RAPID COMMUNICATION

Establishment of Human T-cell Leukemia Virus Type I T-cell Lymphomas in Severe Combined Immunodeficient Mice


Human T-cell leukemia virus type I (HTLV-I) is recognized as the etiologic agent of adult T-cell leukemia (ATL), a disease endemic in certain regions of southeastern Japan, Africa, and the Caribbean basin. Although HTLV-I can immortalize T lymphocytes in culture, factors leading to tumor progression after HTLV-I infection remain elusive. Previous attempts to propagate the ATL tumor cells in animals have been unsuccessful. Severe combined immunodeficient (SCID) mice have previously been used to support the survival of human lymphoid cell populations when inoculated with human peripheral blood lymphocytes (PBL). SCID mice were injected intraperitoneally with PBL from patients diagnosed with ATL, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), or from asymptomatic HTLV-I-seropositive patients. Many of these mice become persistently infected with HTLV-I. Furthermore, after human reconstitution was established in these mice, HTLV-I–infected cells displayed a proliferative advantage over uninfected human cells. Lymphoblastic lymphomas of human origin developed in animals injected with PBL from two ATL patients. The tumor cells represented outgrowth of the original ATL leukemic clone in that they had monoclonal or oligoclonal integrations of the HTLV-I provirus identical to the leukemic clone and predominantly expressed the cell surface markers, CD4 and CD25. In contrast, cell lines derived by HTLV immortalization of T cells in vitro did not persist or form tumors when inoculated into SCID mice, indicating differences between in vitro immortalized cells and ATL leukemic cells. This system represents the first small animal model to study HTLV-I tumorigenesis in vivo.

HUMAN T-CELL leukemia virus type I (HTLV-I) was the first human oncogenic retrovirus to be isolated and characterized, and is the etiologic agent of adult T-cell leukemia (ATL), a malignancy of helper/inducer T lymphocytes. Development of ATL occurs after a characteristically long latency period of as long as 20 to 30 years after initial exposure to the virus. Molecular analysis of HTLV proviral sequences in patient lymphocytes has determined that viral integration progresses from a polyclonal pattern in asymptomatic cases, to an oligoclonal or monoclonal pattern of integration in acute ATL. Viral gene expression is generally undetectable in fresh isolates of ATL cells from patients, suggesting that viral gene products are not required to maintain the malignant phenotype. In addition, chromosomal abnormalities are commonly found in ATL cells and are often indicative of the severity of the disease, implying that somatic mutations may contribute in the progression to tumorigenesis. The development of an animal model system would delineate the role HTLV-I infection plays in the induction of lymphomagenesis and would allow characterization of cellular progression events required in the manifestation of the fully malignant phenotype in vivo. Severe combined immunodeficient (SCID) mice support survival of human lymphoid cell populations when inoculated with human peripheral blood lymphocytes (Hu-PBL–SCID). Because it was previously shown that HTLV-I cannot infect mouse cells, we used the SCID mouse as a small animal model system to investigate HTLV-I tumorigenesis in vivo. HTLV-I–infected human lymphocytes engraft in SCID mice and display a replicative advantage over uninfected human cells. In addition, lymphoblastic lymphoma development occurred in mice inoculated with PBL from two ATL patients. Importantly, in contrast to what is seen in in vitro systems, the HTLV-I–induced tumor cell outgrowth in this model represents the identical leukemic clone found in the patient PBL, suggesting that this model may be valuable for elucidation of progression events important for HTLV-induced lymphomagenesis.

MATERIALS AND METHODS

PBL and cell lines. PBL were obtained from patients by venipuncture and isolated by centrifugation over ficoll-hypaque (Pharmacia, Piscataway, NJ). SLB-I, an HTLV-I–transformed T-cell line, was maintained in Iscove’s media containing 20% fetal calf serum (FCS). Normal PBL were infected with HTLV-I and transformed by cocultivation with lethally irradiated (10,000 rad) SLB-I cells in Iscove’s media containing 20% fetal bovine serum (FBS) supplemented with 10 U/mL interleukin 2 (IL-2) (Amgen, Thou-
Fig 1. Quantitative PCR analysis of DNA from cells recovered from SCID mice. Mice were injected with 1 to 2 × 10^7 PBL from either ATL, HAM/TSP, or asymptomatic HTLV-I-seropositive patients. Control mice were injected with normal uninfected adult donor PBL. The total number of cells recovered by lavage varied between animals. DNA from cells recovered by peritoneal lavage were assayed by quantitative PCR for the presence of HTLV-I tax/rex (nt 7336 to 7495) and human β-globin sequences. Amplified HTLV-I and β-globin products are 159 bp and 110 bp, respectively. PCR amplification of DNA from cells recovered from SCID mice reconstituted with ATL PBL (G.P. no. 5), HAM/TSP PBL (M.W. no. 1 and M.W. no. 3), and HTLV-I-seropositive patient PBL (Sero no. 4) are indicated. Analysis of cells recovered by lavage from two SCID mice that were reconstituted with normal human donor PBL (PBL no. 6 and PBL no. 7) and an unoinoculated mouse (mock) are also presented. All DNA samples were tested at least three times by PCR.

Table 1. Detection of HTLV-I–Infected Cells in SCID Mice

<table>
<thead>
<tr>
<th>Patient Source</th>
<th>Total Number of Patient Samples Inoculated* (total no. of SCID mice)</th>
<th>Number of Patient Samples Resulting in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL</td>
<td>13 (57)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>HAM/TSP</td>
<td>8 (21)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>HTLV-I-seropositive asymptomatic</td>
<td>17 (36)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>HTLV-I–transformed cells</td>
<td>5 (34)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Depending on the number of cells recovered, the majority of patient PBL samples were inoculated into at least two animals (1 to 2 × 10^7 cells/mouse). The total number of mice injected with cells of each clinical category is indicated in parentheses.

† Detection of more than 10 copies of HTLV-I in a lavage at 4 weeks postinoculation was scored as positive for HTLV-I infection.

‡ Total number of patient PBL samples that resulted in development of malignancies in mice. Tumor tissue and organ samples were examined by histologic analysis and classified as lymphoblastic lymphoma.

§ Four PBL samples were infected with HTLV-I by cocultivation with lethally irradiated donor cells. The SLB-I cell line was inoculated directly into SCID mice (described in Materials and Methods).

sand Oaks, CA). Cultures were sustained in vitro for between 1 to 60 days, and the entire coculture (including the irradiated SLB-I cells) was inoculated into SCID mice. It should be noted that establishment of an immortalized lymphoid cell line by coculture (as defined by IL-2–independent growth) requires approximately 3 weeks of growth in vitro. Therefore, the cocultures were inoculated into SCID mice at different stages during the transformation protocol. All cells were prepared by centrifugation, washed once in phosphate-buffered saline (PBS), resuspended in serum-free RPMI 1640, and injected at concentrations ranging from 10^6 to 2 × 10^7 cells/mouse.

Construction and analysis of the Hu-PBL–SCID mice. Homozygous Balb C.B-17 scid/scid (SCID) mice, a gift from Kenneth Dorshkind (University of California, Riverside), were bred and maintained on a regimen of the antibiotics, trimethoprim, and sulfamethoxazole, as described. Before all manipulations, mice were anesthetized with methoxyfluorane. They were injected with 1 to 2 × 10^7 PBL from either ATL, HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or an asymptomatic HTLV-I+ patient. The SLB-I cell line was inoculated at identical concentrations into SCID mice. Three separate HTLV-I–transformed PBL cocultures were inoculated into a total of 11 mice at concentrations between 0.5 × 10^6 and 2 × 10^7 cells per mouse. In addition, three mice were first reconstituted with 2 × 10^6 normal PBL, and after 3 weeks, superinoculated with 10^7 SLB-I cells. Starting at 4 to 5 weeks postinoculation, all mice were subjected to peritoneal lavage using 5 mL of RPMI 1640. Cells recovered by lavage were analyzed by flow cytometry and/or DNA was prepared and analyzed by quantitative PCR.

Antibodies and immunofluorescence analysis. Cells (10^5 to 10^6 cells/sample) were washed once with 1-mL PBS and stained in a cocktail of murine monoclonal antibodies (MoAbs) specific for Leu 3a (CD4) (phycoerythrin (PE)-conjugated), the IL-2 receptor α chain (CD25) (fluorescein isothiocyanate (FITC)-conjugated), and Leu 2a (CD8) (biotin-conjugated) under conditions recommended by the manufacturer. All reagents were obtained from Becton-Dickinson (San Jose, CA). After 30 minutes, the cells were washed twice with PBS and incubated with streptavidin allophycocyanin. All incubations and washes were performed at 4°C in PBS containing 1% FBS and 0.1% sodium azide. To assess nonspecific binding of mouse monoclonal antibodies to human cell surface antigens, the same cell samples were also separately incubated with mouse IgG1 (FITC) and IgG1 (PE) and analyzed (data not shown). Lymphocytes were gated as shown, based on the forward (FSC) versus

![Diagram](https://example.com/diagram.png)
side scatter (SSC) signals, and analyzed for fluorescence on a Becton-Dickinson FACStar Plus flow cytometer. Data analysis was performed using the Lysis II program (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA).

**Quantitative polymerase chain reaction (PCR).** DNA from cells recovered by peritoneal lavage, beginning at 3 weeks postinoculation, were assayed by quantitative polymerase chain reaction (PCR) for the presence of HTLV-I tax/rex (nt 7336 to 7495) and human β-globin sequences. The DNA was extracted from recovered cells by the urea lysis method. Briefly, cells were washed in PBS, lyzed in urea lysis buffer (4.7 mol/L urea, 1.3% [wt/vol] sodium dodecyl sulfate (SDS), 0.23 mol/L NaCl, 0.67 mmol/L EDTA [pH 8.0], 6.7 mmol/L Tris-HCl [pH 8.0]), and subjected to phenol-chloroform extraction and ethanol precipitation. Total DNA was resuspended in 450 μL of H2O, and a 10-μL sample was analyzed by quantitative PCR, as previously described. PCR products resulting from amplification (94°C, 1 minute; 65°C, 2 minutes; 30 cycles) with 32P-end-labeled primers specific for the HTLV-I tax/rex region and for the human β-globin gene were 159 and 110 bp, respectively. Hind III-cleaved HTLV plasmid DNA (pH0neo) was serially diluted 10-fold in the presence of 1.0 μg/mL of human PBL carrier DNA and amplified in parallel to provide virus copy number standards for the reactions. Normal (uninfected) human PBL DNA was serially diluted 10-fold and amplified in parallel as a measure of the number of human cells. Following amplification, radiolabeled products were resolved on a 6% polyacrylamide gel and visualized by autoradiography.

**Southern blots.** High molecular weight DNA was extracted using SDS and proteinase K. Digested DNA was resolved on 1% agarose gel, transferred to a Genescreen Plus nylon membrane (NEN Research Products, Boston, MA), and subsequently hybridized with a 32P HTLV-I-specific probe (pHT3.9). Hybridizations were performed in QuickHyb solution (Stratagene, La Jolla, CA) for 1 to 2 hours at 68°C. The blots were examined by exposure to Kodak XAR film (Eastman Kodak, Rochester, NY) at −70°C.

**RESULTS**

**Establishment of HTLV-I-infected cells in SCID mice.** Eight-week-old SCID mice were inoculated intraperitoneally with 1 to 2 × 107 PBL from patients previously determined, by serology, to be infected with HTLV-I. The presence of HTLV-I DNA in human cells recovered from the peritoneal cavity of reconstituted mice was serially analyzed, beginning at 3 weeks postinoculation, by quantitative PCR. Primers specific for HTLV-I tax/rex sequences were used in conjunction with primers specific for the human β-globin to provide equivalent β-globin signal. Samples were tested by PCR at least three times. (A) ATL patient PBL and cells recovered from SCID mice. Thirty cycles of PCR amplification were performed on DNA from ATL patient PBL and from cells recovered from mice reconstituted with patient PBL. RV407 and RV417 arise from the same patient; however, RV417 PBL DNA was obtained and tested after the patient received chemotherapy for CTCL. Sample RV407 no. 3 was recovered by lavage from an animal that developed an ascites tumor 9 months after inoculation with RV407 PBL (primary tumor). RV417 no. 3 and RV417 no. 4 (primary tumors) were from animals that developed lymphoblastic lymphomas 7 and 11 weeks, respectively, after inoculation with RV417 PBL. RV417 no. 4.1 and RV417 no. 4.2 (secondary tumors) were ascites tumor cells recovered from SCID mice transplanted with between 106 and 107 ascites tumor cells from RV417 no. 4. These mice also developed lymphoblastic lymphomas at 4 and 7 weeks after inoculation, respectively. RV417 no. 4.1-2 (tertiary tumors) were from mice that eventually developed lymphoblastic lymphoma 5 weeks after transplantation with between 106 and 107 ascites tumor cells from mouse RV417 no. 4.1. All animals were assayed at the time of ascites development. (B) HAM/TSP patient PBL and cells recovered from reconstituted mice. DNA from M.W. PBL and cells recovered from two SCID mice reconstituted with M.W. PBL (M.W. no. 1 and M.W. no. 3) at 4 weeks postinoculation were analyzed by PCR, as in A. Dilutions of uninfected PBL DNA and HTLV-I DNA were analyzed in parallel as controls. It should be noted that none of the animals inoculated with HAM/TSP PBL ever developed a detectable malignancy.

**Fig 2.** Comparison of HTLV-I copy load in patient PBL versus infected SCID mice. The relative frequency of HTLV-I–infected cells compared with uninfected human cells was determined by quantitative PCR. DNA was prepared and tested as in Fig 1, but was serially diluted in water to obtain equivalent β-globin signal. Samples were tested by PCR at least three times. (A) ATL patient PBL and cells recovered from SCID mice. Thirty cycles of PCR amplification were performed on DNA from ATL patient PBL and from cells recovered from mice reconstituted with patient PBL. RV407 and RV417 arise from the same patient; however, RV417 PBL DNA was obtained and tested after the patient received chemotherapy for CTCL. Sample RV407 no. 3 was recovered by lavage from an animal that developed an ascites tumor 9 months after inoculation with RV407 PBL (primary tumor). RV417 no. 3 and RV417 no. 4 (primary tumors) were from animals that developed lymphoblastic lymphomas 7 and 11 weeks, respectively, after inoculation with RV417 PBL. RV417 no. 4.1 and RV417 no. 4.2 (secondary tumors) were ascites tumor cells recovered from SCID mice transplanted with between 106 and 107 ascites tumor cells from RV417 no. 4. These mice also developed lymphoblastic lymphomas at 4 and 7 weeks after inoculation, respectively. RV417 no. 4.1-2 (tertiary tumors) were from mice that eventually developed lymphoblastic lymphoma 5 weeks after transplantation with between 106 and 107 ascites tumor cells from mouse RV417 no. 4.1. All animals were assayed at the time of ascites development. (B) HAM/TSP patient PBL and cells recovered from reconstituted mice. DNA from M.W. PBL and cells recovered from two SCID mice reconstituted with M.W. PBL (M.W. no. 1 and M.W. no. 3) at 4 weeks postinoculation were analyzed by PCR, as in A. Dilutions of uninfected PBL DNA and HTLV-I DNA were analyzed in parallel as controls. It should be noted that none of the animals inoculated with HAM/TSP PBL ever developed a detectable malignancy.
HTLV-I T-CELL LYMPHOMAS IN SCID MICE

Table 2. Phenotypes of Ascites Tumor Cell Populations in SCID Mice

<table>
<thead>
<tr>
<th>Tumor No.</th>
<th>Status</th>
<th>Time of Analysis (wks post-inoculation)</th>
<th>CD4/CD8 Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.P. 1</td>
<td>1⁺</td>
<td>10</td>
<td>+/− +/dim</td>
</tr>
<tr>
<td>G.P. 2</td>
<td>1⁺</td>
<td>16</td>
<td>+/− +/dim</td>
</tr>
<tr>
<td>G.P. 3</td>
<td>1⁺</td>
<td>13</td>
<td>+/− +/dim</td>
</tr>
<tr>
<td>G.P. 1</td>
<td>1⁺</td>
<td>10</td>
<td>−/− +/−</td>
</tr>
<tr>
<td>G.P. 2</td>
<td>1⁺</td>
<td>16</td>
<td>−/− +/−</td>
</tr>
<tr>
<td>G.P. 3</td>
<td>1⁺</td>
<td>13</td>
<td>−/− +/−</td>
</tr>
<tr>
<td>G.P. 5.1</td>
<td>2⁺</td>
<td>7</td>
<td>+/− dim</td>
</tr>
<tr>
<td>G.P. 5.2</td>
<td>2⁺</td>
<td>7</td>
<td>+/− dim</td>
</tr>
<tr>
<td>G.P. 1.1</td>
<td>2⁺</td>
<td>8</td>
<td>−/− +/−</td>
</tr>
<tr>
<td>G.P. 1.1-2</td>
<td>3⁺</td>
<td>5</td>
<td>−/− −/−</td>
</tr>
<tr>
<td>RV407 3</td>
<td>1⁺</td>
<td>36</td>
<td>+/− −/−</td>
</tr>
<tr>
<td>RV417 4</td>
<td>1⁺</td>
<td>5</td>
<td>+/− dim</td>
</tr>
<tr>
<td>RV417 4.1</td>
<td>2⁺</td>
<td>4</td>
<td>+/− dim</td>
</tr>
<tr>
<td>RV417 4.2</td>
<td>2⁺</td>
<td>4</td>
<td>+/− dim</td>
</tr>
<tr>
<td>RV417 4.1-3</td>
<td>3⁺</td>
<td>5</td>
<td>+/− −/−</td>
</tr>
<tr>
<td>RV417 4.2-2</td>
<td>3⁺</td>
<td>5</td>
<td>+/− −/−</td>
</tr>
<tr>
<td>RV417 4.1-3(A)</td>
<td>4⁺</td>
<td>5</td>
<td>+/− −/−</td>
</tr>
<tr>
<td>RV417 4.1-3(b)</td>
<td>4⁺</td>
<td>5</td>
<td>+/− −/−</td>
</tr>
</tbody>
</table>

* Malignancies that developed after inoculation with patient PBL are indicated as primary tumors. Subsequent inoculations of ascites cells from a diseased mouse into a naive SCID mouse are indicated as secondary and tertiary, depending on the passage number. Note that mice G.P. nos. 1, 2, and 3 all carried two phenotypically distinct primary tumor cell subpopulations. All tumors contained tax/er genomic DNA sequences, as determined by PCR.

β-globin gene as an internal control for the number of human cells (Fig 1). PBL from patients with ATL, HAM/TSP, or asymptomatic HTLV-I-seropositive carriers resulted in engraftment of HTLV-I-infected cells in SCID mice 4 to 5 weeks postinjection, albeit at different relative frequencies (Table 1). Five out of 13 ATL PBL samples and 3 of 8 HAM/TSP PBL samples resulted in engraftment of HTLV-I-infected cells in SCID mice 4 weeks after inoculation. In contrast, only 1 out of 17 HTLV-I-seropositive asymptomatic carrier PBL resulted in engraftment of HTLV-I-infected cells.

HTLV-I infection correlated with the presence of human cells in all reconstituted mice; in fact, in all animals where successful engraftment of PBL from HTLV-I-infected patients occurred, persistence of HTLV-I-infected cells was also seen. In the majority of mice inoculated, reconstitution with patient PBL was transient and human cells were no longer detected in the peritoneal cavity within 3 to 4 weeks. Time course experiments were performed to determine the timeframe by which HTLV-transformed cell lines and patient PBL that failed to engraft became undetectable by peritoneal lavage. These animals exhibited a steady decline in the number of human cells recovered by lavage, until no human cells or HTLV genomic DNA were detectable by 3 weeks postinoculation in all cases (data not shown). The frequency of engraftment of PBL in SCID mice from ATL and HAM/TSP patients was approximately 35%. For reasons that are unclear, this engraftment rate was significantly lower than that seen with normal donor PBL, where greater than 90% of the animals are engrafted after 4 weeks (Zack, Feuer, and Chen, unpublished observations, January 1992).

A progressive increase in the proportion of HTLV-I genomes relative to human cell number was found to occur in all mice persistently infected with HTLV-I, regardless of the clinical status of the patient donor. An example is shown in Fig 2. Quantitative PCR analysis showed that approximately 3% and 20% of the PBL were HTLV-I-infected in ATL (RV417) or HAM/TSP (M.W.) patients, respectively, in agreement with previously published reports of HTLV-I- and HTLV-II-infected cells found in patients. 16,20,21 SCID mice were subsequently assayed at various timepoints after the cells were injected. An increase in the ratio of HTLV-I genomes relative to human cell number was observed, such that 50% to greater than 90% of the human cells in the peritoneal cavity carried the viral genome by 4 weeks postinoculation. In contrast, preferential proliferation or maintenance of HTLV-I-infected cells occurred relative to uninfected human cells in the peritoneal cavity of these mice.

Phenotypic characterization of HTLV-I-induced lymphoblastic lymphomas. Tumors of human origin formed after reconstitution of mice with PBL from two ATL patients. Injection of four SCID mice with PBL from patient G.P. resulted in rapid proliferation of HTLV-I-infected cells in the peritoneal cavity of each animal inoculated, and solid tumor masses appeared in all four animals within 10 to 16 weeks after inoculation. Moribund animals were killed and histologic analysis of the solid tissue identified the malignancy as a lymphoblastic lymphoma, which infiltrated mesenteric lymph nodes and metastasized to the spleen, lung, liver, thymus, and PB. Tumor cells from the ascites of mice G.P. no. 1 and G.P. no. 5 were inoculated into a naive SCID mouse (approximately 0.5 to 1 X 10^7 cells injected per mouse) and, in both cases, new lymphomas developed that killed the host within 5 to 10 weeks.

Cells recovered from the ascites of SCID mice inoculated with PBL from ATL patient G.P. were stained with a panel of antibodies against human lymphocyte markers and analyzed by flow cytometry. Consistent with the phenotype of ATL cells, 22,23 CD4⁺, CD8⁺, CD25⁺ and CD4⁺, CD8⁺, CD25dim cells were the predominant tumor cell populations arising in three mice that were analyzed, although these mice also supported the growth of a CD4⁺, CD8⁺, CD25⁺ cell subpopulation (G.P. no. 1, G.P. no. 2, and G.P. no. 3) (Table 2). Transplantation of these primary tumor cells into naive SCID mice resulted in engraftment of either the CD4⁺, CD8⁺, CD25dim or the CD4⁺, CD8⁺, CD25⁺ secondary tumor cell populations. PCR analysis showed that both of these tumor cell populations contained HTLV-I DNA. Flow cytometric analysis of patient G.P. PBL was performed independently by a clinical laboratory, which reported that the majority of leukemic cells in this patient had an atypical profile of cell surface markers, similar to the
A. Normal PBL (PHA Stimulated)

B. ATL Patient PBL

C. Primary Tumor

D. Primary Tumor

E. Transplanted Tumor
HTLV-I T-CELL LYMPHOMAS IN SCID MICE

phenotype of one of the tumor cell subpopulations recovered from SCID mice. The majority (90%) of circulating malignant cells in this patient did not express the CD4, CD8, or CD25 (IL-2 receptor α chain) markers, but were positive for the pan-T-cell markers, CD2, CD3, and CD5. Cases in which leukemic cells from ATL and cutaneous T-cell lymphoma (CTCL) patients express unusual surface phenotypes have previously been reported. Confirmation of the flow cytometric analysis, as well as Southern blot analysis of the genomic DNA, was not possible because of the limited amount of cells available from this patient.

Reconstitution of SCID mice with PBL from Epstein-Barr virus (EBV)-seropositive donors has been reported to result in development of B-cell lymphomas in certain instances. None of the lymphomas arising in these animals showed evidence of staining with a B-lymphocyte-specific antibody (anti-CD20).

Injection of SCID mice with PBL of a second patient (RV407/RV417), initially diagnosed with HAM/TSP and subsequently developing CTCL (a clinical condition found in patients with acute ATL), similarly gave rise to lymphoblastic lymphomas in 3 of 6 animals. In the earliest inoculation (RV407), an ascites tumor cell population developed 9 months postinjection in 1 of 2 animals (RV407 no. 3). A second PBL sample (RV417), obtained after the patient received chemotherapy treatment for CTCL, was injected into four additional mice (RV417 no. 1 to RV417 no. 4). Two of these mice developed human lymphoblastic lymphomas 8 (RV417 no. 3) and 11 weeks (RV417 no. 4) after injection. The predominant phenotype of ascites tumor cells recovered from animal RV407 no. 3 was CD4+, CD25dim, with a CD8+, CD25+ subpopulation (Fig 3C and Table 2). A CD8dim, CD25+ minor subpopulation of human lymphocytes was also detectable in this mouse. In comparison, the flow cytometric analysis of the original patient PBL (RV407) showed a normal distribution of CD4+ and CD8+ lymphocyte subpopulations; a dominant clone or subpopulation of malignant cells was not distinguishable in the PBL sample. This was consistent with quantitative PCR analysis of RV407 PBL, which showed that only 1% to 3% of the cells were infected with HTLV-I (Fig 2A). Thus, the HTLV-I–infected cells preferentially proliferated in the SCID mouse.

Flow cytometric analysis of an additional tumor cell population arising in another SCID mouse injected with the PBL of the same patient (RV417 no. 4) showed a slightly different pattern of staining (Fig 3D). The majority of these cells were CD4+, CD25dim, with a CD8+, CD25+ subpopulation. Transfer of ascites tumor cells from this mouse into two naive animals (RV417 no. 4.1 and RV417 no. 4.2) resulted in the selective outgrowth of the CD4+, CD25dim subpopulation (Fig 3E). Both of these mice developed lymphoblastic lymphomas and greater than 90% of the human cells recovered from the peritoneal cavity of these mice were infected with HTLV-I, as determined by PCR analysis (Fig 2A). Subsequent transfer of secondary ascites tumor cells into additional SCID mice resulted in tertiary tumor development. Phenotypically, all tertiary tumor cells were CD4+, although the staining intensity for the CD25 marker varied from dim to high between animals inoculated with the same secondary tumor cell source.

**Molecular characterization of HTLV-I proviral DNA in lymphoblastic lymphomas.** A SCID mouse model of HTLV tumorigenesis would be most meaningful if the tumor cells selected in the mouse represented the same clone as the original ATL cells. The number and clonality of proviral integration sites in the genomic DNA from these cells was analyzed by restriction enzyme analysis. Because the HTLV-I genome has no Eco RI sites, Eco RI restriction digestion was used to compare RV407 and RV417 tumor cell DNA and ATL patient (RV417) PBL DNA (Fig 4B).

DNA from tumors arising from patient RV417 all contained an HTLV-I–specific band of approximately 12 kb, showing a predominant proviral integration site. The same proviral integration event was detected in the patient PBL sample, demonstrating that the malignant cells that developed in SCID mice were derived from the same clone that predominated in the ATL patient. Analysis of the same tumor DNA samples after digestion with Eco RI and Sac I showed hybridization to a 3.6-kb fragment and a minor fragment of 2.5 kb (presumably a proviral junction fragment), and confirmed the clonal origin of these tumors (Fig 4C). Smal I digestion was performed as a gross analysis of the integrity of the env and tax/rex genes of the provirus in these tumor cells (Fig 4D). Hybridization of the probe to a 2.5-kb fragment in RV417 PBL DNA and in all RV417-derived tumors indicates that while these tumor cells carry the viral tax/rex sequences, a deletion of proviral sequences occurred. Additional restriction enzyme analysis showed a deletion of approximately 4 kb of proviral sequences between the 5'-long terminal repeat (LTR) and the tax/rex sequences.
This same deletion was also present in the patient PBL. It was reported previously that a significant proportion of ATL tumors carry deletions of the provirus while retaining the *tax/rex* sequences, similar to those observed here.\(^27\)

Southern analysis of G.P. PBL-induced tumors digested with *Eco* RI as well as *Eco* RI and *Sac* I showed two detectable proviral integrations and hybridization to fragments of relatively large molecular weight (greater than 15 kb) (Fig 4B and C). This oligoclonal pattern may be reflective of the phenotypically distinct primary tumor cell subpopulations that engrafted in SCID mice and were identified by flow cytometric analysis. Indeed, the hybridization pattern of G.P. no. 1 identifies these tumor cells as carrying an additional proviral integration, with respect to the other G.P. PBL-induced lymphomas (Fig 4C and D). *Sma* I Southern
Southern analysis of HTLV proviral DNA in tumor cells. (A) Schematic map of the HTLV-I provirus. Positions of SmaI (S) restriction sites are indicated. The SacI sites are not found in all variants of HTLV-I; SacI sites in the provirus of SLB-I are shown in parentheses. The orientation of the provirus is indicated by 5' and 3', and the location of the HTLV-I-specific probe (pHT3.9, from nt + 4990 to + 8980 labeled with 32P) is indicated. The U3, R, and U5 regions of the LTR and the viral genes are illustrated. (B) Southern hybridization analysis of EcoRI-digested DNA from tumor cells in SCID mice. High molecular weight DNA (6 μg) was extracted from tumor tissue recovered from killed mice and from RV417 PBL. Tumors induced after inoculation with ATL patient G.P. PBL (G.P. no. 1, G.P. no. 2, G.P. no. 5, G.P. no. 5.1, and G.P. no. 5.2-1) and patient RV407/RV417 PBL (RV417 no. 4, RV417 no. 4.2, RV417 no. 4.1-1, and RV417 no. 4.1-2) are represented. G.P. no. 5.1 is a secondary tumor established in mice inoculated with ascites tumor cells from animal G.P. no. 5. Similarly, G.P. no. 5.2-1 is a tertiary tumor that developed after transplantation of ascites tumor cells from G.P. no. 5.2. RV417 no. 4.2 is a secondary tumor that developed in a SCID mouse after inoculation of ascites tumor cells from mouse RV417 no. 4. RV417 no. 4.1-1 and RV417 no. 4.1-2 are tertiary tumors that developed in mice transplanted with ascites tumor cells from mouse RV417 no. 4.1. The fragment representing the major proviral integration site in RV407/RV417 tumor DNA and in DNA from the patient PBL is indicated by an arrow. The blot was deliberately overexposed to detect the proviral integration in RV417 PBL. The apparent lower molecular weight band in RV417 no. 4.1-1 and RV417 no. 4.1-2 is a result of aberrant migration not seen in other experiments. The sizes of the molecular weight markers are indicated in kilobase pairs. (C) High molecular weight tumor cell DNA (6 μg) was digested with EcoRI and SacI and Southern blots were performed and hybridized with the same probe, as described in (B). "M" is the nonradioactive molecular weight marker lane. The molecular weight markers in kilobase pairs are indicated on the left. For comparison, DNA from SLB-I, an HTLV-I-transformed T-cell line, was digested with the same restriction enzymes. The size of the molecular weight markers is indicated. SacI does not cleave within the provirus of many variants of HTLV-I, although it does cut once within each LTR of the provirus in SLB-I cells, resulting in hybridization to an 8.3-kb fragment, indicated by an arrow. The SLB-I cell line retains multiple integrations of deleted viral genomes of HTLV-I, accounting for hybridization to multiple fragments. Hybridization of the 2.5-kb fragment in the RV417 tumor cell lines most likely represents a proviral-genomic DNA junction fragment. (D) High molecular weight DNA (6 μg) was digested with SmaI and Southern blots were performed and hybridized with the same probe, as described in (B). The wild-type HTLV-I proviral 4.4-kb fragments in the SLB-I and the G.P. no. 1 tumor lanes are indicated by an arrow.
injected with RV407/417 PBL was noteworthy in that malignant cells were genotypically identical to leukemic cells found in the ATL patient, with respect to the site of HTLV-I proviral integration. Expression of cell surface markers on malignant cells from SCID mice were also characteristic of leukemic cells from ATL patients. The development of RV407/417 lymphomas in the SCID mouse, in fact, allowed for early detection of a subclinical tumor cell population in a patient initially diagnosed with a nonmalignant condition (ie, HAM/TSP). Although an unequivocal comparison of clonality between G.P. PBL DNA and lymphoblastic lymphomas induced in SCID mice by inoculation of G.P. PBL could not be made, the proviral structure and integration patterns of individual tumors arising in SCID mice were identical, suggesting that leukemic cells from this patient also predominated and expanded after inoculation into SCID mice.

The difficulty in establishing ATL cells in culture suggests that the SCID mouse provides an ideal microenvironment for propagation of these leukemic cells. Attempts to culture human lymphoblastic lymphoma cells from SCID mice in vitro have not been successful, and it will be of interest to identify the factors the SCID mouse provides to support the growth of ATL cells in vivo. Conversely, the relatively rapid clearance of in vitro HTLV-I-infected cells and the SLB-I cell line after inoculation of SCID mice may be used to characterize immune mechanisms that are not of T- or B-lymphoid origin that are involved in cell rejection.

All of the lymphomas that develop in SCID mouse carry defective HTLV-I proviral genomes and retain the tax/rev sequences. Because these viral sequences are invariably retained in leukemic cells from ATL patients, it will be important to determine the role these sequences play in the expansion of malignant cells in vivo. Experiments characterizing the presence or absence of viral gene expression in SCID mouse lymphomas will determine the extent of biologic similarities that these lymphoma cells share with malignant cells found in patients. The Hu-PBL–SCID mouse is the first in vivo model to examine tumorigenic potential of HTLV-I–infected human cells, and should enable one to examine genetic perturbations that result in leukemogenesis. Importantly, this model will also provide an in vivo system for evaluating therapeutic agents.

NOTE ADDED IN PROOF

Similar to tumor cells from ATL patients, the steady state levels of HTLV-I RNA in ATL tumor cells from SCID mice were approximately 20-fold lower in comparison with levels of viral expression seen in SLB-1 and MT-2 cells.

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