Peripheral blood T cells were immortalized in vitro by introduction of the Tax1 gene of human T-cell leukemia virus type 1 (HTLV-1) with a retroviral vector and were characterized for transformation-associated markers. Long-term observation showed that these Tax1-immortalized T cells eventually exhibited very similar features that were characteristic of HTLV-1-immortalized T cells, i.e., increased expression of c-fos, c-jun, egr-1, IL-2Ra, and Lyn and decreased expression of Lck and cell-surface CD3 antigen. Among these changes, an increase in the expression of Lyn and a decrease in the expression of Lck and cell-surface CD3 antigen were observed only in Tax1-immortalized T cells after long-term culture. The expression level of Tax1 protein did not differ significantly between early and late passage of cells, and the celluar clonality was found to be the same by the analysis of the retroviral vector integration site and the T-cell receptor β-chain gene rearrangement pattern. These changes in the expression of Lyn, Lck, and cell-surface CD3 antigen probably resulted from indirect effects of Tax1 that appeared after extended culture.

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MATERIALS AND METHODS

Cells and culture. Primary human T cells derived from the peripheral blood of a healthy donor were infected with a tax1-expressing retroviral vector, DGL-Tax1, or a control vector, DGL, followed by selection with G418. Detailed experimental procedures for establishment of these retroviral vector-infected cells were described previously.19 These infectants were maintained in AIM-V medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (PCS), 10 ng/mL recombinant IL-2 (Gakeda, Osaka, Japan), and 0.05 mmol/L 2-mercaptoethanol. Frozen stocks of these cells from various culturing periods were used in this study. KN6-HT is an HTLV-1-infected human CD4+ helper T-cell clone,19 and FU-KOS-3 is an HTLV-1-uninfected normal human CD4+ helper T-cell clone derived from the peripheral blood of a healthy donor.20 These cells were also maintained in the same medium as described above.

Southern blot analysis. Genomic DNA (10 μg) was digested with restriction endonucleases, separated by electrophoresis in 0.8% agarose, and blotted onto a nitrocellulose membrane. The membrane was hybridized with 32P-labeled Tax1-specific probe (a 1.3-kb BamHI-Sal I fragment from pUC/Tax1), as described previously.18 These infectants were maintained in AIM-V medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (PCS), 10 ng/mL recombinant IL-2 (Gakeda, Osaka, Japan), and 0.05 mmol/L 2-mercaptoethanol. Frozen stocks of these cells from various culturing periods were used in this study. KN6-HT is an HTLV-1-infected human CD4+ helper T-cell clone,19 and FU-KOS-3 is an HTLV-1-uninfected normal human CD4+ helper T-cell clone derived from the peripheral blood of a healthy donor.20 These cells were also maintained in the same medium as described above.

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Dra I fragment of p3-16; human IL-2Ra cDNA, a 1.3-kb HindIII fragment of pKCR. Tac-2.A; human Lck cDNA, a 1.8-kb Neo I-HindIII fragment of YTI; and human Lyn cDNA, nucleotides 264 to 1949 of pLY-30. These DNA fragments were labeled with multiprime DNA labeling system (Amersham, Amersham Place, UK).

Fluorescence-activated cell sorter (FACS) analysis. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD2 (Leu5), CD3 (Leu4), CD4 (Leu3a), CD5 (Leu1), CD25 (IL-2Ra), CD28 (KOLT-2), CD45RA (2H4), and CD45RO (UCHL1) were used to detect the cell-surface marker. Cells were allowed to react with optimal concentrations of these antibodies and were analyzed with flow cytometer, Cytoron-Absolute (Ortho, Raritan, NJ).

T-cell proliferation assay. T-cell proliferation was assayed by measuring the incorporation of "H-thymidine. T cells were cultured in AIM-V medium containing 10% FCS either in the absence or in the presence of IL-2 (10 ng/mL) in flat-bottom 96-well plates (4 x 10^4 cells per well). After 48 hours, each well was pulsed for 18 hours with 1 μCi "H-thymidine. The cells were harvested and the radioactivity of the 5% trichloro acetic acid-insoluble fraction was measured in a liquid scintillation counter. Results are presented as the means of triplicates ± SD.

Immunoblotting. Immunoblotting was performed essentially as described. Briefly, 5 x 10^4 cells were pelleted and lysed in 100 μL of 2% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After quantitating the protein by BCA protein assay reagent (Pierce, Rockford, IL), each lysate corresponding to 5 μg of protein was fractionated on 10% SDS-PAGE, and the proteins were electrophoretically transferred to Immobilon (Millipore, Bedford, MA). After blocking with 3% bovine serum albumin in TBS (10 mmol/L Tris-HCl, pH 7.4, 140 mmol/L NaCl) overnight at room temperature, the membranes were incubated with mouse anti-Tax monoclonal antibody TAXY7 in the blocking buffer for 1 hour. The membranes were then washed extensively with TBS containing 0.1% Tween 20, incubated for 1 hour at room temperature with horseradish peroxidase-conjugated sheep antimouse IgG antibody, washed, and developed with the Amersham ECL chemiluminescence reagent as directed by the manufacturer.

RESULTS

Immortalization of Tax1-transduced peripheral blood lymphocytes (PBLs). Continuously growing cells can be obtained from PBLs after infection with the Tax1-expressing retroviral vector, DGL-Tax1. Two cell lines designated PBL/DGL-Tax1A and PBL/DGL-Tax1B were established from two independent infection experiments. Expression of Tax1 in these cells was confirmed as described previously. PBL/DGL-Tax1A and PBL/DGL-Tax1B have continued to grow in IL-2-containing medium for nearly 4 and 2 years, respectively. Such long-term proliferating cells were not obtainable from the PBLs that were infected with a control vector DGL. Although PBL/DGL could proliferate for several months after infection in IL-2-containing culture medium, its rate of proliferation gradually declined thereafter and finally ceased to grow. Early passage of PBL/DGL-Tax1A did not differ from PBL/DGL in its morphology, but, after culturing over 2 years, a morphologic change in the phenotype of PBL/DGL-Tax1A could be observed. These cells increased in size and formed large, irregular aggregates.

Analysis of the clonality. We maintained the DGL-Tax1-infected PBLs as a bulk population without cell cloning after infection. We later determined the clonal composition of these cells by analyzing the integration sites of the retroviral vector by Southern blotting with Tax1-specific probe. PBL/DGL-Tax1A at 36 months post-infection (PI) and PBL/DGL-Tax1B at 22 months PI were examined (Fig 1). In PBL/DGL-Tax1A, one major and one minor band were detected at 3 months PI (lane 1). At 6 months PI (lane 2), only the major band was seen, and this pattern did not change thereafter (lanes 3 and 4). Thus, PBL/DGL-Tax1A consisted of a mixed population of at least two clones at 3 months after infection that became monoclonal at 6 months and remained so up to at least 36 months. PBL/DGL-Tax1B was monoclonal at 22 months PI (lane 5). The integration pattern of the retroviral vector differed between PBL/DGL-Tax1A and PBL/DGL-Tax1B; therefore, these two were different clones. The same result was obtained from an analysis for clonality by Southern blotting with a probe for the TCR β-chain gene (data not shown).

Cell-surface phenotypes. Cell-surface expression of CD3, CD4, CD25 (IL-2Ra), and CD28 antigens in PBL/DGL-Tax1A and PBL/DGL-Tax1B was analyzed by flow cytometry. PBL/DGL at 6 months PI was also examined as a control. As shown in Fig 2, PBL/DGL, PBL/DGL-Tax1A (except for 36 months PI), and PBL/DGL-Tax1B all had the phenotype of helper T cells, CD3+ and CD4+. In contrast, PBL/DGL-Tax1A at 36 months PI showed a markedly decreased level of CD3 expression and a slight reduction in CD4 expression. Expression of CD25 (IL-2Ra) was higher in all the DGL-Tax1 infected than in PBL/DGL, especially in long-term cultured DGL-Tax1 infected (PBL/DGL-Tax1A at 24 months PI and 36 months PI and PBL/DGL-Tax1B at 22 months PI). Percentages of CD28+ cells were 70% to 80% in PBL/DGL at 6 months PI and in PBL/DGL-Tax1A at 6 and 24 months PI and were 90% to 95% in PBL/DGL-Tax1A at 6 and 24 months PI and were 90% to 95% in PBL/DGL-Tax1B at 22 months PI.
Fig 2. Analysis of cell-surface phenotypes. Cells cultured in IL-2-containing medium were subjected to FACS analysis. Cytofluorometric profiles of cells stained with anti-CD3 antibody, anti-CD4 antibody, anti-CD25 antibody, or anti-CD28 antibody are shown. The names of the cells examined are indicated on the left. The vertical axis indicates relative cell numbers. The horizontal axis indicates relative fluorescence intensities. We confirmed that the fluorescence intensity of the staining with negative control antibody, FITC-conjugated normal mouse IgG, was less than 64 in each case.

DGL-Tax1A at 36 months PI and PBL/DGL-Tax1B at 22 months PI. As for other cell-surface markers, we found that all of these Tax1-immortalized T cells are CD2', CD5', and CD45RO' (data not shown).

IL-2 dependency on cell proliferation. Growth properties of PBL/DGL-Tax1A and PBL/DGL-Tax1B with or without IL-2 were analyzed. KN6-HT, an HTLV-1-immortalized human CD4' helper T-cell clone, and a PBL/DGL culture at 6 months PI were also examined as controls. As shown in Fig 3, all of these cells could proliferate in the presence of IL-2. In the absence of IL-2, significant levels of proliferation were observed in PBL/DGL-Tax1A of late passages (24 and 36 months PI) and in PBL/DGL-Tax1B at 22 months PI, but these levels were much lower than that of KN6-HT. However, these Tax1-immortalized T cells could not be maintained for more than 1 month after complete depletion of IL-2, whereas KN6-HT could (data not shown). Therefore, Tax1-immortalized T cells retained their IL-2 dependency even at 36 months PI.

Analysis of cellular gene expression. We further characterized the Tax1-immortalized T cells by using Northern blot analysis to examine the expression of several cellular genes (Fig 4A). Initially, we focused on genes of egr-1, c-fos, IL-2, and IL-2Ra, which are known to be trans-activated by Tax1. Compared with PBL/DGL, all of the DGL-Tax1 infectants expressed egr-1 and c-fos at a much higher level.
That level was almost the same or even higher than that of an HTLV-1–infected T-cell clone, KN6-HT. None of the cells expressed IL-2 mRNA. The expression level of IL-2Ra was slightly higher in PBL/DGL-Tax1A from an early passage (6 months PI) and much higher in later passages of PBL/DGL-Tax1A (24 and 36 months PI) and in PBL/DGL-Tax1B at 22 months PI. The expression level of these genes in those long-term cultured cells was almost the same as that of KN6-HT.

We then analyzed the expression of src-family tyrosine kinases, Lck and Lyn (Fig 4A), because altered production of these kinases in HTLV-1–infected T cells is reported.13,14 Lck, which is known to be the major tyrosine kinase in normal T cells,13,26 was expressed in PBL/DGL and most of the DGL-Tax1 infectants, except for PBL/DGL-Tax1A at 36 months PI. Expression of Lck was markedly decreased in KN6-HT and PBL/DGL-Tax1A at 36 months PI. We could hardly detect Lck mRNA in PBL/DGL-Tax1A at 36 months PI, whereas slight expression of the gene was evident in KN6-HT. On the other hand, increased expression of Lyn, which is known to be expressed at a very low level in normal T cells,14,26 was observed in KN6-HT and in the long-term cultured DGL-Tax1 infectants, PBL/DGL-Tax1A at 24 and 36 months PI, and PBL/DGL-Tax1B at 22 months PI. We confirmed that the expression levels of Lck and Lyn in a normal helper T-cell clone, FU-KOS-3,20 were quite similar to those of PBL/DGL and markedly different from those of PBL/DGL-Tax1A at 36 months PI (Fig 4B).

Analysis of Tax1 expression. There was a clear difference in the expression of genes for Lck and Lyn between the early and late passages of DGL-Tax1 infectants, as indicated; therefore, we examined the possible role of Tax1 in the altered expression of these genes. There were no significant differences in the amount of Tax1 protein in these DGL-Tax1 infectants (Fig 5). Thus, the difference in the cellular gene expression could not be explained by the level of Tax1 protein.

DISCUSSION

Primary human T cells derived from PBLs were immortalized by transduction of Tax1 using a retroviral vector. Although we maintained the Tax1-transduced PBLs as bulk populations in IL-2–containing medium, the immortalized cells were monoclonal CD4+ T cells at 6 months PI. We estimated the infection efficiency to be around 0.1% (data not shown); therefore, clonal selection of the infected cells should have occurred. Such clonal selection is reported for the course of T-cell immortalization by HTLV-1.27,28 Two Tax1-immortalized T-cell clones were established from two independent infection experiments. These two clones showed different integration patterns of retroviral vector. Therefore, it is less likely that the immortalization was caused by the insertional activation of some cellular oncogene by the retroviral vector at the integration site. Grassmann et al17 also report immortalization of primary human T cells by Tax1. They used Herpesvirus Saimiri vector, which retained most of the large viral genome, including the recently identified genes for cyclin-like protein and G-protein–like protein.29,30 In contrast, the retroviral vector used in this work encodes only Tax1 and neo18 and seems to be preferable for evaluating the effect of Tax1.

T cells immortalized by HTLV-1 are known to have several characteristic features that are distinct from normal T cells. In this study, we showed that Tax1-immortalized T cells eventually exhibited features that were very similar to those characteristic features of T cells immortalized by HTLV-1, ie, increased expression of egr-1, c-fos, IL-2Ra, and Lyn and decreased expression of Lck and cell-surface CD3 antigen. In addition, we previously reported that the Tax1-immortalized T cells resembled HTLV-1–immortalized T cells in the aberrant expression of GD2 ganglioside.31

Investigation of one of the Tax1-immortalized T-cell clones at various times in culture showed that, among these alterations associated with transformation, an increase in the expression of Lyn and a decrease in the expression of Lck and cell-surface CD3 antigen were observed only in long-term cultured cells. This finding was in marked contrast to the increased expression of egr-1, c-fos, and IL-2Ra observed both in early and late passages. However, augmented expression of IL-2Ra was detected in late-passaged cells. Because the Tax1-transduced T cells became monoclonal as early as 6 months PI and retained their clonality as shown...
by analysis of the retroviral vector integration site and the TCR rearrangement pattern, it is unlikely that a preexisting minor clone with a different phenotype from the major population in the early passage became dominant after long-term culture. Moreover, there was no significant difference in the amount of Tax1 protein between the early and late passages of Tax1-transduced T cells. Considering these results, the changes in the expression of Lyn, Lck, and cell-surface CD3 antigen may have been indirect effects of Tax1 that appeared only after the long culturing period. That the induced expression of Tax1 in JURKAT cells stably transfected with tax1 gene does not change the expression levels of Lck and the δ-chain of CD3 also supports the indirect involvement of Tax1 in the decreased expression of these genes.° There are many reports describing the progressive changes in the properties of T cells infected with HTLV-1, phase I and II, are observed. Phase I cells are seen early after infection, proliferate in an IL-2–dependent way, and are not different from the uninfected parental clone in morphology or cell-surface expression of the CD3/TCR complex. Phase II cells, which emerge later, proliferate in the absence of IL-2 in large dense aggregates, have a giant cell-like appearance, do not express the CD3/TCR complex on their surface, and therefore cannot respond to the signals through the CD3/TCR complex. Although the expression of src-family tyrosine kinases in phase II HTLV-I–infected T cells was not investigated in their study, Bolen et al described that decreased IL-2 dependency in HTLV-1–infected T cells correlates with decreased amounts of Lck and significant increases in the expression of Lyn. Based on those results, altered expression of src-family tyrosine kinases is expected to occur in phase II HTLV-1–infected T cells. PBL/DGL-Tax1A showed very similar phenotypic changes during extended time in cultures, as described here. More-
is modulated by these changes in cellular transcriptional factors.

Despite the similarities to phase II HTLV-1–infected T cells, long-term cultured Tax1-immortalized T cells remained IL-2 dependent even at 36 months PI. Grassmann et al. also reported the IL-2 dependency of the T cells immortalized by a Tax1-expressing Herpesvirus Saimiri vector. These results imply that, in addition to tax1, other viral genes, such as env gene and a recently identified p121 gene, may be required to establish a fully IL-2–independent growth state.

In this report, we clearly showed the importance of Tax1 in many aspects of phenotypic transformation by HTLV-1. Further investigation of our Tax1-immortalized T cells will help to unravel the precise molecular mechanisms leading to the transformation of T cells after HTLV-1 infection.

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Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1

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