Radioactive bands were visualized using a Fujix BAS 2000 Bioimage Analyzer (Fugi, Fuji Medical Systems USA Inc., Stamford, CT).

PCR and Sequencing Analysis. The following primers were used. Vγ1: 5'-CCGGCAAAAAGCAAAAAAGT-3'; Jγ4: 5'-GCA-AATATCTTGACCCATGA-3'; and pan Cγ: 5'-CTTATG-GAGATTTGTTTCAGC-3'.

Genomic DNA from hybridoma cells (T and S) was prepared by the proteinase K/phenol extraction/ethanol precipitation method. Sequences spanning the V-J junction were amplified by PCR using primers specific for the Vγ1 and Jγ4 genes. For each sample, two PCRs were performed in parallel, each one containing either one of the primers kinased. To produce single-stranded DNA after PCR amplification, the double-stranded product was digested with λ exonuclease as originally described (32). Both strands were then sequenced by the dideoxy chain termination method using the Sequenase enzyme (United States Biochemical Corporation, Cleveland, OH).

Total cellular RNA from hybridoma cells (BTC) was prepared by the guanidine isothiocyanate/acid phenol extraction method. cDNA was synthetized with oligo-dT using superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Sequences spanning the V-J junction were amplified by PCR using primers specific for the Vγ1 and all

four Cγ genes. After amplification, 4 μl of each PCR reaction mixture was incubated with shrimp alkaline phosphatase and exonuclease I to remove the excess of primers and dNTPs and sequenced by the dideoxy chain termination method using the Sequenase enzyme as described in the Sequenase version 2.0 DNA polymerase for sequencing PCR products kit (Pharmacia-USB).

Results

Characterization of the 2.11 mAb. The mAb 2.11 was selected by its ability to block spontaneous IL-2 production by the Vγ1/Vδ6-expressing γ/δ T cell hybridoma that had been used for immunization. Initial studies showed that this mAb binds to and inhibits spontaneous IL-2 production by 10 of 10 Vγ1/Vδ6-expressing T cell hybridomas, but does not inhibit IL-2 production by α/β-bearing T cell hybridomas (not shown). Furthermore, the 2.11 mAb binds to the immunizing hybridoma cells but not to variants that have lost expression of the γ/δ TCR (not shown), suggesting that the 2.11 mAb recognizes some component of the γ/δ TCR. SDS-PAGE analysis of immunoprecipitates from lysate of ^{125}I–surface-labeled Vγ1 hybridoma cells revealed an apparently identical pattern of two bands for the 2.11 mAb and the pan-γ/δ–specific mAb, 3A10 (Fig. 1). This confirms that the 2.11 mAb is specific for TCR-γ/δ.

To characterize further the specificity of the 2.11 mAb, we stained several γ/δ T cell hybridomas known to express different Vγ and Vδ gene products. The mAb 2.11 did not bind to previously characterized γ/δ T cell hybridomas that are known to express TCRs containing Vγ4, Vγ5, Vγ6, or Vγ7 chains (30, 33). In contrast, this mAb recognized 14 of 14 γ/δ T cell hybridomas that produce IL-2 spontaneously (not shown). Sequence analysis of rearranged γ genes in those 14 hybridomas showed that all contain a functionally rearranged Vγ1 gene (Fig. 2 *A*). These data demonstrate that the 2.11 mAb recognizes the Vγ1-Cγ4 protein but do not

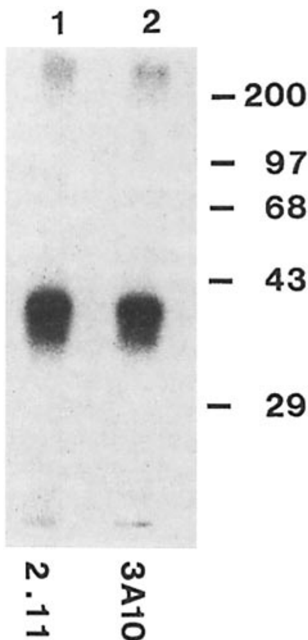

Figure 1. 2.11 and the pan γ/δ specific mAb, 3A10, immunoprecipitate an identical complex from surface-labeled Vγ1-expressing hybridoma cells. Lysate from ^{125}I-labeled cells was immunoprecipitated with 2.11 or 3A10 mAbs and subjected to SDS-PAGE under reducing conditions. 2.11 immunoprecipitate (lane *1*), 3A10 immunoprecipitate (lane *2*). Molecular weight markers are indicated in kilodaltons.

	Vγ1	P	N	P	Jγ4
germline Vγ1	TGT GCA GTC TGG ATA AA	TT			
germline Jγ4				GA	TCA GGC ACA
A					
T3.13.1	TGT GCA GTC TGG				TCA GGC ACA
T4.7.7	TGT GCA GTC TGG ATA AA		GCTC		GGC ACA
T3.23.2	TGT GCA GTC TGG ATA AA		AAGGG	GA	TCA GGC ACA
S3.11.2	TGT GCA GTC TGG ATA		C		CA GGC ACA
T3.41.13	TGT GCA GTC TGG ATA		GGA		GGC ACA
T4.14.1	TGT GCA GTC TGG		CCCC	GA	TCA GGC ACA
S3.17.8	TGT GCA GTC TGG AT		GCA	A	TCA GGC ACA
T4.36.6	TGT GCA GTC TGG A		CCCCTTC	A	TCA GGC ACA
T3.16.1	TGT GCA GTC TGG AT		CC	GA	TCA GGC ACA
S3.29.2	TGT GCA GTC TGG A			GA	TCA GGC ACA
T3.18.9	TGT GCA GTC TGG AT		CAG		A GGC ACA
T3.20.17	TGT GCA GTC TGG		GGG		GGC ACA
T4.41.6	TGT GCA GTC TGG		CCCC	GA	TCA GGC ACA
T4.8.1	TGT GCA GTC TGG		CC	GA	TCA GGC ACA
B					
BTC 1	TGT GCA GTC TGG		GC	A	TCA GGC ACA
BTC 7	TGT GCA GTC TGG AT		T		GGC ACA
BTC 54	TGT GCA GTC TGG AT		TA		GC ACA
BTC 57	TGT GCA GTC TGG		G	GA	TCA GGC ACA
BTC 58	TGT GCA GTC TGG		GTC		TCA GGC ACA
BTC 59	TGT GCA GTC TGG AT		T		TCA GGC ACA
BTC 60	TGT GCA GTC TGG AT		GG		CA GGC ACA
BTC 63	TGT GCA GTC TGG A		AGGGG		GGC ACA
BTC 66	TGT GCA GTC TGG ATA AA	T			TCA GGC ACA
BTC 78	TGT GCA GTC TGG		GT	A	TCA GGC ACA

Figure 2. Vγ1-Jγ4 junctional sequences of 2.11$^+$ T cell hybridomas. DNA (*A*) or cDNA (*B*) was amplified by PCR with Vγ1-Jγ4 or Vγ1-Cγ primers, respectively. The products obtained were directly sequenced as described in Materials and Methods. Hybridomas in *A* were derived from adult thymocytes (*T*) and splenocytes (*S*) activated in vitro with PPD. Hybridomas in *B* were derived from γ/δ thymocytes activated in vitro with the 2.11 mAb. *P*, palindromic nucleotides (54); *N*, nucleotides not present in the germline sequence.

allow us to exclude the possibility that it also recognizes the Vγ2-Cγ2 protein. However, three additional lines of evidence reinforce the conclusion that the 2.11 mAb is specific for the Vγ1-Cγ4 protein. (*a*) 2.11$^+$ hybridomas obtained by fusion of BW5147 cells with CD4$^-$CD8$^-$ thymocytes that had been activated in vitro with the 2.11 mAb all expressed a functionally rearranged Vγ1-Cγ4 mRNA (Fig. 2 *B*), whereas they each expressed one of five different Vδ genes (not shown). (*b*) Immunoprecipitation of the γ/δ TCR by the 2.11 mAb performed with four different γ/δ T cell hybridomas showed a 43-kD γ chain compatible with the Vγ1 chain but incompatible with the Vγ2 chain (1). (*c*) Expression of functionally rearranged Vγ1-Cγ4 mRNA, but not of Vγ2-Cγ2 mRNA, correlates with the 2.11$^+$ phenotype in sorted 2.11$^+$ and 2.11$^-$ γ/δ T cell populations (Pereira, P., D. Gerber, A. Regnault, V. Hermitte, A. Coutinho, and S. Tonegawa, manuscript submitted for publication). Taken together, these data strongly suggest that the 2.11 mAb recognizes only the product of the Vγ1-Cγ4 gene.

Ontogeny of γ/δ T Cells Expressing the Vγ1-Cγ4 Gene Product in the Thymus. Using the 2.11 mAb, we quantitated the number of Vγ1-bearing γ/δ thymocytes in B6 mice as a function of age. For comparison, we also determined the number of γ/δ thymocytes expressing the Vγ4 or the Vγ5 chains using the respective antibodies that had previously been produced (1, 26). The results are summarized in Fig. 3. In agreement with previous results (26), Vγ5-expressing thymocytes constituted the major γ/δ T cell population at day 15 of gestation, but their number decreased thereafter until the first week after birth, when they became nearly undetectable. Vγ4-expressing thymocytes also appeared in fetal life, and their number increased until 1 wk of age, when they represented ~60% of all γ/δ thymocytes. Thereafter, the number of Vγ4$^+$ thymocytes decreased to adult levels

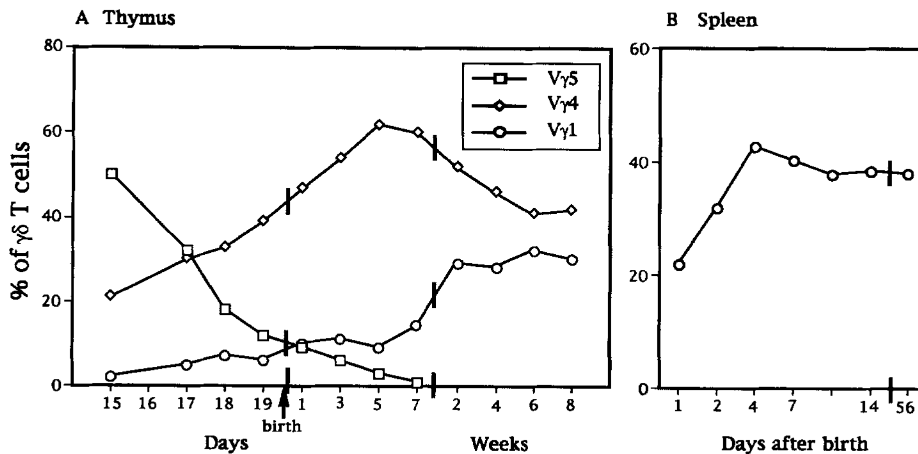

Figure 3. Differential expression of γ/δ chains on thymocytes and splenocytes of C57BL/6 mice of different ages. Cells were stained with FITC-labeled anti-Cδ mAb (3A10) and biotin-labeled anti-Vγ1 (2.11), anti-Vγ5/Vδ1 (536), and anti-Vγ4 (UC3-10A6) mAbs followed by streptavidin PE. 2,000–5,000 γ/δ^+ cells per sample were analyzed with a FACScan®. The percentages of 2.11^+ (○), 536^+ (□), and UC3-10A6$^+$ (◇) cells among 3A10$^+$ cells are shown. Cell pools were always used for the analysis, and each point represents the mean of at least two independent determinations.

(~45% of all γ/δ thymocytes). Vγ1-expressing cells constituted a very minor population during fetal and early postnatal life. Their number increased rapidly 1–3 wk after birth, concomitant with the decrease of Vγ4-expressing thymocytes, and quickly reached the adult levels of around one-third of all γ/δ thymocytes (Fig. 3 *A*).

In spite of the very low numbers of Vγ1-expressing cells in the fetal thymus, these cells are detectable in the spleen at birth, constituting ~20% of all γ/δ T cells in that organ. Here again, their numbers increased very rapidly, and by 1 wk of age they reached the adult level, which is ~40% of all γ/δ splenic T cells in B6 mice (Fig. 3 *B*).

Tissue Distribution of Vγ1-Cγ4–expressing γ/δ T Cells. We then quantitated the proportion of Vγ1-bearing γ/δ T cells in the peripheral lymphoid organs and in different epithelia in adult B6, BALB/c, and C3H mice. The results are summarized in Table 1. As can be seen, Vγ1-bearing cells represent 30–50% of all γ/δ T cells in the spleen and lymph nodes of the three strains studied. Given that Vγ4-bearing cells also represent around half of the γ/δ T cells in the peripheral lymphoid organs in these mouse strains (1, 34), these data show that Vγ1- and Vγ4-bearing cells comprise most of the spleen and lymph node γ/δ T cells.

Table 1. *Expression of Vγ1-Cγ4$^+$ γ/δ T Cells in Different Tissues*

	2.11^+ cells (% of total γ/δ T cells) Strain		
Tissue	C57BL/6	BALB/c	C3H/HeJ
Spleen	34.3 ± 5.3	40.4 ± 3.4	55.4 ± 5.2
LN	38.9 ± 4.4	32.3 ± 6.0	54 ± 5.4
i-IEL	24 ± 3.1	21.4 ± 4.9	36 ± 8.3
DEC	0.7	1.4	1.6

Cells were stained and analyzed as in Fig. 2. At least three determinations were performed except for DEC, for which the average of two experiments is given. A pool of two to five mice was used for each determination.

Although γ/δ T cell clones expressing a TCR composed of Vγ1 and Vδ6 chains have been isolated from the skin epithelium (18), we found that no more than 2% of the γ/δ-bearing DEC cells express the Vγ1-Cγ4 gene product in any of the three strains examined (B6, BALB/c, and C3H).

We also found that close to 30% of the γ/δ i-IEL in B6 mice were recognized by the 2.11 mAb and were thus likely to express the Vγ1 gene product. This observation was surprising because previous reports analyzing mRNA expression of Vγ genes in i-IEL had led to the belief that Vγ1-bearing cells are at most a small minority of γ/δ i-IEL. Stainings of B6 i-IEL with the anti-pan γ/δ mAb 3A10 together with either the 2.11 mAb or an anti-Vδ4 mAb are shown in Fig. 4. The anti-Vδ4 mAb recognizes a fraction of the γ/δ i-IEL that is mainly included in the Vγ7$^+$ population (35). In this particular experiment, the Vγ1-bearing cells represent 28% of the γ/δ i-IEL population. It is worth noting that the level of TCR expression of Vγ1 i-IEL is significantly lower than that of other γ/δ TCR$^+$ i-IEL (compare gate *A* in both stainings). This has been consistently observed in many individual mice and in all three strains. We conclude that Vγ1$^+$ cells comprise a significant population of γ/δ i-IEL.

Vγ1-Cγ4–expressing i-IEL Originate Outside the Thymus. γ/δ i-IEL, as is the case for many α/β i-IEL, have been shown to have a thymus-independent origin, as evidenced by their presence in normal numbers in athymic nude mice and in thymectomized, lethally irradiated, bone marrow–reconstituted chimeras (36–38). Furthermore, thymus-independent γ/δ cells are phenotypically different from thymus dependent, peripheral γ/δ T cells. Thus, whereas most peripheral γ/δ T cells do not express CD4 or CD8 molecules, most of the γ/δ i-IEL express the α but not the β chain of the CD8 molecule (39). Furthermore, although all peripheral γ/δ T cells express the Thy1 and CD5 molecules, only 30–50% of the γ/δ i-IEL express Thy1, and all of them are negative for the expression of the CD5 antigen (40–42). Given the possibility that the Vγ1-bearing i-IEL population could arise from thymus-dependent peripheral Vγ1 cells through migration to the intestinal epithelium, we quantitated the Vγ1-expressing cells in i-IEL isolated from athymic nude mice and analyzed the phenotype of Vγ1-bearing

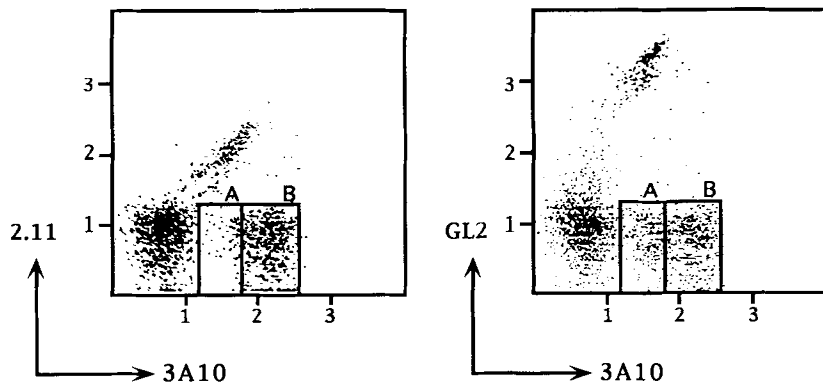

Figure 4. Expression of Vγ1 and Vδ4 in i-IEL from C57BL/6 mice. i-IEL were labeled with 3A10 FITC and either 2.11– or GL4–biotin and analyzed as described in Materials and Methods. Gates *A* and *B* denote TCR^{low} and TCR^{high} γ/δ T cell populations. The fractions of 2.11^+ and GL-2^+ cells among $3A10^+$ i-IEL were 28 and 24%, respectively.

cells in i-IEL preparations isolated from normal animals. The percentage of γ/δ i-IEL marked by the 2.11 mAb in normal and nude B6, BALB/c, and C3H mouse strains is shown in Table 2. As can be seen, the proportion of Vγ1-bearing cells among γ/δ i-IEL was very similar in normal and nude mice of all strains examined.

The expression of Thy1, CD5, CD8α, and Cd8β among total γ/δ TCR^+ or 2.11^+ i-IEL in B6 mice is shown in Table 3. In concordance with previous observations (39, 41, 42), virtually all the cells stained with the anti-δ mAb were negative for the expression of CD5 and CD8β, whereas 80–90% of them expressed the α chain of the CD8 molecule, and close to 40% of them expressed the Thy1 antigen. The 2.11^+ population displayed a very similar phenotype with regard to these markers, with most of the cells being $CD5^-$-$CD8\alpha^+\beta^-$. The only significant difference found between total γ/δ^+ and 2.11^+ i-IEL was the proportion of $Thy1^+$ cells (38 and 50%, respectively). As $V\gamma1^+$ cells represented ~25% of the total γ/δ i-IEL in these experiments, these data imply that the Thy1 antigen is expressed in 50% of the $V\gamma1^+$ cells and in 20% of the $V\gamma1^-$ γ/δ T cell population.

Normal i-IEL are constitutively cytolytic in a redirected lysis assay (6, 43). This assay uses an Fc receptor–expressing target cell and relies on the cross-linking of TCR on effector cells by mAbs against the TCR–CD3 complex that also bind to the Fc receptor to trigger lysis. B6 i-IEL lyse P815 target cells in the presence of the 2.11 mAb (data not shown), indicating that the $V\gamma1^+$ i-IEL population is constitutively cytolytic.

From these experiments we conclude that, similar to the $V\gamma7^+$ i-IEL population, $V\gamma1^+$ i-IEL also mature extrathymically, and that these γ/δ T cell populations are phenotypically and functionally similar.

The Proportion of $V\gamma1^+$ Cells among γ/δ i-IEL Is Independent of the Antigenic Load of the Intestine. The presence of Vγ1 mRNA in the i-IEL population of some individual mice or in mice infected with the parasite *Eimeria* has been reported (44, 45), leading to the suggestion that colonization of the gut could result in antigen-driven accumulation or expansion of a few incipient $V\gamma1^+$ cells. To study the effect of microbial colonization and/or food antigens on the extent of Vγ1-bearing cells among γ/δ i-IEL, we quantitated the proportion of $V\gamma1^+$ cells in i-IEL isolated from SPF and AgF BALB/c mice. The results of one such experiment are shown in Table 4. As can be seen, the proportion of Vγ1-bearing i-IEL was virtually identical in SPF and AgF BALB/c mice. These experiments demonstrate that the presence of the Vγ1-bearing population in the epithelium of the small intestine is independent of the antigenic load of the gut epithelium.

Expression of Vγ1-Cγ4 i-IEL in Different Mouse Strains. We then quantitated the proportion of Vγ1-bearing lymphocytes in the i-IEL population of 12 different strains of mice, including some MHC-congenic strains in different backgrounds. The results are summarized in Fig. 5. Several conclusions can be drawn from this analysis. First, all strains

Table 2. *Expression of $V\gamma1^+$ i-IEL in Euthymic and Athymic (nu/nu) Mice*

	2.11^+ cells (% of total γ/δ T cells)
BALB/c (nu/+)	26.1
BALB/c (nu/nu)	23.4
C57BL/6 (nu/+)	24.5
C57BL/6 (nu/nu)	29.2
C3H (nu/+)	46.1
C3H (nu/nu)	39.8

Cells were stained and analyzed as in Fig. 3. The average of two determinations is given. A pool of two to three mice was used for each determination.

Table 3. *Phenotype of $V\gamma1^+$ i-IEL*

	Percentage of cells expressing			
i-IEL population	Thy1	CD8α	CD8β	CD5
γ/δ^+ ($3A10^+$)	38.6; 38.0	89.9; 86.6	1.6; 1.8	<1
$V\gamma1^+$ (2.11^+)	50.8; 50.0	79.0; 81.9	1.0; 1.4	<1

C57BL/6 IEL were stained with mAbs against the indicated antigen and counterstained with either anti-δ (3A10) or anti-Vγ1 (2.11) mAbs. Numbers represent the percentage of cells expressing the defined antigen among total γ/δ cells (*upper*) or 2.11^+ cells (*lower*). Results from two independent experiments are shown.

Table 4. *Expression of Vγ1$^+$ i-IEL in SPF and AgF BALB/c mice*

	2.11$^+$ Cells (percentage of total γ/δ T cells)
BALB/c SPF	18.1
BALB/c AgF	19.2

Cells were stained and analyzed as in Fig. 3. The average of two determinations is given. Three mice were used for each determination.

tested contained a sizable proportion of Vγ1-bearing γ/δ i-IEL that ranged from 15 to 60% of all γ/δ-bearing i-IEL. Second, the representation of Vγ1-bearing lymphocytes among γ/δ i-IEL seems to be strain specific. It should be pointed out that some of the data presented were obtained using identical strains of mice that were acquired from different sources and housed in different places. The low standard deviation observed within a single strain suggests that genetic rather than environmental factors are responsible for the observed strain-to-strain differences in the proportion of Vγ1-bearing lymphocytes. Third, although differences in the percentage of γ/δ i-IEL expressing the Vγ1 gene product are found between MHC-congenic strains, these differences are relatively small when compared with those found between strains that differ genetically outside of the MHC locus. These data suggest that genes other than MHC genes are involved in the regulation of the number of Vγ1-bearing cells.

Discussion

In this report we describe a new mAb specific for the Vγ1-Cγ4 chain of the γ/δ TCR. The use of this mAb allowed us to quantitate and characterize Vγ1-expessing γ/δ T cells in the thymus of fetal and adult animals, as well as in the peripheral lymphoid organs and in several epithelia. Our data, while confirming some previously drawn conclusions, revealed a few new facts about the composition of various γ/δ T cell subsets in developing and adult mice.

Vγ1-bearing cells constitute a small minority of the γ/δ thymocytes throughout fetal life. Their number increases substantially during the first weeks of postnatal life, concomitant with a decrease in the number of Vγ4-bearing thymocytes. By 3 wk of age, the Vγ1 and Vγ4 subpopulations reach their adult values of ~30 and 50% of the total γ/δ thymocytes, respectively. At that time, Vγ5-bearing cells are virtually absent, and Vγ7-bearing thymocytes represent no more than 5% of total adult γ/δ thymocytes (46; Pereira, P., unpublished observations). Furthermore, although functional rearrangements of Vγ6 and Vγ2 genes have been described in the adult thymus (47), it is believed that γ/δ thymocytes expressing these γ chains constitute a small minority among the total γ/δ thymocytes. Although no other Vγ genes have been identified, a possibility that some minor populations of γ/δ T cells express hitherto unidentified TCR-γ chains cannot be excluded. Taken together, these data are consistent with the possibility that, at least in B6 mice, other Vγ genes could be expressed in the adult thymus.

In the spleen and lymph nodes, Vγ1- and Vγ4-bearing cells are also the major subpopulations of the γ/δ T cell population, constituting close to 90% of all γ/δ lymphocytes in these peripheral lymphoid organs. The actual proportion of Vγ1- and Vγ4-expressing cells varies in different strains of mice. Nevertheless, they constitute the large majority of the splenic and lymph node γ/δ T cells in most of the common laboratory strains (34; Pereira, P., unpublished observations). Thus, it appears that the proportions of the two γ/δ T cell subsets are coordinated; a decrease in the frequency of cells expressing one of these Vγ chains is accompanied by a corresponding increase in the frequency of cells expressing the other Vγ chain. A similar situation can be observed concerning the expression of Vγ1 and Vγ7 gene products within the γ/δ i-IEL population. Together, Vγ1- and Vγ7-bearing cells represent close to 90% of the γ/δ i-IEL population, and their relative frequencies in different strains of mice are also coordinated (Fig. 5; Pereira, P., unpublished observations). This is particularly important to consider in the context of positive and/or negative selection for γ/δ T cells. It has been proposed that the expansion or overrepresentation of T cells using a particular γ/δ receptor could be used as an indication that positive selection has occurred (34, 38). Although such increased representation of a specific γ/δ T cell type may suggest that cellular selection has occurred, the fact that the overrepresentation of T cells using a defined Vγ chain is concomitant to a similar underrepresentation of T cells using another Vγ chain precludes definition of the selection as either positive or negative or both.

Although Vγ1-bearing T cells have been isolated from the skin of C3H mice (18), our data show that Vγ1-bear-

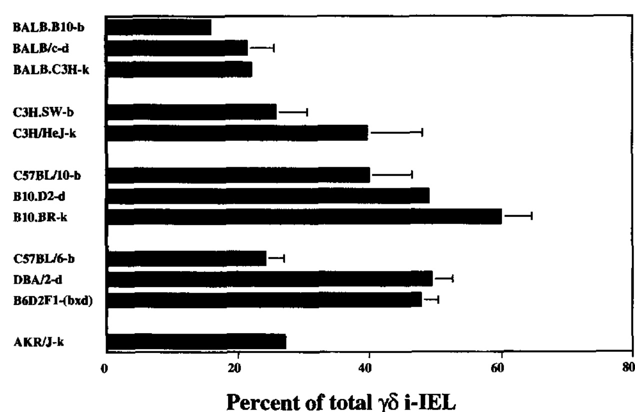

Figure 5. Analysis of Vγ1 usage in inbred mice. i-IEL from the indicated mouse strains were analyzed for 2.11 and 3A10 expression by fluorescence flow cytometry. Data are shown as the percentage of 3A10$^+$ cells that are 2.11$^+$. When standard deviations are shown, at least three determinations were performed; otherwise the mean of two experiments is given. Two to five mice were used for each determination. In one experiment, five B6 mice were analyzed individually. The small letter after each strain denotes the MHC haplotype.

ing cells constitute a minor population (0.5–2%) of total γ/δ DEC in that strain as well as in the other strains tested. On the other hand, it has been shown that the skin of nude mice is colonized by thymus-independent Vγ1-bearing γ/δ T cells (48), demonstrating that these cells can home to the epithelium of the skin. Furthermore, recent analysis of IL-7–transgenic mice that spontaneously develop dermatitis (49) has shown a massive infiltration or expansion of γ/δ T cells other than Vγ5-bearing cells in the skin. It is possible, therefore, that the representation of Vγ1-bearing cells in the skin epithelium is increased upon infection and could, therefore, vary in different mouse colonies.

The most striking finding concerning the tissue localization of γ/δ T cells expressing the Vγ1-Cγ4 chain is that they constitute a substantial proportion of γ/δ i-IEL in normal mice. Vγ1$^+$ cells represent 15–60% of all γ/δ i-IEL in different strains of mice, and their presence is independent of the thymus and of the antigenic load of the gut. The fact that this population has been overlooked is not readily understood, although some plausible explanations can be put forward. To date, four different groups have analyzed TCR-γ rearrangement or TCR-γ mRNA expression in i-IEL, reaching the conclusion that the Vγ1-bearing population is minor, if not absent, in the normal γ/δ i-IEL population. The first group (50) did not analyze Vγ1 rearrangements in i-IEL because their previous work (7) had shown that the major γ protein expressed in i-IEL had a relative molecular mass of 34–35 kD, which is clearly different from the Vγ1-Cγ4 protein (M_r = 41–42 kD [1]). Besides the fact that the immunoprecipitation technique is not very sensitive and is certainly not quantitative (because of the differential labeling rates of different proteins and the specificity of the antisera used), it should also be noted that the size of the Vγ1-Cγ4 chain is very similar to the size of most of the Vδ-Cδ proteins. Thus, it is possible that the δ chain band in the gels masked the Vγ1-Cγ4 protein.

Two other groups (51, 52) analyzed TCR-γ rearrangements in i-IEL by PCR techniques using Vγ- and Jγ-specific primers. To analyze Vγ1 and Vγ2 rearrangements, they used an identical set of primers. The Vγ-specific primer cross-hybridizes with Vγ1 and Vγ2 genes, whereas the Jγ-specific primer cross-hybridizes with all four Jγ sequences. A closer look at the primers used reveals that the Vγ primer has a sequence identical to the Vγ2 gene but contains a single mismatch with respect to the Vγ1 sequence in the second base at its 3′ end. Similarly, the Jγ primer has a sequence identical to the Jγ1 and Jγ2 segments but has two mismatches with respect to the Jγ4 segment, one of which is located in the fourth base of its 3′ end. The Vγ2 gene appears to be rearranged in almost all γ/δ T cells and in many α/β T cells (Pereira, P., D. Gerber, A. Regnault, V. Hermitte, A. Coutinho, and S. Tonegawa, manuscript submitted for publication), whereas, with the exception of a small number of other γ/δ T cells, the Vγ1 gene is almost exclusively rearranged in cells expressing the Vγ1-Cγ4 protein. Thus, it seems very likely that, in the PCR conditions used by these authors, the Vγ2-Jγ2 rearrangements would be preferentially amplified over the Vγ1-Jγ4 rearrangements. Finally, the fourth group analyzed the expression of Vγ mRNA in i-IEL by in situ hybridization using antisense probes but failed to detect any cells expressing either Vγ1 or Vγ2 mRNA (53). Further experiments conducted by these authors were limited by the preparation of cDNAs with a Cγ1-specific primer and subsequent amplification of the cDNA with primers specific for the Vγ4, Vγ6, and Vγ7 genes, thereby excluding Vγ1-Cγ4 and Vγ2-Cγ2 from the analysis. As this report shows no positive control for the hybridization of any of the Vγ-specific probes, it is possible that their failure to detect Vγ1 and Vγ2 mRNA simply reflects the low sensitivity of the in situ hybridization technique.

We thus believe that the normal γ/δ i-IEL population contains a relatively high frequency of cells expressing the Vγ1-Cγ4 gene product. This frequency is comparable to the frequency of cells expressing the Vγ7-Cγ1 chain. The frequency of the Vγ1$^+$ i-IEL varies in different strains of mice but appears to be quite constant among different individuals of the same strain, even when mice are housed in different colonies. This suggests that genetic factors, rather than environmental factors, are primarily responsible for the determination of Vγ1$^+$ i-IEL frequency.

A striking characteristic of γ/δ T cell subsets is the rather strict correlation among their onset of appearance in development, the Vγ (and Vδ) genes used to encode the TCR, and their homing to distinct peripheral sites. Thus, Vγ5- and Vγ6-expressing cells are primarily, if not exclusively, produced early in the fetal thymus, and home to the epithelium of skin and of uterus, vagina, and tongue, respectively (9, 26, 30, 54–57). Vγ4-bearing cells appear later in the fetal thymus, constituting the major γ/δ thymocyte population at birth, and preferentially home to the peripheral lymphoid organs (1, 2, 33). Vγ7-bearing cells, on the other hand, develop from precursors that do not require a thymus for maturation and home to the intestinal epithelium (36–38, 50, 51, 53). Until this study, only a few exceptions to these rules were observed. Thus, ~2% of the γ/δ thymocytes bear the Vγ7 protein (46), and ~5% of γ/δ i-IEL express the Vγ4 chain in all mouse strains examined, including athymic mice (Pereira, P., unpublished observations). In clear contrast to the other γ/δ T cell subsets, the Vγ1-bearing γ/δ T cells seem to develop and home in a more diversified fashion. Thus, they can develop in the thymus and migrate to the peripheral lymphoid organs, where, together with the Vγ4 subset, they represent the vast majority of the γ/δ T cells. On the other hand, Vγ1-bearing cells can also develop extrathymically and constitute, together with the Vγ7 subset, the large majority of γ/δ i-IEL. Thus, the ability to rearrange and express the Vγ1 gene is not limited to a particular γ/δ T cell precursor, nor is the differentiation of Vγ1-bearing γ/δ T cells restricted to a defined milieu. Thymus-independent Vγ1-bearing cells have also been found in the skin of nude mice (48), and CD8α$^+$β$^-$ γ/δ T cells, which are believed to have a thymus-independent origin, have been found in the liver of normal mice (58).

The presence of Vγ1-bearing cells in lymphoid organs and epithelia might be related to their specificity. A large

fraction of Vγ1 cells appears to recognize an endogenous antigen expressed by lymphocytes (11). These cells can be further stimulated in the presence of PPD and HSP proteins or peptides (11, 13), which led to the suggestion that they might recognize autologous stress proteins (57, 59). In some instances, proliferation or accumulation of Vγ1-bearing cells has been shown to occur in vivo after infection of mice with different bacteria or parasites, suggesting a physiological role for those cells in the defense against pathogens (45, 60, 61). A similar reactivity has also been described for a subset of human γ/δ T cells that express the Vγ9 gene product. Cells of this type respond to mycobacterial extracts (and in some cases to mycobacterial and endogenous HSPs) and recognize an undefined antigen on the surface of some tumor cell lines of lymphoid origin (62–70). Cells with these specificities are restricted in the use of Vγ and Vδ genes, but there is considerable diversity in the junctional sequences of their TCR genes (66, 71–74). As in the case of Vγ1 cells in the mouse, Vγ9 cells in humans appear late in ontogeny, but compose a major γ/δ T cell population in adult individuals (75, 76). This parallelism suggests a precise and important function of these cells. A precise definition of the ligands recognized by these cells will certainly help us to understand their physiological role.

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