Double-Negative (CD4− CD8−) T Cells From Adult T-Cell Leukemia Patients Also Have Poor Expression of the T-Cell Receptor αβ/CD3 Complex

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We present four patients with adult T-cell leukemia (ATL) derived from a novel T-cell subset (CD4−, CD8− [double-negative, DN], T-cell receptor [TCR] αβ∗). In the ATL cells of these patients, neither gene nor surface expression of CD4 and CD8 antigens was detected. Clinical and laboratory data showed no difference between DN-ATL and CD4+ATL patients. In contrast to typical CD4+ATL cells, DN-ATL cells were shown to express the protein and messenger RNA (mRNA) for T100β in immunocytochemical assay and the reverse-transcription polymerase chain reaction assay. The mean fluorescence intensity of the TCR/CD3 complex was extremely low in all four DN-ATL patients as well as in typical CD4+ ATL. All four patients had TCR β and γ chain gene rearrangements, with deletion of TCR δ chain gene and mRNA expression for TCR α, β, and CD3 δ but not for TCR γ and δ chain genes. Thus, CD4− TCR αβ T cells are also a target for human T-cell lymphotropic virus type I-induced leukemogenesis. In addition, expression of the TCR αβ/CD3 complex on the DN-ATL cells was further diminished by the addition of anti-CD3 or anti-TCR αβ monoclonal antibody. These results suggest that the decreased expression of the TCR αβ/CD3 complex by ATL cells plays a key role in the development of ATL, irrespective of CD4 expression.

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MATERIALS AND METHODS

Patients and cell line. Cells from four patients with DN-ATL (three acute and one lymphoma type) were extensively studied. The disease of one of these patients (patient no. 4) was previously reported. The diagnosis of ATL was made by the detection of serum anti-HTLV-I antibody and the monoclonal integration of HTLV-I proviral DNA in leukemic cells. Clinical subtyping was performed according to the previously described criteria. Lymph node biopsy was performed after obtaining informed consent according to the guidelines of the Kumamoto University Medical School Committee for the Protection of Human Subjects. The human myeloid leukemia cell line (HL60), human T-cell leukemia cell lines (Jurkat and PEER), and ATL cell line (SKT-1B) used in this study were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Cell preparation and phenotypic analysis. Peripheral blood mononuclear cells (PBMC) were obtained from normal volunteers and the acute ATL by Ficoll-Conray density gradient centrifugation. Normal T cells were then prepared from PBMC by rosette formation with sheep red blood cells (RBCs). Lymph node cells were obtained from lymphoma type ATL by passing lymph node biopsy specimens through a steel mesh.

The following murine monoclonal antibodies (MoAbs) were used for indirect immunofluorescence assays: CD2 (OKT11), CD3 (OKT3), CD4 (OKT4), and CD8 (OKT8) (Ortho Diagnostics, Raritan, NJ); CD3 (Leu4), CD4 (Leu2a), CD8 (Leu2a), and TCR αβ (WT31) (Beckton Dickinson Monoclonal Center, Mountain View, CA); HLA-DR (Nuchi Micros) (Nichirei Co, Ltd, Tokyo, Japan); TCR γδ...
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chirei CO), an anti-CD25 MoAb (Cosmo Bio CO, Ltd, Tokyo, Japan), followed by phenol-chloroform extraction. The DNA was digested with restriction enzymes, subjected to electrophoresis on 0.7% agarose gels, and transferred to nitrocellulose filters (Hybond C extra; Amersham International plc, Buckinghamshire, UK). The filters were hybridized with radiolabeled probes at 42°C for 12 hours, washed, and then exposed to x-ray film at -80°C.

Table 1. Clinical Findings of Untreated Patients With DN-ATL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Subtype</th>
<th>Age/Sex</th>
<th>Sk</th>
<th>Lu</th>
<th>Gl</th>
<th>LN</th>
<th>WBC (×10⁹/L)</th>
<th>LDH (U/L)</th>
<th>Ca (mg/dL)</th>
<th>Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lymphoma</td>
<td>65/M</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>7.2</td>
<td>647</td>
<td>9.2</td>
<td>13&lt;</td>
</tr>
<tr>
<td>2</td>
<td>Acute</td>
<td>89/M</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19.2</td>
<td>1,949</td>
<td>11.7</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Acute</td>
<td>40/M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>41.8</td>
<td>1,812</td>
<td>11.0</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Acute</td>
<td>72/M</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>17.1</td>
<td>4,103</td>
<td>9.5</td>
<td>16</td>
</tr>
</tbody>
</table>

Normal ranges: WBC, 3.5 to 8.5; LDH, 130 to 250; Ca, 8.7 to 10.3.

Abbreviations: Sk, skin; Lu, lung; Gl, gastrointestinal tract; LN, lymph node; WBC, white blood cell count; LDH, lactic dehydrogenase.

(TCR δ1 and δTCS1) (T Cell Science Inc, Cambridge, MA); and CD25 (anti-Tac; provided by Dr T. Uchiyama, Kyoto University). The cells were further incubated with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2 antirabbit IgG (Sigma, St Louis, MO), and analyzed with a FACScan (Becton Dickinson Immunocytometry Systems). VAK5 (anti-p24 of human immunodeficiency virus type 1) was used as isotype-matched control Ab (IgG,) for WT31. PBMNC from three normal volunteers were simultaneously analyzed with OKT3 and WT31 as a control and the relative mean fluorescence intensity (MFI) was calculated as follows:

\[
\text{MFI of the Antigen on Patient Cells} = \frac{\text{MFI of the Antigen on PBMNC From the 3 Normal Volunteers}}{100}. 
\]

Two-color analysis was performed using cells from patient no. 2, a FACStar (Becton Dickinson Immunocytometry Systems), and the following MoAbs: an FITC-labeled anti-CD4 MoAb (Nu-T4; NIH, Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, MD) in a Cetus/Perkin-Elmer thermocycler for 30 cycles under the following conditions: melting at 95°C for 1 second, annealing at 55°C for 1 second, and extension at 72°C for 1 minute. Amplification was performed with 2.5 U Taq polymerase (Thermus aquaticus DNA polymerase; Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, MD) in a Cetus/Perkin-Elmer thermocycler for 30 cycles under the following conditions: melting at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The 5'-sense S100β primer used was 5'- TGC CTC GCA AGA GTA GAA ATG AA-3' (residues 33-52 in the first exon) and the 3'-antisense primer was 5'- GAA CTC GTG GCA GGC AGT AG-3' (residues 303-316 in the third exon). After the reaction, one tenth of each sample was

Table 2. Surface Phenotypes of DN-ATL Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD2 (OKT11)</th>
<th>CD4 (OKT4)</th>
<th>CD8 (OKT8)</th>
<th>CD3 (OKT3)</th>
<th>TCRδ (WT31)</th>
<th>TCRγ (WT31)</th>
<th>HLA-DR (Nu-2a)</th>
<th>CD25 (Tac)</th>
<th>TeT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>15/145:10</td>
<td>0</td>
<td>6</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>11</td>
<td>9</td>
<td>98</td>
<td>1/49:172</td>
<td>0</td>
<td>9</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
<td>1</td>
<td>8</td>
<td>63</td>
<td>6/153:28</td>
<td>14</td>
<td>92</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers are actual percentages of positive cells. Values in parentheses are rMFI/rMFI/rMFI.

Abbreviations: rMFI, relative MFI; rMFI, average MFI value of the amtigin on PBMNC from the three normal volunteers.
subjected to Southern blot analysis. The probes used for hybridization were 20-mer oligonucleotides derived from 3'-primers by S'-juxtaposition and labeled by the 5'-end labeling method.

RESULTS

Clinical features and laboratory data of DN-ATL. The clinical and laboratory data of the patients in this study obtained at diagnosis are shown in Table 1. All patients were males aged 40 to 89 years. Three had acute ATL and the other lymphoma ATL. Proliferation of leukemic cells in the skin, lungs, and/or gastrointestinal tract was observed in three of the four DN-ATL patients. The morphologies of peripheral blood cells corresponded to that of typical ATL cells with convoluted and coarse nuclei. Two of the four DN-ATL patients had hypercalcemia, which is frequently observed in ATL. All four patients received intensive chemotherapy and their survival did not differ from that of patients with CD4+ acute ATL.

Immunophenotypic analysis. The results of phenotypic analysis of DN-ATL cells at diagnosis are shown in Table 2. More than 85% of the cells from all DN-ATL patients were CD2+ and the cells from these patients were also CD4-, CD8-, and TCR γδ- (less than 12%). Lack of CD4 or CD8 antigens was confirmed using two different MoAbs in each case (CD4, OKT4 and Leu3a; CD8, OKT8 and Leu2a). Two-color analysis showed that most of the CD25+ cells of patient no. 2 were CD4+ and CD8+ (Fig 1). Cells from three DN-ATL patients had CD25 antigens, whereas the expression of HLA-DR antigens on cells from any of the four patients was very low, a similar finding to typical acute CD4+ ATL. Cells from all three acute DN-ATL patients were CD3+, but the MFI values were extremely low compared with those for normal T cells. The lymph node cells from the lymphoma type DN-ATL patient were CD3-. Cells from two DN-ATL patients were TCR γδ- (less than 10%). However, MFI value for TCR γδ in patient no. 2 as well as patients no. 3 and 4 was higher than those for VAKS, isotype-matched negative control Ab. Similar to the results for CD3 antigen expression, the MFI values for TCR γδ chain expression by DN-ATL cells were significantly decreased. These observations were confirmed by the expression of TCR γδ chain with the other MoAb.
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Fig 3. Northern hybridization analysis of total cellular RNA from DN-ATL cells using constant region probes for the TCR α, β, γ, and δ chain genes, the CD3 δ chain gene, and the CD4 and CD8 antigen genes. RNA from Jurkat, SKT-1B, and PEER cells served as the positive control. The lane numbers correspond to the patient numbers listed in the tables.

βF1. Over 90% of cells in patients no. 2 and 4 were reacted weakly with βF1. TdT was negative (less than 15%) in cells from all DN-ATL patients.

TCR α, β, γ, and δ chain gene rearrangements. To characterize further the TCR molecules in DN-ATL cells, we first examined the gene configuration of each chain of the TCR by Southern blot analysis. In patients no. 1 and 2, whose cells did not show surface expression of the TCR, the TCR δ and γ chain genes were rearranged, as they also were in patients no. 3 and 4 (Fig 2). The TCR δ chain gene, which lies between the Va and Ja loci, showed biallelic deletion in all cases, suggesting that TCR α chain genes were assembled. The profile of rearrangements of the TCR β and γ chain genes were different in each case (Fig 2).

Expression of TCR, CD3 δ, CD4, and CD8 genes. To verify whether the absent or decreased surface expression of TCR, CD4, and CD8 antigens on DN-ATL cells was caused by a lack of gene expression, we examined the mRNA for these molecules by Northern blot analysis (Fig 3). Mature transcripts for the TCR α and β chain genes were detected in DN-ATL, but TCR γ and δ chain mRNA could not be found in any of the DN-ATL cells. CD3 δ mRNA was also expressed in all cases. These results indicate that abnormal surface expression of the TCR/CD3 complex by DN-ATL cells was not caused by defective transcription of this complex. CD4 and CD8 mRNA were not detectable in any of the DN-ATL cells (Fig 3), confirming the lack of surface expression of these antigens.

Effect of anti-CD3 MoAb on surface expression of the TCR/CD3 complex. In CD4+ ATL cells, expression of the TCR/CD3 complex on the cell surface decreases following stimulation with anti-CD3 MoAb. To examine whether expression of the TCR/CD3 complex by DN-ATL cells would also be diminished despite their lack of CD4 and CD8 antigens, cells from patient no. 4 were used. Similar to CD4+ ATL cells, the surface expression of CD3 antigen was diminished.

Fig 4. Representative histograms of CD3 antigen expression after stimuli with anti-CD3 MoAb. PBMC from patient no. 4 and normal PBMNC were cultured with 1 μg/mL OKT3 MoAb. After 18 hours, the cells were stained with Leu4 and analyzed as described in Materials and Methods. The MFI values for CD3 antigen expression are shown in the upper right-hand part of each histogram.
by the addition of 1 μg/mL OKT3 MoAb for 18 hours to cells from patient no. 4 or normal PBMNC (Fig 4). We also obtained a similar result after the addition of 1 μg/mL WT31 MoAb (data not shown).

Expression of the gene and protein of S100β by DN-ATL cells. S100β protein was recently reported to be expressed by the cells from some patients with aggressive T-cell chronic lymphoproliferative disease (T-CLPD), which showed a double-negative phenotype.45 To examine whether DN-ATL cells also expressed S100β, we analyzed the product and mRNA for this gene in DN-ATL and CD4+ ATL by immunocytochemical assay and the RT-PCR. We examined the cytoplasmic S100 protein in PBMNC from patients no. 1 and 2 using the PAP-staining technique. In the majority of the cells from both patients, S100 protein was detected with strong or weak intensity in each cell (Fig 5C and D). In contrast, this protein was not detected in either the cells from a patient with CD4+ATL or normal PBMNC (Fig 5A and B). A band corresponding to the amplified S100β gene was clearly shown in DN-ATL patients no. 1 through 3, and was barely detectable in CD4+ ATL (Fig 6). However, these faint bands were considered to be caused by contamination with normal S100β-positive cells, because no band was detected in SKT-1B cells derived from the original CD4+ ATL cells.

DISCUSSION

Most ATL cells have the CD3+, CD4+, CD8-, and TCR αβ- phenotype, so that ATL has been considered to be a mature T-cell leukemia.6 This report is of four patients with ATL who express an aberrant phenotype of CD4−, CD8+. It is unlikely that this double-negative phenotype was caused by lack of the OKT4 epitope. The lack of CD4 and CD8 mRNA expression in these patients confirmed that their leukemic cells were derived from a double-negative subset. It is also unlikely that this double-negative phenotype was derived from immature T cells because of its expression of the CD3 antigen but not TdT. Most mature lymphocytes having a double-negative phenotype are known to bear the TCR γδ chain. However, leukemic cells from all the DN-ATL patients did not react with anti-TCR γδ MoAbs. On the other hand, cell surface expression of TCR αβ was observed in two patients with acute ATL (patients no. 3 and 4), although the MFI values were low. The leukemic cells from all DN-ATL patients had rearrangements of the TCR β and γ chain genes associated with allelic deletion of the TCR δ chain gene. This genotype is usually observed in CD3+ TCR αβ+ mature T-cell neoplasia, including ATL.39,46 Furthermore, leukemic cells from all the DN-ATL patients showed expression of TCR αβ and CD3 δ mRNA but not TCR γδ chain mRNA. These findings indicate that the DN-ATL cells were derived from a TCR αβ-positive but TCR γδ-negative subset. Some ATL patients with an aberrant phenotype have been reported previously, such as CD2−, CD4+ CD8+ (double-positive), or CD4− CD8+ (CD8 single-positive).47−49 Similar to typical ATL, the ATL cells of these patients also showed surface expression of the TCR αβ chain, and no ATL cells expressing the TCR γδ chain have ever been reported to our knowledge. Taken together with these observations, our DN-ATL patients show
that TCR $\alpha\beta^+$ T cells with or without the CD4 phenotype are the sole target of HTLV-I-induced leukemogenesis.

A subset of TCR $\alpha\beta$ lymphocytes that lacks CD4 and CD8 expression has been detected in normal human peripheral blood; these cells responded to interleukin-2 (IL-2), IL-3, and IL-4, and had lytic activity when their TCR complex was activated.29 The DN-ATL cells we examined only responded to IL-2 and IL-4, similar to typical ATL cells.30 CD4$^-$, CD8$^-$, TCR$\alpha\beta^+$ lymphocytes have also been detected in normal human skin, and they produced IL-2, tumor necrosis factor $\alpha$ (TNF-$\alpha$), and interferon gamma (IFN-$\gamma$) when they were stimulated with anti-CD3 MoAb and phorbol myristate acetate.30 Interestingly, they had the same surface phenotype as DN-ATL cells (CD2$^+$, CD7$^+$, CD25$^+$, except for HLADR$^-$) after they were cultured with IL-2 for 4 weeks, and the leukemic cells from one of our DN-ATL patients expressed mRNA of TNF-$\alpha$ and IFN-$\gamma$ without any stimulation (Suzushima et al, unpublished data). However, in this study only one of the four DN-ATL patients had prominent skin invasion by ATL cells. Therefore, it remains unclear whether DN-ATL cells originate from DN-T cells residing in the skin. Several cases of DN-T-cell lymphoma have been reported, which had many features in common with our DN-ATL cases.51 They were widespread at presentation, with frequent cutaneous, pulmonary, and bone marrow involvement, and they have a rapid course even with aggressive combined chemotherapy. However, HTLV-I or TCR studies were not performed in those lymphomas. DN-T-CLPD associated with massive hepatosplenomegaly but without significant lymphadenopathy or cutaneous involvement have been reported recently, and leukemic cells from those patients expressed S100$\beta$ protein, which is known to be expressed by less than 3% of normal circulating lymphocytes.45 Although these S100$\beta^+$ normal lymphocytes have a suppressor immunophenotype, with expression of CD2, CD8, and CD11b, their role in normal T-cell function remains obscure.52-53 We showed that cells from the DN-ATL we examined expressed the product and mRNA for the S100$\beta$ gene. Although HTLV-I proviral DNA was not detected in S100$\beta^+$-positive CLPD,45 it seems that the normal counterpart of the leukemic cells in DN-ATL is the same as that in S100$\beta$-positive CLPD.

In typical CD4$^+$ ATL cells, the MFI values of TCR $\alpha\beta$ and CD3 antigens are specifically decreased.7,54 Furthermore, surface expression of the TCR/CD3 complex by leukemic cells from lymphoma type ATL is lower than in acute ATL.7,10,11 In addition, the relative proportion of TCR $\alpha\beta$ antigen-positive cells is lower than that of CD3 antigen-positive cells using our antibodies,7,10,11 resulting in a CD3', TCR $\alpha\beta$' phenotype.11 Among the four DN-ATL patients, the MFI values for TCR $\alpha\beta$ and CD3 antigens were decreased in two cases of acute ATL (patients no. 3 and 4). Leukemic cells from the lymphoma type ATL patient (patient no. 1) had no surface expression of either TCR $\alpha\beta$ or CD3 antigens, whereas cells from the remaining acute ATL patient (patient no. 2) reacted with anti-CD3 but not anti-TCR $\alpha\beta$ MoAb. Thus, surface expression of the TCR/CD3 complex was specifically decreased in DN-ATL as well as in typical ATL. This supports our hypothesis that diminished expression of this complex plays a key role in the development of ATL. Because expression of the TCR/CD3 complex on DN-ATL cells was further diminished by the addition of anti-CD3 or anti-TCR $\alpha\beta$ MoAbs similar to that on CD4$^+$ ATL cells,8 it seems that the TCR/CD3 complex is downmodulated by some in vivo stimuli, although these cells have neither the CD4 nor CD8 antigens that are necessary for assembling MHC-TCR complexes.

In mice, a subset of TCR $\alpha\beta$ lymphocytes without CD4 or CD8 antigen expression has been detected among mature thymocytes,17-22 which preferentially expresses TCR $\gamma\delta$ instead of TCR $\alpha\beta$. Moreover, they have been reported in autoimmune mice homozygous for the lpr/lpr (lpr) and its non-autoimmune counterparts,23-27 which preferentially expresses TCR $\alpha\beta$. These lpr DN-T cells had low surface levels of TCR $\alpha\beta$ protein and normal or increased amounts of TCR $\alpha$ and $\beta$ mRNA.23 These similarities between lpr DN-T cells and DN-ATL cells raise a possibility that both types of cells respond to unknown stimuli acting via the TCR $\alpha\beta$ and causing downmodulation of TCR/CD3 complex expression on the cell surface. The TCR $\alpha\beta$ is considered to recognize antigens presented by MHC molecules acting with CD4 or CD8, and it is not known whether DN-T cells that bear TCR $\alpha\beta$ are able to recognize antigens. However, lpr DN-T cells are reported to be involved in the generation of autoantibodies by interaction with surface Igs on Ly1+ B cells through the TCR $\alpha\beta$.26 The proliferation of DN TCR $\alpha\beta$ cells was also reported in a patient with combined immunodeficiency.26 These DN-T cells recognized cu-
taneous tissue and were responsible for the reactions resembling those of graft-versus-host disease. Further analysis of TCR αβ ligands is considered to provide some insight into the mechanism of the possible downmodulation of surface expression of the TCR/CD3 complex in DN-ATL.

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