

## DISTINCT MOLECULAR FORMS OF HUMAN T CELL RECEPTOR $\gamma/\delta$ DETECTED ON VIABLE T CELLS BY A MONOCLONAL ANTIBODY

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It is now clear that there are two types of T lymphocytes. Classical T cells recognize non-self antigens in the context of molecules of the MHC by means of the TCR, consisting of disulphide-linked  $\alpha$  and  $\beta$  chains (1–3). A recently defined type of T cells, of which it is unclear what they recognize, uses a TCR encompassing the  $\gamma$  chain (4, 5). All three chains,  $\alpha$ ,  $\beta$ , and  $\gamma$ , have a variable and a constant domain, encoded in different gene segments that join through rearrangement during T cell ontogeny (6–8). As compared with TCR- $\alpha$  and  $\beta$ -genes, the germline diversity of the TCR- $\gamma$  gene is more limited (9). The human genome contains 11 variable, 5 joining, and 2 constant (C) gene segments in tandem (10–12). The  $\gamma$  gene is rearranged and expressed at an early stage in T cell development, coinciding with or preceding rearrangement and expression of respectively TCR- $\beta$  and TCR- $\alpha$  genes (13–15). The TCR- $\gamma$  chain forms either disulphide-linked or non-disulphide-linked dimers with the so-called  $\delta$  protein, which is structurally distinct from  $\gamma$  (16). The gene encoding this protein is positioned in the TCR- $\alpha$  locus (17) and thus organized in a similar fashion as  $\alpha$ ,  $\beta$ , and  $\gamma$  genes, but its exact genomic organization has not yet been unravelled. Before cell surface expression, either type of TCR associates with the CD3 protein complex, consisting of four invariant subunits (18, 19), which may play a role in signal transduction (20).

Upon in vitro activation, TCR- $\gamma/\delta$ -expressing cells can exert MHC nonrestricted cytolytic activity towards allogeneic cells (21–23). In this and other functional aspects, TCR- $\gamma/\delta^+$  cells share features with CD3<sup>-</sup> NK cells. On the other hand, they share features with CD3<sup>+</sup>/TCR- $\alpha/\beta^+$  MHC-restricted cells in the expression of CD3 and a potentially variable receptor.

The existence of the TCR- $\gamma$  protein has first been demonstrated biochemically

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using antipeptide sera, reactive with denatured protein. It was found in peripheral blood (PB)<sup>1</sup> of normal individuals on cells derived from a small subset of CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> cells (4, 21, 24) and on CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> thymocytes in both mouse and man (5, 25), which represent a small population in adults. Human TCR- $\gamma/\delta$  has first been identified on CD3<sup>+</sup> lymphocytes, which failed to react with the mAb WT31 (26), thought to recognize a common determinant on TCR- $\alpha/\beta$ . Thus, TCR- $\gamma/\delta$ <sup>+</sup> cells may be identified using CD3 expression and lack of reactivity with WT31 mAb as criteria. However, it was important to make antibodies that positively identify TCR- $\gamma/\delta$ -expressing cells, particularly since we have recently found that TCR- $\gamma/\delta$ <sup>+</sup> cells may bind WT31 mAb under certain conditions (26a). We here report an mAb, which reacts with human TCR- $\gamma/\delta$  in native as well as denatured states. This is in contrast to previously reported antipeptide sera, which exclusively detected TCR- $\gamma/\delta$  after denaturation. With this reagent one can assess TCR- $\gamma/\delta$  expression in lymphoid tissues and blood, isolate TCR- $\gamma/\delta$  expressing cells, manipulate their function and use it for isolation of the protein: studies needed to elucidate the role TCR- $\gamma/\delta$ <sup>+</sup> cells play in the immune system.

### Materials and Methods

**Cells.** PB samples were derived from healthy volunteers or from children hospitalized for afflictions other than infectious diseases or disorders of the immune system. Thymocyte samples were from children undergoing cardiac surgery and from an 18-wk-old human fetus (abortion on medical indication). These human samples were obtained with permission of the Committee of Medical Ethics of the Hospital Dijkzigt, Erasmus University, Rotterdam, The Netherlands. PBL were isolated from blood by density separation using Ficoll-Hypaque. Cloned lymphocytes were generated from PB of normal individuals in the case of AK 4, AK 781 (donor X; 22), 1012 (donor Y) and JS-93,-132,-228 (donor JS; 27), from pleura exudate of a patient with a mamma carcinoma in the case of AK 119, from PB of a patient with T $\gamma$  lymphocytosis in the case of NK 77 (28), and from PB of a patient with subacute sclerosing encephalitis in the case of Wi.K (29). Clones AK 4, AK 781 and AK 119 were derived from PBL directly by limiting dilution, clone 1012 was derived from CD4<sup>-</sup>8<sup>-</sup> sorted PBL of donor Y, clone JS-228 was derived from WT31<sup>-</sup> sorted PBL of donor JS. All clones were cultured in Yssel's medium (30), supplemented with 2% pooled human serum in round-bottom microwell plates (Greiner Labor Technik, Plidelsheim, FRG) in the presence of PHA, irradiated allogeneic PBL and EBV-transformed B cells of the line JY (feeder cells), as described in detail elsewhere (31). The JY line (HLA-A2,2; B7,7; DR4,w6) was cultured in Yssel's medium supplemented with 2% pooled human serum. The human T leukemic cell line PEER (32), the Burkitt's B lymphoma cell line Daudi, the melanoma cell line MEWO, and the erythroid/myeloid cell line K562 were cultured in Iscove's medium containing 5% FCS under standard conditions.

**Antibodies.** The purified mAbs WT32 (IgG2a) (anti-CD3) and WT31 (IgG1) used for immunofluorescence, were kindly provided by Dr. W. Tax, Nijmegen, The Netherlands. Purified anti-CD3, -CD4, and -CD8 mAbs anti-Leu-4, -Leu-3a, and -Leu-2a, as well as phycoerythrin (PE)-conjugates of anti-Leu-4, -Leu-3a, -Leu-2a, and goat anti-mouse Ig were from Becton Dickinson & Co., Mountain View, CA. For immunoprecipitation of CD3/TCR complexes the anti-CD3 mAb 2G3 (IgG1) was used, which was a kind gift from Dr. Chang Geanwu, Dr. L. Willems Instituut, Diepenbeek, Belgium. Anti-C $\gamma$  serum was raised in rabbits against a synthetic peptide encompassing amino acids 137-157 of the human TCR- $\gamma$  protein (4) and was kindly provided by Dr. J. G. Seidman, Harvard Medical School, Boston, MA.

<sup>1</sup> Abbreviations used in this paper: IPB, immunoprecipitation buffer; PB, peripheral blood.

*Immunization Protocol and Generation of Hybridomas.* For immunization, anti-CD3 immunoprecipitates were prepared as described below from a digitonin-solubilized mixture of cloned cells expressing disulphide-linked TCR- $\gamma/\delta$ . Antigen derived from  $50 \times 10^6$  cells in the form of protein A-Sepharose/anti-CD3/antigen complex was injected subcutaneously into BALB/c mice three times at 4–6-wk intervals. The fourth injection was done 7 wk after the third, directly into the spleen, using the same immunogen. The spleen was removed 4 d later. Spleen cells were fused to myeloma cells of the line SP2/0 at a ratio of 4:1 using polyethylene glycol. Hybridomas were seeded into 96-well microtiter plates (Costar, Cambridge, MA) at  $10^5$  spleen cell equivalents per well and grown and selected under standard conditions (hypoxanthin-aminopterin-thymidin selection).

*Enzyme-linked Immunosorbent Assay (ELISA).* ELISA was used for screening of reactivity of hybridoma supernatants with intact cells: cells were fixed in microwells using poly-L-lysine and 0.025% glutaraldehyde. Specific binding of mAbs to the cells was determined by incubation with biotinylated horse anti-mouse Ig, followed by incubation with biotinyl-peroxidase/avidin complex (Vector Inc., Burlingame, CA) and addition of 5-aminosalicylic acid as a substrate and  $H_2O_2$  as a catalyst.

*Immunofluorescence.* Hybridoma supernatants were screened by indirect immunofluorescence using FITC-conjugated goat anti-mouse F(ab) $_2$  Ig (Tago, Inc., Burlingame, CA) for staining and a fluorescence microscope for evaluation. For identification and phenotyping of TCR- $\gamma/\delta^+$  cells in PB and thymus of normal individuals described in Table IV, PBL were first incubated with WT31 or anti-TCR- $\gamma/\delta$ -1 mAb followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse Ig. Free binding sites of the second-step reagent were blocked with normal mouse serum and cells were incubated with FITC-conjugated mAb directed against CD3, CD4, or CD8 (Becton Dickinson & Co.). Samples were analyzed by fluorescence microscopy. For determination of the percentages CD3 $^+$ , WT31 $^+$ , and anti-TCR- $\gamma/\delta$ -1 $^+$  cells, 1,000 cells were inspected for each sample. For determination of the CD4/CD8 phenotype, 100 anti-TCR- $\gamma/\delta$ -1 $^+$  cells were inspected for each sample. As a control, normal mouse Ig was used as the first-step reagent. Other analyses were performed as indicated in the legends of the appropriate figures.

*Cytotoxicity Assays.* Assays were carried out in serum-free medium. Cytotoxicity was measured in triplicate as percentage of  $^{51}Cr$ -release in a 4-h assay using round-bottom microtiter plates (Greiner Labor Technik). Effector cells were incubated with anti-TCR- $\gamma/\delta$ -1 mAb for 1 h at 37°C before the assay at a dilution of ascites fluid of 1:100 for induction or inhibition of cytotoxicity. The antibody remained present during the assay at a dilution of ascites fluid of 1:200.

*Radiolabeling.* For cell surface radioiodination  $\sim 20 \times 10^6$  viable cells were washed with and resuspended in PBS, and labeled with 1–2 mCi  $Na^{125}I$  (Amersham Co., Amersham, United Kingdom) using 1,3,4,6 tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenyl glycoluril (Iodogen; Pierce Chemical Co., Rockford, IL) as a catalyst.

*Immunoprecipitation.* Cells were lysed in immunoprecipitation buffer (IPB), consisting of 0.01 M triethanolamine-HCl, pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, 0.02 mg/ml ovomucoid trypsin inhibitor, 1 mM TLCK, 1 mM TPCK, 0.02 mg/ml leupeptin, supplemented with either 1% digitonin or 1% NP-40 as detergents. Nuclear debris was removed by centrifugation for 15 min at 13,000 g. Lysates were centrifuged for 30 min at 100,000 g and precleared by three subsequent incubations with 30  $\mu$ l of a 10% vol/vol suspension of protein A-CL 4B Sepharose beads (Pharmacia, Uppsala, Sweden) coated with normal mouse Ig. Specific immunoprecipitation was carried out for 2–4 h with mAb, coupled covalently to protein A beads by means of dimethylpimelimidate-HCl (Pierce Chemical Co.). Beads were subsequently subjected to five washes in IPB with digitonin or NP-40 and samples were analyzed on 10–15% SDS-polyacrylamide gradient gels. Autoradiography was done using Kodak XAR-5 film in combination with intensifier screens (Cronex; Dupont Chemical Co., Newtown, CT).

*Electronmicroscopy.* PBL were obtained from a normal donor. One-half of the cell sample was incubated with 0.01 mg/ml purified WT31 mAb and one-half with a 1:250 dilution of anti-TCR- $\gamma/\delta$ -1 ascites fluid, followed by washing and incubation with purified

rabbit anti-mouse Ig. Next, cells were washed and labeled with protein A-colloidal gold probes (9 nm). Gold particles were prepared by the tannic acid/citrate reduction procedure (33) and complexed to protein A (Pharmacia). All incubations were performed for 30 min at 4°C in the presence of sodium azide. Subsequently, cells were washed with ice-cold medium and fixed in a mixture of paraformaldehyde (2% wt/vol) and glutaraldehyde (2.5% vol/vol) in 0.1 M sodium cacodylate, pH 7.4, for 24 h at 4°C. Cells were pelleted in 3% agar (Agar Noble, Difco Laboratories, Inc., Detroit, MI) at 80°C. After postfixation in OsO<sub>4</sub> (1% wt/vol) in cacodylate buffer for 2 h, slices of agar were rinsed, dehydrated in graded ethanol, and embedded in Epon 812 using a LKB Ultra Processor (LKB Produkter, Bromma, Sweden). Ultrathin sections (70 nm) were cut with an LKB-Nova ultramicrotome, contrasted with uranyl magnesium acetate and lead citrate staining and examined in a JEOL-1200 EX electron microscope (JEOL, Tokyo, Japan).

## Results

*Generation of TCR- $\gamma/\delta$ -specific mAb.* Human TCR- $\gamma/\delta$  proteins can be isolated in two ways. The intact CD3/TCR- $\gamma/\delta$  complex can be recovered with anti-CD3 mAb from cells solubilized in the mild detergent digitonin (34), or from cells solubilized after chemical crosslinking of TCR and CD3 components (35). In addition, rabbit antisera have been described raised against synthetic peptides encompassing TCR- $\gamma$  sequences (4, 36). Such sera only detect TCR- $\gamma$  protein after denaturation. To prepare mAbs, which would detect TCR- $\gamma/\delta$  proteins in cell lysates as well as on intact, viable cells, we chose to immunize mice with CD3/TCR- $\gamma/\delta$  complexes isolated from digitonin-solubilized cells. A mixture of clones, expressing disulphide-linked dimers of TCR- $\gamma$  and  $\delta$  chains, was used.

In the relevant fusion, Ig was detected by ELISA in 344 hybridoma culture supernatants. These supernatants were assayed for reactivity in immunofluorescence with viable cells of clone 1012, which expresses disulphide-linked TCR- $\gamma/\delta$ . Only one supernatant contained reactivity with clone 1012, while no fluorescence was observed with the TCR- $\alpha/\beta^+$  clone JS-132 or the EBV-transformed B cell line JY. This supernatant also specifically reacted with glutaraldehyde-fixed cells of clone 1012 in ELISA. This hybridoma was subcloned to give a stable clone, anti-TCR- $\gamma/\delta$ -1, which was used for further studies. The subclass of the mAb was IgG1.

*Reactivity of Anti-TCR- $\gamma/\delta$ -1 mAb with Viable Clones of Various Phenotypes.* To assess the specificity of anti-TCR- $\gamma/\delta$ -1 mAb, first its reactivity was tested in immunofluorescence with PBL of 18 normal donors. Positive PBL ranged from undetectable to 5% as analyzed with a FACS. Such percentages of reactivity would be expected for a TCR- $\gamma/\delta$ -specific mAb, given previous results with WT31 mAb, and the suggestion that predominantly the small CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> T cell subset would express TCR- $\gamma/\delta$  (37). Next, we determined reactivity in immunofluorescence of anti-TCR- $\gamma/\delta$ -1 mAb on a number of cloned cells of different phenotypes, representing different lymphocyte subsets and cells with different configurations of TCR- $\gamma/\delta$ : the NK clone NK 77 (CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup>), the nonlytic clone AK 781 (CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>/TCR- $\alpha/\beta^+$ ), the cytolytic clones JS-132 (CD3<sup>+</sup>4<sup>-</sup>8<sup>+</sup>/TCR- $\alpha/\beta^+$ , class I MHC specific) and JS-93 (CD3<sup>+</sup>4<sup>+</sup>8<sup>-</sup>/TCR- $\alpha/\beta^+$ , class II MHC specific), and the cytolytic clones Wi.K, JS-228, AK 119, and AK 4 (CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>, unknown specificity). It has been shown in immunoprecipitation experiments

using anti-C $\gamma$  peptide serum that the latter four clones express the TCR- $\gamma$  protein, be it in different configurations: non-disulphide-linked (Wi.K, JS-228) or disulphide-linked (AK 119, AK 4), and without (Wi.K, AK 119) or with (JS-228, AK 4) direct evidence for participation of a  $\delta$  chain in the receptor (see below). Also tested was the T leukemic cell line PEER, which was previously shown to express a non-disulphide-linked TCR- $\gamma$  chain of  $\sim$ 55 kD (24, 38), which is a different molecular mass than found for the TCR- $\gamma$  chains on Wi.K or JS-228 (see below).

As can be seen in Fig. 1, neither the CD3<sup>-</sup> NK clone, nor any of the TCR- $\alpha$ /

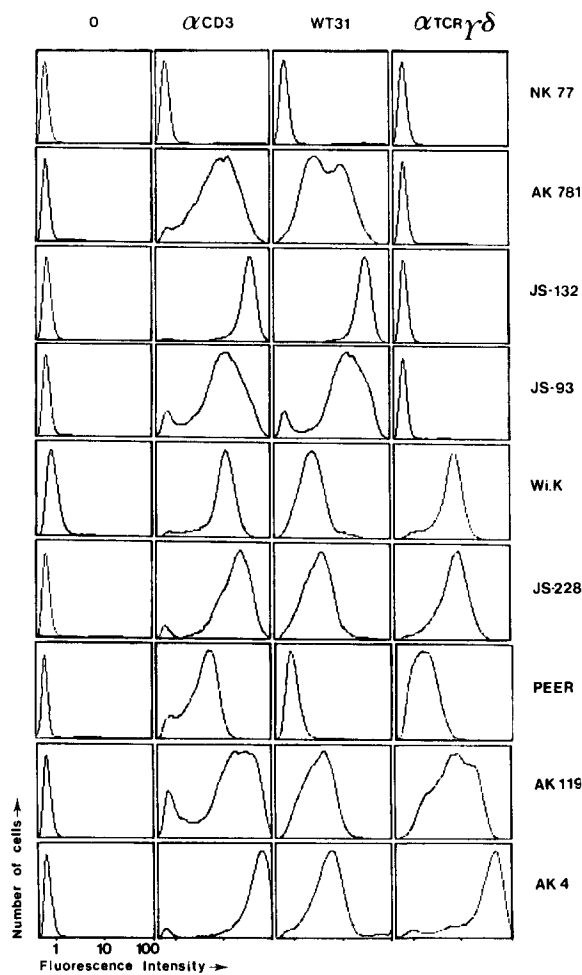


FIGURE 1. Immunofluorescence of various representative lymphocyte clones with anti-TCR- $\gamma$ / $\delta$ -1 mAb. Cells were incubated with 0.01 mg/ml purified WT32 (anti-CD3) or WT31 mAb, or with anti-TCR- $\gamma$ / $\delta$ -1 mAb at 1:500 dilution of ascites fluid, followed by incubation with FITC-conjugated goat anti-mouse F(ab)<sub>2</sub> Ig. Fluorescence analysis was performed using a FACS IV (Becton Dickinson & Co., Sunnyvale, CA). The characteristics of the cell samples used are indicated in the text. Fluorescence intensity is plotted on a logarithmic scale. For exact comparison, fluorescence indexes, defined as: [(fluorescence intensity sample) - (fluorescence intensity negative control)] / (fluorescence intensity negative control), are given:

	NK77	AK781	JS-132	JS-93	Wi.K	JS-228	PEER	AK119	AK4
Anti-CD3	0	15	49	20	8.2	11	4.5	31	59
WT31	0	9	42	22	1.3	1.5	0.3	4.4	7.8
Anti-TCR- $\gamma$	0	0.2	0.2	0.2	4	3.5	1.1	14	43

$\beta^+$  clones reacted with anti-TCR- $\gamma/\delta$ -1 mAb. In contrast, all TCR- $\gamma/\delta$ -expressing clones were positive. For comparison, fluorescence profiles obtained with anti-CD3 and WT31 mAbs are also shown. Fluorescence intensities are given in the legend of Fig. 1. Except NK 77, all clones expressed CD3. TCR- $\alpha/\beta^+$  clones reacted strongly with WT31 mAb, with intensities comparable to those obtained with anti-CD3 mAb. TCR- $\gamma/\delta^+$  clones also bound WT31 mAb, but stained at low intensities, while on these clones fluorescence intensities with anti-TCR- $\gamma/\delta$ -1 mAb were more comparable to those obtained with anti-CD3 mAb. These results clearly indicate that reactivity with anti-TCR- $\gamma/\delta$ -1 mAb, rather than lack of reactivity with WT31 mAb is a proper criterion for the identification of TCR- $\gamma/\delta$ -expressing cells.

*Anti-TCR- $\gamma/\delta$ -1 mAb Reacts with Different Molecular Species of TCR- $\gamma/\delta$  in Immunoprecipitation.* In contrast to TCR- $\alpha/\beta$ , TCR- $\gamma/\delta$  occurs in various molecular configurations. We have previously described (21) cytolytic clones such as AK 4, expressing a TCR consisting of a 40-kD  $\gamma$  chain disulphide linked to a 43-kD  $\delta$  chain. Clone 1012 and eight other clones derived from the same culture express a similar receptor (not shown). Clone AK 119 was an exception, in that it expressed a disulphide-linked receptor, including a differentially glycosylated  $\gamma$  chain migrating at 36 and 40 kD, while no  $\delta$  chain could be detected by cell surface iodination (21). Non-disulphide-linked TCR- $\gamma/\delta$  have been demonstrated on a growth factor-dependent cell line derived from PB of an immunodeficiency patient (4), a leukemic T cell line (24, 38), and PBL from a leukemic patient (39). We have derived several T cell clones expressing non-disulphide-linked TCR- $\gamma/\delta$  from PB of a normal individual, one of which was used in these studies (JS-228). In addition, a clone expressing a non-disulphide-linked receptor has been isolated from PB of a patient with autoimmune disease (clone Wi.K). Fig. 2 demonstrates the presence of the  $\gamma$  chain in the non-disulphide-linked CD3-associated receptors found on clones Wi.K (lanes A and B) and JS-228 (lanes D and E), which was identified by immunoprecipitation with anti-C $\gamma$  peptide serum (lanes C and F). The mol mass of the  $\gamma$  chain was  $\sim$ 41 kD for clone Wi.K and  $\sim$ 44 kD for clone JS-228. This in contrast to the  $\sim$ 55-kD mol mass of the  $\gamma$  chain found on the cell line PEER (lanes G, H, and I; references 24, 38). In clone JS-228 a  $\delta$  chain could be detected, migrating at 37 kD under nonreducing and 40 kD under reducing conditions (lanes D and E, arrows). In clone Wi.K a  $\delta$  chain could not be demonstrated by cell surface iodination. Table I summarizes the different molecular forms of TCR- $\gamma$  ( $\delta$ ).

To prove that anti-TCR- $\gamma/\delta$ -1 mAb reacted with TCR- $\gamma/\delta$ , we have carried out reprecipitation experiments. Anti-CD3 immunoprecipitates were prepared from digitonin solubilized,  $^{125}$ I-labeled TCR- $\gamma/\delta$ -expressing clones. Next, TCR- $\gamma/\delta$  was dissociated from CD3 by incubation of the immunoprecipitate in buffer with 1% NP-40 and 1% SDS, for 30 min at room temperature. The solubilized material was subjected to immunoprecipitation with anti-TCR- $\gamma/\delta$ -1 as detailed in the legend of Fig. 3. In Fig. 3 the CD3/TCR- $\gamma$  ( $\delta$ ) complexes as isolated by anti-CD3 mAb are shown, as well as TCR- $\gamma$  ( $\delta$ ) reprecipitated with anti-TCR- $\gamma/\delta$ -1 mAb. The antibody could be used to reprecipitate both types of disulphide-linked receptors from TCR- $\gamma$  ( $\delta$ ) $^+$  clones 1012 (lanes A and B) and AK 119 (lanes C, D, G, and H), as well as the non-disulphide-linked receptors from TCR- $\gamma$  ( $\delta$ ) $^+$  clones Wi.K (lanes E and F) and JS-228 (not shown).

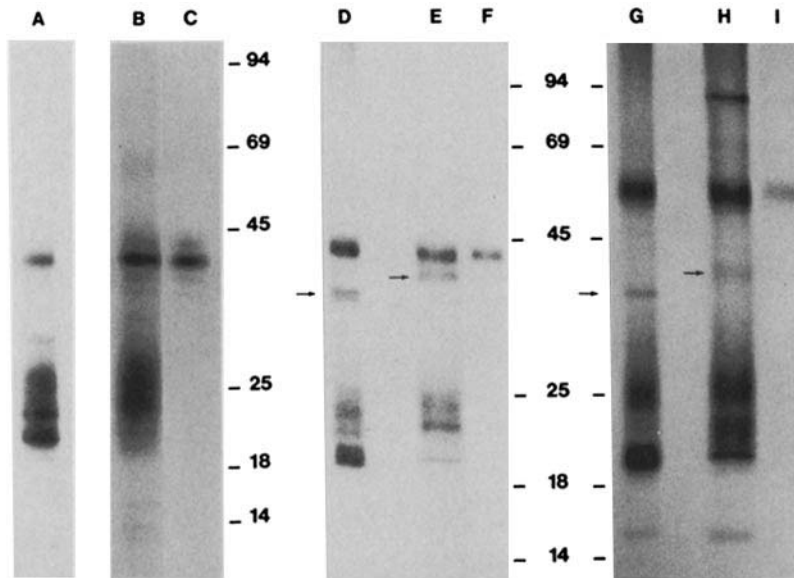


FIGURE 2. Characteristics of non-disulphide-linked TCR- $\gamma$  ( $\delta$ ). Cells were labeled with  $^{125}\text{I}$ , anti-CD3 immunoprecipitates were made from digitonin lysates and analyzed by SDS-PAGE under nonreducing (NR) or reducing (R) conditions. For reprecipitation of TCR- $\gamma$  chains, anti-CD3 precipitates were incubated in IPB with 1% SDS and 2 mM DTT for 5 min at 68°C. The supernatant was diluted five times with IPB containing 1.5% NP-40, 20 mM iodoacetamide and 25  $\mu\text{g}$  myoglobin, precleared once with normal mouse Ig-coated beads and subjected to immunoprecipitation with anti-C $\gamma$  serum. Arrows indicate the positions of the  $\delta$  chain. (A) Clone Wi.K, anti-CD3, NR; (B) clone Wi.K, anti-CD3, R; (C) clone Wi.K, anti-C $\gamma$ , R; (D) clone JS-228, anti-CD3, NR; (E) clone JS-228, anti-CD3, R; (F) clone JS-228, anti-C $\gamma$ , R; (G) PEER, anti-CD3, NR; (H) PEER, anti-CD3, R; (I) PEER, anti-C $\gamma$ , R.

Thus, anti-TCR- $\gamma/\delta$ -1 mAb reacts with all different types of TCR- $\gamma$  ( $\delta$ ) described thus far, in native state as present on intact viable cells, as well as in denatured state after detergent solubilization.

*Anti-TCR- $\gamma/\delta$ -1 mAb Affects the Function of Cytolytic Clones.* We have demonstrated previously (21, 22) that TCR- $\gamma$  ( $\delta$ )-expressing clones can exert cytolytic

TABLE I  
Molecular Forms of TCR  $\gamma(\delta)$

Clone	Receptor type	Interchain S-S bond	Mol mass of $\gamma$ chain	Mol mass of $\delta$ chain
			kD	kD
AK 119	$\gamma(\delta)$	+	$\sim 40 + \sim 36$	Not detected
AK 4	$\gamma \delta$	+	$\sim 40 (+ \sim 36)^*$	$\sim 43$
1012	$\gamma \delta$	+	$\sim 40 (+ \sim 36)$	$\sim 43$
Wi.K	$\gamma(\delta)$	—	$\sim 41$	Not detected
JS-228	$\gamma \delta$	—	$\sim 44$	$\sim 40$ (R), <sup>‡</sup> $\sim 37$ (NR) <sup>‡</sup>
PEER	$\gamma \delta$	—	$\sim 55$	$\sim 40$ (R), $\sim 37$ (NR)

\* Occasionally detected.

<sup>‡</sup> R, reducing conditions; NR, nonreducing conditions.

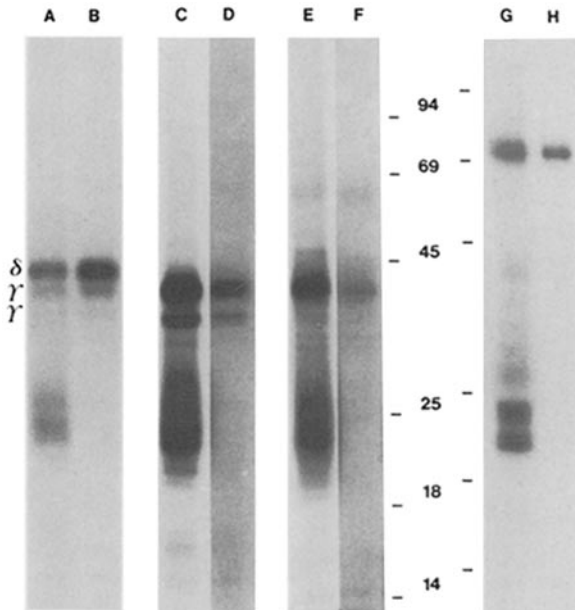


FIGURE 3. Biochemical proof that anti-TCR- $\gamma/\delta$ -1 mAb detects TCR- $\gamma$  ( $\delta$ ). Anti-CD3 precipitates were prepared as described for Fig. 2. TCR- $\gamma$  ( $\delta$ ) was dissociated from CD3 by incubation of one-half of the anti-CD3 precipitates in IPB with 1% NP-40 and 1% SDS for 30 min at room temperature. The supernatant was diluted five times with IPB containing 1.5% NP-40 and 25  $\mu$ g myoglobin, precleared once with normal mouse Ig-coated beads, and subjected to immunoprecipitation with anti-TCR- $\gamma/\delta$ -1 mAb and analyzed by SDS-PAGE under nonreducing (NR) or reducing (R) conditions. (A) Clone 1012, anti-CD3, R; (B) clone 1012, anti-TCR- $\gamma/\delta$ -1, R; (C) clone AK 119, anti-CD3, R; (D) clone AK 119, anti-TCR- $\gamma/\delta$ -1, R; (E) clone Wi.K, anti-CD3, R; (F) clone Wi.K, anti-TCR- $\gamma/\delta$ -1, R; (G) clone AK 119, anti-CD3, NR; (H) clone AK 119, anti-TCR- $\gamma/\delta$ -1, NR.

activity upon in vitro culture, without evidence for involvement of polymorphic domains of MHC molecules on the target cells. The activity could be regulated by mAbs directed at CD3, which opened the possibility that the CD3-associated TCR- $\gamma/\delta$  was involved in the recognition of these target cells. Another option would be that the cytotoxic spectrum of the clones did not reflect the specificity of the receptor and that the effect of anti-CD3 mAb reflected the regulatory capacity of the TCR- $\gamma/\delta$ /CD3 complex.

It had been observed that lymphokines, such as IL-2 and IFN- $\beta$  enhanced the cytotoxicity of TCR- $\gamma/\delta$ -expressing clones (22). Therefore, it was investigated in the first place, whether the lymphokines produced by the feeder cell mixture used to culture TCR- $\gamma/\delta^+$  clones, affected the cytotoxic potential of the clones. For this purpose, two TCR- $\gamma/\delta$ -expressing clones, 1012 and Wi.K, were cultured for 5 d in the presence of irradiated feeder cells. Next, when feeder cells had disintegrated, clones were washed and cultured for an additional 72 h in Yssel's medium with 2% pooled human serum, lacking lymphokines. To one part of the cells, deprived of feeder cell factors for 48 h, rIL-2 was added at 1,000 U/ml for 24 h. Control cells of the same batch were kept in culture for 8 d without changing the medium. Then, cytotoxicity was tested towards four allogeneic tumor cell lines of different histological origin. In Table II it can be seen that clone 1012, when harvested directly from the 8-d culture in the presence of feeder cells and their products (control), displayed significant MHC nonrestricted cytotoxicity towards all target cells. The cytotoxicity of factor-deprived cells of clone 1012 towards three of four target cells was drastically reduced, while it could be enhanced to a level comparable to or higher than that of control cells by the addition of IL-2. Clone Wi.K displayed significant cytotoxicity



TABLE II  
*Influence of Feeder Cell Factors on the Cytotoxicity of TCR  $\gamma/\delta^+$  Clones*

Clone	Percent cytotoxicity							
	K562*		JY		Daudi		MEWO	
	10:1 <sup>‡</sup>	2:1	10:1	2:1	10:1	2:1	10:1	2:1
1012: Control <sup>§</sup>	52	32	35	26	69	67	39	22
– factors <sup>¶</sup>	8	0	19	10	73	37	11	2
+ IL-2 <sup>¶¶</sup>	66	46	55	44	71	73	53	31
Wi.K: Control	32	9	8	3	4	2	13	3
– factors	7	0	11	3	0	0	1	0
+ IL-2	31	18	13	14	0	0	9	4

\* Target cells used.

<sup>‡</sup> ET cell ratio

<sup>§</sup> Control: culture with feeder cells for 8 d.

<sup>¶</sup> – factors: culture as in “control,” but harvested and washed on day five and subsequently cultured in absence of lymphokines for 72h.

<sup>¶¶</sup> + IL-2: culture as in “– factors,” but cultured with 1,000 U/ml IL-2 during the last 24h.

only towards K562 cells, where similar effects of factor depletion and IL-2 addition were observed as for clone 1012. IL-2 could not induce significant cytotoxicity of Wi.K towards the other three target cell lines.

Secondly, it was determined whether anti-TCR- $\gamma/\delta$ -1 mAb affected the function of the clones. Clones 1012, Wi.K, and as a control, the TCR- $\alpha/\beta^+$  cytotoxic clone JS-132, specific for a determinant on the HLA-A2 molecule, were used. To discriminate between factor-induced cytotoxicity and possible cytotoxicity reflecting the specificity of the TCR molecules, the assay was carried out with clones kept in culture with feeder cells for 8 d (control), as well as with clones harvested and washed on day six and cultured in the absence of lymphokines as described above, for 48 h. The results of this experiment are shown in Table III. Clone JS-132 displayed nonspecific cytotoxicity towards K562 and Daudi, which was strongly reduced in the absence of factors, while its specific activity towards JY and MEWO involving recognition of HLA-A2 by the TCR was retained. Again, the effect of factors produced by the feeder cell mixture on the cytotoxicity of clone 1012 was evident. In this experiment also the cytotoxicity of clone 1012 towards the cell line Daudi was reduced in the absence of factors, in contrast to the results shown in Table II. Clone Wi.K did not display any cytotoxicity in this experiment. However, anti-TCR- $\gamma/\delta$ -1 mAb induced significant cytotoxicity of clone Wi.K towards K562 and Daudi, but not to JY or MEWO. The antibody had no effect on the cytotoxicity of clone 1012 towards K562, but reduced its activity towards JY, Daudi, and MEWO. As expected, anti-TCR- $\gamma/\delta$ -1 had no effect on the activity of clone JS-132.

It is concluded that the MHC-nonrestricted cytotoxicity of TCR- $\gamma/\delta^+$  clones 1012 and Wi.K is induced by the culture conditions, most likely by growth factors such as IL-2 produced by the feeder cell mixture. Addition of mAb towards TCR- $\gamma/\delta$  can specifically induce cytotoxicity. The antibody can also specifically

TABLE III  
*Influence of Anti-TCR- $\gamma/\delta$ -1 mAb on the Cytotoxicity of TCR- $\gamma/\delta^+$  Clones*

Clone	Percent cytotoxicity							
	K562*		JY		Daudi		MEWO	
	10:1 <sup>‡</sup>	2:1	10:1	2:1	10:1	2:1	10:1	2:1
1020: Control <sup>§</sup>	37	11	23	10	55	21	42	17
Control + mAb	28	18	7	4	13	5	7	2
– factors <sup>  </sup>	0	0	10	5	26	7	11	4
– factors + mAb	10	6	1	1	3	2	1	1
Wi.K: Control	3	0	0	0	2	0	3	1
Control + mAb	57	46	4	2	63	33	13	6
– factors	0	0	0	0	2	1	1	0
– factors + mAb	30	25	0	0	28	13	2	1
JS-132: Control	55	32	88	84	26	9	64	43
Control + mAb	61	38	81	84	36	9	52	47
– factors	15	6	90	86	2	1	54	43
– factors + mAb	20	10	86	81	3	1	48	40

\* Target cells used.

<sup>‡</sup> ET cell ratio.

<sup>§</sup> Control: culture with feeder cells for 8d.

<sup>||</sup> – factors: clones harvested and washed on day six and cultured in absence of lymphokines for 48 h.

inhibit cytotoxicity, despite the fact that this activity must be categorized as factor induced.

*Distribution of TCR- $\gamma$  ( $\delta$ )-bearing Cells in Normal PBL and Thymus.* Since we had demonstrated the reactivity of anti-TCR- $\gamma/\delta$ -1 mAb with all forms of TCR- $\gamma$  ( $\delta$ ) described thus far, we felt confident to use this antibody to assess the occurrence of this second type of TCR in PB and thymus of normal individuals. In an initial experiment described above, using FACS analysis of PBL from 18 normal adult donors, variable percentages of TCR- $\gamma/\delta^+$  cells had been found, ranging from undetectable to 5%. In a few cases, including that of donor CM, a distinct subpopulation of TCR- $\gamma/\delta^+$  cells could be observed. PBL from this donor were used to determine the percentage of CD3<sup>+</sup>/TCR- $\gamma/\delta^+$  cells and the CD4/CD8 phenotype of this population by two-label immunofluorescence, followed by FACS analysis. As can be seen in Fig. 4, 7.2% of CD3<sup>+</sup> cells in this individual expressed TCR- $\gamma/\delta$  (panel A). The majority of these cells were of the CD4<sup>-</sup>CD8<sup>-</sup> phenotype as previously described. No TCR- $\gamma/\delta^+$  cells expressing the CD4 marker were detectable (panel C). However, 21% of TCR- $\gamma/\delta^+$  cells was CD8<sup>+</sup> (panel D). In Fig. 4 it is also shown that freshly isolated TCR- $\gamma/\delta^+$  cells stain very weakly or not with WT31 mAb (panel B).

To determine the percentage of TCR- $\gamma/\delta^+$  cells and their phenotype accurately, PBL samples were stained by two-label immunofluorescence and analyzed by fluorescence microscopy. In these cases, PBL were derived from donors ranging in age from 2 mo to 16 y in order to investigate whether age affected the size of the TCR- $\gamma/\delta^+$  population. In these samples, the percentages of

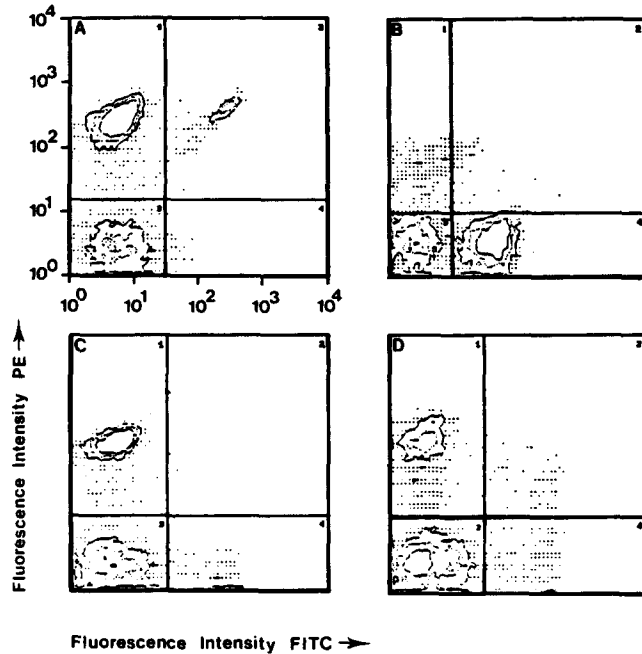


FIGURE 4. Phenotypic analysis of TCR- $\gamma/\delta^+$  cells in PB of a normal donor by two-label immunofluorescence. PBL were first incubated with anti-TCR- $\gamma/\delta$ -1 mAb, followed by goat anti-mouse Ig-FITC conjugate (A, C, and D) or goat anti-mouse Ig-PE conjugate (B). Free binding sites of the second-step reagent were blocked with normal mouse serum and cells were incubated with PE-conjugated mAb (A, C, and D) or FITC-conjugated WT31 (B). Fluorescence analysis was performed using a FACStar (Becton Dickinson & Co.). Fluorescence intensities for all panels are plotted identically on a  $^{10}\log$  scale as indicated in A. (A) Anti-Leu-4 (anti-CD3) PE; anti-TCR- $\gamma/\delta$ -1 FITC. (B) Anti-TCR- $\gamma/\delta$ -1 PE; WT31 FITC. (C) Anti-Leu-3a (anti-CD4) PE; anti-TCR- $\gamma/\delta$ -1 FITC. (D) Anti-Leu-2a (anti-CD8) PE; anti-TCR- $\gamma/\delta$ -1 FITC.

Panel	Percent fluorescence			
	Quadrant			
	1	2	3	4
A	64.0	4.58	31.0	0.50
B	3.62	1.07	31.4	63.9
C	45.3	0.01	49.7	4.99
D	20.8	1.14	73.8	4.25

CD3<sup>+</sup>, WT31<sup>+</sup>, and TCR- $\gamma/\delta^+$  cells were determined by examination of 1,000 cells for each marker. The CD4/CD8 phenotypes of TCR- $\gamma/\delta^+$  cells were determined by examination of 100 TCR- $\gamma/\delta^+$  cells. The results from 10 donors are enumerated in Table IV. The percentages of TCR- $\gamma/\delta^+$  cells ranged from 1.9 to 7.2% within the CD3<sup>+</sup> cell population. The fluorescence of the TCR- $\gamma/\delta^+$  population was virtually mutually exclusive with the fluorescence of the WT31<sup>+</sup> population. In 4 of 10 donors CD4<sup>+</sup>/TCR- $\gamma/\delta^+$  cells were detected, constituting up to 3.0% of TCR- $\gamma/\delta^+$  cells. In all donors but one, CD8<sup>+</sup>/TCR- $\gamma/\delta^+$  cells were found, ranging from 1.0 to 17% of TCR- $\gamma/\delta^+$  cells. No obvious correlation between age of the donor and the size of the TCR- $\gamma/\delta^+$  population could be found. In addition, Table IV lists data obtained with thymocytes derived from three children. The relative size of the TCR- $\gamma/\delta^+$  population in these thymuses was significantly smaller than in PBL, ranging from 0.1 to 1.0% of CD3<sup>+</sup> cells. Also, most TCR- $\gamma/\delta^+$  thymocytes were of the CD4<sup>-</sup>8<sup>-</sup> phenotype, while signifi-

TABLE IV  
*Percentage and Phenotype of Anti-TCR- $\gamma/\delta$ -1<sup>+</sup> Cells in PB and Thymus of Normal Donors*

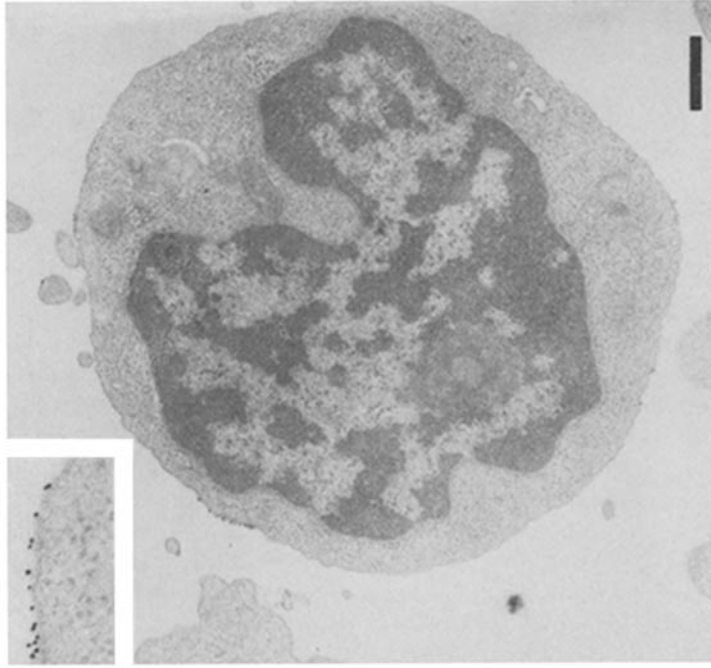
Donor	Age	CD3 <sup>+</sup>	WT31 <sup>+</sup> per CD3 <sup>+</sup>	Anti- TCR- $\gamma/\delta$ -1 <sup>+</sup> per CD3 <sup>+</sup>	CD4 <sup>+</sup> per anti- TCR- $\gamma/\delta$ -1 <sup>+</sup>	CD8 <sup>+</sup> per anti- TCR- $\gamma/\delta$ -1 <sup>+</sup>
PB		%	%	%	%	%
samples						
HM	2 mo	47	98	1.9	0	10
PvH	2.5 mo	62	98	1.9	3	12
WB	3 mo	38	96	2.4	3	17
KD	13 mo	44	94	5.6	0	9
SB	27 mo	38	95	4.7	0	1
MH	6 y	73	98	1.9	1	5
MJ	7 y	61	90	7.2	0	0
SN	8 y	61	94	3.9	0	6
AvdH	12 y	71	96	4.4	1	1
EH	16 y	63	96	4.1	NT*	6
Thymus						
sample						
A	3 wk	76	99.2	1.0	NT	NT
B	5 y	63	99.5	0.2	0	8
C	10 y	73	99.9	0.1	0	28
D <sup>‡</sup>	18 wk <sup>‡</sup>	78	99.0	0.7	NT	NT

\* NT, not tested.

<sup>‡</sup> Fetal thymus.

cantly more CD8<sup>+</sup>/TCR- $\gamma/\delta$ <sup>+</sup> than CD4<sup>+</sup>/TCR- $\gamma/\delta$ <sup>+</sup> cells were found. The same analysis was done for thymocytes derived from an 18-wk-old fetus. At this early stage in human development, the percentage of TCR- $\gamma/\delta$ <sup>+</sup> thymocytes was 0.7% of CD3<sup>+</sup> cells.

*Ultrastructure of TCR- $\gamma$  ( $\delta$ )<sup>+</sup> Cells in PB.* To study the morphological features of TCR- $\gamma$  ( $\delta$ )<sup>+</sup> cells in PB, electronmicroscopy was used. PBL were derived from a healthy individual, who had previously been found by FACS analysis to contain a relatively high percentage of TCR- $\gamma/\delta$ <sup>+</sup> cells. To identify these TCR- $\gamma/\delta$ <sup>+</sup> cells in electron microscopy, the total cell population was stained with anti-TCR- $\gamma/\delta$ -1 mAb, followed by rabbit anti-mouse Ig and gold-conjugated protein A. For comparison, cells were labeled in the same fashion using WT31 mAb, which in a freshly isolated PB sample would allow predominantly the identification of TCR- $\alpha/\beta$ <sup>+</sup> cells. The gold labeling was specific as indicated by the fact that 40% of the cells were labeled using WT31 mAb, while 4% of the cells were labeled using anti-TCR- $\gamma/\delta$ -1 mAb, figures corresponding to the results of immunofluorescence stainings. Fig. 5 shows two cells, representative of the anti-TCR- $\gamma/\delta$ -1<sup>+</sup> population (*left*) or the WT31<sup>+</sup> population (*right*). No significant overall differences in ultrastructural morphology between these two populations were observed. Both cell types appeared to be medium sized lymphocytes,  $\sim 7-8 \mu\text{m}$  in diameter. Moreover, like TCR- $\alpha/\beta$ <sup>+</sup> cells, TCR- $\gamma/\delta$ <sup>+</sup> cells were resting lymphocytes as determined by the following features: a high nucleus/cytoplasm ratio, a single "resting" nucleolus (not seen in the left hand photo) in a heterochromatin-rich nucleus, and a small amount of finely vesicular smooth-surfaced membranes; ribosomes existed as single units rather than as polysomal



subsequently fixed and embedded in Epon. The two insets show a higher magnification ( $\times 16,000$ ) to illustrate labeling of the antigens with gold particles. Bar = 1  $\mu\text{m}$ .

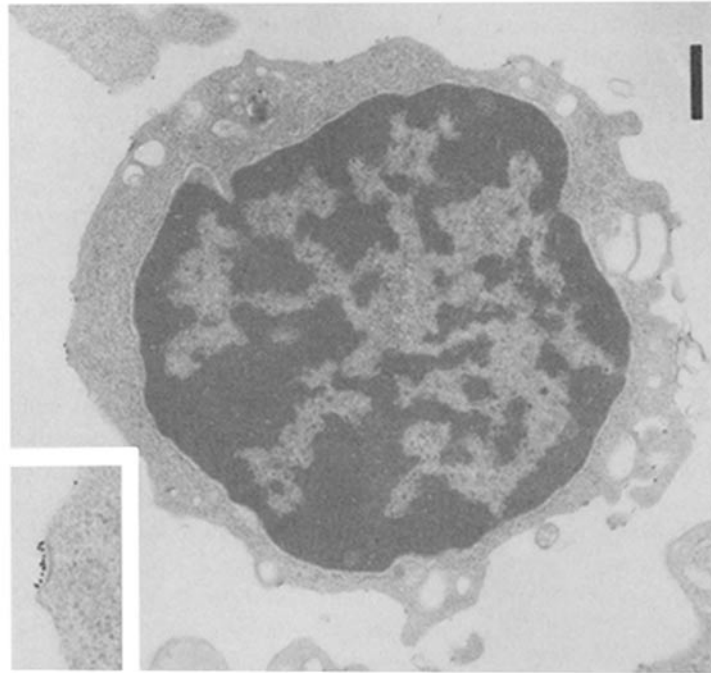


FIGURE 5. Ultrastructure of TCR- $\gamma$  ( $\delta$ )<sup>+</sup> cells in PB of a normal donor. Cells were labeled with either anti-TCR- $\gamma$ / $\delta$ -1 mAb (*left*) or WT31 mAb (*right*) and protein A-gold at 4°C in the presence of sodium azide. Cells were

aggregates; rough endoplasmatic reticulum and Golgi system were almost completely absent and only some small mitochondria and a few lysosomes were observed. There was no evidence for the presence of significant numbers of "granular structures" as can be identified in activated cytolytic T cells.

### Discussion

An mAb specific for human TCR- $\gamma$  ( $\delta$ ) was generated, using as immunogen CD3/TCR complexes isolated from digitonin-solubilized clones. The major advantage of this mAb over the already available anti-TCR- $\gamma$  peptide sera is that it reacts with intact, viable cells. This allows positive identification of TCR- $\gamma/\delta$ -expressing cells by immunofluorescence. Previously, human TCR- $\gamma/\delta^+$  T cells have been identified using lack of reactivity with WT31 mAb as an indication and immunoprecipitation with anti-C $\gamma$  peptide serum as confirmation. This procedure is more time consuming than immunofluorescence, requires a relatively large number of cells, and is not fail-safe, since TCR- $\gamma/\delta^+$  cells may react with WT31 mAb (see above and reference 26a). Reactivity of anti-TCR- $\gamma/\delta$ -1 mAb with viable cells also allows their selective isolation from heterogeneous cell populations and their subsequent use in functional or biochemical studies.

Unlike TCR- $\alpha/\beta$ , TCR- $\gamma$  ( $\delta$ ) occurs in different molecular configurations. In the first place, TCR- $\gamma$  ( $\delta$ ) can occur in either disulphide-linked or non-disulphide-linked forms. The molecular basis of this difference in organization has been unravelled (16, 39, 40). Of the two C gene segments in the  $\gamma$  locus, C $\gamma$ -1 encodes a cysteine residue in its second exon, while C $\gamma$ -2 does not (11). This cysteine residue is thought to be responsible for the formation of an interchain disulphide bond (6). Secondly, significant differences in size of TCR- $\gamma$  chains are detected between non-disulphide-linked receptors on different cells. The leukemic line PEER (24, 38) and the line IDP2 (4, 24) express a  $\gamma$  chain of  $\sim$ 55 kD, while on clones Wi.K and JS-228, as well as on PBL from a leukemic patient (39), we have found a  $\gamma$  chain of 40–44 kD. In PEER, a triplication of the second exon of the C gene segment has occurred, rather than a duplication as in other cells using C $\gamma$ -2, which seems to be a polymorphic feature (40). However, since this exon contains only 48 bp, this is not sufficient to explain the size difference between the 55-kD and the 40–44-kD  $\gamma$  chains. Posttranslational modifications of the  $\gamma$  chain may play a role. A third aspect in which TCR- $\gamma$  ( $\delta$ ) shows organizational variation, is the appearance of the  $\delta$  chain. In more than 10 clones expressing disulphide-linked receptors analyzed thus far, we have clearly identified a  $\sim$ 43-kD  $\delta$  chain (unpublished results). Clone AK 119 is an exception in that no  $\delta$  chain can be detected after cell surface labeling with  $^{125}\text{I}$ . Moingeon et al. (23) have also reported disulphide-linked TCR- $\gamma$  in which no  $\delta$  chain could be detected. A similar problem is encountered in the analysis of non-disulphide-linked receptors. In PEER, Weiss et al. (38) observed only a 55-kD  $\gamma$  chain, while Brenner et al. (24) in addition found a  $\delta$  chain that migrated at 40 kD under reducing conditions and at a lower mol mass under nonreducing conditions. Such a  $\delta$  chain was also found in the line IDP2 (4). Our results are consistent with those of Brenner et al. In PEER as well as in clone JS-228 derived from PB of a normal individual a  $\delta$  chain was expressed, which, however, did not label consistently well by cell surface iodination. In clone Wi.K as well

as in TCR- $\gamma^+$  leukemic T cells (39) no  $\delta$  chain could be detected by cell surface labeling with  $^{125}\text{I}$ . Possibly, the  $\delta$  chain present in non-disulphide-linked receptors, as well as in the disulphide-linked receptor expressed on clone AK 119, cannot be labeled efficiently by this technique. However, to account for such dramatic effect on  $^{125}\text{I}$ -labeling this chain must differ significantly in primary structure and/or conformation from the  $\delta$  chain found in the disulphide-linked receptors. We are currently investigating the occurrence of the  $\delta$  chain using metabolic labeling. Preliminary results indicate that all types of receptors contain a  $\delta$  chain. It should be remarked that it is not known, whether the  $\delta$  chain in disulphide-linked receptors and the  $\delta$  chain in non-disulphide-linked receptors are products of the same gene.

Anti-TCR- $\gamma/\delta$ -1 mAb reacts with all types of TCR- $\gamma/\delta$ . It is not clear on which chain of TCR- $\gamma/\delta$  the epitope recognized by anti-TCR- $\gamma/\delta$ -1 mAb is located. The mAb does not react in Western blotting, nor after separation of either disulphide-linked or non-disulphide-linked  $\gamma$  and  $\delta$  chains by reductive alkylation and/or denaturation in SDS at high temperatures. Therefore, this point is difficult to resolve.

TCR- $\gamma/\delta$ -expressing cells can display MHC-nonrestricted cytotoxicity (21–24, 36). As was shown earlier for T cells activated in mixed lymphocyte cultures (40a) and cloned TCR- $\alpha/\beta^+$  T cells (40b), and as is shown here for TCR- $\gamma/\delta^+$  T cell clones, such cytolytic activity is induced by the culture conditions. Cells are stimulated weekly with irradiated allogeneic PBL and JY cells, and PHA. Growth factors are produced, such as IL-2, which previously has been shown to enhance cytolytic activity of TCR- $\gamma/\delta$ -expressing clones (22). A TCR- $\alpha/\beta$ -expressing clone also displayed MHC-nonrestricted cytotoxicity, which was lost upon factor depletion. However, its specific activity, dependent on expression of the HLA-A2 molecule on the target cells, was retained. Similarly, cytotoxicity of TCR- $\gamma/\delta$  cells, reflecting the specificity of the receptor, would be expected to remain after factor depletion, particularly since expression of the receptor is not affected. Therefore, TCR- $\gamma/\delta$  most likely does not play a role in target cell recognition in the case of in vitro-induced MHC-nonrestricted activity, which has also been suggested by others (41). Recently, evidence has indeed been presented that MHC molecules may be the ligand of TCR- $\gamma/\delta$  (42). However, the receptor plays a role in the regulation of T cell activity in the in vitro systems used. mAbs directed at the receptor may induce nonspecific cytotoxicity (this paper and reference 43), and proliferation (23), as shown previously for TCR- $\alpha/\beta$ -expressing cells, which is dependent on the presentation of the mAb via Fc receptors on the target cells (44, 45). Also, inhibition of nonspecific cytotoxicity was reproducibly found. In one case, cytotoxicity of clone 1012 towards Daudi, which bears Fc receptors for IgG1, was inhibited, while cytotoxicity of clone Wi.K was induced. Perhaps the effect depends on the nature of the conformational change induced in the receptor by binding of the mAb.

It is unknown what contribution TCR- $\gamma/\delta^+$  cells make to the immune system. Therefore, it is of interest to determine in which tissues and in what relative numbers TCR- $\gamma/\delta^+$  lymphocytes are present in man, in health and disease. In this paper, we have reported the percentages of TCR- $\gamma/\delta^+$  cells in PB and thymus of normal individuals. In 11 donors, TCR- $\gamma/\delta^+$  cells constituted 1.9–7.2%

of CD3<sup>+</sup> lymphocytes in PB. It has not yet been determined whether these percentages are constant with time in a given healthy donor. In thymus samples from three children, the percentage of TCR- $\gamma/\delta$ <sup>+</sup> thymocytes ranged from 0.1 to 1.0% of CD3<sup>+</sup> cells, which is significantly lower than in PB. These data are consistent with those reported for CD3<sup>+</sup>/WT31<sup>-</sup> cells in PB and thymus (37). WT31 expression is indeed very low or nondetectable on freshly isolated TCR- $\gamma/\delta$ <sup>+</sup> cells. Thymocytes from an 18-wk-old human fetus were included in this study, since Pardoll et al. (46) had reported the expression of TCR  $\gamma/\delta$  on all fetal thymocytes at day 15 of gestation in mouse. No higher percentage of TCR- $\gamma/\delta$ <sup>+</sup> cells were detected in human fetal thymus than in thymi derived from children. It may be relevant to study earlier samples.

The phenotype of the majority of TCR- $\gamma/\delta$ <sup>+</sup> cells in PB and thymus was CD4<sup>-</sup>8<sup>-</sup>, in concordance with previously published data. However, in the total pool of TCR- $\gamma/\delta$ <sup>+</sup> cells, as we have been able to study now, a significant proportion may bear the CD8 antigen, ranging from 0 to 28% between 13 donors. CD8<sup>+</sup>/TCR- $\gamma/\delta$ <sup>+</sup> clones have indeed been reported (47). We have found that CD4<sup>+</sup>/TCR- $\gamma/\delta$ <sup>+</sup> cells are much more infrequent, but do occur. TCR- $\gamma/\delta$ <sup>+</sup> cells of the CD4<sup>+</sup> phenotype have not previously been reported. In two-label immunofluorescence using FACS analysis as well as fluorescence microscopy, we have observed some TCR- $\gamma/\delta$ <sup>+</sup> cells, which did not react with anti-CD3 mAb. Although it is an unlikely possibility, it will have to be investigated whether TCR- $\gamma/\delta$  can be expressed at the cell surface in the absence of the CD3 molecule.

Electron microscopic analysis has pointed out that TCR- $\gamma/\delta$ <sup>+</sup> cells in PB are resting lymphocytes, like TCR- $\alpha/\beta$ <sup>+</sup> cells and unlike NK cells. This is consistent with our previous observation that CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> cells derived from PB need *in vitro* activation in order to become functionally active (48). It will be investigated whether more subtle morphological features discern TCR- $\gamma/\delta$ <sup>+</sup> cells from TCR- $\alpha/\beta$ <sup>+</sup> cells. For instance, CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> cells have previously been selectively isolated on continuous density gradients, which would imply certain differences in cellular structure (48).

Further studies will involve the identification of human TCR- $\gamma/\delta$ <sup>+</sup> cells in healthy and diseased states, their isolation and characterization, in which the developed mAb will be an important tool.

### Summary

A second type of TCR molecule has been identified on human and murine T lymphocytes, which involves the protein products of the  $\gamma$  and  $\delta$  genes. T lymphocytes bearing this receptor may constitute a separate cell lineage with a distinct immune function. We have produced an mAb, which specifically detects human TCR- $\gamma/\delta$  in native as well as denatured states, this in contrast to previously used anti- $\gamma$  chain peptide sera, which only reacted with denatured protein. The receptor occurs in different molecular forms, with or without interchain disulphide bonds, in which a  $\delta$  chain may or may not be detected by cell surface iodination. The mAb is reactive with all these receptor forms. Therefore, this antibody could be used to determine the expression of TCR- $\gamma/\delta$  on viable human T lymphocytes. In normal individuals, TCR- $\gamma/\delta$  was found on a subset composing 2–7% of CD3<sup>+</sup> lymphocytes in peripheral blood and 0.1–1.0% in



thymus. The majority of these cells do not express the CD4 or CD8 antigens, although a significant percentage of CD8<sup>+</sup> cells was found. TCR- $\gamma/\delta$ <sup>+</sup> cells in peripheral blood are resting lymphocytes, as judged by ultrastructural analysis. T cell clones with different receptor types can display MHC-nonrestricted cytolytic activity, which is shown to be induced by the culture conditions, most likely by growth factors such as IL-2. This strongly suggests that TCR- $\gamma/\delta$  does not play a role in target cell recognition in MHC-nonrestricted cytotoxicity. The anti-TCR- $\gamma/\delta$  antibody can specifically induce cytotoxic activity in clones expressing the receptor, but in addition inhibit growth factor induced cytotoxicity, which indicates a regulatory role of the TCR- $\gamma/\delta$ /CD3 complex in MHC-nonrestricted cytotoxicity.

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### References

1. Allison, J. P., B. W. McIntyre, and D. Bloch. 1982. Tumor-specific antigen of murine T lymphoma defined with monoclonal antibody. *J. Immunol.* 129:2293.
2. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157:1149.
3. Meuer, S. C., K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function: relationship to the T3 molecular complex. *J. Exp. Med.* 157:705.
4. Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel. 1986. Identification of a putative second T-cell receptor. *Nature (Lond.)* 322:145.
5. Lew, A. M., D. M. Pardoll, W. L. Maloy, B. J. Fowlkes, A. Kruisbeek, S.-F. Cheng, R. N. Germain, J. A. Bluestone, R. H. Schwartz, and J. E. Coligan. 1986. Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. *Science (Wash. DC)* 234:1401.
6. Kronenberg, M., G. Siu, L. E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.* 4:529.
7. Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa. 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature (Lond.)* 309:757.
8. Murre, C., R. A. Waldmann, C. C. Morton, K. F. Bongiovanni, T. A. Waldmann, T. B. Shows, and J. G. Seidman. 1985. Human  $\gamma$ -chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. *Nature (Lond.)* 316:549.
9. Kranz, D. M., H. Saito, M. Heller, Y. Takagaki, W. Haas, H. N. Eisen, and S. Tonegawa. 1985. Limited diversity of the rearranged T-cell  $\gamma$  gene. *Nature (Lond.)* 313:752.
10. LeFranc, M.-P., A. Forster, R. Baer, M. A. Stinson, and T. H. Rabbitts. 1986. Diversity and rearrangement of the human T cell rearranging  $\gamma$  genes: nine germ-line variable genes belonging to two subgroups. *Cell* 45:237.

11. Strauss, W. M., T. Quertermous, and J. G. Seidman. 1987. Measuring the human T cell receptor  $\gamma$ -chain locus. *Science (Wash. DC)*. 237:1217.
12. Forster, A., S. Huck, N. Ghanem, M.-P. Lefranc, and T. H. Rabbitts. 1987. New subgroups in the human T cell rearranging  $V_\gamma$  gene locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1945.
13. Snodgrass, H. R., Z. Dembić, M. Steinmetz, and H. Von Boehmer. 1985. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature (Lond.)*. 315:232.
14. Raulat, D. H., R. D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature (Lond.)*. 314:103.
15. Van Dongen, J. J. M., T. Quertermous, C. R. Bartram, D. P. Gold, I. L. M. Wolvers-Tettero, W. M. Comans-Bitter, H. Hooijkaas, H. J. Adriaansen, A. De Klein, A. Raghavachar, A. Ganser, A. D. Duby, J. G. Seidman, P. Van den Elsen, and C. Terhorst. 1987. T cell receptor-CD3 complex during early T cell differentiation. *J. Immunol.* 138:1260.
16. Krangel, M. S., H. Band, S. Hata, J. McLean, and M. B. Brenner. 1987. Structurally divergent human T cell receptor  $\gamma$  proteins encoded by distinct  $C_\gamma$  genes. *Science (Wash.DC)*. 237:64.
17. Chien, Y., M. Iwashima, K. B. Kaplan, J. F. Elliott, and M. M. Davis. 1987. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature (Lond.)*. 327:677.
18. Borst, J., J. E. Coligan, H. Oettgen, S. Pessano, R. Malin, and C. Terhorst. 1984. The  $\delta$ - and  $\epsilon$ -chains of the human T3/T-cell receptor complex are distinct polypeptides. *Nature (Lond.)*. 312:455.
19. Weissman, A. M., L. E. Samelson, and R. D. Klausner. 1986. A new subunit of the human T-cell antigen receptor complex. *Nature (Lond.)*. 324:480.
20. Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of the T3/antigen receptor complex in T-cell activation. *Annu. Rev. Immunol.* 4:593.
21. Borst, J., R. J. Van de Griend, J. W. Van Oostveen, S.-L. Ang, C. J. Melief, J. G. Seidman, and R. L. H. Bolhuis. 1987. A T-cell receptor  $\gamma$ /CD3 complex found on cloned functional lymphocytes. *Nature (Lond.)*. 325:683.
22. Van de Griend, R. J., W. J. M. Tax, B. A. Van Krimpen, R. J. Vreugdenhil, C. P. M. Ronteltap, and R. L. H. Bolhuis. 1987. Lysis of tumor cells by  $CD3^+4^-8^-16^+$  T cell receptor  $\alpha\beta^-$  clones, regulated via CD3 and CD16 activation sites, recombinant interleukin 2 and interferon  $\beta$ . *J. Immunol.* 138:1627.
23. Moingeon, P., S. Jitsukawa, F. Faure, F. Troalen, F. Triebel, M. Graziani, F. Forestier, D. Bellet, C. Bohuon, and T. Hercend. 1987. A  $\gamma$ -chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity. *Nature (Lond.)*. 325:723.
24. Brenner, M. B., J. McLean, H. Scheft, J. Riberdy, S.-L. Ang, J. G. Seidman, P. Devlin, and M. S. Krangel. 1987. Two forms of the T-cell receptor  $\gamma$  protein found on peripheral blood cytotoxic T lymphocytes. *Nature (Lond.)*. 325:689.
25. Bank, I., R. A. DePinho, M. B. Brenner, J. Cassimeris, F. W. Alt, and L. Chess. 1986. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature (Lond.)*. 322:179.
26. Spits, H., J. Borst, W. Tax, P. J. A. Capel, C. Terhorst, and J. E. De Vries. 1985. Characteristics of a monoclonal antibody (WT31) that recognizes a common epitope on the human T cell receptor for antigen. *J. Immunol.* 135:1922.
- 26a. Van de Griend, R. J., J. Borst, W. J. M. Tax, and R. L. H. Bolhuis. 1988. Functional reactivity of WT31 monoclonal antibody with T cell receptor- $\gamma$  expressing  $CD_3^+4^-8^-$  T cells. *J. Immunol.* 140:1107.

27. Borst, J., H. Spits, A. Voordouw, E. De Vries, A. Boylston, and J. E. De Vries. 1986. A family of T-cell receptor molecules expressed on T-cell clones with different specificities for allo major histocompatibility antigens. *Hum. Immunol.* 17:426.
28. Van de Griend, R. J., and R. L. H. Bolhuis. 1985. In vitro expansion and analysis of cloned cytotoxic T cells derived from patients with chronic T $\gamma$  lymphoproliferative disorders. *Blood.* 65:1002.
29. Ang, S.-L., J. G. Seidman, G. M. Peterman, A. D. Duby, D. Benjamin, S. J. Lee, and D. A. Hafler. 1987. Functional  $\gamma$  chain-associated T cell receptors on cerebrospinal fluid-derived natural killer-like T cell clones. *J. Exp. Med.* 165:1453.
30. Yssel, H., J. E. De Vries, M. Koken, W. Van Blitterswijk, and H. Spits. 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
31. Van de Griend, R. J., and R. L. H. Bolhuis. 1984. Rapid expansion of allospecific cytotoxic T cell clones using nonspecific feeder cell lines without further addition of exogenous IL2. *Transplantation (Baltimore).* 38:401.
32. Ravid, Z., N. Goldblum, R. Zaizov, M. Schlesinger, T. Kertes, J. Minowada, W. Verbi, and M. Greaves. 1980. Establishment and characterization of a new leukaemic T-cell line (PEER) with an unusual phenotype. *Int. J. Cancer.* 25:705.
33. Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87.
34. Oettgen, H. C., C. L. Pettey, W. L. Maloy, and C. Terhorst. 1986. A T3-like protein complex associated with the antigen receptor on murine T cells. *Nature (Lond.).* 320:272.
35. Brenner, M. B., I. S. Trowbridge, and J. L. Strominger. 1985. Cross-linking of human T-cell receptor proteins: association between the T cell idiotype  $\beta$  subunit and the T3 glycoprotein heavy subunit. *Cell.* 40:183.
36. Lanier, L. L., N. A. Federspiel, J. J. Ruitenber, J. H. Phillips, J. P. Allison, D. Littman, and A. Weiss. 1987. The T cell antigen receptor complex expressed on normal peripheral blood CD4<sup>-</sup>, CD8<sup>-</sup> T lymphocytes. A CD3<sup>-</sup> associated disulfide-linked  $\gamma$  chain heterodimer. *J. Exp. Med.* 165:1076.
37. Lanier, L. L., and A. Weiss. 1986. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. *Nature (Lond.)* 324:268.
38. Weiss, A., M. Newton, and D. Crommie. 1986. Expression of T<sub>3</sub> in association with a molecule distinct from the T-cell antigen receptor heterodimer. *Proc. Natl. Acad. Sci. USA.* 83:6998.
39. Van Dongen, J. J. M., I. L. M. Wolvers-Tettero, J. G. Seidman, S.-L. Ang, R. J. Van de Griend, E. F. R. De Vries, and J. Borst. 1987. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. *Eur. J. Immunol.* 17:1719.
40. Littman, D. R., M. Newton, D. Crommie, S.-L. Ang, J. G. Seidman, S. N. Gettner, and A. Weiss. 1987. Characterization of an expressed CD3- associated Ti  $\gamma$ -chain reveals C $\gamma$  domain polymorphism. *Nature (Lond.).* 326:85.
- 40a. Bolhuis, R. L. H., C. P. M. Ronteltap, M. A. W. de Rooy-Braam, B. A. van Krimpen, and H. Schellekens. 1982. Phytohaemagglutinin-induced susceptibility to lysis by natural and activated killer cells of autologous, allogenic and xenogeneic target cells. *Mol. Immunol.* 19:1347.
- 40b. Van de Griend, R. J., M. J. Giphart, B. A. van Krimpen, and R. L. H. Bolhuis. 1984. Human T cell clones exerting multiple cytolytic activities show heterogeneity in susceptibility to inhibition by monoclonal antibodies. *J. Immunol.* 133:1222.
41. Phillips, J. H., A. Weiss, B. T. Gemlo, A. A. Rayner, and L. L. Lanier. 1987. Evidence that the T cell antigen receptor may not be involved in cytotoxicity mediated by  $\gamma/\delta$  and  $\alpha/\beta$  thymic cell lines. *J. Exp. Med.* 166:1579.
42. Matis, L. A., R. Cron, and J. A. Bluestone. 1987. Major histocompatibility complex-

- linked specificity of  $\gamma\delta$  receptor-bearing T lymphocytes. *Nature (Lond.)*. 330:262.
43. Moingeon, P., A. Ythier, G. Goubin, F. Faure, A. Nowill, L. Delmon, M. Rainaud, F. Forestier, F. Daffos, C. Bohuon, and T. Hercend. 1986. A unique T-cell receptor complex expressed on human fetal lymphocytes displaying natural-killer-like activity. *Nature (Lond.)*. 323:638.
  44. Spits, H., H. Yssel, J. Leeuwenberg, and J. E. De Vries. 1985. Antigen-specific cytotoxic T-cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. *Eur. J. Immunol.* 15:88.
  45. Tax, W. J. M., F. F. M. Hermes, R. W. Willems, P. J. A. Capel, and R. A. P. Koene. 1984. Fc receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody-induced T cell proliferation. *J. Immunol.* 133:1185.
  46. Pardoll, D. M., B. J. Fowlkes, J. A. Bluestone, A. Kruisbeek, W. L. Maloy, J. E. Coligan, and R. H. Schwartz. 1987. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature (Lond.)*. 326:79.
  47. Nowill, A., P. Moingeon, A. Ythier, M. Graziani, F. Faure, L. Delmon, M. Rainaut, F. Forrestier, C. Bohuon, and Th. Hercend. 1986. Natural killer clones derived from fetal (25 wk) blood. *J. Exp. Med.* 163:1601.
  48. Van de Griend, R. J., H. G. De Bruin, H. C. Rümke, R. Van Doorn, D. Roos, and A. Astaldi. 1982. Isolation and partial characterization of a novel subset of human T lymphocytes defined by monoclonal antibodies. *Immunology*. 47:313.