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Human T-Cell Leukemia Virus Infection of Human Hematopoietic Progenitor Cells: Maintenance of Virus Infection during Differentiation In Vitro and In Vivo

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Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia and lymphoma and HTLV-1-associated myelopathy-tropical spastic paraparesis. We examined whether HTLV could productively infect human hematopoietic progenitor cells. $CD34^+$ cells were enriched from human fetal liver cells and cocultivated with cell lines transformed with HTLV-1 and -2. HTLV-1 infection was established in between 10 and >95% of the enriched $CD34^+$ cell population, as demonstrated by quantitative PCR analysis. HTLV-1 p19 Gag expression was also detected in infected hematopoietic progenitor cells. HTLV-1-infected hematopoietic progenitor cells were cultured in semisolid medium permissive for the development of erythroid (BFU-E), myeloid (CFU-GM), and primitive progenitor (CFU-GEMM, HPP-CFC, or CFU-A) colonies. HTLV-1 sequences were detected in colonies of all hematopoietic lineages; furthermore, the ratio of HTLV genomes to the number of human cells in each infected colony was 1:1, consistent with each colony arising from a single infected hematopoietic progenitor cell. Severe combined immunodeficient mice engrafted with human fetal thymus and liver tissues (SCID-hu) develop a conjoint organ which supports human thymocyte differentiation and maturation. Inoculation of SCID-hu mice with HTLV-1-infected T cells or enriched populations of $CD34^+$ cells established viral infection of thymocytes 4 to 6 weeks postreconstitution. Thymocytes from two mice with the greatest HTLV-1 proviral burdens showed increased expression of the CD25 marker and the interleukin 2 receptor alpha chain and perturbation of $CD4^+$ and $CD8^+$ thymocyte subset distribution profiles. Hematopoietic progenitor cells and thymuses may be targets for HTLV infection in humans, and these events may play a role in the pathogenesis associated with infection.

Adult T-cell leukemia and lymphoma (ATL) is characterized by an aggressive clonal malignancy of human T-cell leukemia virus type 1 (HTLV-1)-infected CD4-bearing T lymphocytes, and the disease manifests itself decades after infection. HTLV-1-associated myelopathy (HAM)-tropical spastic paraparesis (TSP) is a neurological disorder which generally develops within a few years after infection with HTLV-1. Previous investigations have shown that viral isolates from ATL and from HAM-TSP patients do not differ significantly at the sequence level (7, 39). It has therefore been argued that the time and route of infection may play significant roles in the pathology induced by HTLV-1 infection. A characterization of the cell types that are susceptible to HTLV-1 infection is clearly important in the understanding of the biology of virus infection and in identifying the determinants of pathogenesis in the host. HTLV-1 exhibits a preferential tropism for $CD4^+$ lymphocytes in vivo, as demonstrated by cells isolated directly from either ATL or HAM-TSP patients (5, 14, 35). The infection of T cells is clearly important for the ultimate development of ATL (45). Peripheral and cord blood T cells can be infected and immortalized by HTLV-1 in vitro, and transformed cells express high levels of CD25, the interleukin 2 (IL-2) receptor alpha chain (30, 46). Although the HTLV-1-HTLV-2 family of viruses has receptors on a wide range of cell types other than T cells (40), the involvement of other cell types in the pathogenesis of disease is less clear. Many cell types are susceptible to HTLV-1 infection in vitro, including $CD8^+$ T lymphocytes (38), B lymphocytes (25, 42), fibroblasts (47), natural killer cells (24), endothelial cells (16), glial cells (43), epithelial cells (48), and macrophages (8, 21). In patients infected with HTLV-2, a virus closely related to HTLV-1, viral DNA can be found in $CD4^+$ and $CD8^+$ T cells (17, 36, 37), B cells (4), monocytes/macrophages, and natural killer cells (22).

Human hematopoietic progenitor cells bearing the CD34 antigen have the capability of differentiating into mature end-stage cells. $CD34^+$ cells are able to form erythroid (burst-forming units [BFU-E]) myeloid (granulocyte-monocyte CFU and macrophage CFU [CFU-GM and CFU-Mac]), and primitive progenitor (multi-lineage CFU [CFU-GEMM or CFU-A] and high proliferative potential CFU [HPP-CFU] [29]) colonies in vitro. Hematopoietic colony assays are, however, not capable of assessing lymphoid cell differentiation from hematopoietic progenitor cells. The severe combined immunodeficient (SCID) mouse implanted with human fetal thymus and liver cells (SCID-hu) develops a functional human thymus (Thy/Liv), and has recently been shown to provide a model with which human T-lymphocyte differentiation in vivo can be studied. Introduction of exogenous $CD34^+$ hematopoietic progenitor cells into sublethally irradiated SCID-hu mice results in lymphoid cell repopulation of the thymic graft (1, 34).

In this study, we test the ability of HTLV-1 to establish infection in an enriched human $CD34^+$ hematopoietic progenitor cell population and determine whether HTLV-1 is main-

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tained after cells differentiate into mature hematopoietic lineages in vitro and in vivo.

MATERIALS AND METHODS

Preparation of $CD34^+$ cells. Human $CD34^+$ were prepared as previously described (1). Briefly, fragments of fetal liver tissues were washed in phosphate-buffered saline (PBS), and a single-cell suspension was obtained by digestion in collagenase (507 U/ml)–hyaluronidase (2,400 U/ml)–DNase (300 Kunitz units per ml) in serum-free AIM-V medium (GIBCO, Grand Island, N.Y.) for 4 h at 37°C. Erythrocytes were removed from the cell suspension by centrifugation over Ficoll-Hypaque (Sigma, St. Louis, Mo.), and the mononuclear cells were incubated with anti-CD34 monoclonal antibody conjugated with biotin (Cellpro Inc., Bothell, Wash.) for 1 h at room temperature. $CD34^+$ cells were purified by passage of the cell suspension through an avidin affinity column (Cellpro Inc.).

Cell lines. HTLV-1-transformed cell lines, SLB-1 and MT-2, were maintained in Iscove's medium containing 20% fetal calf serum (FCS). An HTLV-2-transformed cell line, 96/pH6neo, was established in culture (unpublished results) and was maintained in Iscove's medium containing 20% FCS.

HTLV infection of hematopoietic progenitor cells and cell sorting. Enriched $CD34^+$ cell populations were infected with HTLV-1 or HTLV-2 by cocultivation with lethally irradiated (10,000 rads) SLB-1 or 96/pH6neo cells, respectively, in Iscove's medium containing 20% FCS, 100 ng of interleukin 3 (IL-3) per ml, 100 ng of IL-6 per ml, and 10 ng of stem cell factor per ml (all cytokines were provided by Amgen, Thousand Oaks, Calif.). Irradiation of 10 to 10^7 SLB-1 cells at 10,000 rads is lethal, and no growth was observed in culture. After 3 to 4 days, the cell coculture was incubated with human-specific monoclonal antibodies against CD34 and CD4 conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin, respectively, and live cell sorting was performed for high-density expression of the antigens with a FACStar^{plus} flow cytometer (Becton Dickinson, Mountain View, Calif.), as previously described (2). The purity of the $CD34^+$ population after performing fluorescence-activated cell sorting (FACS) was not determined. Murine monoclonal antibodies specific for human CD2, CD8, and CD25 (FITC conjugated) and for CD3, CD4, and CD34 (phycoerythrin conjugated) were obtained from Becton Dickinson (San Jose, Calif.). To assess nonspecific binding of mouse monoclonal antibodies to human cell surface antigens, the same cell samples were also separately incubated with mouse FITC-conjugated immunoglobulin G and phycoerythrin-conjugated immunoglobulin G and analyzed. The Lysis II program (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) was used for data analysis.

Quantitative PCR and flow cytometry. The DNAs from cell populations isolated by FACS were extracted by the urea lysis method (49, 50). Quantitative DNA PCRs for HTLV *tax-rex* sequences (nucleotides 7336 to 7495) and human β-globin sequences and immunofluorescence analysis of cell surface markers were performed as previously described (2, 10, 11).

In vitro assays of hematopoietic progenitor cells. Clonogenic progenitor assays were performed in MethoCult medium H4433 (StemCell Technologies, Vancouver, British Columbia, Canada). $CD34^+$ cells (2×10^3) were cultured in 2 ml of MethoCult at 37°C in a humidified atmosphere with 5% CO_2 in air. Clonogenic granulocyte-macrophage (CFU-GM), macrophage (CFU-Mac), erythroid bursts (BFU-E), mixed cell, and primitive progenitor (CFU-GEMM, HPP-CFC, and CFU-A) colonies were identified by morphology at 14 to 16 days postplating, counted under an inverted microscope, and isolated by aspiration from the methylcellulose medium. DNAs from individual colonies were processed as previously described (11), with the exception that cells were lysed in a solution of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, instead of urea lysis buffer.

Preparation and reconstitution of SCID-hu mice. Purified $CD34^+$ cells (3×10^6) were infected with HTLV-1 by coculture with 6×10^6 lethally irradiated (10,000 rads) SLB-1 cells, as described above. After 3 to 4 days, the $CD34^+$ cell population was fractionated by immunodepletion with a mouse monoclonal antibody against the human CD4 antigen, as previously described (44). Briefly, the cell coculture was incubated with a monoclonal antibody against human CD4. Cells (10^6) were pipetted through 100-μm-pore-size nylon mesh onto a 60-mm-diameter petri dish coated with goat antimouse polyclonal antibody (Cappell, Durham, N.C.). After 1 h, unbound cells were collected and pooled by washing with PBS plus 5% FCS. The purity of the $CD34^+$ cell population enriched by immunodepletion was not determined. C.B.-17 SCID mice were bred at the University of California, Los Angeles, and were housed in a biosafety level-3 animal facility. SCID-hu mice were constructed by implantation with human fetal liver and thymus pieces under their murine kidney capsules, as previously described (2, 20, 28, 32). For reconstitution of the T-lymphoid lineage, animals were sublethally irradiated (200 or 400 rads) 4 to 6 months postimplantation, and implants were directly injected with $CD34^+$ cells. SCID-hu mice inoculated with HTLV-1-transformed T-cell lines received 1×10^6 to 3×10^6 mixed, lethally irradiated (10,000 rads) SLB-1 and MT-2 cells. SCID-hu mice inoculated with HTLV-1-transformed cell lines were not irradiated. Sequential wedge biopsies of thymic implants were obtained at between 4 and 24 weeks postreconstitution, as previously described (2).

RESULTS

HTLV infection of hematopoietic progenitor cells. An enriched human $CD34^+$ cell population was isolated from fetal liver tissue and cocultivated with lethally irradiated SLB-1 cells, an HTLV-1-transformed T-cell line. Cocultivation was used, since efficient HTLV-1 infection requires cell-to-cell contact (6). After 3 to 4 days of cocultivation, cells were coincubated with monoclonal antibodies against CD34 (FITC conjugated) and CD4 (phycoerythrin conjugated). $CD34^+$ cells were separated from $CD4^+$ SLB-1 cells by FACS. Uninfected $CD34^+$ cells were mixed with equal numbers of SLB-1 cells and subjected to FACS to demonstrate the level of contaminating HTLV-transformed cells not removed by FACS. DNA was isolated from separated cell populations and tested for the presence of HTLV sequences by quantitative PCR. Primers specific for HTLV-1 *tax-rex* sequences were used in conjunction with primers specific for the human β-globin gene as an internal control for the number of human cells. Data from six experiments demonstrated that between 10 and >95% of $CD34^+$ cells separated from the coculture were infected with HTLV-1, whereas $CD34^+$ cells separated by FACS from the control mixture showed <2% infection, presumably because of the presence of a small number of contaminating HTLV-transformed donor cells. A representative experiment is presented in Fig. 1A. Cocultivation of hematopoietic progenitor cells with an HTLV-2-transformed T-cell line, 96/pH6neo, also resulted in infection of the cells (Fig. 1B).

To determine whether HTLV-1-infected hematopoietic progenitor cells were permissive for viral gene expression, HTLV-1 Gag protein (p19) expression was analyzed on HTLV-1-infected enriched $CD34^+$ cells separated by FACS by indirect immunofluorescence analysis. In one experiment showing a particularly high level of HTLV-1 infection (>95%) by PCR, we detected p19 Gag by fluorescence microscopy in 13% of the enriched $CD34^+$ cell population (data not shown).

Differentiation of HTLV-1-infected hematopoietic progenitor cells in vitro. Hematopoietic progenitor cells can differentiate in culture and form clonal colonies when provided with appropriate cytokines. To determine whether HTLV-1 infection was maintained after progenitor cells were induced to differentiate in vitro, HTLV-1-infected hematopoietic progenitor cells were isolated by FACS for CD34 expression and cells were cultured in a semisolid medium permissive for propagation of erythroid (BFU-E), myeloid (CFU-GM and CFU-Mac), and primitive progenitor (CFU-GEMM, HPP-CFC, or CFU-A) colony-forming cells. Colonies were identified by morphology at 12 to 14 days postplating and randomly isolated by aspiration, and DNAs from cells were purified. Quantitative PCR analysis indicated that some colonies were infected with HTLV-1, and the ratio of HTLV-1 genomes to the number of human cells, as measured by number of β-globin copies, was 1:1 in each colony, suggesting that all cells within the colony were infected and that each colony arose from a single infected hematopoietic progenitor cell (Fig. 2). Cumulative results of five experiments indicated that 8% (6 infected colonies from 75 myeloid-macrophage colonies assayed), 17% (8 infected colonies from 47 erythroid colonies assayed), and 29% (7 infected colonies from 24 primitive progenitor colonies assayed) were infected with HTLV-1. We ensured that the colonies were not SLB-1 cells, since viable SLB-1 cells have a characteristic morphology when grown in methylcellulose cultures. No SLB-1 colonies were detected in the methylcellulose cultures in these experiments.

Reconstitution of SCID-hu mice with HTLV-1-infected hematopoietic progenitor cells. Hematopoietic colony assays are

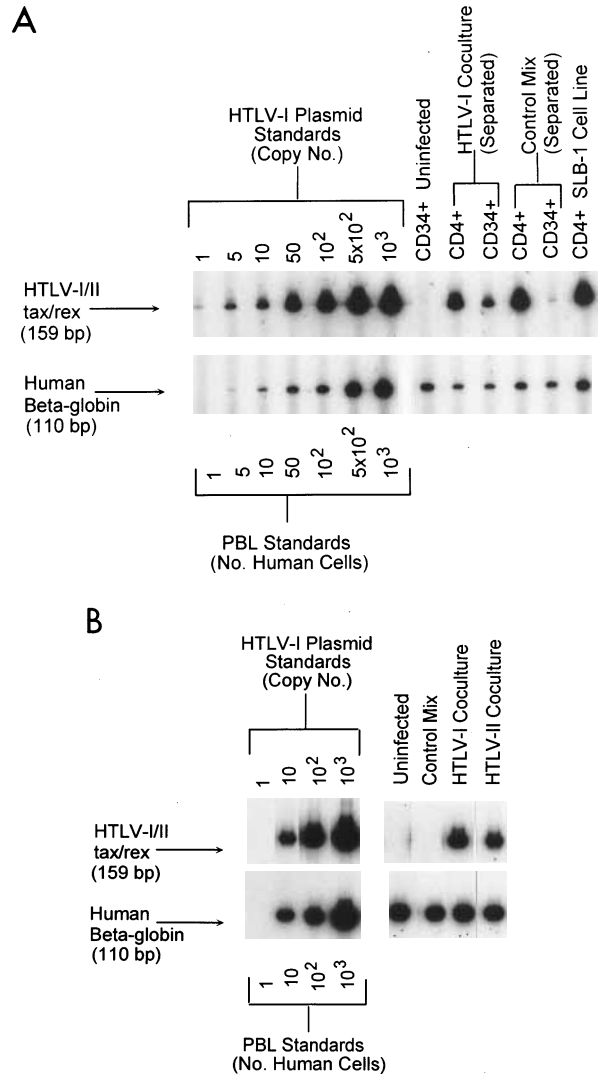

FIG. 1. HTLV infection of an enriched $CD34^+$ cell population. (A) Quantitative PCR analysis of DNA from cells separated by FACS. Purified $CD34^+$ cells (10^6) were infected with HTLV-1 by cocultivation for 3 days with lethally irradiated (10,000 rads) SLB-1 cells (3×10^6) in Iscove's medium supplemented with 20% FCS, 100 ng of IL-3 per ml, 100 ng of IL-6 per ml, and 10 ng of stem cell factor per ml. The T-cell coculture was incubated with human-specific monoclonal antibodies against CD4 and CD34 conjugated with FITC and phycoerythrin, respectively. Live sorting was performed on a Becton Dickinson FACStar^{plus} flow cytometer. DNAs recovered from separated cell samples were assayed by quantitative PCR for the presence of HTLV *tax-rex* (nucleotides 7336 to 7495) and human β-globin sequences, as previously described (11). Amplified HTLV-1 and globin products are 159 and 110 bp, respectively. PCR amplifications of individual cell populations of uninfected $CD34^+$ cells and $CD4^+$ SLB-1 cells separated by FACS are indicated. $CD34^+$ cells separated from $CD4^+$ cells after coculture and from a culture containing uninfected $CD34^+$ and SLB-1 cells mixed immediately prior to FACS (the control mixture) are also presented. Uninfected human peripheral blood lymphocyte (PBL) DNAs and linearized HTLV plasmid DNAs were serially diluted and analyzed in parallel as controls. Additional experiments showed that 20, 25, 10, and >95% of separated $CD34^+$ cells were infected with HTLV-1. $CD34^+$ cells separated from control mixtures showed 1, <1, and <1% HTLV-1 infections in three experiments. (B) HTLV-2 infection of an enriched $CD34^+$ cell population. $CD34^+$ cells were cocultured either with lethally irradiated SLB-1 or with 96/pH6neo cells, an HTLV-2-transformed cell line, under the conditions described in panel A. After 3 days of coculture, $CD34^+$ cells were separated by FACS and DNAs were isolated and analyzed by PCR. Dilutions of uninfected PBL DNA and HTLV-1 DNA were analyzed in parallel as controls.

not capable of assessing lymphoid cell differentiation from hematopoietic progenitor cells. We therefore employed the SCID-hu mouse model to determine whether HTLV-1-infected enriched $CD34^+$ cells were capable of establishing infection in the lymphoid compartment in vivo. Sublethal irradiation of SCID-hu mice results in depletion of the endogenous population of human thymocytes in the Thy/Liv organs. Subsequent inoculation of exogenous enriched $CD34^+$ cells has been shown to result in lymphoid cell repopulation of the thymic graft, including $CD4^+$ and $CD8^+$ T lymphocytes (1, 34). An enriched $CD34^+$ cell population (3×10^6 cells) was cocultivated with SLB-1 cells (6×10^6) for 4 days, and $CD34^+$ cells were fractionated by immunodepletion with a monoclonal antibody against CD4. To assess whether reconstitution with HTLV-1-infected enriched $CD34^+$ cells could establish viral infection in SCID-hu mice, the enriched $CD34^+$ cell population was inoculated directly into the Thy/Liv organs. In the first experiment, five SCID-hu mice were sublethally irradiated (200 rads) and inoculated with enriched $CD34^+$ cells (5×10^5 per mouse) directly into the thymic graft. Implants from mice receiving enriched $CD34^+$ cells infected with HTLV-1 and from two control mice (which received radiation treatment but no exogenous $CD34^+$ cells) were biopsied at 4.5 weeks postreconstitution. DNA was extracted from biopsied tissue and tested for HTLV-1 by quantitative PCR. Four mice that had received HTLV-1-infected enriched $CD34^+$ cells demonstrated viral infection at levels ranging from 5 to 250 copies of HTLV per 10^3 human cells in their Thy/Liv organs, and one mouse, 65-18, displayed high levels of HTLV-1 ($\sim 10^3$ virus copies per 10^3 human cells) (Fig. 3 and Table 1). HTLV-1 sequences were not detected in the two mock-inoculated mice (Table 1).

Flow cytometric analyses of the distribution of CD4-, CD8-, and CD25-bearing cells were carried out on cells from the Thy/Liv implants. The four SCID-hu mice with lower levels of HTLV-1 infection displayed distribution patterns of $CD4^+$ $CD8^+$, $CD4^+$ $CD8^-$, and $CD4^-$ $CD8^+$ T cells that are typical of patterns seen in normal human thymuses and in mock-

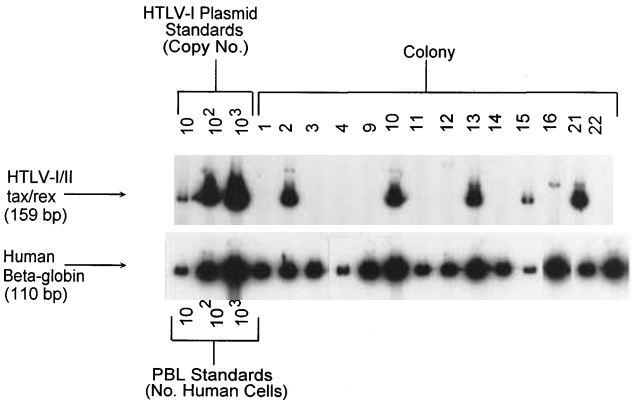

FIG. 2. Analysis of clonogenic colonies established from HTLV-1-infected hematopoietic progenitor cells by quantitative PCR. Clonogenic progenitor assays were performed in MethoCult medium H4433 (Stem Cell Technologies). HTLV-1-infected $CD34^+$ cells (3×10^3) were cultured in 2 ml of MethoCult at 37°C in a humidified atmosphere with 5% CO_2 in air. Clonogenic colonies were identified by morphology at 14 to 16 days postplating and isolated by aspiration from the methylcellulose medium with a pipetman under an inverted microscope, and DNAs were processed for PCR analysis (11). The number of cells in each colony varied and is reflected by the β-globin signal obtained. Colonies infected with HTLV-1 and their identities, as determined by morphology, are as follows: colony numbers 2, 15, and 21, CFU-GM; 10, CFU-A; and 13, HPP-CFC. PBL, peripheral blood lymphocyte.

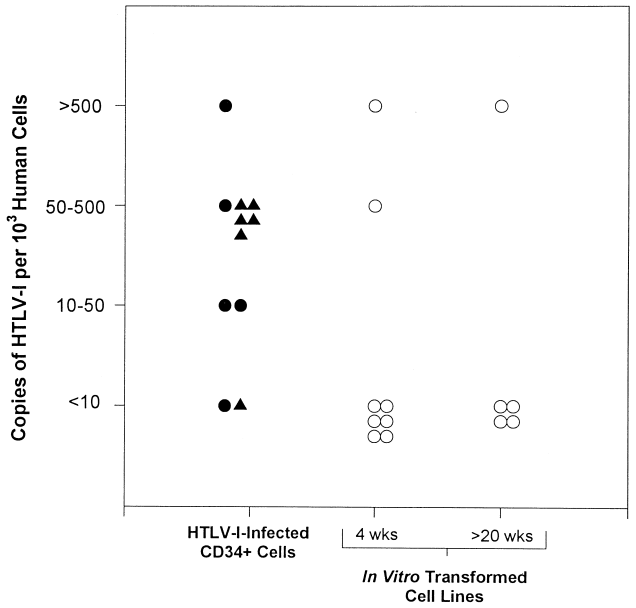

FIG. 3. HTLV-1 copy loads in SCID-hu mice reconstituted with HTLV-1-infected hematopoietic progenitor cells or inoculated with HTLV-1-transformed T-cell lines. HTLV-1-infected hematopoietic progenitor cells were fractionated by immunodepletion, and cells were inoculated into sublethally irradiated SCID-hu mice. Mice represented by filled circles were irradiated with 200 rads and reconstituted with 5×10^5 enriched $CD34^+$ cells, and those represented by triangles received 400 rads and were reconstituted with 2.5×10^5 $CD34^+$ cells. Implants of reconstituted SCID-hu mice were biopsied 4 to 5 weeks postinoculation, and DNAs were extracted from whole tissues (filled circles) or from $CD2^+$ $CD3^+$ lymphocytes separated by FACS (triangle) and assayed by quantitative PCR. Lethally irradiated HTLV-1-transformed cell lines (SLB-1 and MT-2) were injected into two sets of unirradiated SCID-hu mice (1×10^6 to 3×10^6 cells per animal) (unfilled circles). Mice were biopsied at 4 weeks postinoculation from one set and at 20 to 24 weeks postinoculation from the second set, and HTLV-1 proviral burdens were determined by quantitative PCR of DNAs extracted from whole tissues. All virus loads are expressed as number of HTLV genomes per 10^3 human cells, as determined by human β-globin copies.

inoculated SCID-hu mice (Table 1), as previously reported (2, 20). Although sublethal irradiation depletes the endogenous population of human thymocytes, endogenous stem cells can persist in the Thy/Liv organ in some instances, as demonstrated by a mock-inoculated SCID-hu mouse which displayed normal thymocyte distribution patterns (Fig. 4A and Table 1). In contrast, mouse 65-18 displayed an elevated $CD4^+$ $CD8^-$-to-$CD4^-$ $CD8^+$ ratio (Fig. 4A). A majority of $CD4^+$ thymocytes from this mouse also expressed the CD25 antigen, the marker for the IL-2 receptor alpha chain. The phenotype displayed by thymocytes of mouse 65-18 is unique and has not been observed in over 40 other SCID-hu mice reconstituted with uninfected $CD34^+$ cells under similar experimental conditions (data not shown).

Biopsied Thy/Liv tissues from SCID-hu mice contain cells of multiple phenotypes, including hematopoietic progenitor cells. Thus, it could be argued that HTLV-1 sequences detected in biopsied tissues in the first experiment were due to the persistence of exogenous HTLV-1-infected cells. We tested whether HTLV-1 sequences could be detected in differentiated thymocytes from SCID-hu mice reconstituted with HTLV-1-infected hematopoietic progenitor cells. The CD2 marker is one of the first surface antigens expressed on lymphoid progenitor cells that colonize thymuses, after which the CD3–T-cell receptor complex appears (15). Hematopoietic progenitor cells do not express either the CD2 or the CD3 marker. In a second experiment, seven SCID-hu mice were sublethally irradiated at a higher dosage (400 rads) and inoculated with 2.5×10^5 HTLV-1-infected hematopoietic progenitor cells. Mice were biopsied at 5 weeks postreconstitution, and mature thymocytes were separated by FACS. Mature ($CD2^+$ $CD3^+$) thymocyte populations were separated, and quantitative PCR analysis was carried out on extracted DNAs. PCR analysis revealed that mature thymocytes from six of seven mice were infected with HTLV-1 (Fig. 3 and Table 1). Five mice displayed between 50 and 500 copies of the HTLV genome per 10^3 $CD2^+$ $CD3^+$ thymocytes, and one mouse displayed five viral copies per 10^3 cells. PCR analysis on one mouse was inconclusive because of the recovery of an insufficient amount of DNA. These data demonstrate that HTLV-1 infection detected by PCR in reconstituted SCID-hu mice involved the mature thymocyte population and was not due to persistence of the infected input hematopoietic progenitor cells. Flow cytometric analyses of the distribution of the CD4-, CD8-, and CD25-bearing cells of SCID-hu mice in the second experiment did not reveal any mice with aberrant distribution patterns, in comparison with mock-inoculated mice (Table 1).

Inoculation of SCID-hu mice with cell lines transformed in vitro with HTLV-1. We tested whether SCID-hu mice could be infected with HTLV-1 by direct inoculation of cell lines transformed in vitro with HTLV-1 into the Thy/Liv implants. Eight mice were injected with a cell suspension of lethally irradiated (10,000 rads) SLB-1 and MT-2 cells (1×10^6 to 3×10^6 cells per mouse). HTLV-1 sequences were not detected in the Thy/Liv tissues of six mice, while of two other mice, one displayed 60 and the other displayed >500 HTLV-1 copies per 10^3 human cells at 4 weeks postinoculation (Fig. 3). In a second

TABLE 1. Thymocyte subset distribution in individual implants reconstituted with an enriched $CD34^+$ cell population infected with HTLV-1

Expt	Mouse	% of indicated cell population(s) in implant from specified mouse[a]				Virus load[b]
		$CD4^+$	$CD8^+$	$CD4^+$ $CD8^+$	$CD4^+$ $CD25^+$	
1[c]	26-11 (mock)[d]	15	7	77	4	<10
	26-22 (mock)	9	4	87	3	<10
	65-13	18	5	76	8	50–500
	65-14	12	5	83	3	10–50
	65-16	11	4	85	3	10–50
	65-17	12	4	83	3	<10
	65-18	48	8	38	54	>500
2[e]	66-10 (mock)	19	61	2	11	<10
	66-34 (mock)	38	37	23	7	<10
	66-36	44	37	15	5	50–500
	66-38	35	41	23	11	50–500
	66-39	39	36	22	11	ND[f]
	66-40	22	44	32	7	50–500
	66-41	19	10	69	2	<10
	66-42	20	10	69	2	50–500
	66-43	17	2	74	3	50–500

[a] Thymocytes from implants were analyzed for CD4, CD8, and CD25 surface markers.

[b] Copies of HTLV proviral sequences per 10^3 human cells, as determined by quantitative PCR. Proviral burdens were determined on unsorted cells in experiment 1 and on $CD2^+$ $CD3^+$ cells purified by FACS in experiment 2.

[c] Mice received 200 rads prior to reconstitution, and implants were biopsied at 4.5 weeks.

[d] Mock-infected animals were irradiated but did not receive exogenous cells.

[e] Mice received 400 rads prior to reconstitution, and implants were biopsied at 5 weeks.

[f] ND, not determined because of an insufficient amount of DNA.

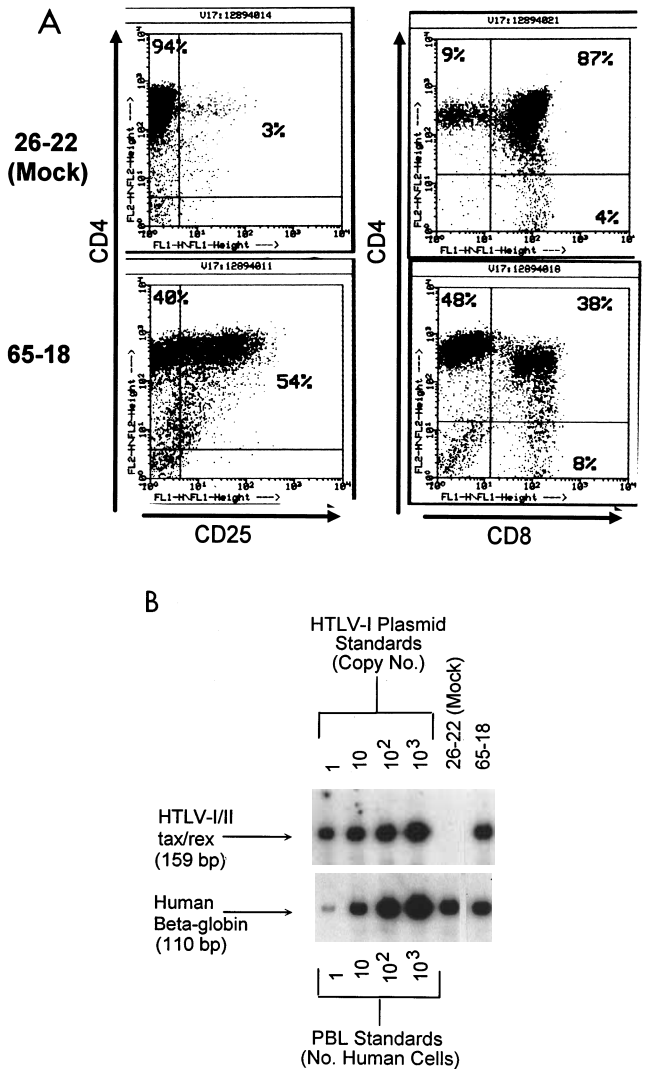

FIG. 4. Analysis of a SCID-hu mouse reconstituted with HTLV-1-infected hematopoietic progenitor cells. (A) Two-color flow cytometric analysis of Thy/Liv cells recovered from wedge biopsies of thymic implants 4.5 weeks postinoculation from a mock-reconstituted SCID-hu mouse receiving no exogenous $CD34^+$ cells or a mouse reconstituted with HTLV-1-infected $CD34^+$ cells (65-18). The left panels illustrate flow cytometric analysis for human CD4 (vertical axis) and CD25 (horizontal axis) antigens, and the right panels illustrate CD4 (vertical axis) and CD8 (horizontal axis) antigens. For the major subpopulations of cells, the percentage of cells in each quadrant is indicated. (B) DNAs from Thy/Liv biopsied tissues from a mock-inoculated mouse and from mouse 65-18 were analyzed by PCR, as described in the legend to Fig. 1. Dilutions of uninfected peripheral blood lymphocyte (PBL) and HTLV-1 DNAs were analyzed in parallel as controls.

experiment, five SCID-hu mice were inoculated with a mixture of lethally irradiated SLB-1 and MT-2 cells (1×10^3 to 3×10^3 cells per mouse) and were analyzed at 20 to 24 weeks postinoculation. Four of five displayed between 1 and 10 HTLV-1 copies per 10^3 cells. Of interest, one mouse from this group demonstrated very high levels of infection (10^3 HTLV-1 copies per 10^3 human cells) (Fig. 3 and Table 2). Flow cytometric analysis of cells recovered from the biopsied implant of this mouse revealed a marked increase in the proportion of $CD8^+$ cells in comparison with mice displaying lower levels of HTLV infection. Furthermore, a majority of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes (57%) from this mouse also displayed increased expression levels of the CD25 antigen. In comparison, only 1 to 10% of the thymocytes from the four mice displaying lower levels of HTLV-1 infection showed expression of the CD25 antigen, similar to patterns seen with uninfected mice.

DISCUSSION

Characterization of the cell types that harbor HTLV in vivo is crucial for understanding the pathogenesis of HTLV-1-associated disease. The establishment of HTLV infection and detection of viral gene expression in hematopoietic progenitor cells, including primitive multipotent progenitor cells, demonstrate that these cells are competent to support productive HTLV-1 and -2 infection and indicate that hematopoietic progenitor cells may be important targets for establishing infection early in life. In a previous clinical study, HTLV-1 sequences could not be detected in hematopoietic $CD34^+$ progenitor cells from the bone marrows of ATL patients (31). However, $CD34^+$ cells from HTLV-1-infected children or from adults at early time points following virus infection, when higher infectious virus loads may be present, were not examined.

The persistence of HTLV-1 in differentiated hematopoietic colonies implies that virus tropism may encompass nonlymphoid cells. Productive infection of primary human macrophages with HTLV-1 has previously been demonstrated to occur ex vivo, and HTLV-1 infection of macrophages, in particular, has been suggested to be involved in the neurological lesions of HAM-TSP patients (8, 19, 22). Infection of hematopoietic progenitor cells by HTLV-1 may be an important means by which T cells as well as other hematopoietic lineages are infected by HTLV-1 in humans.

Infection of neonatal thymuses may be directly linked to the development of ATL. Previous studies have shown that HTLV-1 infection in rabbits (23) or HTLV-1 *tax* expression in transgenic mice (12, 33) can result in the development of thymic abnormalities. For humans, a clinical study of ATL patients noted the occurrence of thymus depletion (13), and a recent report implicated HTLV-1 infection in the development of thymic lymphoma (18). Recent reports have also demonstrated that mature and immature thymocytes can be infected in vitro with HTLV-1 (26, 27) and that infected thymocytes exhibited IL-2-dependent proliferation in vitro. HTLV-1 infection of hematopoietic progenitor cells is an effective route to establishing HTLV-1 infection in SCID-hu mice and may be a mechanism whereby virus infection can spread and localize to the thymus in humans. We cannot formally exclude the possibility that HTLV-1 infection of SCID-hu mice reconstituted with

TABLE 2. Thymocyte subset distribution in implants inoculated with cell lines transformed in vitro with HTLV-1[a]

Mouse	% of indicated cell population(s) in implant from specified mouse				Virus load[b]
	$CD4^+$	$CD8^+$	$CD4^+$ $CD8^+$	$CD4^+$ $CD25^+$	
6-8	36	13	19	2.6	<10
6-16	26	7	20	1.6	<10
8-G1	45	33	13	2.6	<10
9-19	5	38	18	18	>500
13-9	33	28	22	5	<10

[a] Thymocyte subsets were determined as described for Table 1. Mice were inoculated with a mixture of lethally irradiated SLB-1 and MT-2 cells (1×10^6 to 3×10^6 cells per mouse), and implants were biopsied 20 to 24 weeks postinoculation.

[b] Copies of HTLV proviral sequences per 10^3 human cells, as determined on unsorted cells.

HTLV-1-infected hematopoietic progenitor cells occurred after inoculation of cells into mice, because of the presence of contamination with SLB-1 cells in the inoculum. However, the relatively low levels of HTLV-1 infection demonstrated by direct inoculation of SLB-1 and MT-2 cell lines into the Thy/Liv tissues argues against this being a significant route of infection in SCID-hu mice. Future experiments will directly establish whether exogenous HTLV-1-infected $CD34^+$ cells differentiate into the T-lymphoid lineages in vivo.

It is noteworthy that the two mice with the highest levels of HTLV-1 infection displayed perturbation of thymocyte subset distributions; in one case, an increased proportion of $CD8^+$ cells was seen, and in the other, an increased proportion of $CD4^+$ cells was seen. Both had increased levels of IL-2 receptor (CD25) expression, a feature characteristic of HTLV-1-transformed T cells and ATL leukemic T cells. A third animal with a high level of HTLV (Fig. 3) could not be analyzed for cell phenotype. This result suggests that the development of transforming events in the progression to ATL may result from increased viral replication occurring in lymphoid tissues such as the thymus. Similar observations have been made for human immunodeficiency virus type 1, for which high levels of viral replication are observed in lymph nodes throughout the course of the disease, ultimately leading to destruction of the lymph node architecture (9). We and others previously reported that human immunodeficiency virus type 1 infection of the Thy/Liv implants in SCID-hu mice results in pathology characterized by depletion of CD4-bearing cells (2, 3, 20, 41). In the case of HTLV, a transforming noncytopathic virus, increased viral replication may lead to the selection of clones of T cells with an increased proliferative advantage which, in combination with other genetic changes, may ultimately lead to development of ATL. Efficient infection of thymocytes and the disregulation of thymopoiesis observed in some SCID-hu mice displaying high levels of HTLV-1 infection implicate the human thymus as a reservoir for HTLV infection and suggest that lymphoproliferation and the disregulation of thymopoiesis may be predisposing events in the development of leukemia. The SCID-hu mouse provides an in vivo model in which to further characterize virus tropism and pathogenesis associated with HTLV infection.

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