Clin. exp. Immunol. (1980) **80**, 114–121

Characterization of normal human $CD3^+CD5^-$ and $\gamma\delta$ T cell receptor positive T lymphocytes

E. F. SROUR*†, T. LEEMHUIS*, L. JENSKI‡, R. REDMOND*†, D. FILLAK* & J. JANSEN*† *Department of Medicine, Division of Hematology/Oncology, and †Margaret Ball-Petty Laboratory for Experimental Bone Marrow Transplantation, Indiana University School of Medicine, Indianapolis, IN; and ‡Department of Biology, Purdue University School of Science, Indianapolis, IN, USA

(Accepted for publication 19 October 1989)

SUMMARY

The functional and phenotypic properties of normal human $CD3^+CD5^-$ T cells which have a higher frequency of cytotoxic cells than $CD3^+CD5^+$ T lymphocytes have been described. Using three- and four-colour immunofluorescence flow cytometric cell sorting, the $CD3^+CD5^-$ and $CD3^+CD5^+$ populations were subdivided into $\alpha\beta$ or $\gamma\delta$ T cell receptor positive cells. The four subsets were examined for the *in vitro* cytotoxic activity and were also stimulated with mitogens in limiting-dilution assays to measure the frequencies of proliferating and interleukin-2 (IL-2) producing cells. $CD3^+CD5^-\alpha\beta^+$, $CD3^+CD5^-\gamma\delta^+$ and $CD3^+CD5^+\gamma\delta^+$ cells had lower frequencies of proliferating and IL-2-producing cells than did $CD3^+CD5^+\alpha\beta^+$ cells. However, the cytotoxic activity of the different phenotypes was higher in the $CD3^+CD5^-$ subsets, especially when these cells were $\gamma\delta^+$. Expression of $\gamma\delta$ or lack of expression of CD5 appeared to be associated with the acquisition of cytolytic potentials. CD8 was expressed on 20% of fresh $CD3^+\gamma\delta^+$ cells. Cultured $\gamma\delta^+$ cells retained the expression of $\gamma\delta$, but quickly lost that of CD8 and with time modulated the expression of CD5. The expression of CD5 was found to be higher on sorted $CD3^+CD5^+\gamma\delta^-$ than on $CD3^+CD5^+\gamma\delta^+$cells. These observations indicate that $\gamma\delta$ is preferentially expressed on CD5-negative or weakly positive T lymphocytes and that $CD3^+CD5^-\gamma\delta^+$ cells appear to constitute a discrete small subset of mature T lymphocytes which are cytotoxic in nature. However, the exact immunological function of these cells and their place in T cell ontogeny are yet to be elucidated.

Keywords $CD3^+CD5^-$ $\gamma\delta$ T cell receptor

INTRODUCTION

Recently, several groups (Bierer *et al.*, 1988; van de Griend *et al.*, 1987) including ours (Jansen *et al.*, 1987; Srour *et al.*, 1988), described a small T cell subset expressing CD3 but not the CD5 marker. These cells were found in normal peripheral blood and bone marrow (Srour *et al.*, 1988), in CD5 plus complement-depleted bone marrow (Rozans *et al.*, 1986; Jansen *et al.*, 1987) and in bone marrow transplant recipients (Anderson *et al.*, 1985). The functional role of the CD5 molecule is still unknown. However, it is well documented that monoclonal antibodies (MoAbs) directed against the 67-Kd CD5 antigen augment T cell proliferation in response to antigens and mitogens and causes an increase in the production of interleukin-2 (IL-2) (Ledbetter *et al.*, 1985, 1986; Ceuppens & Baroja, 1986). The available literature clearly indicates that $CD3^+CD5^-$ T lymphocytes are cytotoxic in nature (Srour *et al.*, 1988; Bierer *et al.*, 1988).

Correspondence: Dr E. F. Srour, Department of Medicine, Indiana University School of Medicine, Medical Research and Library Building, Room 442, 975 West Walnut, Indianapolis, IN 46202-5121, USA.

The T cell receptor (TcR) molecule is composed of a disulphide-linked heterodimer (Ti) which is non-covalently associated with the CD3 complex (T3) (Koning *et al.*, 1988). A small population of T cells expresses a different TcR termed $\gamma\delta$ (Borst *et al.*, 1987) which is a part of the Ti/T3 complex on $\alpha\beta^-$ cells (Weiss, Newton & Cromie, 1986). It has been shown that $\gamma\delta^+$ T cells display different types of cytotoxicity (Borst *et al.*, 1987; Moretta *et al.*, 1987; Janeway, Jones & Hayday, 1988) and that the murine epidermis contains $\gamma\delta^+$ cells (Koning *et al.*, 1987) which mediate non-MHC-restricted cytotoxicity (Klein, 1986).

These properties of the $\gamma\delta$ T cells coupled with the functional characteristics of $CD3^+CD5^-$ cells (Srour *et al.*, 1988) prompted us to investigate whether $CD3^+CD5^-$ cells are $\gamma\delta^+$ and vice versa. The *in vitro* behaviour of different $\gamma\delta$-bearing T cells including $CD3^+CD5^-\gamma\delta^+$ lymphocytes were examined and compared with those of $CD3^+CD5^+$ and $\alpha\beta^+$ T lymphocytes.

MATERIALS AND METHODS

MoAbs and cell staining

Ficoll/Hypaque-isolated peripheral blood lymphocytes (PBL) were stained on ice with the various MoAbs listed in Table 1. PBL washed twice in PBS + 2% human serum albumin were first incubated with purified δ1 TcR. Texas red (TR) conjugated, affinity-purified, subclass-specific goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) was then added to develop the δ1 TcR. Free active sites on the second-step reagent were blocked by mouse myeloma IgG1. Next, biotinilated Leu-4, fluorescein isothiocyanate (FITC) WT31 and phycoerythrin (PE) Leu-1 were added simultaneously. Finally, streptavidin-conjugated with allophycocyanin (APC) (Molecular Probes, Eugene, OR) was added to develop the Leu-4. Cells were washed once after each incubation step (20 min) except after the addition of the myeloma protein and three times at the end of the staining procedure.

Table 1. CD and cell reactivity of the monoclonal antibodies (MoAbs) used in this study

Antigen cluster designation	MoAb	Forms of MoAbs used	Predominant reactivity
CD5	Leu-1	F, PE	T cells and B cell subsets
CD8	Leu-2a	F, PE	T cytotoxic/suppressor
CD4	Leu-3a	F, PE	T helper/inducer
CD3	Leu-4	F, PE, B	T cells
CD2	Leu-5	P	T cells/NK cells
CD16	Leu-11	PE	Fc IgG receptor/NK, neutrophils
—	Leu-19	PE	NK/cytotoxic T cell subsets
—	WT31	F	αβ TcR
—	δ1 TcR	P	γδ TcR

P, purified antibody; F, fluorescein conjugate; PE, phycoerythrin conjugate; B, biotin conjugate.
δ1 TcR was obtained from T Cell Sciences, Cambridge, MA. The remaining MoAbs were obtained from Becton Dickinson, Mountain View, CA.

Flow cytometry and cell sorting

Previously published procedures (Srour *et al.*, 1988) with some modifications were used. All experiments were performed on a Coulter Epics 753 dual laser flow cytometer (Coulter Electronics, Hialeah, FL). For four-colour immunofluorescence cell analysis and sorting, custom-made 550 nm, 600 nm and 650 nm long-wave pass dichroic filters were used for the reflection of the FITC, PE and TR signals, respectively, towards the appropriate photomultiplier tubes while 525 nm, 575 nm, 625 nm and 675 nm narrow-band pass filters selected the FITC, PE, TR and APC signals, respectively. Dual-fluorescence histograms gated on forward angle light scatter and the two remaining fluorescence signals, and single-parameter histograms gated on forward angle light scatter only were used for data analysis. Non-specific staining with isotype-matched myeloma proteins was used to determine the background fluorescence for each fluorochrome. Cell sorting was performed as described in Fig. 1. Cells intended for the cytotoxicity assays were first cultured at 2×10^6/ml with 5% T cell growth factor (TCGF) (Cellular Products, Buffalo, NY) and 1% PHA-HA15 (Wellcome Diagnostics, Research Triangle Park, NC). On day 3, the cells were washed, stained and sorted to yield the four groups B, C, D and E (described in Fig. 1 and Table 2). Group A consisted of the unstained, unsorted cultured cells.

Measurement of the frequency of proliferative T lymphocytes

The measurement of the frequency of proliferating T cells was performed as previously described (Srour *et al.*, 1988). Round-bottomed 96-well plates were seeded with 10^5 irradiated (5000 rad) human spleen cells. Responder cells in a two-fold dilution

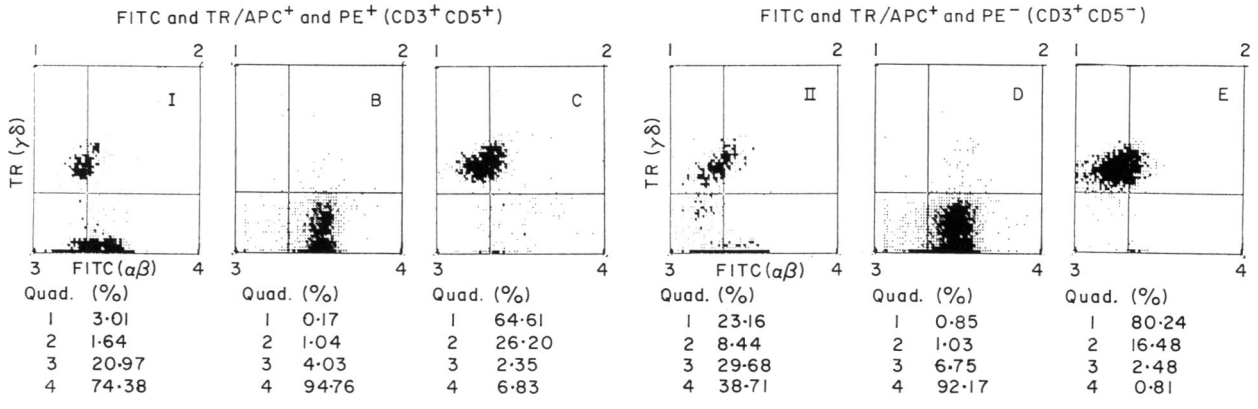

Fig. 1. A representative four-colour immunofluorescence cell-sorting experiment. Cell sorting was performed by establishing sorting windows around the populations of choice on a gated two-parameter histogram (two fluorescence signals) displaying events positive or negative for the two remaining fluorescence signals. The six panels show the immunofluorescence analyses of pre- and post-sort peripheral blood lymphocytes (PBL) stained for CD3, CD5, αβ and γδ. Gated $CD3^+CD5^+$ and $CD3^+CD5^-$ pre-sort cells are shown in panels I and II, respectively. Sorting of the cells of panel I yielded two groups of cells (B and C in panels B and C, respectively) as did the sorting of the cells of Panel II (D and E in Panels D and E, respectively). In this study, unlabelled, unsorted PBL will be referred to as group A. The phenotypes of groups B, C, D and E are described in Table 2, set iv. In this experiment $CD3^+CD5^+$ cells constituted 65·5% of all cells analysed, whereas while the $CD3^+CD5^-$ cells constituted only 6·3%. Quadrant (Quad.) statistics are given below each histogram. The percent positive for CD3, CD5, αβ and γδ from the single-colour control samples were 71·8, 65, 67 and 4·6, respectively. The values obtained from the four color sample were 71·5, 63, 65 and 5·8%, respectively. Post-sort cell viability by trypan blue exclusion was always >96%.

Table 2. Frequencies of proliferating and interleukin-2 (IL-2) producing cells of different subpopulations of T lymphocytes

Set/group	Phenotype	Relative size*	Frequency/10^3 cells: Proliferating cells	Frequency/10^3 cells: IL-2-producing cells
i	PBL	—	152·5	ND
	$\alpha\beta^+, \gamma\delta^-$	96·9	290·0	ND
	$\alpha\beta^-, \gamma\delta^+$	2·3	19·6	ND
ii	PBL	—	64·7	8·9
	$CD3^+, \alpha\beta^+, \gamma\delta^-$	96·0	328·3	54·0
	$CD3^+, \alpha\beta^-, \gamma\delta^+$	1·7	19·4	15·3
iii	PBL	—	70·2	11·0
	$CD2^+, CD3^+, CD5^+, \alpha\beta^+$	90·2	198·5	23·9
	$CD2^+, CD3^+, CD5^-, \alpha\beta^+$	5·0	10·3	7·7
	$CD2^+, CD3^+, CD5^-, \alpha\beta^-$	1·9	9·1	1·6
iv A	PBL	—	195·3	32·4
B	$CD3^+, CD5^+, \alpha\beta^+, \gamma\delta^-$	81·2	275·3	51·9
C	$CD3^+, CD5^+, \alpha\beta^-, \gamma\delta^+$	7·1	16·3	0·2
D	$CD3^+, CD5^-, \alpha\beta^+, \gamma\delta^-$	6·6	33·4	4·2
E	$CD3^+, CD5^-, \alpha\beta^-, \gamma\delta^+$	3·9	16·3	2·1

* Relative size of each phenotype of sorted cells as a percentage of the total number of $CD3^+$ cells.

PBL, peripheral blood lymphocytes; ND, not determined

scheme were delivered in medium containing 5% TCGF and 1% PHA-HA15 as final concentrations. The plates were incubated at 37°C, fed with 10 μl/well of TCGF on day 7 and examined microscopically 12–14 days later. A well was scored positive if definitive growth of a colony (or colonies) was observed.

Measurement of the frequency of IL-2-producing lymphocytes

Conical-bottomed, 96-well plates with 10^4 irradiated JY cells per well and concanavalin A at a final concentration of 3 μg/ml were used for this assay. After 6 days of incubation at 37°C, 100 μl of the supernatant of each well were tested for their IL-2 content in a CTLL assay. Frequencies of proliferating and IL-2-producing lymphocytes were calculated using the χ^2 minimalization method. In the assay for frequency of IL-2-producing lymphocytes, wells were scored positive if their ct/min were higher than 3 s.d. of the mean spontaneous incorporation calculated from the wells containing JY cells only.

Microcytotoxicity assays

The microcytotoxicity assay was performed as previously described (Jenski & Kleyle, 1989). Effector cells were suspended in 80 μl of RPMI 1640 medium with 20% defined fetal bovine serum. P815 mastocytoma cells to be used as targets for lectin-dependent killing were labelled for 1 h at 37°C with 150 μCi of ^{51}Cr (sodium chromate; New England Nuclear, Boston, MA). After washing, the targets were serially diluted in medium plus 20 μg PHA-HA16/ml (PHA-HA16 is a form more purified than PHA-HA15). Five microlitres of targets and 5 μl of effectors were pipetted into microtest wells to produce five target concentrations in duplicate. The plates were incubated for 4 h and then centrifuged. A 2-μl aliquot of supernatant from each well was spotted onto thick blotting paper which was air dried. Autoradiography was accomplished by exposing X-ray film (X-Omat AR, Eastman Kodak, Rochester, NY) to the filter paper for 7 days at −80°C. The autoradiogram was scanned at 490 nm with a Bio-Tek EL307C microplate reader. Killing velocity was absorbance/effector cell number/4h. A curve defined by the Hill equation was fitted to each plot of killing velocity *versus* target concentration by weighted non-linear regression (IBM-PC program ENZFITTER; Biosoft, Milltown, NJ). The maximum killing velocity (at an infinite target concentration) determined from the curve was used to compare the velocity of equal numbers of effector cells.

Expansion of T cell subpopulations

T cells were expanded *in vitro* as described (Bierer *et al.*, 1988). Briefly, 5×10^3–10^4 cells were seeded in 1 ml in a 24-well plate with $1–2·5 \times 10^5$ irradiated JY cells. The cells were stimulated every 7–10 days with JY cells for the first 4 weeks. Following that, the cells were stimulated weekly with irradiated JY cells and 5% TCGF. When these cells were used in microcytotoxicity assays, they were first activated with 1% PHA-HA15 for 3 days.

RESULTS

Phenotypic characterization of $\gamma\delta$ TcR^+ T lymphocytes

The expression of CD3 and CD5 on $\gamma\delta^+$ and $\gamma\delta^-$ PBL was examined through the reanalysis of sorted T lymphocytes (Fig. 2). Post-sort analysis clearly indicated that $\gamma\delta^+$ cells displayed an attenuated expression of both CD3 and CD5, as compared with $\gamma\delta^-$ cells. The diminution of expression of CD5 on $\gamma\delta^+$ cells was much more prominent than that of CD3. Interrelations between CD3, CD5 and $\gamma\delta$ TcR on fresh PBL were studied in a series of 10 experiments. The entire population of $\gamma\delta$ T cells

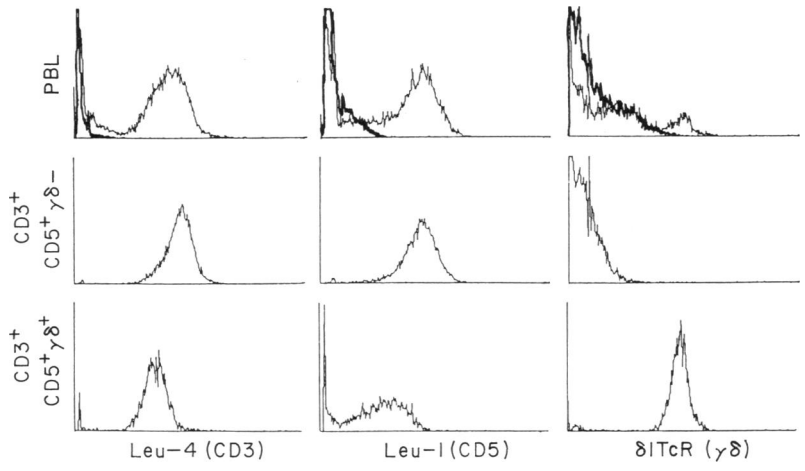

Fig. 2. Phenotypic analysis of the level of expression of CD3, CD5 and γδ on sorted CD3⁺CD5⁺γδ⁺ and CD3⁺CD5⁺γδ⁻ cells along with that of unfractionated peripheral blood lymphocytes (PBL). Gated CD3⁺CD5⁺ cells were sorted for the expression or lack thereof of γδ TcR. The darker histograms in the PBL panels represent the non-specific staining obtained with the respective isotype controls.

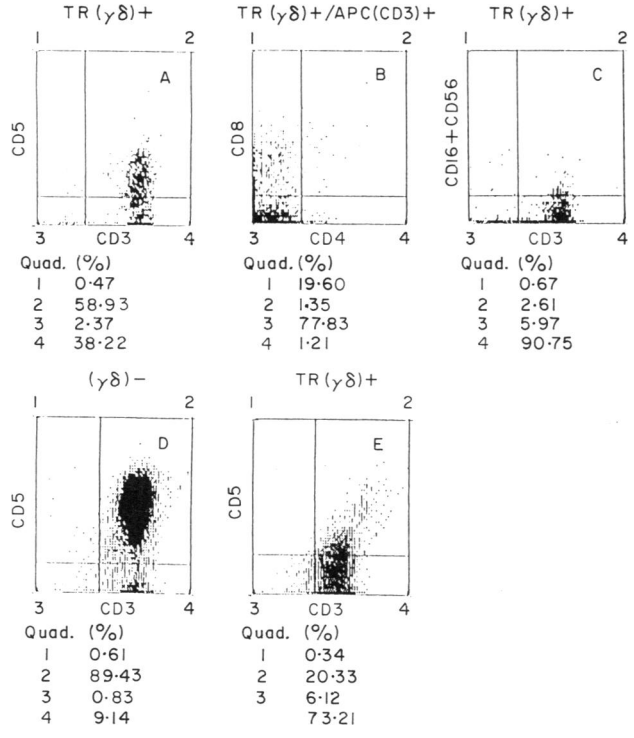

Fig. 3. Phenotypic analysis of fresh peripheral blood lymphocytes (PBL) (A,B,C) and of CD3⁺γδ⁻ and CD3⁺γδ⁺ (panels D and E, respectively) that were expanded *in vitro* for 5 months. In panels A, C and E cells were stained for γδ and the two other markers as indicated. Only γδ⁺ cells were considered for the analysis shown in these panels. The cells of panel B were positive for the expression of γδ and CD3. Panel D depicts the CD3 and CD5 fluorescence distribution of cultured CD3⁺γδ⁻ cells.

expressed CD3. However, only 2/3 to 3/4 of these cells expressed CD5. A discrete CD3⁺CD5⁻γδ⁺ subpopulation equal in size to 1/4 to 1/3 of all the γδ⁺ T lymphocytes was thus identified (Fig. 3A). Whereas the CD3⁺CD5⁻ population constituted 7·5% ± 2·5 (mean ± s.d., n = 10) of the cells analysed or 11% of CD3⁺ cells, the γδ⁺ cells made up 5·3% ± 2·4 of all cells analysed or 7·8% of CD3⁺ cells. CD3⁺CD5⁻γδ⁺ cells were 2·4% ± 0·8 of all cells or 3·5% of CD3⁺ T lymphocytes. The distribution of CD4, CD8, CD16 and Leu-19 (CD56) on fresh γδ TcR T cells was also examined (Fig. 3B, C). CD8 was expressed on approximately 20% of the γδ⁺ cells, while the remaining markers (CD4, CD16 and CD56) showed very minimal or no expression (< 3%).

Similar phenotypic studies were performed on *in vitro* expanded CD3⁺γδ⁺ and CD3⁺γδ⁻ cells. CD3⁺γδ⁺ cells cultured for 21 days continued to express CD8 on 20% of the cells. However, after a period of 5 months, these cells were completely CD8⁻CD4⁻ (data not shown). At this same stage, the CD3⁺γδ⁻ cells continued to express CD5 (Fig. 3D) while only 20% of the CD3⁺γδ⁺ cells maintained a weak expression of CD5 (Fig. 3E).

Frequencies of proliferating and IL-2-producing cells

The data from four different sets of experiments measuring the frequencies of proliferating and IL-2-producing lymphocytes of different T cell subpopulations are shown in Table 2. The first series of experiments showed that αβ⁺γδ⁻ cells were more clonogenic than αβ⁻γδ⁺ cells. Having established that, we isolated CD3⁺ T lymphocytes expressing an αβ⁺γδ⁻ or αβ⁻γδ⁺ phenotype and examined both groups along with unfractionated PBL. Once again, it was established that αβ cells were more clonogenic than γδ T cells. In addition, CD3⁺αβ⁺γδ⁻ cells had a higher frequency of IL-2-producing lymphocytes than did CD3⁺αβ⁻γδ⁺ cells. We then examined whether the CD2⁺CD3⁺CD5⁻ T cells could be divided into αβ⁺ and αβ⁻ cells. It was observed that such a division was possible and that the behavioral characteristics of both subpopulations (CD2⁺CD3⁺CD5⁻αβ⁺ and CD2⁺CD3⁺CD5⁻αβ⁻) were different from those of CD2⁺CD3⁺CD5⁺αβ⁺. When compared with CD2⁺CD3⁺CD5⁺αβ⁺ cells, both groups had reduced frequencies of proliferating and IL-2-producing cells. A small difference in the frequencies of IL-2-producing cells was observed between the αβ⁺ and αβ⁻ subpopulations of CD2⁺CD3⁺CD5⁻ cells. In the last series of experiments, αβ⁺γδ⁻ and αβ⁻γδ⁺ cells were sorted on a background of CD3⁺CD5⁺ and CD3⁺CD5⁻ T lymphocytes to yield the four

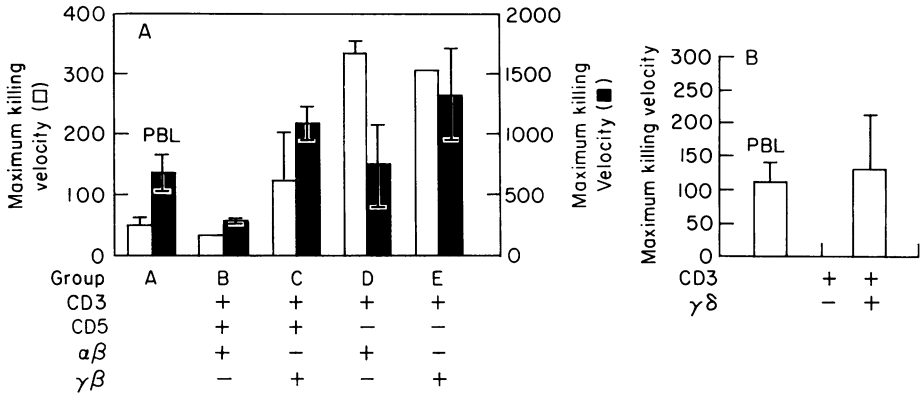

Fig. 4. (A) Lectin-dependent killing by five groups of T cell subpopulations activated *in vitro* for 3 days (□) or expanded in culture for 10 weeks (■). Maximum velocity of killing (ordinate) was determined by nonlinear regression analysis of dose-response data generated in the microcytotoxicity assay. (B) Lectin-dependent killing by PBL, $CD3^+\gamma\delta^-$ and $CD3^+\gamma\delta^+$ cells that were expanded *in vitro* for 5 months. The phenotypic analysis of the $CD3^+\gamma\delta^-$ and $CD3^+\gamma\delta^+$ cells used in this assay is shown in Fig. 3, D and E.

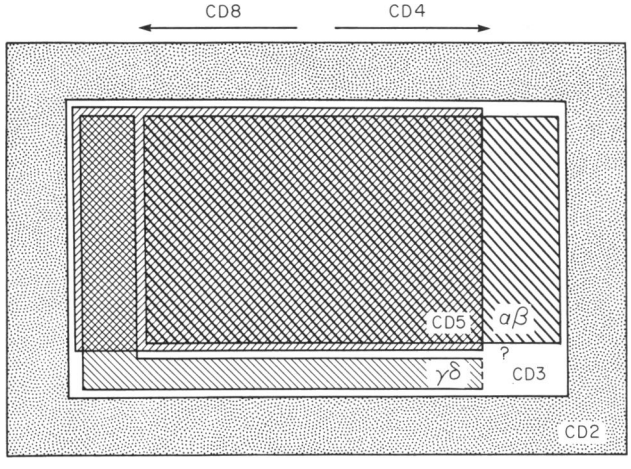

Fig. 5. A schematic representation of the hypothetical inter-relations between the CD2, CD3, CD4, CD5, CD8, $\alpha\beta$ and $\gamma\delta$ T lymphocytes as determined by this study. The relative sizes of the different phenotypes were not drawn to scale.

different subpopulations of T cells (groups B, C, D, and E in Fig. 1). The lowest frequencies of proliferating and IL-2-producing lymphocytes were detected among the subpopulations expressing $\gamma\delta$ TcR regardless of whether these cells expressed CD5 (groups C and E). Within the $CD3^+CD5^-$ cells, slightly higher frequencies were observed among the cells that do not express $\gamma\delta$ TcR (group D).

Cytotoxic activity of T cell subpopulations

Cells used in these assays were prepared in two different ways. The first method was activating fresh PBL *in vitro* for 3 days, then sorting them to yield groups A–E. In the second method, the five groups of cells were expanded *in vitro* for 10–12 weeks with JY cells and TCGF (Bierer *et al.*, 1988), activated with 1% PHA-HA15 for 3 days and then used in the micro-cytotoxicity assay. The growth pattern of the expanded five groups of cells continued to reflect the frequency of proliferating T lymphocytes observed in the limiting-dilution assays. The doubling time of groups C and E was more than double that of groups A, B and D. The results of two separate experiments in which the killing of P815 cells by effectors prepared according to the two methods described above are shown in Fig. 4A. Among the cells that were in culture for 3 days only (open bars), the highest degree of killing was found in groups D and E, both of which share the phenotype $CD3^+CD5^-$. Within this phenotype, $\alpha\beta^+$ and $\gamma\delta^+$ cells mediated comparable levels of cytotoxic activity. However, within the $CD3^+CD5^+$ cells, the degree of cytolytic activity of group C ($\gamma\delta^+$) was higher than that of group B ($\alpha\beta^+$). When the cytolytic capacity of the five groups of cells that were expanded *in vitro* for several weeks was measured, an enhanced activity in groups A and C was observed (Fig. 4A, solid bars). In a separate series of experiments, the lectin-dependent cytotoxicity of *in vitro* expanded, PHA-activated unfractionated PBL, $CD3^+\gamma\delta^-$ and $CD3^+\gamma\delta^+$ sorted cells was measured. Although $CD3^+\gamma\delta^+$ and the unfractionated PBL displayed comparable levels of cytotoxicity, there was no detectable cytotoxic activity in the $CD3^+\gamma\delta^-$ cells (Fig. 4B). The phenotypic analysis of the $CD3^+\gamma\delta^-$ and $CD3^+\gamma\delta^+$ cells used in these experiments is shown in Fig. 3 (D and E, respectively).

Cytolytic activity was also examined in individual wells that had colonies of approximately equal sizes chosen from the plates prepared for the frequency of proliferating cells of the five groups. Colonies were selected from rows seeded with 32 responding cells or less. Data from three separate experiments showed that in groups A, B, C and D; 56 ± 17, 35 ± 4, 43 ± 30 and $55\pm3\%$ (mean $\pm$ s.e.m.) of the colonies tested displayed cytotoxicity. However, in group E, $78\pm12\%$ of the tested colonies were positive for cytotoxic activity. Colonies were scored positive for cytotoxicity when killing was >3 s.d. above mean spontaneous radiolabel release.

Phenotypic relations between the different T cell subsets

A hypothetical scheme depicting the inter-relations between the CD2, CD3, CD5, $\alpha\beta$ and $\gamma\delta$ markers and receptors on T lymphocytes only was constructed based on the data generated in this study (Fig. 5). The $CD2^+$ T lymphocyte population encompasses all CD3 positive cells. CD3 in turn encompasses all $CD5^+$ T lymphocytes generating a small population of cells with the phenotype $CD2^+CD3^+CD5^-$. Approximately 90% of T lymphocytes expressing CD2 and CD3 express CD5 as well. The

remaining 10% of T cells with the phenotype CD2⁺CD3⁺CD5⁻ are further subdivided based on the type of TcR molecule they express. αβ TcR is found on 2/3 of the CD2⁺CD3⁺CD5⁻ cells and γδ TcR⁺ cells make up the remaining 1/3. Similarly, 2/3 and 1/3 of the γδ TcR⁺ cells have the phenotype CD3⁺CD5⁺ and CD3⁺CD5⁻, respectively. The question mark in Fig. 5 refers to areas where the relations among these different markers or the existence of such cells is still ambiguous. The arrows for CD4 and CD8 cells indicate that in the areas to the left of this diagram, where the γδ⁺ cells have been placed, only CD8⁺ cells were detected.

DISCUSSION

CD3⁺ T lymphocytes lacking the expression of CD5 (Bierer *et al.*, 1988; Srour *et al.*, 1988), or closely related cells (Van de Griend *et al.*, 1987; Moretta *et al.*, 1987; Pantaleo *et al.*, 1988), have been reported to mediate cytolytic functions. Likewise, cells that are either negative for the expression of αβ TcR (Snodgrass *et al.*, 1985; Bank *et al.*, 1986) or that do express γδ (Janeway *et al.*, 1988; Goodman & Lefrancois, *et al.*, 1988) can mediate different forms of cytotoxicity. Based on this information, we aimed to study the nature of the TcR on CD3⁺CD5⁻ T cells and to examine the functional properties of the supopulations of CD3⁺CD5⁻ cells. Cells within the CD3⁺CD5⁻ T cell subset expressed either αβ or γδ TcR (groups D and E, respectively). Approximately 2/3 of the CD3⁺CD5⁻ cells expressed αβ, whereas 1/3 of these cells expressed the γδ TcR. Phenotypic analysis of sorted γδ⁺ and γδ⁻ cells demonstrated that the density of CD5 on γδ⁺ cells was considerably lower than that on γδ⁻ cells, indicating that γδ TcR is preferentially expressed on CD5 'dull' or negative cells. Functional assays indicated that CD3⁺γδ⁺ T lymphocytes and CD3⁺CD5⁻γδ⁺ cells have lower frequencies of proliferating and IL-2-producing cells than do CD3⁺CD5⁺αβ⁺ cells. The lower frequencies of proliferating and IL-2-producing cells was associated with the lack of expression of CD5, the expression of γδ TcR or both conditions simultaneously. However, it should be noted that slightly higher frequencies in both assays were observed among the αβ⁺ cells of the CD3⁺CD5⁻ population compared with the γδ⁺ lymphocytes.

CD5 has been associated with the execution of T helper functions of CD4⁺ cells (Thomas *et al.*, 1984) and with positive regulatory mechanisms involving T cell proliferation (Ledbetter *et al.*, 1985). Moreover, when CD5 MoAbs were reacted with T cells, an increased production of IL-2 was observed (Ledbetter *et al.*, 1985). Taken together, the available data and the data presented in this study suggest that the low frequencies of proliferating and IL-2-producing cells observed in the CD3⁺CD5⁻γδ⁺ and the γδ⁺ cells could be attributed to the low or absent expression of CD5 on these two populations of T cells.

Recently, Faure *et al.*, (1988) described a CD2⁻CD3⁺γδ⁺ PBL subpopulation with low proliferative capacity attributed to an impaired interaction between the CD2⁻ T cells and the LFA3⁺ feeder layer cells. Even though this is an attractive explanation for reduced proliferative potentials, it cannot explain the results we report in this study. Our current (Table 2, set iii) and previous data (Srour *et al.*, 1988), have demonstrated low frequencies of proliferating lymphocytes among CD2⁺ cells. In our hands, the low frequency of proliferating cells was associated with the expression of γδ or lack of expression of CD5. Suppressed proliferation of CD3⁺WT31⁻ compared with that of CD3⁺WT31⁺ cells was reported by Vilmer *et al.*, (1988) in the PBL of a post-allogeneic bone marrow transplantation patient.

Human γδ cells are expanded *in vitro* by prolonged stimulation with feeder layers and IL-2 (Bank *et al.*, 1986; Brenner *et al.*, 1987). This technique may enrich for non-specific cytolytic cells (Marusic-Galesic *et al.*, 1988) as was observed with CD3⁺, γδ⁺ large granular lymphocytes following 14 days of exposure to recombinant IL-2 (Colamonici *et al.*, 1988). In order to avoid the artificial selection of cytotoxic cells by *in vitro* expansion, cells were activated for 3 days, sorted and assayed for lectin-dependent cytotoxicity. This technique made it possible to isolate the five groups immediately before the assay and was adequate for activating the cells as was seen from their cytolytic activity. It also established a baseline cytolytic activity for each of the five groups of cells. The cytotoxic activity of the five groups of cells which were cultured *in vitro* for several weeks was measured and compared with the baseline activity established with fresh cells. It was found that the highest and lowest cytotoxic activities were still those of groups E and B, respectively. The cells of group C following the *in vitro* expansion displayed an increased cytolytic activity and were still γδ⁺, with a majority expressing a CD3⁺CD5⁻ phenotype (data not shown). The appearance of the CD3⁺CD5⁻ cells occurred even though these cells were originally CD5⁺. Similar changes were detected in the CD3⁺γδ⁺ cells upon expansion *in vitro* (Fig. 4B). Two phenotypic properties may explain why CD3⁺γδ⁺ cells were more cytotoxic than CD3⁺γδ⁻ cells: firstly, 90% of the CD3⁺γδ⁻ cells were CD4⁺, a marker that is not associated with cytotoxicity; and, secondly, the majority of the CD3⁺γδ⁺ cells (which had remained γδ⁺) were CD3⁺CD5⁻ (73%) whereas only 9% of the CD3⁺γδ⁻ cells were CD3⁺CD5⁻. It is possible that the conditions utilized in this study may induce γδ TcR⁺ cells to down-modulate the expression of CD5 in conjunction with the continued expression of γδ TcR and CD3 thus allowing for the selection of cytotoxic cells. γδ⁺ and CD3⁺CD5⁻ cells have been shown to be cytotoxic in nature (Borst *et al.*, 1987; Moretta *et al.*, 1987; Janeway *et al.*, 1988; Srour *et al.*, 1988).

The failure of a murine CD3⁺ variant of the T cell hybridoma 2BR.11 to synthesize normal quantities of IL-2 has been linked to the absence of expression of the CD3ζ component of the CD3 molecule (Sussman *et al.*, 1988). The loss of CD3ζ led to a decreased expression of the CD3/Ti complex. It is unlikely that such an incomplete CD3 molecule is the cause of the low frequency of IL-2-producing cells observed in groups C, D and E. The observed diminution in the density of CD3 molecules on γδ⁺ cells was very minimal and would not account for the low frequency of IL-2-producing lymphocytes. The low frequency of IL-2-producing lymphoyctes was again correlated with the expression of γδ TcR, lack of expression of CD5, or both conditions at the same time.

The observations made in this study support the hypothesis that γδ⁺ cells are a separate entity of mature T lymphocytes (Weiss *et al.*, 1986; Brenner *et al.*, 1987; Janeway *et al.*, 1988). γδ⁺ cells expressed T cell markers associated with 'mature' T lymphocytes such as CD3, CD5 and CD8. It appears that γδ⁺ or CD5⁻ T lymphocytes are functionally specialized, terminal T cells with minimal proliferative capacity. This thesis may explain the lower frequency of proliferating cells seen among the CD3⁺CD5⁻γδ⁺ T cells described in this report. The detection of

$\gamma\delta^+CD8^+$ cells in normal PBL is contradictory to reports showing that $\gamma\delta^+$ cells are almost exclusively $CD4^-CD8^-$ (Bank *et al.*, 1986; Moretta *et al.*, 1987). The apparent reason for this finding in some of these reports is that T cells were depleted with CD4 and CD8 plus complement to enrich for $CD3^+\gamma\delta^+$ cells. Consistent with our results are studies that describe T cell clones expressing both $\gamma\delta$ and CD8 (Brenner *et al.*, 1986) or murine $CD3^+\gamma\delta^+$ intestinal epithelium T cells that are also $CD4^+CD8^+$ (Goodman & Lefrancois, 1988). Furthermore, lectin-dependent cytotoxicity which is a function normally attributed to mature T cells was mediated by all the $\gamma\delta^+$ T cell subpopulations. However, we report functional similarities between $\gamma\delta$ TcR^+ and $CD3^+CD5^-$ T cells which have been thought of as precursors of the more abundant $CD3^+CD5^+$ T lymphocytes (Smith *et al.*, 1987; Bierer *et al.*, 1988). It remains to be seen whether this observation lends support to the thesis that $\gamma\delta^+$ cells are indeed T cell precursors.

This report describes $CD3^+CD5^-\gamma\delta^+$ T cells which seem to possess specialized functions in the immune system. The exact role of these cells would be further defined when a better understanding of the function of CD5 and $\gamma\delta$ T cells is achieved. At this point, we propose a functional role for these cells in transplantation immunology. $CD3^+CD5^-\gamma\delta^+$ cells might be involved in mediating rejection and graft-*versus*-host disease in the organ and bone marrow transplantation settings, respectively. Early indications from the work of Filipovich, Polich & Vallera (1988) and Bierer, Burakoff & Smith (1989) make a connection between $CD3^+CD5^-$ cells and graft-*versus*-host disease. We are currently investigating this notion in our bone marrow transplant patients. It is important to note that T cell depletion of donor bone marrow with CD5 and complement, which has been used heavily as a regimen for the control of graft-*versus*-host disease (Jansen *et al.*, 1987), would not only spare $CD5^-$ cells, but that $\gamma\delta^+$ cells may also escape killing because of their low or lack of expression of CD5.

ACKNOWLEDGMENT

We thank Charlotte Franklin for preparing the manuscript.

REFERENCES

ANDERSON, M.J., RAPPAPORT, J.M., AULT, K.A., BURAKOFF, S.J. & SMITH, B.R. (1985) Clinical correlates of unusual circulating lymphocytes appearing post marrow transplantation (BMT). *Blood*, **66,** (Suppl.), 257a.

BANK, I., DEPINHO, R.A., BRENNER, M.B., CASSIMERS, J., ALT, F.W. & CHESS, L. (1986) A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature*, **322,** 179.

BIERER, B.E., BURAKOFF, S.J. & SMITH, B.R. (1989) A large proportion of T lymphocytes lack CD5 expression following bone marrow transplantation. *Blood*, **73,** 1359.

BIERER, B.E., NISHIMURA, Y., BURAKOFF, S.J. & SMITH B.R. (1988) Phenotypic and functional characterization of human cytolytic T cells lacking expression of CD5. *J. clin. Invest.* **81,** 1390.

BORST, J., VAN DE GRIEND, R.J., VAN OOSTVEEN, J.W., ANG, SL., MELIEF, C.J., SEIDMAN, J.G. & BOLHUIS, R.L.H. (1987) A T-cell receptor $\gamma\delta$/CD3 complex found on cloned functional lymphocytes. *Nature*, **325,** 683.

BRENNER, M.B., MCLEAN, J., DIALYNAS, D.P., STROMINGER, J.L., SMITH, J.A., OWEN, F.L., SEIDMAN, J.G., IP, S., ROSEN, F. & KRANGEL, M.S. (1986) Identification of a putative second T-cell receptor. *Nature*, **322,** 145.

BRENNER, M.B., MCLEAN, J., SCHEFT, H., RIBERDY, J., ANG, S.L., SEIDMAN, J.G., DEVLIN, P. & KRANGEL, M.S. (1987) Two forms of the T-cell receptor protein found on peripheral blood cytotoxic T lymphocytes. *Nature*, **325,** 689.

CEUPPENS, J.L. & BAROJA, M.L. (1986) Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-base-bound OKT3. *J. Immunol.* **137,** 1816.

COLAMONICI, O.R., ANG, S., QUINONES, R., HENKART, P., HEIKKILA, R., GRESS, R., FELIX, C., KIRSCH, I., LONGO, D., MARTI, G., SEIDMAN, J.G. & NECKERS, L.M. (1988) IL-2-dependent expansion of $CD3^+$ large granular lymphocytes expressing T cell receptor-$\gamma\delta$. *J. Immunol.* **140,** 2527.

FAURE, F., JITSUKAWA, S., MEUER, S., BOHUON, C., TRIEBEL, F. & HERCEND, T. (1988) Identification of a $CD2^-CD3^+$ T cell receptor-γ^+ peripheral blood lymphocyte subpopulation. *J. Immunol.* **140,** 2128.

FILIPOVICH, A.H., POLICH, D.M. & VALLERA, D. (1988) T depletion with anti CD5 immunotoxin (IT): residual CD5 negative T cells in donor bone marrow (BM) predicts subsequent graft vs. host disease (GVHD). *Blood*, **72** (Suppl.), 387a.

GOODMAN, T. & LEFRANÇOIS, L. (1988) Expression of γ-δ T-cell receptor on intestinal $CD8^+$ intraepithelial lymphocytes. *Nature*, **333,** 855.

JANEWAY, C.A. JR, JONES, B. & HAYDAY, A. (1988) Specificity and function of T cells bearing $\gamma\delta$ receptors. *Immunol. Today*, **9,** 74.

JANSEN, J., SROUR, E., COOK, C., WALKER, D. & REICHERT, T.H. (1987) T-cell depletion with anti-Leu-1 for allogeneic bone marrow grafting. *Blood*, **68** (Suppl.), 289a.

JENSKI, L.J. & KLEYLE, R.M. (1989) Kinetic assay of cytotoxic T lymphocyte clones. I. Methodology and data analysis. *J. immunol. Methods*, **117,** 181.

KLEIN, J.R. (1986) Ontogeny of the Thy-1^-, Lyt-2^+ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa *J. exp. Med.* **164,** 309.

KONING, F., STINGL, G., YOKOYAMA, W.M., YAMADA, H., MALOY, W.L., TSCHACHLER, E., SHEVACH, E.M. & COLIGAN, J.E. (1987) Identification of a T3-associated $\gamma\delta$ T cell receptor on Thy-1^+ dendritic epidermal cell lines. *Science*, **236,** 834.

KONING, F., LEW, A.M., MALOY, W.L., VALAS, R. & COLIGAN, J.E. (1988) The biosynthesis and assembly of T cell receptor α- and β-chains with the CD3 complex. *J. Immunol.* **140,** 3126.

LEDBETTER, J.A., JUNE C.H., MARTIN, P.J., SPOONER, C.E., HANSEN, J.A. & MEIER, K.E. (1986) Valency of CD3 binding and internalization of the CD3 cell-surface complex control T cell responses to second signals: Distinction between effects of protein kinase C, cytoplasmic free calcium and proliferation. *J. Immunol.* **136,** 3945.

LEDBETTER, J.A., MARTIN, P.J., SPOONER, C.E., WOFSY, D., TSU, T.T., BEATTY, P.G. & GLADSTONE, P. (1985) Antibodies to Tp67 and Tp44 augment and sustain proliferative responses of activated T cells. *J. Immunol.* **135,** 2331.

MARUSIC-GALESIC, S., PARDOLL, D.M., SAITO, T., LEO, O., FOWLKES, B.J., COLIGAN, J., GERMAIN, R.N., SCHWARTZ, R.H. & KRUISBEEK, A.M. (1988) Activation properties of T cell receptor-$\gamma\delta$ hybridomas expressing diversity in both γ- and δ-chains. *J. Immunol.* **140,** 411.

MORETTA, L., PENDE, D., BOTTINO, C., MIGONE, N., CICCONE, E., FERRINI, S., MINGARI, M.C. & MORETTA, A. (1987) Human $CD3^+4^-8^-WT31^-$ T lymphocyte populations expressing the putative T cell receptor γ-gene product. A limiting dilution and clonal analysis. *Eur. J. Immunol.* **17,** 1229.

PANTALEO, G., ZOCCHI, M.R., FERRINI, S., POGGI, A., TAMBUSSI, G., BOTTINO, C., MORETTA, L. & MORETTA, A. (1988) Human cytolytic cell clones lacking surface expression of T cell receptor α/β or γ/δ: evidence that surface structures other than CD3 or CD2 molecules are required for signal transduction. *J. exp. Med.* **168,** 13.

ROZENS, M.K., SMITH, B.R., EMERSON, S., CRIMMINS, M., LAURENT, G., REICHERT, T., BURAKOFF, S.J. & MILLER, R.A. (1986) Functional assessment of T cell depletion from bone marrow prior to therapeutic transplant using limiting dilution culture methods. *Transplantation*, **42,** 380.

SMITH, B.R., RAPPEPORT, J.M., BURAKOFF, S.J. & AULT, K.A. (1987) Clinical correlates of unusual circulating lymphocytes appearing post marrow transplantation. In *Recent Advances in Bone Marrow Transplantation* (ed. by R.P. Gale & R. Champlin) p. 53, 659. Alan R. Liss, New York.

SNODGRASS, H.R., DEMBIC, Z., STEINMETZ, M. & VON BOEHMER, H. (1985) Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature*, **315,** 232.

SROUR, E.F., WALKER, E.B., WALKER, D.E. & JANSEN, J. (1988) Functional and phenotypical studies of the Leu-4 $(CD3)^+$, Leu-1 $(CD5)^-$ T lymphocytes. *Clin. exp. Immunol.* **73,** 34.

SUSSMAN, J.J., BONIFACINO, J.S., LIPPINCOTT-SCHWARTZ, J., WEISSMAN, A.M., SAITO, T., KLAUSNER, R.D. & ASHWELL, J.D. (1988) Failure to synthesize the T cell CD3-ζ chain: structure and function of a partial T cell receptor complex. *Cell*, **52,** 85.

THOMAS, Y., CLICKMAN, E., DEMARTINO, J., WANG, J., GOLDSTEIN, G. & CHESS, L. (1984) Biologic functions of the OKT1 T cell surface antigen. I. The T1 molecule is involved in helper function. *J. Immunol.* **133,** 724.

VAN DE GRIEND, R.J., TAX, W.J.M., VAN KRIMPEN, B.A., VREUGDENHIL, R.J., RONTELTAP, C.P.M. & BOLHUIS, R.L.H. (1987) Lysis of tumor cells by $CD3^+4^-8^-16^+$ T cell receptor αβ- clones, regulated via CD3 and CD16 activation sites, recombinant interleukin 2, and interferon β. *J. Immunol.* **138,** 1627.

VILMER, E., GUGLIELMI, P., DAVID, V., LECA, G., RABIAN, C., DEGOS, L., BOIRON, M. & BENSUSSAN, A. (1988) Predominant expression of circulating $CD3^+$ lymphocytes bearing gamma T cell receptor in a prolonged immunodeficiency after allogeneic bone marrow transplantation. *J. clin. Invest.* **82,** 755.

WEISS, A., NEWTON, M. & CROMMIE, D. (1986) Expression of T3 in association with a molecule distinct from the T-cell antigen receptor heterodimer. *Proc. natl Acad. Sci. USA*, **83,** 6988.