Technical Report Series

Flow-cytometric Measurement of $CD4^{-}8^{-}$ T Cells Bearing T-cell Receptor $\alpha\beta$ Chains: 1. Results for a Normal Population Including Two Cases with Unusually High Frequencies

Yoichiro Kusunoki, Ph.D.; Yuko Hirai, Ph.D.; Seishi Kyoizumi, Ph.D.; Mitoshi Akiyama, M.D.

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Table 3 on page 9 lacks a footnote f, for which the author agreed not to distribute an errata sheet because it is explained in the text. This might have occurred because a table frame happened to change during the final output by using the other PC.

業績報告書シリーズ

T細胞受容体αβ鎖を有する末梢血$CD4^-8^-$T細胞のフローサイトメトリーによる測定 1. 正常人集団および異常に高頻度を示した2例に関する結果§

Flow-cytometric Measurement of $CD4^-8^-$ T Cells Bearing T-cell Receptor αβ Chains: 1. Results for a Normal Population Including Two Cases with Unusually High Frequencies

楠 洋一郎 平井裕子 京泉誠之 秋山實利

要 約

まれでおそらく異常と思われるT細胞，すなわち，T細胞受容体（TCR）$\alpha\beta$鎖およびCD3細胞表面抗原を有し，CD4およびCD8抗原を欠く，T細胞（フローサイトメトリーではTCR $\alpha\beta^+CD4^-8^-$ 細胞として検出される）の検出を行った。TCR $\alpha\beta^+CD4^-8^-$ T細胞は，119名の正常人の末梢血TCR $\alpha\beta^+$ 細胞中，平均0.63 ± 0.35％の頻度で検出された。上記119名以外に，非常に高いTCR $\alpha\beta^+CD4^-8^-$ T細胞頻度（TCR $\alpha\beta^+$ 細胞全体の5～10％および14～19％）を示す異常な例が2名認められた。両名は，その他の点では生理学的に全く健康な男性で重篤な疾患の既往歴もなかった。さらにこのような高頻度は，2年あるいは8年前に採血した試料においても認められた。この2名のTCR $\alpha\beta^+CD4^-8^-$ T細胞は，三重染色フローサイトメトリーの解析によれば，成熟T細胞マーカーのCD2，3および5抗原のみならず，ナチュラルキラー（NK）細胞マーカーのCD11b，16，56および57抗原を発現していた。レクチン依存性あるいは逆方向抗体依存性細胞障害活性は，分離したばかりのTCR $\alpha\beta^+CD4^-8^-$ T細胞および試験管内で樹立されたクローンのいずれにも認められた。にもかかわらずNK様活性は検出されなかった。さらに，試験管内で樹立されたすべてのTCR $\alpha\beta^+CD4^-8^-$ T細胞クローンについて，TCR βおよびγ鎖遺伝子のサザンブロット解析は

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著者の所属は放射線生物学部。

すべて同一の再構成パターンを示した。以上の結果は，この2名の男性のTCR $\alpha\beta^{+}CD4^{-}8^{-}$ T細胞は独特な特徴を有し，生体内でクローン性に増殖していることを示している。

Technical Report Series

Flow-cytometric Measurement of $CD4^-8^-$ T Cells Bearing T-cell Receptor αβ Chains: 1. Results for a Normal Population Including Two Cases with Unusually High Frequencies§

Yoichiro Kusunoki, Ph.D.; Yuko Hirai, Ph.D.; Seishi Kyoizumi, Ph.D.; Mitoshi Akiyama, M.D.

Summary

In this study we detected rare, possibly abnormal, T cells bearing CD3 surface antigen and T-cell receptor (TCR) αβ chains but lacking both CD4 and CD8 antigens (viz., $TCR\alpha\beta^+CD4^-8^-$ cells, as determined by flow cytometry). The $TCR\alpha\beta^+CD4^-8^-$ T cells were detected at a mean frequency of 0.63 ± 0.35% (mean ± standard deviation) in peripheral blood $TCR\alpha\beta^+$ cells of 119 normal persons. Two unusual cases besides the 119 normal persons showed extremely elevated frequencies of $TCR\alpha\beta^+CD4^-8^-$ T cells, viz., approximately 5% to 10% and 14% to 19% in whole $TCR\alpha\beta^+$ cells. Both individuals were males who were otherwise physiologically quite normal with no history of severe illness, and these high frequencies were also observed in blood samples collected 2 or 8 years prior to the current measurements. The $TCR\alpha\beta^+CD4^-8^-$ T cells of the two individuals were found to express mature T-cell markers such as CD2, 3, and 5 antigens, as well as natural killer (NK) cell markers, viz., CD11b, 16, 56, and 57 antigens, when peripheral blood lymphocytes were subjected to three-color flow cytometry. Lectin-dependent or redirected antibody-dependent cell-mediated cytotoxicities were observed for both freshly sorted $TCR\alpha\beta^+CD4^-8^-$ cells and in vitro established clones. Nevertheless, NK-like activity was not detected. Further, Southern blot analysis of TCRβ and γ genes revealed identical rearrangement patterns for all the $TCR\alpha\beta^+CD4^-8^-$ clones established in vitro. These results suggest that the $TCR\alpha\beta^+CD4^-8^-$ T cells from these two men exhibit unique characteristics and proliferate clonally in vivo.

§*This technical report is based on Research Protocol 3-87. The complete text of this report will not be available in Japanese. Approved 11 July 1991; printed September 1992.*

Note: all authors are with the Department of Radiobiology, RERF.

Introduction

A significant but not severe reduction of immune function as a result of exposure to atomic bomb (A-bomb) radiation has been observed among survivors more than 35 years after the bombings.[1–3] Two kinds of approaches have been used to interpret the immunological dysfunction in A-bomb survivors: (1) attempts to prove the involution of major functional cells and (2) efforts to identify the existence and/or expansion of abnormal cells. Results obtained from studies taking the first approach suggest that the mature T cells only partly recovered from injuries to the peripheral immune system caused by A-bomb radiation, since the thymus, in which major T cells differentiate, had degenerated with aging.[2,3]

Most peripheral blood T cells express either CD4 or CD8 molecules on their surface. However, small numbers of $CD4^-8^-$ T cells bearing TCRα and β chains ($TCR\alpha\beta^+CD4^-8^-$) have also been detected in murine[4–13] and human organs.[14–21] While partial characterization of $TCR\alpha\beta^+CD4^-8^-$ T cells has been reported,[16–19] the developmental and physiological properties of these T cells are still unknown. Despite the lack of information about these T cells in human organs, abnormal increases in the number of such T cells in peripheral lymphoid organs of *lpr* and *gld* mice have been extensively cited with respect to the mechanisms of development of autoimmune diseases.[22,23] Thus, evaluations of these T cells in A-bomb survivors may demonstrate abnormal T-cell differentiation after exposure to the A-bomb, as did one study using this approach.[2]

A flow-cytometric method for measuring the frequency of $TCR\alpha\beta^+CD4^-8^-$ T cells was developed using peripheral blood from normal individuals before studies on A-bomb survivors were begun. These T cells were detected at a mean frequency of 0.63 ± 0.35% in samples of peripheral blood $TCR\alpha\beta^+$ cells from 119 persons. In addition, two healthy males showed extremely elevated frequencies of these cells (5% to 10% and 14% to 19%). We analyzed the cell-surface antigen expression of the $TCR\alpha\beta^+CD4^-8^-$ T cells from these two individuals using three-color flow cytometry and established clones in vitro from these subsets in order to characterize their T cells.

Materials and Methods

Blood samples

Peripheral blood mononuclear cells (PBMCs) were obtained after defibrination of peripheral blood samples from 32 healthy volunteers (20 males and 12 females) and from 89 participants (12 males and 77 females) of the Adult Health Study (AHS) who had been exposed to <0.005 Gy of A-bomb radiation. The PBMCs were separated by Ficoll-Hypaque density gradient centrifugation and were washed twice with Earle's balanced salt solution (EBSS, Nissui, Tokyo) supplemented with 2.5% fetal calf serum and antibiotics.[24]

Monoclonal antibodies

The monoclonal antibodies (MAbs) used in this study were anti-Leu6 (CD1), anti-Leu5b (CD2), Leu4 (CD3), anti-Leu3a (CD4), anti-Leu1 (CD5), anti-Leu9 (CD7), anti-Leu2a (CD8), anti-Leu15 (CD11b), anti-Leu11c (CD16), anti-Leu18

(CD45RA), anti-UCHL1 (CD45RO), anti-Leu19 (CD56), anti-Leu7 (CD57), anti-δTCS1, anti-TCRδ1, anti-HLADR, and anti-IL2R (IL2 receptor α chain). Anti-Leu, anti-TCR1 (WT31), anti-HLADR, and anti-IL2R MAbs were purchased from Beckton Dickinson Immunocytometry Systems (San Jose, Calif.). Anti-UCHL1 was obtained from Seikagaku Kogyo Co. (Tokyo). Anti-TCRδ1 and anti-δTCS1 were purchased from T Cell Science, Inc. (Cambridge, Mass.).

Flow-cytometric analysis of cell-surface antigen expression

Flow cytometry was performed as described previously.[25] Cell lines were reacted with fluorescein isothiocyanate–coupled (FITC-coupled) MAbs followed by phycoerythrin-coupled (PE-coupled) MAbs, and expression of cell-surface antigens was analyzed using a FACScan (Beckton Dickinson Immunocytometry Systems).

For three-color flow cytometry, PBMCs were reacted with FITC-coupled anti-CD4 plus anti-CD8 MAbs, biotin-conjugated anti-TCRαβ MAb sandwiched with Texas red-coupled streptoavidin (Duochrome, Beckton Dickinson Immunocytometry Systems), and the appropriate PE-coupled MAbs, such as anti-CD3, CD11b, CD16, CD56, and IL2R. The $TCR\alpha\beta^{+}CD4^{-}8^{-}$ cells were sorted into FITC-negative and Duochrome-positive fractions and further analyzed for orange (PE) fluorescence. Another combination of MAbs used for staining PBMCs consisted of PE-coupled anti-CD4 plus anti-CD8 MAbs, biotin-conjugated anti-TCRαβ MAb sandwiched with Duochrome, and the appropriate FITC-coupled MAbs, such as anti-CD2, CD5, CD45RA, CD45RO, CD57, and TCRδ1. The PE-negative, Duochrome-positive cell population ($CD4^{-}8^{-}$ $TCR\alpha\beta^{+}$) was sorted and subsequently analyzed for green (FITC) fluorescence.

Sorting and culture of $TCR\alpha\beta^{+}CD4^{-}8^{-}$ T cells

$CD4^{-}8^{-}$ (FITC-negative) $TCR\alpha\beta^{+}$ (PE-positive) cell populations were sorted with a FACStar (Beckton Dickinson Immunocytometry Systems). Approximately 5,000 cells were sorted and subsequently cultured in GIT medium (Wako Pure Chemical Industry, Osaka) supplemented with 10% fetal calf serum (M.A. Bioproducts, Walkersville, Md.), 1:6400 phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.), 2 ng/mL of human recombinant interleukin-2 (rIL-2; Takeda Chemical Industries, Osaka), and feeder cells (i.e., 5×10^5 allogeneic PBMCs and 10^5 lymphoblastoid cells, OKIB, irradiated with 50 and 100 Gy of X rays, respectively) in each well of 24-well plates (Corstar, Cambridge, Mass.). The culture was maintained by weekly refeeding of growth medium and irradiated feeder cells as described above. It should be noted that feeder cells disappear within 1 week. Immediately after sorting, the cells used for T-cell cloning were distributed into round-bottomed, 96-well microtest plates (Corstar) at a mean frequency of 1 cell per well, using the same medium but one-fifth the number of feeder cells as used for the 24-well plates. Clones derived from these cultures were expanded in number with feeder cells in the 24-well plates for further analysis.

Cell-mediated cytotoxicity assay

Cytotoxic activity of the TCRαβ$^+$CD4$^-$8$^-$ T cells against K562 or U937 cells was measured using the 4 hr ^{51}Cr-release assay, as described previously.[26] An effector-to-target-cell ratio of 10:1 was used with or without PHA (1:1600), anti-Leu4 (0.5 μg/mL), or anti-TCR1 MAbs (1:20).

Triplicate samples per experimental group were used, and the percentage of specific lysis was calculated using the formula:

$$\text{Percentage of specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} .$$

Spontaneous release denotes supernatant ^{51}Cr radioactivity in a target cell culture incubated with medium only; *maximum release* denotes the supernatant radioactivity of a target cell culture incubated with 1% (weight per volume) Triton x-100. Throughout the experiments, spontaneous release was always less than 10% of maximum release. Standard deviations for triplicate wells were consistently below 5% and were therefore not shown.

Southern blot analysis

Southern blot analysis of TCR genes was performed as described previously.[26] In brief, high-molecular-weight genomic DNA was extracted from T cells grown to >3 × 10^6 cells. DNA samples (5 μg) were digested to completion with *Bam*HI, *Hin*dIII, or *Eco*RI (Toyo Soda Ltd. & Co., Osaka), fractionated on a 0.7% agarose gel in Boyer's buffer (50 mM Tris-HCl, pH 8.0; 20 mM sodium acetate, 2 mM EDTA, 10 mM NaCl), transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany), and probed with ^{32}P-labeled TCR Cβ[27] and TCR Jγ.[28]

Results

Frequency of TCRαβ$^+$CD4$^-$8$^-$ T cells in PBMCs

Rare T cells exhibiting a phenotype of TCRαβ$^+$CD4$^-$8$^-$ were detected using flow cytometry, and the frequency of these cells in PBMCs was examined on a total of 121 healthy persons. Two men, aged 45 and 47 (PD and FJ), showed extremely elevated frequencies of TCRαβ$^+$CD4$^-$8$^-$ T cells, where as the frequency of these T cells in the others was less than 2.3%, with a mean of 0.63 ± 0.35% (mean ± standard deviation) among TCRαβ$^+$ cells (Figures 1 and 2). Good reproducibility has been observed in the measurement of frequency of these T cells, when samples collected at the same time were examined repeatedly (Table 1), and when examinations were repeated at different times on 10 normal donors (Figure 3). Further, lymphocyte samples from PD and FJ taken 2 or 8 years ago were found to show consistently high frequencies (Table 2). However, these two men had no history of severe diseases and no serum antibodies against human T-cell leukemia virus-1 (HTLV-1). Moreover, the sorted TCRαβ$^+$CD4$^-$8$^-$ T cells showed no sign of blasts but did show typical resting lymphocytes (data not shown).

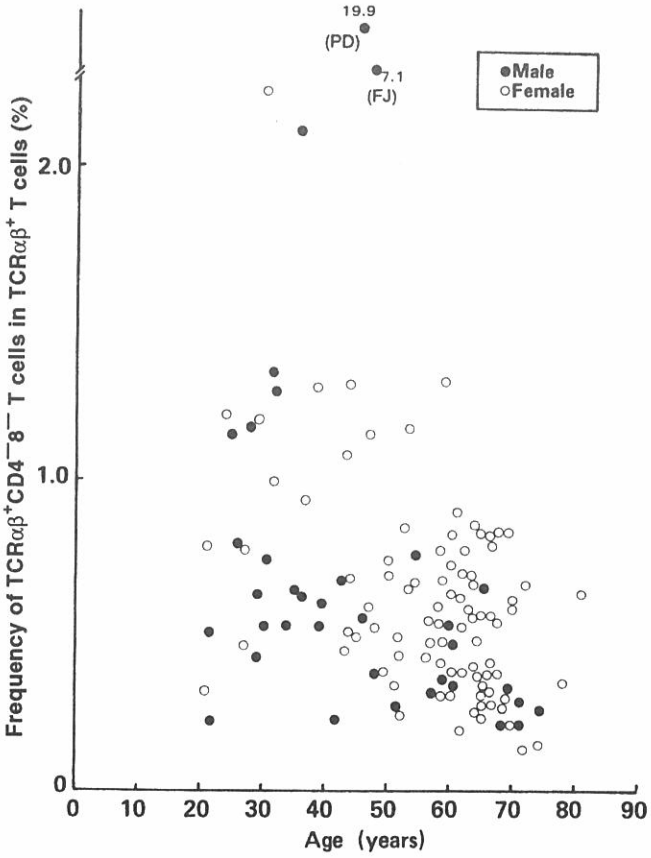

Figure 1. Frequency of TCRαβ$^+$CD4$^-$8$^-$ cells among TCRαβ$^+$ cells in 121 healthy persons. Blood samples from 32 males and 89 females were analyzed. Two men (cases PD and FJ) showed enormously elevated frequencies of TCRαβ$^+$CD4$^-$8$^-$ cells.

Characterization of TCRαβ$^+$CD4$^-$8$^-$ T cells

Three-color flow-cytometric analyses showed that TCRαβ$^+$CD4$^-$8$^-$ T cells from donors PD and FJ were brightly positive for CD2, CD3, and CD5 antigens in addition to NK-cell-like phenotypes such as CD11b$^+$16$^+$56$^+$57$^+$. The cells were also CD45RA$^+$ and CD45RO$^+$ (Figure 4).

Sorted TCRαβ$^+$CD4$^-$8$^-$ cell fractions from the two subjects were immediately distributed into 96-well plates for colony formation at a mean frequency of one cell per well. The cloning efficiencies were calculated to be 61% (PD) and 55% (FJ). Twenty-six out of 44 (PD) and 21 out of 46 (FJ) colonies derived from the donors were successfully analyzed for their cell-surface phenotype using flow cytometry. All of the clones were found to bear the same phenotype as shown in Figure 5, viz., TCRαβ$^+$CD2$^+$3$^+$4$^-$5$^+$8$^-$11b$^+$56$^+$, similar to the results of three-color flow cytometry of peripheral blood lymphocytes from these two individuals. However, some differences were also observed. For example, uncultured TCRαβ$^+$CD$^-$8$^-$ cells were

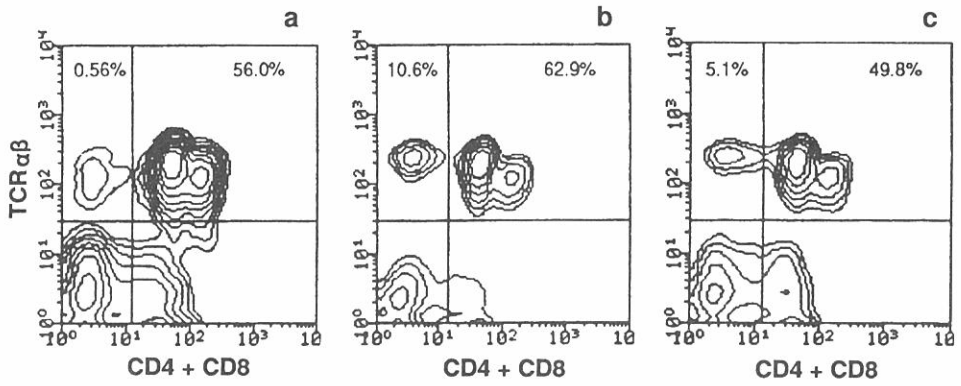

Figure 2. Flow-cytometric analyses of peripheral blood mononuclear cell (PBMC) frequencies in three individuals. a: A representative case with a normal frequency of $TCR\alpha\beta^+CD4^-8^-$ cells in PBMCs. Cases PD (b) and FJ (c) have abnormally elevated frequencies of $TCR\alpha\beta^+CD4^-8^-$ T cells. Approximately 5×10^5 PBMCs were stained with FITC-conjugated anti-CD4 (Leu3a) and anti-CD8 (Leu2a), and biotin-conjugated anti-TCRαβ, followed by phycoerythrin-conjugated streptoavidin, and were then analyzed with a FACScan. Contours differed by 50% logarithmic order as calculated using the Lysis software (Beckton Dickinson Immunocytometry Systems).

Table 1. Frequency of $TCR\alpha\beta^+CD4^-8^-$ cells among $TCR\alpha\beta^+$ peripheral blood mononuclear cells from three individuals sampled at the same time

Case	Exp. no.	$TCR\alpha\beta^+CD4^-8^-$ cell frequency* %	Mean (*SD*)
NI	1	1.5	
	2	1.5	1.4 (0.13)
	3	1.3	
WA	1	0.40	
	2	0.33	0.36 (0.04)
	3	0.35	
YA	1	0.74	
	2	0.84	0.80 (0.06)
	3	0.82	

*The frequency was calculated as the percentage (%) of $TCR\alpha\beta^+CD4^-8^-$ cells in the entire $TCR\alpha\beta^+$ cell population..

$CD16^+45RA^+57^+$ while cultured clonal cells were $CD16^-45RA^-57^-$. These markers were most likely lost during cell culture in vitro, as the cloning efficiency exceeds 50% and hence it is unlikely that $CD16^-45RA^-57^-$ cells had been positively selected. None of them reacted with anti-TCRδ1 and anti-δTCS1 MAbs.

Cytotoxic activities of $TCR\alpha\beta^+CD4^-8^-$ T cells were examined using leukemia cell lines as targets. Freshly isolated $TCR\alpha\beta^+CD4^-8^-$ cells, their mass-culture derivatives, and clonal derivatives from PD and FJ all showed cytotoxic activity

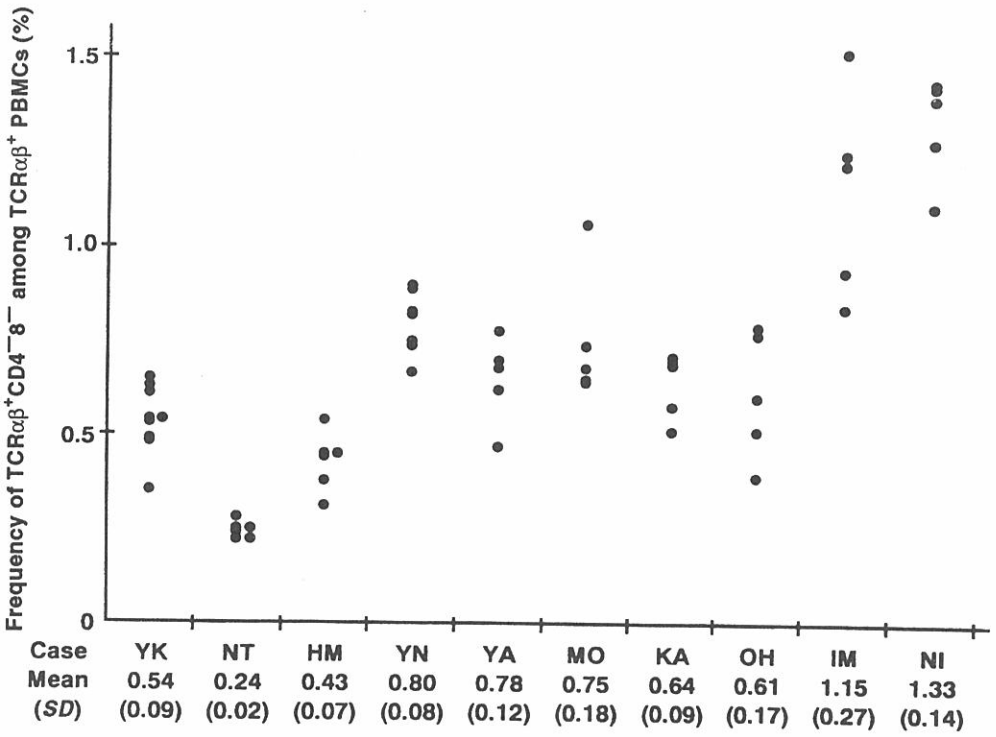

Figure 3. Reproducibility of the measurement on 10 normal individuals. Blood samples were analyzed repeatedly for a period of 1 to 2 years on each donor; PBMC = peripheral blood mononuclear cell.

Table 2. Frequency of TCRαβ$^+$CD4$^-$8$^-$ cells among TCRαβ$^+$ peripheral blood mononuclear cells (PBMCs) from two abnormal cases sampled at different times

Case	Date of blood sampling	TCRαβ$^+$CD4$^-$8$^-$ cell frequency
PD (45-year-old male)	20 Dec. 1982*	16.7
	14 Dec. 1987	15.1
	2 Aug. 1988	17.7
	14 Feb. 1989	18.7
	19 Jan. 1990	14.4
FJ (47-year-old male)	26 June 1988	9.6
	13 Sept. 1988	5.4
	19 Jan. 1990	9.8
	2 April 1990	6.9

*Cryopreserved PBMCs were assessed for the analysis.

against K562 cells in the presence of PHA, but not in its absence (Table 3). Further, the cultured cell lines and all the clones tested effectively lysed U937 cells coated with anti-CD3 or anti-TCRαβ MAbs.

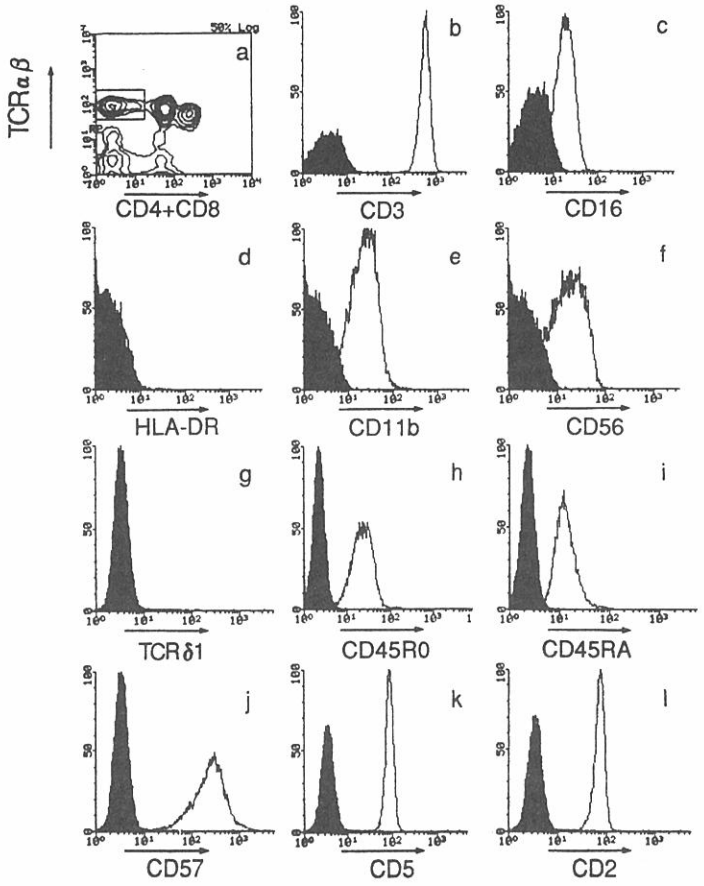

Figure 4. Three-color flow cytometry of TCRαβ$^+$CD4$^-$8$^-$ cells from case PD. Peripheral blood mononuclear cells (PBMCs) were reacted with a combination of fluorescein isothiocyanate-coupled (FITC-coupled) anti-CD4 plus anti-CD8 monoclonal antibodies (MAbs), biotin-conjugated anti-TCRαβ MAbs sandwiched with Texas red-coupled streptoavidin (Duochrome), and the following phycoerythrin-coupled (PE-coupled) MAbs: anti-CD3 (b), anti-CD16 (c), anti-HLA-DR (d), anti-CD11b (e), and anti-CD56 (f). CD4$^-$8$^-$ populations were gated for positive TCRαβ expression (a). PBMCs were reacted with PE-coupled anti-CD4 plus anti-CD8 MAbs, and with biotin-coupled anti-TCRαβ MAb followed by Duochrome. TCRαβ$^+$CD4$^-$8$^-$ populations were gated as described for (a), and were analyzed for reactivity to FITC-coupled anti-TCRδ1 (g), anti-CD45RO (h), anti-CD45RA (i), anti-CD57 (j), anti-CD5 (k), and anti-CD2 (l) MAbs. The black areas indicate fluorescence of a PE-coupled control MAb (b–f) and an FITC-coupled control MAb (g–l). The relative frequency of each fluorescence channel was calculated using the Lysis software.

Table 3. Cytotoxic activity (% ^{51}Cr release) of TCRαβ$^+$CD4$^-$8$^-$ T cells on K562 and U937 target leukemic cell lines

Subject	Effector cells	K562		U937			
		No stimulus	PHA[a]	No stimulus	Anti-CD3[b]	Anti-TCRαβ[b]	Anti-CD8[b]
PD	Unfractionated PBMCs[c]	7.1	16.2	ND	ND	ND	ND
	Freshly isolated TCRαβ$^+$CD4$^-$8$^-$ fraction[d]	–0.7	18.2	ND	ND	ND	ND
	Mass-cultured TCRαβ$^+$CD4$^-$8$^-$ fraction[e]	0.1	30.8	2.5	49.5	21.3	1.9
	Clone[f]						
	PD57	ND	55.6	ND	73.2	70.9	ND
	PD71	3.3	37.5	ND	ND	ND	ND
	PD76	2.7	36.9	ND	ND	ND	ND
	PD77	3.3	60.3	ND	ND	ND	ND
	PD78	3.5	49.9	ND	ND	ND	ND
FJ	Unfractionated PBMCs[c]	22.0	13.8	ND	ND	ND	ND
	Freshly isolated TCRαβ$^+$CD4$^-$8$^-$ fraction[d]	–0.5	25.6	ND	ND	ND	ND
	Mass-cultured TCRαβ$^+$CD4$^-$8$^-$ fraction[e]	0.2	34.6	0.2	51.0	53.0	ND
	Clone[f]						
	FJ22	–3.4	49.0	0.6	58.4	44.9	0.2
	FJ33	2.3	52.8	1.0	74.7	68.3	1.4
	FJ34	–1.8	53.2	0.6	77.8	58.1	1.0
	FJ36	–2.2	49.8	1.0	58.5	40.4	–0.4

NOTE: Cytotoxic activity was measured at an effector-to-target ratio of 10:1. ND = not done.

[a]Phytohemagglutinin (PHA, 1:1600) was added to the test wells 30 min prior to the addition of target cells.

[b]Anti-CD3 (Leu 4, final concentration = 0.5 μg/mL), anti-TCRαβ (TCR1, 1:20 dilution at final volume), and anti-CD8 (Leu2a, final concentration = 0.5 μg/mL) were added 30 min prior to the addition of target cells.

[c]Peripheral blood mononuclear cells (PBMCs) were treated with monoclonal antibodies (MAbs) for sorting by a FACStar as described in the Materials and Methods section. The cells were incubated in medium containing 10% fetal calf serum (FCS) at 37°C overnight to modulate the antigen-MAbs interaction.

[d]TCR αβ$^+$CD4$^-$8$^-$ fractions were sorted by a FACStar (purity was >97% in each case) and incubated in medium containing 10% FCS at 37°C overnight to moderate the antigen-MAbs interaction.

[e]Approximately 5,000 TCRαβ$^+$CD4$^-$8$^-$ T cells sorted by a FACStar were cultured in the presence of PHA, rIL-2, and feeder cells for 3 to 5 weeks with weekly stimulation. More than 95% of the cultured cells exhibited the TCRαβ$^+$CD4$^-$8$^-$ phenotype.

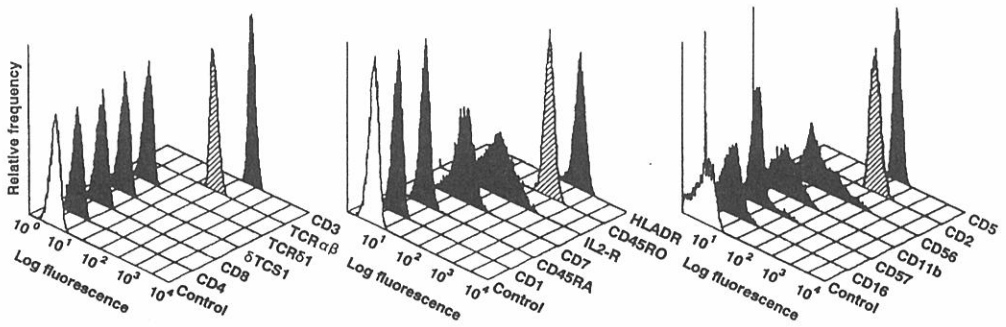

Figure 5. Cell surface phenotype of a typical TCRαβ$^+$CD4$^-$8$^-$ T-cell clone (PD57) established from donor PD. The relative frequency for each fluorescence channel was calculated using the Lysis software.

Analyses of TCR gene rearrangement patterns of TCRαβ$^+$CD4$^-$8$^-$ T cells for PD and FJ

Southern blot analyses of TCR gene rearrangement patterns of the T-cell clones were performed to determine the clonality of each TCRαβ$^+$CD4$^-$8$^-$ cell. Unexpectedly, the TCRβ gene of 26 TCRαβ$^+$CD4$^-$8$^-$ clones derived from donor PD showed identical rearrangement patterns after testing with three different restriction enzymes, viz., *Eco*RI, *Hin*dIII, and *Bam*HI (Figure 6). Identical rearrangement patterns were also observed for the TCR γ gene. These results clearly demonstrate that the TCRαβ$^+$CD4$^-$8$^-$ cells are all clonal derivatives of a single cell, since T-cell cloning was done immediately after cell sorting without preculture. Furthermore, identical rearrangement patterns were also observed in clones established at different times. However, six CD4 single-positive and five CD8 single-positive T-cell clones established from the same individual showed different rearrangement patterns.

In the case of donor FJ, one mass-cultured cell line and 20 clones established from the TCRαβ$^+$CD4$^-$8$^-$ fraction were examined, and again, all of them showed identical patterns of TCRβ and TCRγ gene rearrangements (Figure 7). However, the rearrangement patterns of the TCRαβ$^+$CD4$^-$8$^-$ T cells from the two donors were different from each other. Further, neither the presence of TCRδ genes, the rearrangement of immunoglobulin heavy chain genes, nor the integration of the Epstein-Barr (EB) virus or HTLV-1 genome were observed in the DNA extracted from these cells (data not shown).

Discussion

Rare TCRαβ$^+$CD4$^-$8$^-$ cells were detected in peripheral blood samples of normal individuals using flow cytometry and were found to appear at a mean frequency of 0.63 ± 0.35% in the TCRαβ$^+$ cells among 119 donors. The observation of such rare cells in A-bomb survivors may give us some information on the alteration of the composition of T-cell subpopulations affected by A-bomb radiation; that is, abnormal T-cell differentiation due to thymic dysfunction at the time of the

bombings may be reflected in hyper- or hypoproduction of these cells even at the present time. Working from this hypothesis, we have started to enumerate the frequency of TCRαβ$^+$CD4$^-$8$^-$ T cells among A-bomb survivors. Unexpectedly, however, we found that two nonexposed individuals had TCRαβ$^+$CD4$^-$8$^-$ cell frequencies that were approximately 15 to 30 times higher than those of the other subjects. Therefore, we established TCRαβ$^+$CD4$^-$8$^-$ T-cell clones from these two subjects in order to characterize their T cells. Each donor's in vitro established clones were identical both in phenotype and in their TCR gene rearrangement patterns. Since three restriction enzymes (*Bam*HI, *Hin*dIII, and *Eco*RI) were used for the analyses of TCRβ and γ genes, it was highly unlikely that the same patterns of restriction fragments were observed by chance. Because cloning was performed immediately after sorting without preculture, and the cloning efficiency of the variant T cells exceeded 50%, it was also unlikely that such clones were in vitro artifacts. Therefore, the present results can be best explained by assuming that the TCRαβ$^+$CD4$^-$8$^-$ T cells were in vivo derivatives of a single cell.

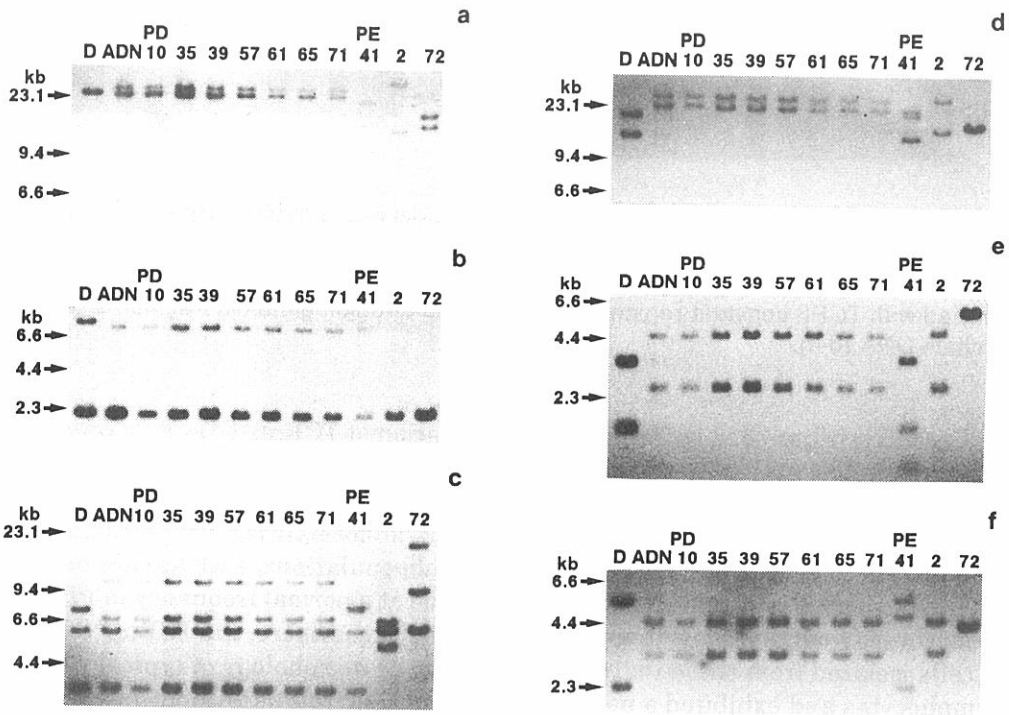

Figure 6. Southern blot patterns of TCR genes of a Daudi (D) B-cell line; a TCRαβ$^+$CD4$^-$8$^-$ T-cell line (ADN) from case PD; seven TCRαβ$^+$CD4$^-$8$^-$ T-cell clones (PD10, PD35, PD39, PD57, PD61, PD65, and PD71) in which PD10, PD35, and PD39 had been established at different times from the others; and two TCRαβ$^+$CD4$^-$8$^+$ clones (PE41, PE2) and one TCRαβ$^+$CD4$^+$8$^-$ clone (PE72) established from the same case. Genomic DNA digested with *Bam*HI (a,d), *Eco*RI (b,e), and *Hin*dIII (c,f) were probed with TCRβ constant region cDNA (a–c) and J-region genomic fragments of TCR γ-chain DNA (d–f).

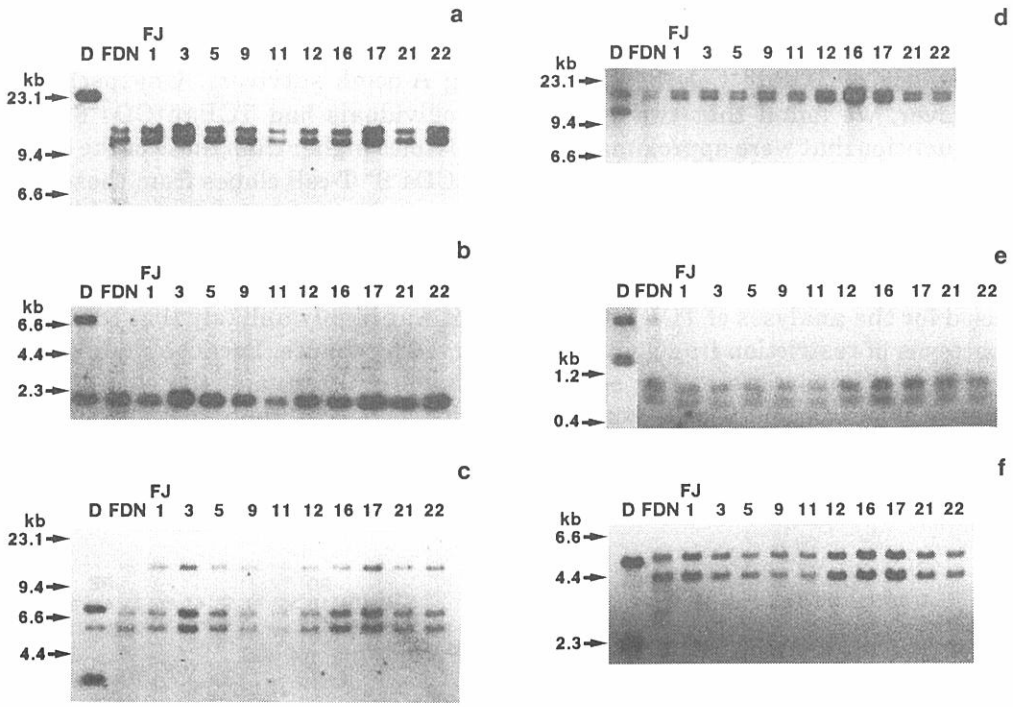

Figure 7. Southern blot patterns of a Daudi (D) B-cell line, a TCRαβ$^+$CD4$^-$8$^-$ T-cell line (FDN) from case FJ, and 10 TCRαβ$^+$CD4$^-$8$^-$ T-cell clones established from the same case. Genomic DNA digested with *Bam*HI (a,d), *Eco*RI (b,e), and *Hin*dIII (c,f) were probed with TCRβ constant-region cDNA (a–c) and J-region genomic fragments of TCR γ-chain DNA (d–f).

Recently, it has been reported that proliferation of TCRαβ$^+$CD4$^-$8$^-$ T cells was observed in one patient bearing combined immunodeficiency[20] and in one case of adult T-cell leukemia.[21] However, the two men examined here are not carriers of the HTLV-1 virus and have neither clinical abnormalities nor a history of hemopoietic diseases. The other lymphocyte subpopulations, such as CD4 or CD8 single-positive T cells and B cells, were observed at a normal frequency in PBMCs from these two individuals (unpublished data). Furthermore, the TCRαβ$^+$CD4$^-$8$^-$ T cells isolated from these individuals showed the morphology of typical resting lymphocytes and exhibited a normal chromosome karyotype without integration of EB virus genome (unpublished data). In addition, high frequencies of the TCRαβ$^+$CD4$^-$8$^-$ T cells have been stably observed for periods of 2 and 8 years. Thus, there are no indications that this clonal proliferation was associated with leukemic or preleukemic progression of a single cell. Lastly, it is unlikely that these T cells were stimulated by a certain antigen to expand *in vivo,* since they bore CD45RA antigen, which is expressed only in T cells devoid of previous sensitization by any antigen.[29] Indeed, the CD45RA epitope was lost after stimulation with a mitogen PHA and IL-2 *in vitro* (Figure 5).

TCRαβ$^+$CD4$^-$8$^-$ T-cell clones established in vitro showed a phenotype of CD2$^+$3$^+$5$^+$11b$^+$16$^-$56$^+$57$^-$ (Figure 5), whereas three-color flow-cytometric studies of the cells from the TCRαβ$^+$CD4$^-$8$^-$ fraction of fresh PBMCs were positive for both CD16 and CD57 antigens. As it has been reported that lymphocytes bearing CD16 and CD57 antigens lose their expression during culture,[30–32] it is conceivable that these TCRαβ$^+$CD4$^-$8$^-$ T-cells had expressed them *in vivo*. The T cells reported here expressed both mature T-cell markers (CD2$^+$3$^+$5$^+$) and NK-cell markers (CD11b$^+$16$^+$56$^+$57$^+$), and are distinct from the TCRαβ$^+$CD^4$^-$8$^-$ population reported by Seki et al.,[15] Shivakumar et al.,[16] Londei et al.,[18] Wirt et al.,[20] and Hattori et al.[21] Lanier et al.[33] have reported two cases of normal individuals who possessed 23.6% and 9.4% of CD3$^+$4$^-$8$^-$16$^+$ lymphocytes in PBMCs. The phenotype and cytolytic ability of those cells resemble our observation that those cells were positive for CD2, CD5, CD11b, and CD16 antigens and showed little NK-like activity but significant antibody-dependent cell-mediated cytotoxic activity. Although they did not describe the expression of TCR molecules, their findings would be quite consistent with ours if those cells had borne bear αβ heterodimers.

The origin of the TCRαβ$^+$CD4$^-$8$^-$ T cells reported here is not understood as yet. Sykes[11] and Levitsky et al.[12] reported that NK1$^+$TCRαβ$^+$CD4$^-$8$^-$ cells in mice are exclusively derived from the thymus and preferentially localize to bone marrow. Although the TCRαβ$^+$CD4$^-$8$^-$ T cells in our cases bear NK-cell markers, it is not clear whether these human NK markers correspond to the murine one. On the contrary, Ohteki et al.[34] reported evidence that TCRαβ$^+$CD4$^-$8$^-$ T cells proliferate in the liver of MRL-*lpr* mice and suggested a possibility that these cells directly come from bone marrow without any association with the thymus. More studies will be required to understand the meaning of the clonal proliferation of the TCRαβ$^+$CD4$^-$8$^-$ T cells. In addition, the clonal analyses of these T cells from A-bomb survivors and from the other healthy donors now in progress may elucidate both the in vivo developmental pathways of these cells and the effects of A-bomb radiation on the developmental process.

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