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Spontaneous Loss and Alteration of Antigen Receptor Expression in Mature $CD4^+$ T Cells

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1989年から，放射線影響研究所の業績報告書は，従来の日英両文を併記した方式では発行しない．主要な報告書については，今後も日英両文で印刷するが，それぞれ別に発行する．内容が高度に専門的であり，一般の関心が少ないと思われる報告書については英文のみとし，日本文の要約を添付する．

これにより，広島・長崎の原爆電離放射線被曝の人体に及ぼす晩発性生物学的影響に関する最近の知見を今までよりも速やかにお知らせできることと思う．

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財団法人　放射線影響研究所
業　績　報　告　書

RERF TR 22-89
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成熟CD4^{+}T細胞における抗原受容体発現の自然欠損と変異[§]

Spontaneous Loss and Alteration of Antigen Receptor Expression in Mature CD4^{+} T Cells

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要　約

高齢者や放射線被曝者にみられる免疫系の変化を明らかにする調査の一環として，T細胞レセプター（TcR）/CD3抗原複合体の発現について調査した．この複合体は抗原認識及び成熟T細胞の活性化に中心的な役割を果たしており，複合体の発現異常は種々の抗原刺激に対するT細胞の不応答性をもたらすと考えられる．

我々はフローサイトメトリーを用いて健常人（127名）やDNA修復障害及びT細胞系免疫異常が指摘されている種々の遺伝性疾患者（5名）において，成熟T細胞（CD4陽性T細胞）のうち，細胞表面TcR/CD3複合体の発現異常（欠損または減少）を持つ変異T細胞の頻度を測定した．更に複合体発現異常の性状を，4名の健常人から得られた計37個の異常クローンを用いて免疫学的並びにDNAレベルでの解析をした．末梢血での変異T細胞頻度は健常人では平均2.5×10^{-4}であり，加齢に伴い統計学的に有意に増加していた（$P<0.0001$）．2名のファンコニー貧血症患児では同年齢の健常児の平均値と比べて，この変異体頻度は約2～3.5倍高く，2名の毛細血管拡張性運動失調患者では約7倍も高い16.6×10^{-4}と17.9×10^{-4}であった．一方，ダウン症患者は正常範囲内の頻度であった．

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§本報告にはこの要約以外に訳文はない．

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変異クローン細胞の免疫学的並びに遺伝子レベルでの解析の結果は，CD3分子ではなくTcR蛋白発現の欠損及び蛋白の部分欠失などの変異が原因であることが分かった．また，TcR遺伝子の異常は末梢において誘発されていることが判明した．

このようなTcRの発現異常は老化や遺伝性疾患に認められるT細胞機能不全に重要な因子であり得ることが示唆された．

一方，現在，我々は原爆放射線を含む種々の変異原物質に被曝した人々や患者について，TcR/CD3複合体発現異常の頻度を調査しており，この遺伝子は変異原物質のモニタリングに新しいインディケーターとして応用できる可能性が示されている．

Spontaneous Loss and Alteration of Antigen Receptor Expression in Mature CD4+ T Cells§

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Summary

The T-cell receptor CD3 (TCR/CD3) complex plays a central role in antigen recognition and activation of mature T cells, and therefore abnormalities in the expression of the complex should induce unresponsiveness of T cells to antigen stimulus. Using flow cytometry, we detected and enumerated variant cells with loss or alteration of surface TCR/CD3 expression among human mature $CD4^+$ T cells. The presence of variant $CD4^+$ T cells was demonstrated by isolating and cloning them from peripheral blood, and their abnormalities can be accounted for by alterations in TCR expression such as defects of protein expression and partial protein deletion. The variant frequency in peripheral blood increased with aging in normal donors and was highly elevated in patients with ataxia telangiectasia, an autosomal recessive inherited disease with defective DNA repair and variable T-cell immunodeficiency. These findings suggest that such alterations in TCR expression are induced by somatic mutagenesis of TCR genes and can be important factors related to age-dependent and genetic disease-associated T-cell dysfunction.

Introduction

The CD3-associated α, β T-cell receptor (TCR$\alpha\beta$) heterodimer is expressed on the surface of the vast majority of mature $CD4^+$ or $CD8^+$ T cells in the peripheral blood (PB) and lymphoid organs.[1–3] A second class of TCRs consisting of γ and δ chains (TCR$\gamma\delta$), also CD3-associated, is found mainly in the double negative ($CD4^-8^-$) T-cell population.[4–6] The TCR and CD3 are thought to form a functional unit in antigen recognition and signal transduction, and the association of all the components appears to be essential for cell surface expression of

§**The complete text of this report will not be available in Japanese.**

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the molecular complex (for a review, see reference 7). Thus, inactivation of a gene encoding a protein of the TCR/CD3 complex should lead to loss of surface TCR/CD3 expression and thereby result in defective antigen recognition and cell activation. Many mutant cell lines lacking surface expression of the TCR/CD3 complex have been obtained from human T-lymphoma cells exposed to radiation or chemical mutagens.[8–17] These cells failed to respond to various mitogen and antibody stimuli.[9,12–15] Most of these mutant lines were found to have defects in TCR expression (mostly TCRβ) rather than CD3 expression and intracellular accumulation of the incompletely assembled complexes. This suggests that TCR genes are more susceptible to mutagenesis than CD3 genes.

These four TCR genes are all located on chromosomes 7 (β, γ) and 14 (α, δ).[18] Similar to immunoglobulin (Ig) genes in B cells, but unlike ordinary autosomal genes, only one of two TCR alleles in T cells is thought to be active in protein expression.[19] Although the mechanism of allelic exclusion in TCR expression is not fully understood at the molecular level, such a mechanism may be essential in order for a given T-cell clone to have only one idiotype on its TCR, which is necessary for the maintenance of an ordered immune network. As a result of allelic exclusion, it is expected that TCR expression-loss variants are frequently generated by a single inactivation event even though TCR genes are autosomally located. In fact, many mutant cell lines lacking the TCR have been established in vitro from T-lymphoma cell lines[8–17] as mentioned above, although the mutation rates have not been determined yet. In this report, we will address the question of whether such variant cells with alterations of TCR/CD3 expression exist in detectable numbers among in vivo mature T-cell populations. Aberrant immature T cells with nonproductive rearrangements of both TCR alleles are assumed to die in the thymus.[19,20] It is unknown whether a similar selection mechanism depletes such aberrant mature T cells induced in peripheral by somatic mutations. If such variant T cells do exist, by what molecular alterations and at what frequencies have they been generated?

For this study, we detected and isolated variants with loss or alteration of TCR/CD3 expression from a human PB $CD4^+$ T-cell population using a cell sorter. Most of these variants had abnormalities in the expression of either the TCRα or β chain. The TCR abnormalities probably were caused by inactivation or alteration of the TCR genes, which resulted in defect or decrease in membrane transport of the incomplete TCR/CD3 complex. The variant frequency in $CD4^+$ T cells from PB was approximately 1×10^{-4} per TCR locus, which was much higher than the ordinary mutant frequency (10^{-5} to 10^{-6}). The variant frequency in patients with genetic diseases showing somatic gene instability was also measured.

Materials and Methods

Monoclonal antibodies

Monoclonal antibodies (MonAbs) used were anti-Leu4 (CD3), NU-T3 (CD3),[21] anti-Leu3a (CD4), anti-Leu6 (CD1), anti-Leu5b (CD2), anti-Leu1 (CD5), anti-Leu2a (CD8), anti-Leu7 (CD57), anti-Leu11a (CD16), anti-Leu16 (CD20), anti-

LeuM3 (CD14), anti-IL-2R (CD25), WT31 (TCR$\alpha\beta$),[22] βF1(TCRβ),[23] αF1(TCRα),[§] anti-TCRδ1 (TCRδ),[24] and anti-TCR$\gamma\delta$1 (TCRγ or $\gamma\delta$ complex).[25] Anti-Leu and WT31 (TcRI) MonAbs were purchased from Becton Dickinson Immunocytometry Systems, (San Jose, Calif). NU-T3 MonAb was obtained from Seikagaku Kogyo Co. (Tokyo). Anti-TCRδ1 MonAb was purchased from T Cell Science Inc. (Cambridge, Mass), and anti-TCR$\gamma\delta$1 MonAb was kindly provided by Dr. J. Borst, The Netherlands Cancer Institute, Amsterdam.

Sorting and culture of variant T cells

PB samples were derived from healthy volunteers and from children with genetic disease such as ataxia telangiectasia (AT), Fanconi's anemia, and Down's syndrome. PB mononuclear cells (PBMC) were isolated from blood by density separation using Ficoll/Hypaque. PBMCs were stained with fluorescein-labeled anti-Leu3a (FL-anti-Leu3a) and phycoerythrin-labeled anti-Leu4 (PE-anti-Leu4) antibodies as described by the supplier. Before flow cytometry, propidium iodide (PI) was added at a final concentration of 10 μg/ml to gate out dead cells. For sorting variant CD4$^+$ T cells with loss or decreased expression of CD3, a sorting window was set to the region where the surface CD3 level was less than 1/25 of that of normal CD4$^+$ cells. Approximately 1,000 cells were sorted and cultured for two weeks with GIT medium (Wako Pure Chemical Industry, Osaka) containing 10% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, Md) phytohemagglutinin (PHA) (1:1600, Difco Laboratories, Detroit, Mich), recombinant IL-2 (2 ng/ml, Takeda Chemical Industries, Osaka) and feeder cells. A mixture of allogeneic PBMCs from three normal donors and from an allogeneic B-cell line, OKIB, established by us was used as feeder cells after irradiation with 50 Gy. For cloning variant T cells, immediately after sorting, cells were distributed into 96-well plastic plates at a mean frequency of approximately one cell per well and were cultured for four weeks under the same condition described above. Normal CD4$^+$ clones were also established from normal donors and AT patients. Clones derived from these culture were expanded with feeder cells for further analysis.

Cytoplasmic immunofluorescence

Variant and normal T-cell clones were cytocentrifuged onto a glass slide and fixed with acetone for 10 minutes at –20°C. The fixed cell specimens were incubated with the first antibodies (anti-Leu4, WT31, αF1, βF1, TCRδ1, and TCR$\gamma\delta$1) or control myeloma proteins (MOPC21, IgG_1, UPC10, IgG_{2a}) for one hour at room temperature. After being washed three times with phosphate-buffered saline (PBS), the specimens were stained with F(ab)'2 fragment of antimouse immunoglobulin (Tago Inc., Burlingame, Calif) for 45 minutes. After extensive washing, the samples were fixed with 1% paraformalin for observation under a fluorescence microscope (Nikon, Tokyo).

[§] *Preparation and characterization of αF1 antibody is described in a technical data sheet provided by T Cell Science Inc., Cambridge, Mass, USA*

Immunoprecipitation analysis

Radiolabeling and immunoprecipitation were performed using the following methods: T-cell clones were biosynthetically labeled with 37 MBq [^{35}S]-methionine ([^{35}S]-Met) (New England Nuclear [NEN], Boston, Mass) in RPMI 1640 Met-free medium at 37°C for six hours. For ^{125}I-surface labeling, cells were incubated with 74 mBq Na ^{125}I (NEN) at 30°C for four minutes in 150 μl PBS containing 100 μg lactoperoxidase (Sigma Chemical Co., St. Louis, MO, USA) and 20 μl of 0.03% H_2O_2, and the mixture was further incubated at 25°C for 10 minutes.[26] After radiolabeling, cells were washed with PBS five times and lysed in the extraction buffer (10 mM triethanolamine, 0.15 M NaCl, pH 7.8, containing as inhibitors, 10 mM iodacetamide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, antipain, pepstatin, and chymostatin) containing 1% digitonin or 1% NP-40, as described in a previous report.[27] Immunoprecipitations were performed by incubating the detergent extracts with 2 μg of antibody on ice for 3–4 hours, then adding a 20 μl packed volume of protein A-Sepharose (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and further incubating the mixture for one hour. For anti-TCRδ1 and MOPC21 antibodies, protein A-Sepharose was precoated with rabbit-antimouse immunoglobulin. Immunoprecipitates were washed five times in the same buffer used for cell extraction and were solubilized in reducing or nonreducing Laemmli sample buffer subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Southern blotting

High-molecular-weight genomic DNA was extracted from variant T-cell clones and a B-cell line, as described in a previous report.[28] DNA samples (10 μg) were digested with *Bam*HI, electrophoresed through 0.7% agarose, transferred to nitrocellulose filters, and probed with cDNA of the TCRβ constant region gene.[29] Phosphorus-32 labeling was performed using a random primer method (Amersham International, Amersham, UK). Filters were washed with 0.1 × saline sodium citrate buffer (SSC) and 0.1% SDS at 65°C.

Chromosome analysis

Variant T-cell clones were examined for karyotype analysis using the G-banding method.[30] Briefly, after exposure of T cells to colcemid (0.05 μg/ml) for four hours, chromosome specimens were prepared by an air-dried method. The slides were treated with a 0.1% trypsin solution (GIBCO, Grand Island, NY) for 15–20 sec at 30°C and then stained with 2% Giemsa solution for 15 minutes. In each clone, more than 10 metaphases were analyzed.

Results

Detection of variant T cells

Flow cytometry is useful for detecting and isolating cells with rare phenotypes in vitro[31] and in vivo[32–34] at frequencies of more than 10^{-6}. To detect variant CD4$^+$ T cells with altered TCR/CD3 expression, two-color fluorescence analysis

of normal PB lymphocytes (PBL) stained with FL-anti-CD4 and PE-anti-CD3 antibodies was performed using a cell sorter. Using WT31 antibody to react with the TCR$\alpha\beta$ complex did not work for detecting variants; the resolution between positive and negative cells was not great enough. The sorting window was set in the region for variant CD4$^+$ cells as shown in Figure 1A. Variant cells showed a discrete cluster in the window and were observed in normal donors at a frequency of several cells per 10,000 CD4$^+$ cells (Figure 1A). All events occurring in the variant window were sorted onto a glass slide and were observed under a fluorescence microscope to determine whether they exhibitied an FL green fluorescence and not PE orange fluorescence. Sorted cells were also stained with PI to examine the shapes of their nuclei. Such experiments revealed that more than 95% of events appearing in the variant window were apparently mononuclear cells not polymorphonuclear cells or debris. Other surface markers expressed in the variant cells were examined by two-color analysis using FL-labeled or PE-labeled anti-CD4 and anti-CD3 with or without a third fluorescence-labeled antibody. Calculation of the difference between the frequencies of cells in the window with and without the third antibody staining showed that more than 90% of the variants expressed pan-T-cell markers (CD2 and CD5), but not TCR$\alpha\beta$, monocyte marker (CD14) or pan-B-cell marker (CD20). These results strongly suggested that most events in the variant window are mature CD4$^+$ T cells with altered TCR$\alpha\beta$/CD3 expression.

To confirm the presence of CD4$^+$ T cells with altered TCR/CD3 expression, approximately 1,000 cells were sorted from the window and expanded by bulk culture for two weeks with feeder cells, PHA, and recombinant IL-2. Most of cells grown in this culture were found to lack or have decreased surface expression of the TCR$\alpha\beta$/CD3 complex with minor contamination by normal CD4$^+$ cells (Figure 1B and C). These cultured cells expressed CD2 antigens (Figure 1D) and IL-2 receptors (data not shown), suggesting that PHA may activate some populations of TCR/CD3 negative T cells via CD2 molecules to induce the expression of the IL-2 receptor, as discussed below. Other surface marker characteristics of these cells were CD1$^-$, 5$^+$, 8$^-$, 11$^-$, 16$^-$, 20$^-$, and 57$^-$, indicating that these variant cells have the surface phenotype of typically mature CD4$^+$ T cells except for altered in TCR/CD3 expression. Previous studies using a TCR-defective T-lymphoma cell line have demonstrated the accumulation of CD3 molecules in cytoplasm.[7–17] Similar cytoplasmic expression of CD3 was observed in these in vivo-derived variant T cells by immunofluorescence staining of fixed cells (Figure 2).

Isolation and characterization of variant clones

Next, we established cloned variant CD4$^+$ T-cell lines from PB for further molecular analysis of TCR/CD3 expression. Although the cloning efficiency for variant cells was about 5%–10% (much lower than the 50%–80% for normal CD4$^+$ T cells), 37 variant T-cell clones could be established from four normal healthy donors by limiting dilution of sorted cells. These variant clones were classified into three types, according to the surface staining characteristics with anti-CD3 (Leu4) and anti-TCR$\alpha\beta$ (WT31) antibodies (Figure 3, Table 1). Type

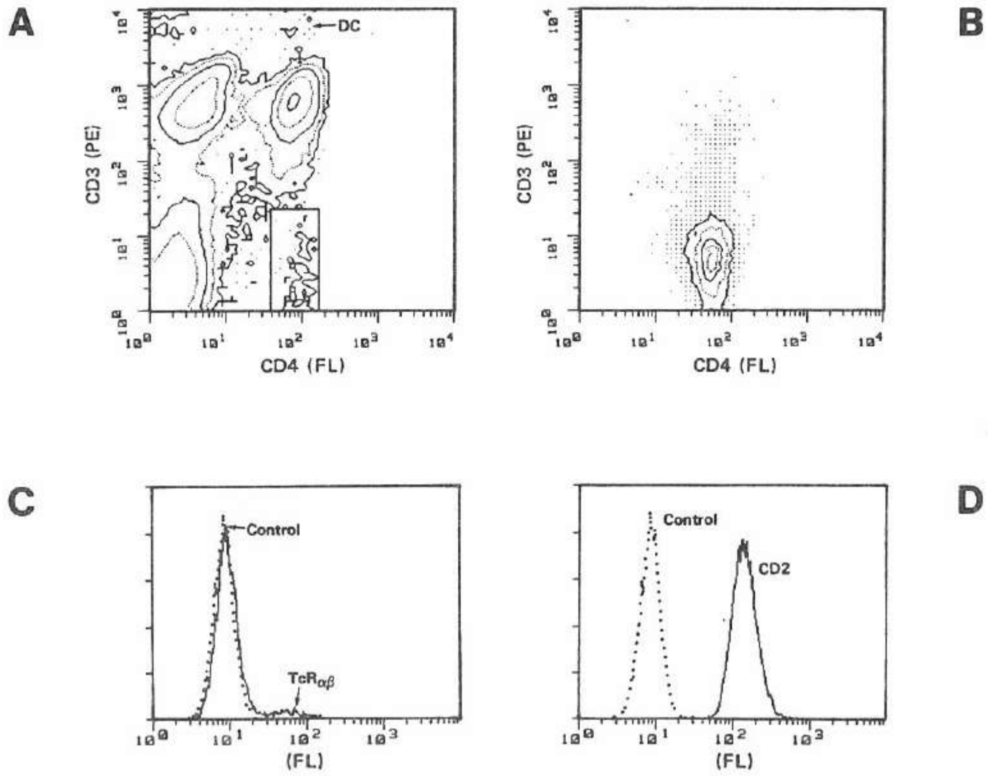

Figure 1. Detection and isolation of variant CD4⁺ T cells with defective or decreased CD3 expression. (A) Flow distribution of 10^6 peripheral blood lymphocytes stained with FITC-anti-CD4 and PE-anti-CD3 antibodies. Before flow cytometry, propidium iodide was added to gate out dead cells (DC). For two-color analysis, the lymphocyte fraction was gated by forward and right-angle light scatter. Contours differ by a factor of 10 in events per channel with the lowest contour representing one event per channel. To sort variant T cells, a sorting window was set in the region shown in this figure. Approximately 1×10^3 cells were sorted and cultured with PHA, r-IL2, and feeder cells for two weeks. Cultured T cells were analyzed for their CD3 and CD4 expression with FITC-Leu3 and PE-Leu4 antibodies (B), TCR$\alpha\beta$ expression with FITC-WT31 antibody (C), and CD2 expression by indirect immunofluorescence with anti-Leu5 (D). Broken lines represent control staining with myeloma IgG antibody.

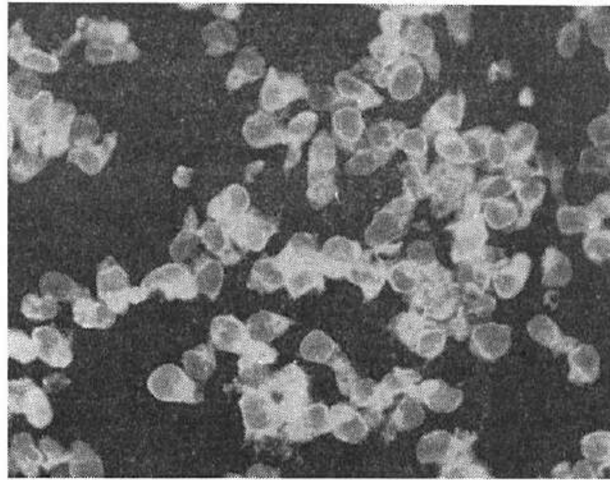

Figure 2. Cytoplasmic expression of CD3 antigens in CD4$^+$ variant T cells by indirect immunofluorescence staining

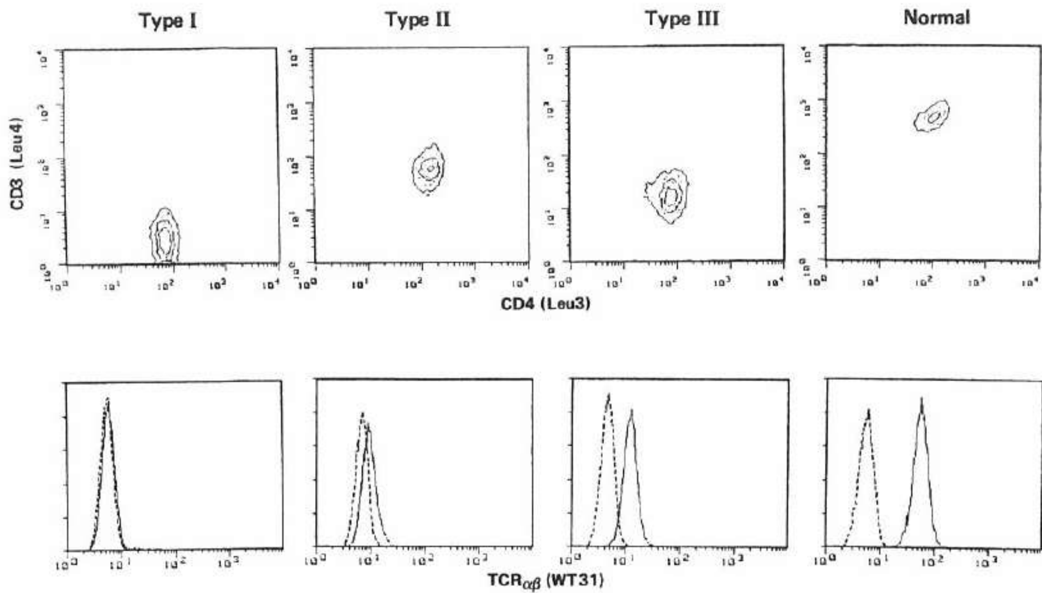

Figure 3. Surface expression of the TCR/CD3 complex in variant T-cell clones. Variant T-cell clones were stained with either PE-Leu3 and FL-Leu4 (upper lane) or FL-WT31 antibodies (lower lane). Broken lines represent control staining using FITC-labeled myeloma IgG antibody.

Table 1. Surface and cytoplasmic expression of the TCR/CD3 complex in variant $CD4^+$ T-cell clones[a]

Variant type	Antigens: (antibodies):	Surface		Cytoplasm				No. of clones
		CD3 (Leu4)	TCR$\alpha\beta$ (WT31)	CD3 (Leu 4)	TCR$\alpha\beta$ (WT31)	TCRα (αF1)	TCRβ (βF1)	
I		–	–	++	–	+	–	22
				++	–	–	+	6
II		L[b]	–	++	–	+	+	3[c]
				++	–	–	–	1[d]
III		L	L	++	+	+	+	5
Total								37

[a]Surface and cytoplasmic immunofluorescence was performed by the methods described in the Materials and Methods section.

[b]Low expression (see Figure 3)

[c]Partial deletions of either TCRα or β chain were detected by immunoprecipitation analysis using anti-CD3 antibody (see Figure 4).

[d]This variant clone was found to be reactive with TCRδ1 antibody, but not with anti-TCR$\gamma\delta$1 antibody.

–: negative; +: positive; ++: strongly positive

I clones lacked membrane expression of both CD3 and TCR$\alpha\beta$. Type II showed low expression of CD3, but the reactivity with WT31 antibody was faint or almost negative. Type III demonstrated a low level of expression of both CD3 and TCR$\alpha\beta$. These variant phenotypes have been stable in culture for more than six months.

Expression of CD3, TCRα, and TCRβ of the variant clones was analyzed by cytoplasmic immunofluorescence and immunoprecipitation using various antibodies against CD3 (Leu4 or NU-T3 antibody), TCR$\alpha\beta$ dimer (WT31), TCRα (αF1), TCRβ (βF1), TCRδ (anti-TCRδ1), and TCR$\gamma\delta$ complex (anti-TCR$\gamma\delta$1). As shown in Table 1, strong expression of CD3 in the cytoplasm was observed for all of the variant clones. On the other hand, the expression of TCRα and β chains was variable. All of the Type I clones were found to lack cytoplasmic expression of either TCRα or β chains as detected by αF1 or βF1 antibodies, respectively. SDS-PAGE analysis of [^{35}S]-Met-labeled polypeptides immunoprecipitated with anti-CD3 antibody (NU-T3) under nonreduced conditions also demonstrated that these clones expressed only monomeric (40 kilodalton [kDa]), not dimeric, TCR (Figure 4A). No abnormalities in the expression of CD3δ and ε were detected for these variants by SDS-PAGE, although other CD3 subunits could not be labeled with [^{35}S]-Met in this labeling condition.[35] These TCRα or β chain monomers could be associated with CD3 molecules, but the molecular complex was not successfully transported to the membrane. Thus, lack of surface TCR/CD3 complex expression in Type I clones can be explained simply by a defect of either TCRα or β chain expression, as previously reported in variant T-cell lines.[7–15] Preliminary experiments using Northern blotting analysis of several Type I variants also

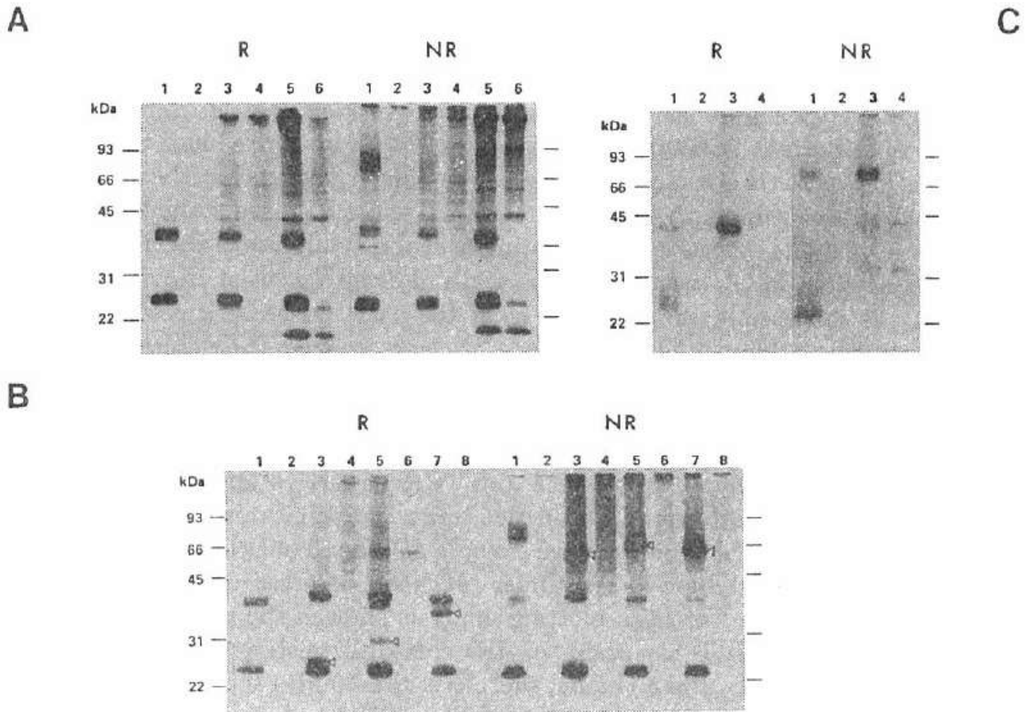

Figure 4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the TCR/CD3 complex in variant T-cell clones. (A) Anti-CD3 immunoprecipitate from Type I variant clones. After labeling with [^{35}S]-Met, a normal CD4^{+} T-cell clone (lanes 1 and 2), a TCRα-defective variant clone (lanes 3 and 4), and a TCRβ-defective variant clone (lanes 5 and 6) were solublized with digitonin buffer. Immunoprecipitates were prepared with either anti-CD3 antibody, NU-T3 (IgG2a) (lanes 1, 3, and 5) or control IgG2a antibody, UPC10 (lanes 2, 4, and 6), and analyzed by SDS-PAGE on 10%–20% gradient gels under reduced (R) or nonreduced (NR) conditions. (B) Immunoprecipitation of digitonin lysates from ^{35}S-labeled normal CD4^{+} clone (lanes 1 and 2) and three Type II variants (lanes 3–8) with either anti-CD3 (lanes 1, 3, 5, and 7) or control (lanes, 2, 4, 6, and 8) antibodies. Arrowheads indicate shortened TCR monomers (R) or dimers (NR). (C) Immunoprecipitation of digitonin (lanes 1 and 2) or NP-40 (lanes 3 and 4) extracts from ^{125}I-surface labeled TCRδ^{+} Type II variant. Immunoprecipitations were obtained with anti-CD3 (lane 1), anti-TCRδ1 (IgG1) (lane 3), control IgG2a (lane 2), and control MOPC21 (IgG1) (lane 4) antibodies.

confirmed the defect of full-length mRNA synthesis from either the TCRα or β gene.

Type II variants, which expressed CD3 on their cell surfaces at low levels, were rather complicated, having various TCR abnormalities. In Type II variants, significant reactivity with WT31 antibody could not be detected either on the cell surface or in the cytoplasm (Figure 3, Table 1). But protein bands corresponding to TCR chains were detected by SDS-PAGE analysis of immunoprecipitates of ^{125}I-labeled surface protein with anti-CD3 antibody (data not shown). However, since TCR protein bands in SDS-PAGE were very weak for three out of four Type II clones listed in Table 1, these three clones were labeled internally with [^{35}S]-Met for further immunoprecipitation experiments (Figure 4B). Those clones,

which were reactive with both αF1 and βF1 antibodies, showed two TCR protein bands under reduced conditions; one was normal in size, but the other TCR chain had a relatively low molecular mass (indicated by the arrowheads in Figure 4B). These TCR chains could form TCR heterodimers, with lower molecular mass (60–65 kDa) than normal TCR dimers (80 kDa), as shown by nonreduced SDS-PAGE analysis. Using Northern blot analysis, it was demonstrated that these variants expressed either full-length TCRα or β mRNA and a relatively short length of TCRβ or α mRNA, respectively, suggesting that the small size of the TCR chains observed by SDS-PAGE was due to partially deleted forms of the TCRα or β proteins. It is possible that some part of the TCR framework recognized by WT31 antibody is truncated in this type of clone, so that the association with CD3 required for surface expression of the complex cannot occur normally.

Another Type II clone (Table 1), which was not reactive with either αFl or βF1 antibodies, was found to express anti-TCRδ1-reactive antigens on its cell surface. SDS-PAGE patterns of ^{125}I-labeled immunoprecipitates with either anti-CD3 or anti-TCRδ1 antibodies revealed a single TCR band (40 kDa) under reduced conditions and a disulphide-linked dimer (80 kDa) detected under nonreduced condition (Figure 4C). Northern blotting analysis showed the production of full-length mRNA for both TCRγ and δ in this clone, suggesting that this variant synthesized both TCRγ and δ chains; therefore, the 40 kDa band under reduced conditions was most likely an overlap of the TCRγ and δ chains. However, this clone was found to be nonreactive both on the cell surface and in the cytoplasm with anti-TCR$\gamma\delta$1 antibody recognizing the TCRγ or TCR$\gamma\delta$ complex.[25] All these data suggested that this CD4$^+$ variant expressed disulphide-linked TCR$\gamma\delta$ heterodimer and that its TCRγ or δ chain had a partial deletion or mutation in a region which may be recognized by anti-TCR$\gamma\delta$1 antibody.

Type III variants were found to express both TCRα and β chains in the cytoplasm by using immunofluorescence, but surface expression of the TCR/CD3 complex was much lower than that of normal clones (Table 1, Figure 3). TCR expression of these Type III clones were also examined by immunoprecipitation of [^{35}S]-Met-labeled proteins with anti-CD3 antibodies. Although these clones appeared to produce both TCRα and β chains at normal levels, the protein band corresponding to the TCR dimer was very weak by SDS-PAGE analysis under nonreduced conditions (data not shown). We speculate that these variants have some mutations in the framework of either the TCRα or β protein, which did not alter the epitope structure recognized by αF1, βF1 or WT31 antibodies. It is likely that such abnormalities in the TCR chains consequently inhibit either the formation of the TCR dimer or its association with CD3 molecules, so that surface expression of TCR/CD3 complex is considerably diminished.

Rearrangement of TCR genes in variant clones

To examine the possibility that the defect of TCRα or β chain expression of Type I variants is due to the lack of TCR gene rearrangement, we analyzed the

TCR genes of the variant clones by Southern blot. Figure 5 reveals the restriction patterns of the TCRβ genes from TCRβ-defective Type I variants, demonstrating that TCRβ genes were rearranged in all these variants. No evidence for loss of the entire TCRβ constant (Cβ) gene could be observed by Southern analysis using the Cβ cDNA probe.

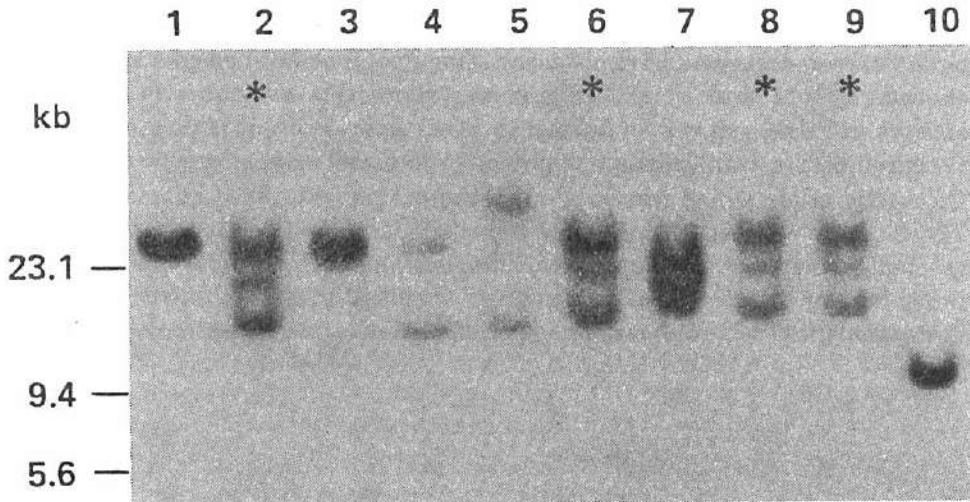

Figure 5. Southern blot analysis of TCRβ gene rearrangements in Type I variant clones (lanes 2–10) with defective TCRβ expression. EB virus-transformed B cells from a normal donor served as a control for the germ line configuration (lane 1). For a clone (lane 3), rearrangement was not apparent with *Bam*HI digestion, but clear rearranged bands were detected in *Eco*RI restriction pattern. Four variant clones, as indicated by an asterisk (•), represent identically rearranged bands.

Among the 37 variant clones examined here, 11 clones derived from one of the donors showed identical TCRβ gene rearrangement patterns (Figure 5 shows four of these clones), using three different restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI). The TCRγ gene for these variants also revealed identical rearrangements (data not shown). This reflects in vivo clonality, since T-cell cloning had been done immediately after cell sorting without preculture. The TCRβ gene restriction patterns by *Bam*HI digestion of these clones exhibited three rearranged bands (Figure 5). This suggested the occurrence of gross alterations of the TCRβ gene induced by partial gene duplication or unequal sister chromatid exchange.[19] It can be presumed that such inactivation occurred in a TCRβ gene of a proliferating T cell and the variant was clonally expanded in vivo.

Chromosome aberration in variants

It has long been observed that translocations between chromosomes 7 and 14 are frequent in T cells from normal donors.[36] The breakpoints on these chromosomes are also highly nonrandom, involving three regions, i.e., 7 p13-15,

7 q32-35, and 14 q11-12, where TCRγ, TCRβ, and TCRα/δ genes, respectively, are located, according to recent studies.[18] The frequency of 7-14 translocations[36] has been reported to be 4×10^{-4}, which is very similar to the frequency of TCR variants as described below. It is possible that 7-14 translocation is accompanied by inactivation of TCR genes. If so, the TCR variants cloned here might frequently bear aberrations of chromosomes 7 and 14.

When we analyzed the chromosomes of 14 Type I and 5 Type II variant clones, no t(7;14) translocations were observed. One Type I clone showed a translocation betweeen 14 q11 and 13 q32 (Figure 6). Since this variant was found to be defective in TCRα expression but not in TCRβ expression, it is very possible that this chromosome translocation might have induced nonexpression in an active TCRα gene allele which was located at the breakpoint. TCR gene inactivation, however, accompanied chromosome translocations less frequently than we had expected. It may be possible that variants bearing such chromosome aberrations cannot be cloned in culture due to their inability to grow. Intrachromosomal rearrangements, i.e., duplications, deletions or insertions, also may be involved.

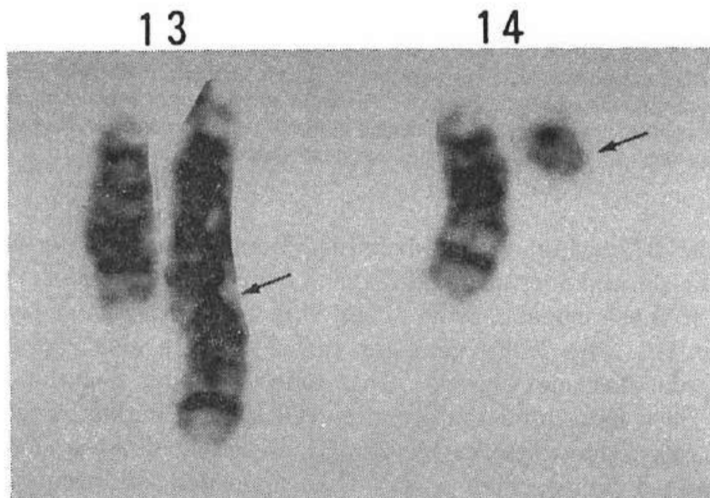

Figure 6. Translocation between chromosomes 13 and 14 seen in the Type I variant clone lacking TCRα expression. Eighteen metaphases were examined and all of them exhibited a reciprocal translocation t(13;14) (q32; q11). Arrows indicate the break points.

Variant frequency in vivo and in vitro

As shown above, partial characterization of TCR/CD3 variants in PB exhibited a variety of TCR abnormalities. Next, we measured the frequency of variant cells in either PB or culture by flow cytometry in normal donors and in patients with hereditary disease showing instability of somatic genes.

The variant frequency was found to be almost constant in each donor, when five normal donors were examined three or more times during one year (coefficients of variation for each donor were less than 10%). The mean frequency for 127 normal donors was about 2.5×10^{-4} on average, and it significantly ($p < 0.0001$) increased with age (Figure 7). Since these variants should have a defect in antigen recognition and activation for specific immune responses, their increasing frequency may be one of the factors related to age-dependent decrease in T-cell function (for a review, see reference 37).

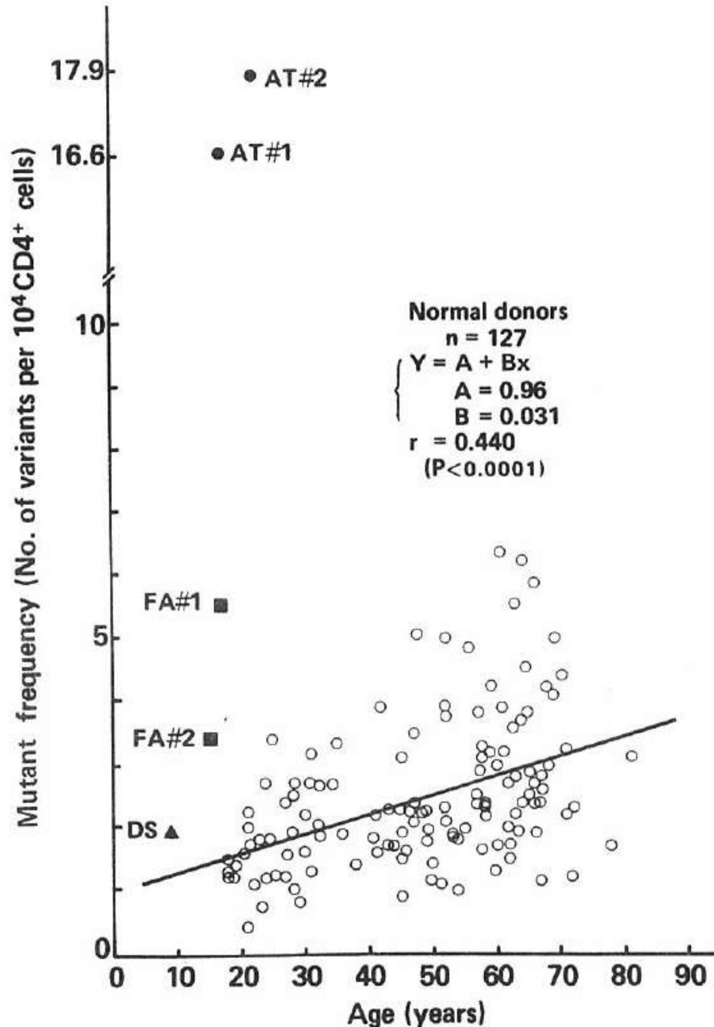

Figure 7. Relationship between donor age and variant T-cell frequency in peripheral blood from 127 normal donors and five patients with genetic disease such as ataxia telangiectasia (AT), Fanconi's anemia (FA), and Down's syndrome (DS). Variant T cells detected are shown in Figure 1A. Variant frequency equals the value of the number of variants occurring in the window divided by the total number of $CD4^+$ cells. The line corresponds to a linear regression calculated from the variant frequencies for the normal donors.

Fanconi's anemia patients showed higher variant frequencies than those of normal young donors, but a Down's syndrome patient had a normal frequency (Figure 7). Much higher variant frequencies were observed in patients with AT. On the other hand, the heterozygous parents of the AT patient #2 (in Figure 7) were found to have frequencies of 3.4×10^{-4} and 3.5×10^{-4}, which are within the normal range. Thus, measurement of the TCR/CD3 variants in PB can be applied to the diagnosis of AT. AT is a recessive inherited disease characterized by defective DNA repair following ionizing radiation,[38,39] and it is known to have a high frequency of spontaneous somatic mutations of erythrocytes[40] and of lymphocyte chromosome aberrations,[38] especially in chromosomes 7 and 14.[41,42] High radiation sensitivity of T cells from these two AT patients has been confirmed. We also found an elevation of in vivo frequency of deletion-type mutants at the glycophorin A locus of erythrocytes for AT patient #1 (unpublished observation).

It is also known that AT patients have a defect in thymus development[43] and various dysfunctions in T-cell response.[38,44–47] Such abnormal thymus development might result in the defective maturation of T cells and provide a source of variant T cells with defective TCR/CD3 expression to the PB. However, as shown in Figure 8, normal T-cell clones ($CD3^+4^+$) derived from AT patients revealed high frequencies ($15–50 \times 10^{-4}$) of spontaneous loss of surface TCR/CD3 expression in vitro, whereas clones from normal donors had low frequencies ($1–5 \times 10^{-4}$). In addition, the normal $CD8^+$ T-cell clone ($CD3^+8^+$) from AT patient #1 also exhibited a high frequency of spontaneous loss of surface TCR/CD3 in vitro (although the data are not shown). These results suggest that the high frequency of in vivo variants in AT patients is not attributable to abnormal differentiation of T cells in the thymus, but to the intrinsic instability of TCR genes in mature T cells.

Discussion

In this paper, we have demonstrated the presence of variant $CD4^+$ T cells with alterations of TCR/CD3 expression in human PB. These variant clones were found to have abnormalities in the expression of one of the chains in the TCR heterodimer, and they expressed the incomplete TCR/CD3 complex in the cytoplasm or the cell surface at various levels. We assume that the in vivo variant is produced not by aberrant rearrangements of TCR genes in the thymus, but by spontaneous somatic mutation, such as inactivation, partial deletion, and point mutation of TCR genes in the peripheral mature T cells. The first reason for this consideration is the increase of variant frequency with age (Figure 7), which indicates the accumulation of abnormal clones in the PB following thymus involution. Second, a high variant frequency was observed in AT patients (Figure 7) who also have a high frequency of erythrocyte mutation and lymphocyte chromosome aberrations. Third, spontaneous inactivation of TCR/CD3 expression occurred at a similar frequency in cloned mature T lymphocytes in vitro (Figure 8). Fourth, ionizing radiation could induce TCR gene inactivation in vitro in a dose-dependent fashion in T-lymphoma cells (manuscript in preparation), and significant elevation of the variant frequency was observed in the Thorotrast

patients who were chronically exposed to α-particles from an internal deposit of radioactive thorium (manuscript in preparation).

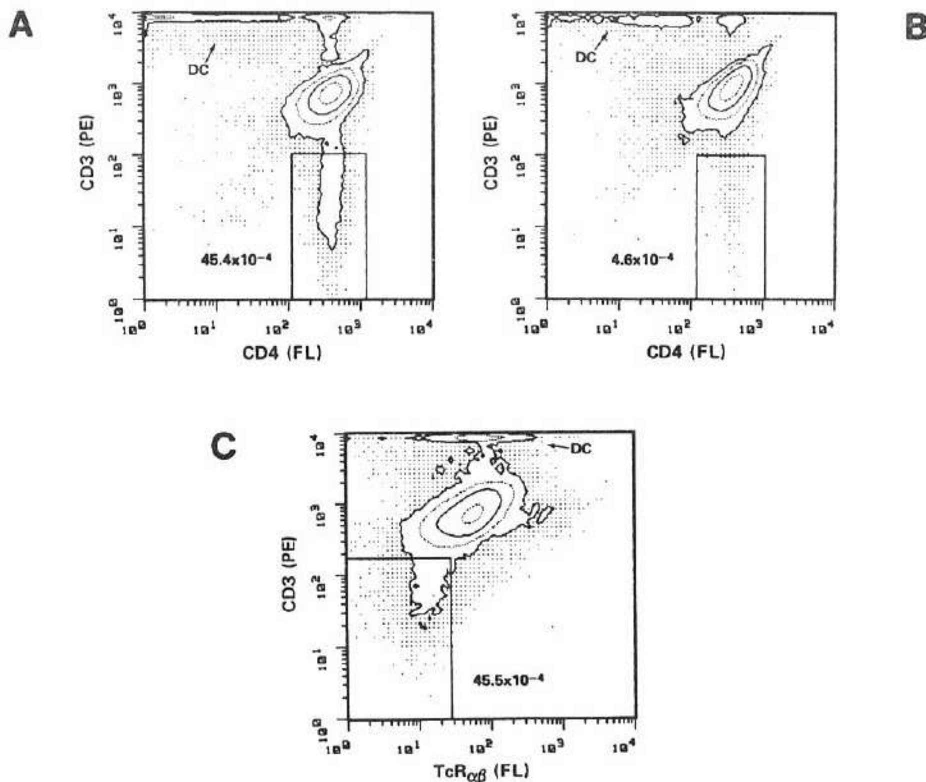

Figure 8. Spontaneous loss of TCR/CD3 expression in vitro in $CD4^+$ T-cell clones derived from ataxia telangiectasia patients. $CD4^+$ T-cell clones normally expressing the TCR/CD3 were established from AT patient #1 (A, C) listed in Figure 7 and a normal donor (B). T cells logarithmically proliferating in culture were stained with either FITC-Leu3 and PE-Leu4 (A, B) or FITC-WT31 and PE-Leu4 (C). After staining dead cells (DC) with propidium iodide, 5×10^5 cells were analyzed, as described in the caption for Figure 1. Contours differ by a factor of 10 in events per channel with a minimal dot indicating one event per channel. The cell frequencies in the windows are shown in the figure.

If TCR gene abnormalities are the results of somatic mutation, the variant frequency of 2.5×10^{-4} that is equal to 1.3×10^{-4} per TCRα or β locus (a simple calculation made by considering the dimeric structure of TCR and an allelic exclusion in TCR genes) is about 10–100 times higher than that of other gene loci such as T-cell hypoxanthine guanine phosphoribosyltransferase,[48–50] erythrocyte glycophorin A,[32–34] and T-cell HLA class I antigen.[51] But it is comparable to those of B-cell Ig genes.[52–57] It has been reported that inactivation of Ig heavy (H) or light chain genes in myeloma and hybridoma cell lines[52–55] and reversion of the amber mutation of the IgH gene in the pre-B-cell line[56,57] occurred at a high rate (10^{-3} to 10^{-5} per cell per generation). These Ig mutational events

include many types of genetic changes such as deletions, point mutations, and frameshifts.[55,58–61] Such a high rate of alteration of the Ig genes is believed to be reflected by the instability of Ig genes in the normal process of B-cell differentiation, including the rearrangements of the variable-region (V-region) gene, the class switch of the IgH gene, and the induction of point mutations in the V-region genes for the generation of diversity.[62–64] The Ig recombination system in B cells may also participate in mediating translocation of the Burkitt's lymphoma chromosome at breaking points 2p11, 14q32, and 22q11, where the Igκ IgH, and Igλ genes are located.[65] Like the Ig genes of B cells, the TCR genes are programmed to dynamically rearrange in the thymus during T-cell ontogenesis, so that the TCR gene of T cells are presumed to be inherently unstable. A recent report has shown that an immature T-cell line can undergo secondary rearrangements in vitro replacing the preexisting productive Vα-Jα rearrangements,[66] although the frequency of such rearrangements is unknown. It is presumed that such secondary rearrangements of TCR genes might be occasionally or frequently nonproductive, and this would be the candidate for one of the TCR inactivation mechanisms.

Many studies using variant T-cell lines lacking a chain of the TCR/CD3 complex have shown that incompletely assembled receptor complexes do not reach the plasma membrane.[7–15] The present results also demonstrated that in vivo-derived T-cell variants lacking TCRα or β protein (Type I variants) did not express the TCR/CD3 complex on the surface. Further, it is noteworthy that three Type II variants bearing a shortened TCRα or β chain, probably a partial deletion, were able to express a low amount of the TCR/CD3 complex on the surface. The shortened TCR chain was able to form a disulfide-linked heterodimer with the other normal TCR chain, as shown in Figure 4, indicating the retention of the cystein residue in the constant region proximal to the transmembrane domain.[7,19] Since the transmembrane domain of TCR, especially the region including some basic amino acid residues, appears to be essential for the association with CD3 molecules,[7,67] the deletion of TCR should not include this region in the membrane domain. In addition, this incomplete heterodimer was reactive with αF1 or βF1 antibodies but not with WT31 antibody. All these results suggested that the shortened TCR chains possessed an internal deletion in the portion of the constant region, which is recognized by WT31 antibody and probably is distal to the transmembrane domain. The small internal deletion in the constant region might directly or indirectly induce conformational changes of the CD3-binding portion, including the transmembrane domain, resulting in a decreased affinity for molecular assembly. Thus, most of the incomplete TCR chains would not form a stable molecular complex in the endoplasmic reticulum to be exported to the plasma membrane, and seems to enter the degradative pathway. Sequence analysis of the cDNA from the shortened TCR mRNA will localize the deletions and their role of the deleted portion in molecular assembly and tranport.

The variants include the $CD4^+$ clone bearing altered TCR$\gamma\delta$ expression. The TCR$\gamma\delta$ dimer is mainly expressed in $CD4^-8^-$ non-MHC-restricted cytotoxic T

cells,[4–6] and infrequently in intestinal cytotoxic $CD8^+$ T cells.[68] Recent reports have suggested the presence of peripheral $CD4^+$ T lymphocytes bearing $TCR\gamma\delta$ at a very low frequency.[25,69] We have also confirmed these observations and established 17 $CD4^+$ $TCR\gamma\delta^+$ clones from PB. All were found to be strongly reactive with both anti-$TCR\delta1$ and anti-$TCR\gamma\delta1$ antibodies and expressed CD3 antigen at a higher level than that of $TCR\alpha\beta$-bearing $CD4^+$ T cells. SDS-PAGE analysis of ^{125}I-labeled immunoprecipitates from these clones revealed $TCR\gamma$ and δ chains (37–40 kDa) (submitted for publication). Since the surface expression of CD3 antigens on the $TCR\delta1^+$ variant established here was much lower than that of normal $CD4^+$ lymphocytes expressing the $\gamma\delta$ heterodimer, we presumed that the mutation which occurred in the $TCR\gamma$ or δ chain also affected the surface expression of the $TCR\gamma\delta$/CD3 complex, an effect similar to that seen on the $TCR\alpha\beta$/CD3 complex.

Only a minor population (5%–10%) of the $CD4^+$ variants detected by flow cytometry could be cloned in vitro by culturing with PHA and IL-2. The majority of variants could not be cloned. This inability suggested that the surface expression of the TCR/CD3 complex affected the activation pathway initiated by PHA to enter the growth cycle from the G_0 state in association with the induction of IL-2 receptor expression. The CD2 molecule appears to be a PHA-binding protein[70] and may be involved in the growth activation pathway. Many studies using mutant T-lymphoma cell lines have provided evidence for functional interaction between the CD2 antigen and the TCR/CD3 complex in stimulation of IL-2 production by anti-CD2 antibody.[9,12–15] A recent report has also shown the physical association between these two structures on the cell surface.[71] We speculate that without TCR/CD3 expression the CD2 molecule cannot transmit the growth signal after PHA binding for the majority of T cells. In contrast, about 10% of the sorted variants did respond to PHA and did grow in culture with IL-2. Possibly, these variants have an alternative pathway for growth stimulation after CD2-PHA binding. We presume that other hypothetical CD2-associated molecules,[71] which can substitute for TCR/CD3 and function in CD2-mediated growth triggering, might be expressed by a minor T-cell population. The variant clones established here might be derived from this minor population. Similar results were obtained by Moretta et al[12] using variants derived from the IL-2-producing Jurkat cell line. They showed that about 90% of the $TCR/CD3^-$ variant clones (eight of nine clones) did not respond to PHA in spite of their CD2 expression, whereas only one clone could be activated by PHA to produce IL-2. The latter appears to correspond to our clonable variants.

Of particular interest is the abnormally high frequency of variant T lymphocytes in vivo and in vitro in AT patients carrying translocations and inversions in chromosome 7 and 14.[18,38,41,42] Analyses of T-cell leukemia and peripheral T cells derived from AT patients have demonstrated the direct involvement of the $TCR\alpha$ and β genes in the translocation of chromosomes 7 and 14.[72,73] However, the surface expression of the TCR gene in T cells and T lymphomas carrying such chromosome translocations from AT patients has not been reported. We are

now conducting chromosome analyses for the TCR/CD3 variant clones derived from AT patients. Causative relationship between the translocations and the developmental mechanism of T lymphoma is still unknown. Furthermore, many studies have shown abnormalities of T-cell function in most AT patients,[44-47] including decreased responses to mitogens, impaired production of lymphokines, and defects of the T-cell helper function in immunoglobulin production. It was also reported that AT patients have abnormal thymus development.[43] Since the TCR/CD3 complex must be directly or indirectly involved in both T-cell functions and development, spontaneous inactivation of the TCR gene might significantly contribute to the array of T-cell-mediated defects. We presume that in AT patients there might be a causal relationship between the high frequency of the TCR gene inactivations observed here, the high rate of t(7;14) translocations, the high incidence of T-lymphoma development, and the immunodeficiency of T cells. Similarly, in normal individuals, the observed increment of the variant frequency with aging might be related to the age-dependent increase in the incidence of chronic lymphocytic leukemia,[74] including T lymphoma which is frequently associated with t(7;14) translocations,[18] and the age-dependent decrease of T-cell functions for immune response.[37]

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