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Time Course and Cytokine Dependence of Human T-Cell Lymphotropic Virus Type 1 T-Lymphocyte Transformation as Revealed by a Microtiter Infectivity Assay

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Human T-cell lymphotropic virus type 1 (HTLV-1) enhances the growth of T lymphocytes, allowing the generation of T-lymphocyte cell lines. This report describes a limiting-dilution assay system which uses low input numbers of HTLV-1-producing cells for generation of T-lymphocyte cultures. The HTLV-1 transformants generated with this assay system produced high levels of HTLV-1 p24 antigen and required exogenous cytokines for maintenance. Clonal populations of CD4- or CD8-positive HTLV-1 transformants were generated with transformation efficiency rates as high as 78%. An exogenous cytokine is necessary for HTLV-1 T-lymphocyte transformation, and cytokine dependence is the most likely outcome of infection and transformation. HTLV-1 T-lymphocyte transformation can occur in the presence of cytokines other than interleukin-2 (IL-2), such as IL-4 or IL-7. IL-4- or IL-7-dependent HTLV-1 transformants underwent T-lymphocyte mitogenesis in response to their homologous cytokines but proliferated best in the presence of IL-2. Since the receptors for IL-2, IL-4, and IL-7 share the IL-2 gamma chain, this component may be the common element in the signaling pathway for HTLV-1-associated transformation.

Human T-cell lymphotropic virus type 1 (HTLV-1), the etiologic agent of adult T-cell leukemia (ATL) (6, 12, 13, 22, 34) and HTLV-1-associated myelopathy or tropical spastic paraparesis (9, 21), causes T-lymphocyte transformation in vitro (4, 18, 30, 33). Classically, cytokine-independent T-lymphocyte mitogenesis has often been used as the hallmark for identifying the HTLV-1-transformed phenotype (2). T-lymphocytes transformed by HTLV-1 undergo DNA synthesis and mitogenesis for years without the need for mitogenic or antigenic stimulation (4, 18, 30). In some HTLV-1 transformants, DNA synthesis and mitogenesis occur in the absence of interleukin-2 (IL-2) (2). In contrast, nontransformed T-lymphocytes require periodic antigenic or mitogenic stimulation and cease to undergo DNA synthesis after a finite number of cell divisions because of decreased synthesis of IL-2 and downregulation of its receptor (IL-2R) (1, 3, 11). The mechanisms underlying the altered regulation of DNA synthesis and mitogenesis in HTLV-1-transformed T lymphocytes remain unclear. No typical oncogene has been identified within the genome of HTLV-1, and no preferred proviral integration site consistent with a promoter insertion model has been identified (27, 28).

Transmission of HTLV-1 is largely cell associated (2, 30). Standard methods for generating HTLV-1-transformed lymphocytes in vitro involve cocultivation of large numbers of activated T lymphocytes with inactivated HTLV-1-infected cells (2, 5, 16, 23). Bulk culture techniques for transforming T lymphocytes with HTLV-1 have several shortcomings. The time course and the full phenotypic potential of HTLV-1 T-lymphocyte transformation are masked, and the generation of multiple HTLV-1 transformants is limited. In addition, early identification of newly infected cells by production of viral core antigen in the culture supernatant is difficult because of residual p24 antigen from the large inoculum of HTLV-1-infected cells used as a source of infecting virus. Sugamura et al. (31) described a microplate method for HTLV transformation of normal T cells in which limiting numbers of peripheral blood lymphocytes were cocultivated with a fixed number of HTLV-infected cells. A high T-cell transformation efficiency was apparent in this system, and multiple transformants could be established; however, the high ratio of infected to normal T cells (20:1) precludes characterization of viral transformation, as outlined above. To better study the time course, efficiency, cytokine requirements, and cellular range of HTLV-1 T-lymphocyte transformation, a microtiter plate assay for transforming human T lymphocytes in vitro with low input numbers of HTLV-1-infected cells is described. With this transformation assay, the enhanced growth potential, the phenotypic range of HTLV-1 infectivity, and the cytokine responses of HTLV-1 transformants are readily apparent. Early identification of HTLV-1 transformants is possible, and multiple HTLV-1 transformants can be reproducibly generated. In addition, HTLV-1 T-lymphocyte transformation can also be accomplished in the presence of cytokines other than IL-2 that share the IL-2R gamma chain (γ_c), namely, IL-4 and IL-7.

MATERIALS AND METHODS

Viruses and cells. All cells were grown in RPMI 1640 medium (Biowhittaker, Walkersville, Md.) supplemented with heat-inactivated 20% fetal calf serum (Gibco Laboratories, Grand Island, N.Y.), glutamine (2 mM), and antibiotics at 37°C in a 5% CO_2 atmosphere. HTLV-1-producing cell lines MT2 and C91/PL (kindly provided by Robert Gallo, National Cancer Institute, Bethesda, Md.) were used as sources of HTLV-1. The cell lines were passaged biweekly at a 1:5 dilution in growth medium.

Human peripheral blood mononuclear cells (PBMC) were collected from healthy HTLV-1-seronegative donors and obtained after Ficoll-Hypaque centrifugation from heparinized whole blood. The PBMC were resuspended in growth medium, plated at 2×10^6 per well in a 24-well plate, and activated with either 2 μg of purified phytohemagglutinin (Burroughs Wellcome, Research Triangle Park, N.C.) per ml or 1 ng of phorbol-12-myristate-13-acetate (Calbio-

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chem, San Diego, Calif.) per ml–0.5 μM ionomycin (Sigma, St. Louis, Mo.) for 36 h. After stimulation, the cells were washed in Hanks balanced salt solution (GIBCO) and resuspended in growth medium at 10^5/ml with human recombinant IL-2 (rIL-2; 20 U/ml), rIL-4 (10 U/ml; Boehringer Mannheim Corp., Indianapolis, Ind.), or rIL-7 (20 ng/ml; Genzyme Diagnostics, Cambridge, Mass.). Ten thousand activated PBMC in medium containing a cytokine (0.1 ml) were plated per well in multiple wells of a 96-well round-bottom microtiter plate.

C91/PL or MT-2 cells were resuspended in medium, placed on ice, and then exposed to 11,700 rads from a gamma radiation source (390 rads/min for 30 min). The irradiated cells were then washed in Hanks balanced salt solution and resuspended in growth medium at various cell concentrations. Various concentrations of irradiated C91/PL or MT-2 cells (10, 100, and 1,000 cells per well) were cocultured with the 10^4 activated PBMC in the presence of the various cytokines. The cells were split weekly at 1:4; the cells were resuspended in the 96-well plate with an eight-channel micropipettor, and 50 μl of the cell-medium mixture was transferred to a fresh 96-well plate containing 150 μl of the respective cytokine-containing medium. Final concentrations of cytokines were as follows: IL-2, 10 U/ml; IL-4, 5 U/ml; IL-7, 10 ng/ml. Control wells containing 10^4 activated PBMC in the various cytokine-containing media and no HTLV-1-producing cells were prepared to monitor the loss of the initial T-cell activation. In addition, control wells containing irradiated C91/PL or MT-2 cells (10^4) and no activated PBMC were prepared to document the killing of C91/PL or MT-2 cells by the irradiation process and to monitor the disappearance of the input p24 antigen. Transformed cell lines were identified as cells that continued to proliferate beyond 6 weeks, the point at which most of the cells not exposed to the HTLV-1-producing cells no longer proliferate in medium containing IL-2, IL-4, or IL-7, and also by the continued production of p24 antigen (enzyme-linked immunosorbent assay; Coulter Immunology, Hialeah, Fla.) in the culture supernatant (>1,000 pg/ml). After 9 weeks of coculture, all cultures (each well) were transferred to individual 15-ml Corning culture tubes containing 0.5 ml of the appropriate cytokine-containing medium. Cultures were split weekly at 1:2 in cytokine-containing medium in the culture tube and transferred to T-25 flasks when the medium turned acidic and the total volume of the cell-medium mixture was approximately 1.5 ml. Cells exposed to HTLV-1 that continued to proliferate in the presence of medium containing a cytokine alone and that continued to produce p24 antigen in the culture supernatant were identified as HTLV-1 transformed. Efficiency of transformation was calculated on the basis of the percentage of the total cultures in the various cytokines that continued to proliferate in medium containing a cytokine and continued to produce p24 antigen at 12 weeks after the initial coculture.

Flow cytometry. The cultures were analyzed by one- or two-color fluorescence in a FACSTAR PLUS cell sorter (Becton-Dickinson, Mountain View, Calif.) for T- and B-cell surface marker expression with monoclonal antibodies to CD2, CD3, CD4, CD8, B1, and B4 (Becton-Dickinson) and the gamma-delta T-cell receptor (TCR) (δ1; T Cell Diagnostics, Cambridge, Mass.).

HLA typing. HLA-DR antigen expression was analyzed (Pablo Rubenstein, New York Blood Center) with a microcytotoxicity assay.

Proliferation assays. Proliferative responses to IL-2, IL-4, and IL-7 were tested by ^{3}H incorporation. The cells were removed from medium containing the cytokine 2 days before the experiment. On the day of the experiment, the cells were washed twice in Hanks balanced salt solution and resuspended in RPMI 1640–20% fetal calf serum. Twenty thousand cells were seeded in triplicate wells in a 96-well round-bottom plate. Growth medium (RPMI 1640, 20% fetal calf serum) or medium containing various concentrations of rIL-2, rIL-4, or rIL-7 was added to the wells. The cells were then incubated at 37°C for 48 to 72 h, and [^{3}H]thymidine (1 μCi per well) was added to the culture for the final 4 h of incubation. The cells were harvested, and the radioactivity was counted with a scintillation counter.

TCR rearrangement studies. TCR rearrangement studies were performed with selected lymphocyte cultures by using Southern blot analysis and a ^{32}P-labelled 0.42-kb fragment of the constant region of the beta chain (CTβ; Oncor, Gaithersburg, Md.) of the TCR complex. DNA was purified from the lymphocyte cultures by using a nonorganic DNA extraction method (Oncor). Ten micrograms of DNA per sample was digested with restriction enzyme *Bam*HI or *Hin*dIII and fractionated in a 0.7% agarose gel. Following transfer (NEAT System; Oncor) to a nylon membrane, the membrane was cross-linked in a Stratalinker 2400 (Stratagene, La Jolla, Calif.) in the auto cross-link mode (254 nm, 120,000 μJ/cm^2). Hybridization was conducted overnight at 45°C in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% (wt/vol) dextran sulfate–1% (vol/vol) sodium dodecyl sulfate and other blocking solutions (Hybridization Solution I; Oncor). The blots were washed twice in 0.1× SSC–0.1% sodium dodecyl sulfate at room temperature for 15 min and once at 52°C for 1 h in the same buffer. The membranes were exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) for 1 to 14 days at −70°C.

RESULTS

Generation of HTLV-transformed cell lines. To generate HTLV-1 transformants, 10^4 stimulated PBMC were cocultivated with various numbers (10 to 1,000) of irradiated HTLV-1$^+$ cells in the presence of 10 U of IL-2 per ml. Wells which exhibited growth were expanded in IL-2-containing medium and tested for production of p24 antigen in the culture supernatant. HTLV-1 transformants were defined as cultures exposed to HTLV-1 that continued to proliferate in the presence of IL-2 without the need for mitogenic stimulation and that continuously produced p24 antigen (>1,000 pg/ml) for 12 weeks after the initial coculture. Control wells, which contained 10^4 activated PBMC in the absence of HTLV-infected cells, showed a limited amount of IL-2-dependent proliferation. However, the cells in these wells rarely proliferated for more than 4 to 6 weeks and never exhibited p24 production. Thus, all of the T-lymphocyte immortalization in this assay was associated with HTLV-induced transformation.

TABLE 1. HTLV-1 transformation efficiency and reproducibility

Expt	Transformation efficiency[a] with C91/PL input cell no. of:		
	10	100	1,000
A	2/20 (10)	7/20 (35)	20/20 (100)
B	6/30 (20)	15/30 (50)	22/30 (73)
C	3/20 (15)	9/20 (45)	19/20 (95)
D	0/10 (0)	2/10 (20)	7/10 (70)
Total	11/80 (15)	33/80 (41)	68/80 (85)

[a] Results are expressed as number of antigen-positive (p24 concentration, >1,000 pg/ml) cultures/total number of wells seeded that could be established as cell lines 9 to 16 weeks after cocultivation with different numbers (10, 100, and 1,000) of irradiated C91/PL cells. Percentages of antigen-positive cultures are in parentheses.

Control wells which contained only HTLV-1$^+$ irradiated cells continued to produce demonstrable quantities of viral core antigen in the supernatant for several weeks following irradiation. Antigen persistence was dependent on the number of input cells. Control wells with 10 input cells were p24 negative by 2 weeks postirradiation; wells with 100 or 1,000 cells were persistently p24 antigen positive for several weeks longer but were generally antigen negative by 6 weeks postirradiation. Therefore, even though these cultures appeared to be senescent by light microscopy and to contain primarily cellular debris, these cultures were maintained until the disappearance of p24. Outgrowth of an irradiated cell line was never observed in this system.

Efficiency and reproducibility of HTLV-1 T-lymphocyte transformation. To quantify HTLV-induced T-lymphocyte transformation in vitro, limiting numbers (0 to 1,000) of irradiated C91/PL cells were cocultivated with a fixed number (10,000) of activated PBMC in the presence of IL-2 (10 U/ml). The results of four different experiments, each using C91/PL cells as a source of virus and a different donor source of PBMC, are summarized in Table 1. The proportion of lymphocyte cultures that continued to proliferate 9 to 16 weeks after cocultivation depended on the input number of HTLV-producing cells: with 10 input cells, an overall transformation efficiency of 15% was noted; with 100 and 1,000 cells, transformation efficiency increased to 41 and 85%, respectively. This dose-dependent effect of the input number of HTLV-1-producing cells on the efficiency of HTLV-1 T-lymphocyte transformation was highly reproducible. By linear regression analysis, the log input number of C91/PL cells correlated with the efficiency of transformation with an R value of 0.91 and P value of <0.0001. The efficiency of HTLV-1 T-lymphocyte transformation was also dependent on the input number of activated PBMC. In a representative experiment, varying the numbers of phorbol-12-myristate-13-acetate–ionomycin-activated PBMC (0, 1, 10, 100, 1,000, and 10,000) in the presence

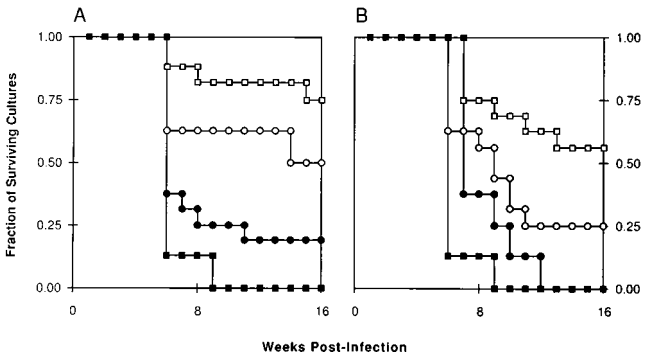

FIG. 1. Kaplan-Meir plots of HTLV-1-associated T-lymphocyte proliferation. Ten thousand activated PBMC were exposed to different input numbers (0 [■], 10 [●], 100 [○], and 1,000 [□]) of irradiated C91/PL (A) or MT-2 (B) cells, and the percentages of surviving cocultivations (i.e., transformants) were plotted as a function of time (weeks). C91/PL or MT-2 cells were irradiated, and cultures were examined and medium was added or changed biweekly as described in the text.

of 10^4 C91/PL cells transformed 0, 0, 13, 25, 38, and 63% of the wells tested, respectively. Thus, HTLV-1 transformants were noted with as few as 10 PBMC per well.

Time course of HTLV-1 T-lymphocyte transformation. To study the time course of HTLV-1-mediated T-cell transformation, limiting numbers (0 to 1,000) of irradiated, HTLV-1-infected C91/PL or MT-2 cells were cocultivated with a fixed number (10,000) of activated PBMC in the presence of IL-2. As shown in Fig. 1, a Kaplan-Meir plot of HTLV-1-enhanced T-lymphocyte proliferation, the proportion of lymphocyte cultures that continued to proliferate in IL-2-containing growth medium 9 to 16 weeks after cocultivation with various numbers of C91/PL or MT-2 cells depended on the input number of HTLV-1-producing cells and on the type of HTLV-1-producing cell used. Six weeks after coculture, the cells in most of the control wells containing only phytohemagglutinin-activated T lymphocytes were no longer proliferating; by 9 weeks, none remained. All of the C91/PL transformants remaining after 9 weeks were stable and continued to proliferate for several months. The MT-2 transformants were initially not as stable, and some transformants could not be expanded. However, all of the transformants established by 14 weeks continued to proliferate.

In addition, the efficiency of HTLV-1 T-lymphocyte transformation also depended on the type of HTLV-1-producing cell used. As shown in Fig. 1, at any cell concentration, C91/PL cells more efficient than MT-2 cells at inducing HTLV-1 T-lymphocyte transformation. Sixteen weeks after coculture with 10, 100, and 1,000 C91/PL or MT-2 cells, 20, 48, and 78% and 0, 22, and 58%, respectively, of the lymphocyte cultures continued to proliferate in the presence of IL-2-containing medium.

HTLV-1 transformants are therefore readily identifiable at 9 weeks after coculture by their sustained ability to proliferate in response to exogenous IL-2 and by the continued production of viral core antigen in the culture supernatant. HTLV-1 T-lymphocyte transformation can be accomplished with very low input numbers (10) of HTLV-1-infected cells or activated PBMC.

Effect of phorbol esters on transformation. Treatment of HTLV-1-infected T-cell lines with 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester) has been shown to stimulate virus release (7, 32). To assess whether treatment of C91/PL cells with phorbol ester would result in enhanced rates of HTLV-1 T-lymphocyte transformation, C91/PL cells were treated with phorbol ester for 36 h before irradiation and coculture. With 10, 100, or 1,000 C91/PL cells cocultured with a fixed input number of activated PBMC in the presence of IL-2-containing medium, treatment of the C91/PL cells with phorbol ester had no effect on the efficiency of HTLV-1 T-lymphocyte transformation. In contrast, treatment of the PBMC with phorbol ester or phorbol-12-myristate-13-acetate before coculture with C91/PL resulted in a marked increase in HTLV-1 T-lymphocyte transformation. In the absence of treatment of the PBMC with phorbol ester, less than 25% of the lymphocyte cultures cocultured with 1,000 C91/PL cells in the presence of IL-2 were transformed, compared with a maximum of 70% of the cultures cocultured with PBMC treated with phorbol ester. The facts that actively dividing cells are more susceptible to transformation by HTLV-1 (16) and that transformation has been found only in HTLV-1-positive cultures with this transformation assay support the notion of a role for viral integration in the process of transformation.

TABLE 2. Cell surface phenotypes of HTLV-1 transformants

Phenotype[a]	No. of cultures with phenotype at C91/PL input cell no. of:			Total no. (%) of cultures with phenotype
	10	100	1,000	
$CD2^+$ or $CD3^+$	9[b]	14[c]	21[d]	44
CD4	5	6	10	21 (48)
CD8	2	2	1	5 (11)
Mixed	2	6	10	18 (41)

[a] Cell lines were designated $CD4^+$ or $CD8^+$ if >95% of the cells were positive for the subtype by flow cytometric analysis as described in the text. All HTLV-1-positive transformants in these experiments were B1 negative.
[b] From 11 positive cultures.
[c] From 33 positive cultures.
[d] From 68 positive cultures.

Phenotypes of HTLV-1 transformants. The profile of the HTLV-1 transformants was characterized by cell surface marker analysis. Table 2 summarizes the cell surface phenotypes of 44 HTLV-1-transformed lymphocyte cultures generated in the presence of various numbers of C91/PL cells. The HTLV-1-transformed lymphocytes were positive for T-lymphocyte markers CD2 and/or CD3, and none expressed the B1 cell surface antigen. Fifty-nine percent of the HTLV-1 transformants generated consisted of pure populations of $CD4^+$ or $CD8^+$ T lymphocytes: 48% (21 of 44) of the HTLV-1-transformed cultures were predominantly $CD4^+$ (>95% $CD4^+$ cells), 11% were predominantly $CD8^+$ (>95% $CD8^+$ cells), and the remaining 31% consisted of mixed populations of $CD4^+$ and $CD8^+$ cells. As expected $CD4^+$ HTLV-1 transformants were more likely (24); however, $CD8^+$ HTLV-1 transformants were also readily established with this transformation assay. Pure CD4- or CD8-positive cell cultures were more likely to be generated at the lower input concentrations of C91/PL cells, and mixed phenotypes were more likely at the higher input concentrations of HTLV-1-infected cells.

Clonality and haplotype of HTLV-1 transformants. The mixed phenotypes observed at the higher HTLV-1 input and the pure phenotypes observed at the lower HTLV-1 input suggest that the cell cultures established at the lower HTLV-1 input are clonal. Clonality of T-cell populations can be determined by investigating rearrangement of the TCR beta chain gene (TCRβ) (17, 29). Southern blots of clonal populations of T cells, when probed with a TCRβ probe, demonstrate one or two unique, non-germ line restriction length fragments, corre-

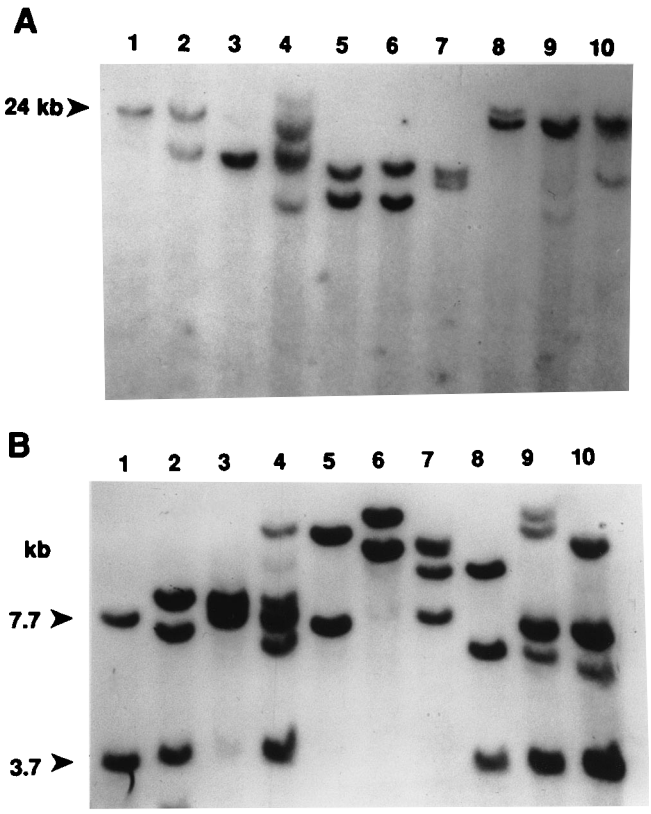

FIG. 2. TCR rearrangement as a measure of clonality. DNAs from selected HTLV-1 transformants were digested with *Bam*HI (A) and *Hin*dIII (B), subjected to Southern blotting as described in the text, and hybridized with a probe to the constant region of the β chain of the TCR (CTβ). The germ line control (lane 1) consisted of DNA from normal peripheral blood lymphocytes and gave the predicted bands of 24 kb with the *Bam*HI digest and 3.7 and 7.7 kb with the *Hin*dIII digest. Lanes: 2, C91/PL; 3, 4, 6, 8, 9, and 10, from cocultivation assay with 10^3 irradiated C91/PL cells per well; 5 and 7, from cocultivation with 10 C91/PL cells. As determined by cell surface analysis, lanes 2, 3, 6, and 8 are $CD4^+$, lane 10 is $CD8^+$, and lanes 4 and 9 are mixed populations (lane 4 is 20% $CD4^+$, 69% $CD8^+$, and lane 9 is 67% $CD4^+$, 36% $CD8^+$. All of the samples, with the exception of those in lanes 4 and 9, were identified as clonal populations by this analysis.

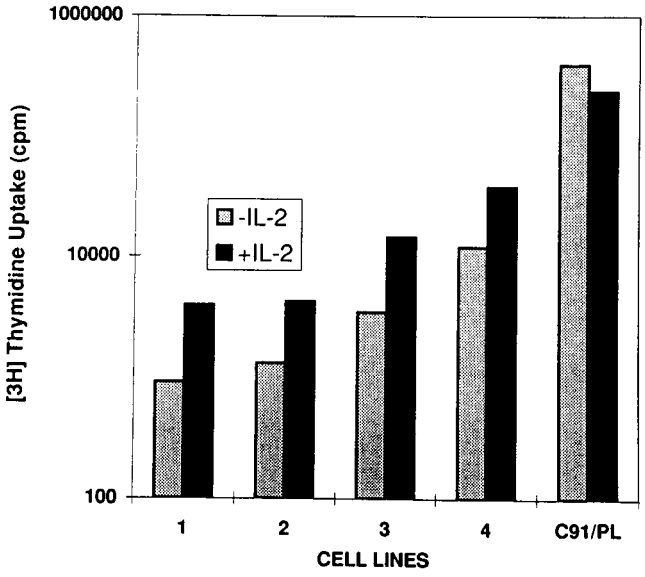

FIG. 3. Proliferative responses to IL-2 of four newly established HTLV-1-infected cell lines (1 to 4) 10 weeks after coculture with C91/PL cells and proliferation of C91/PL cells in the absence or presence of IL-2. Assays were done as described in Materials and Methods, and results are expressed as ^3H incorporation on a semilogarithmic scale.

sponding to TCRβ rearrangement of a dominant clone. Oligoclonal T-cell populations exhibit a number of different non-germ line bands, and only germ line fragments are apparent in polyclonal populations. A total of 18 HTLV-1 transformants were analyzed for TCRβ rearrangement; a representative blot is shown in Fig. 2. As expected, all cultures were confirmed to be T cells by virtue of TCRβ rearrangement. Notably, the clonal pattern demonstrated by C91/PL (Fig. 2, lanes 2) was distinct from that of the other transformants analyzed, indicating that the cultures generated in the infectivity assay were indeed de novo transformants and not the result of outgrowth due to insufficient irradiation of the infecting cell. Transformants which consisted of mixed populations as determined by flow cytometry (lanes 4 and 9) could be classified as oligoclonal populations by this analysis. While most of the transformants which were >95% $CD4^+$ or $CD8^+$ by flow cytometric analysis were confirmed to be clonal by TCRβ rearrangement, four samples were not. These probably represent parallel transformants of like subsets of lymphocytes, e.g., transformation of two (or more) different $CD4^+$ (or $CD8^+$) populations.

In addition, by HLA-DR antigen expression, lymphocyte cultures derived from four different individuals differed from C91/PL cells and were therefore not an outgrowth of the irradiated C91/PL cells.

Cytokine dependence of HTLV-1 T-lymphocyte transformation. To determine whether exogenous IL-2 is essential for HTLV-1 T-lymphocyte transformation, 1,000 activated PBMC were cocultured with 10 to 1,000 C91/PL cells in the absence or presence of exogenous IL-2. In the absence of IL-2, activated T lymphocytes exposed to HTLV-1 ceased to proliferate after 3 weeks of coculture and no HTLV-1 transformants were generated. In contrast, HTLV-1 transformants were readily generated in the presence of IL-2. The exogenous cytokine is therefore essential for HTLV-1 T-lymphocyte transformation.

To confirm that newly established (8 to 16 weeks after coculture) HTLV-1-transformed cultures were undergoing IL-2-mediated DNA mitogenesis, the proliferative responses to IL-2 of four cell lines (1 to 4) generated in the presence of IL-2 were compared with that of C91/PL cells. As shown in Fig. 3, the four newly established lymphocyte cultures tested proliferated in response to IL-2. C91/PL cells, in contrast, did not show enhanced proliferation in response to IL-2, and the level of C91/PL cell proliferation observed was much higher than that of the newly established HTLV-1 transformants. Initially, all of the lymphocyte cultures generated with this microtiter assay are dependent on exogenous cytokines for continued cellular proliferation, and most remained so for their long-term maintenance. A small percentage of the lymphocyte cultures could be propagated in culture in the absence of IL-2 for a few months; however, these were short-lived. Our results suggest that IL-2 dependence is the more likely outcome of HTLV-1 T-lymphocyte transformation and is an early general event. IL-2 independence is less likely and appears to be a late phenomenon that follows a period of IL-2 dependence.

IL-4 and IL-7 mediate HTLV T-lymphocyte transformation. To further explore the ability of cytokines other than IL-2 to support HTLV-1 T-lymphocyte transformation, exogenous IL-4 or IL-7 was substituted for IL-2 at the time of coculture. Table 3 summarizes the percentages of lymphocyte cultures

TABLE 3. Efficiency of transformation with IL-2, IL-4, or IL-7

No. of C91/PL cells	% of cultures[a] with growth in:		
	IL-2	IL-4	IL-7
20	55	0	0
200	90	5	5
2,000	100	15	5

[a] Data are expressed as percentages of 20 HTLV-1-infected lymphocyte cultures that could be established as cell lines 12 weeks after coculture with 20, 200, or 2,000 C91/PL cells in the presence of IL-2 (10 U/ml), IL-4 (10 U/ml) or IL-7 (10 ng/ml).

transformed in the presence of IL-2, IL-4, or IL-7 with various numbers of irradiated C91/PL cells. IL-2 was much more efficient at generating HTLV-1 transformants than either IL-4 or IL-7. For example, at low input numbers of C91/PL cells (20 per well), no transformants were established in the presence of IL-4 or IL-7, compared with 55% of the lymphocyte cultures grown in the presence of IL-2. Similarly, with higher numbers of C91/PL cells (200 and 2,000), 90 and 100% of the lymphocyte cultures were transformed in the presence of IL-2, respectively. However, in the presence of IL-4, only 5% of the cultures were transformed at 200 C91/PL cells per well and 15% were transformed at 2,000 cells per well, and in the presence of IL-7, 5% of the cultures were transformed at 200 and at 2,000 C91/PL cells per well. The IL-4- and IL-7-dependent HTLV-1 transformants were also productively infected with HTLV-1, as evidenced by the continued production of p24 antigen in the culture supernatant. Again, virus-nonproductive transformants were not generated. Despite the relatively low rate of HTLV-1 transformation in the presence of cytokines other than IL-2, both IL-4- and IL-7-dependent transformants could be propagated in long-term culture in the presence of their respective cytokines. The IL-4- and IL-7-dependent HTLV-1 transformants clearly depend on their respective cytokines for cytokine-mediated mitogenesis, as evidenced by the failure of cell growth when the cytokines were excluded from the growth media.

Phenotypically, all of the IL-4-dependent HTLV-1 transformants were positive for CD2, and the majority were $CD4^+$ $CD8^-$. A small proportion of $CD2^+$ transformants expressed neither CD4 nor CD8 cell surface markers and were also negative for the B1 cell surface marker and for the gamma-delta TCR. The IL-7-dependent transformants consisted of pure populations of $CD2^+$ $CD4^+$ cells. No $CD8^+$ transformants were noted in the presence of IL-7.

Proliferative responses of the various HTLV-1 transformants to IL-2, IL-4, and IL-7. To further test the ability of these HTLV-1 transformants to undergo DNA synthesis and mitogenesis in response to the cytokines IL-2, IL-4, and IL-7, the proliferative responses of the various HTLV-1 transformants grown in the presence of IL-2, IL-4, or IL-7 were tested. As shown in Fig. 4, all of the HTLV-1 transformants tested with IL-2, IL-4, and IL-7 had low levels of cellular proliferation in the absence of a cytokine (medium only) and exhibited cytokine-dependent T-lymphocyte mitogenesis in response to the two concentrations of the respective cytokines tested. IL-4-dependent HTLV-1 transformants and IL-7-dependent HTLV-1 transformants were therefore generated by using this assay. All of the HTLV-1 transformants proliferated vigorously in response to IL-2. The IL-4-dependent HTLV-1 transformants, in addition to proliferating in response to IL-2 and IL-4, also proliferated in response to IL-7. Similarly, the IL-7-dependent HTLV-1 transformants, in addition to proliferating in response to IL-7 and IL-2, also proliferated in response to IL-4. Also, for the IL-4- and IL-7-dependent HTLV-1 transformants, the proliferative responses to IL-2 were much greater than those to their respective cytokines. IL-2-dependent HTLV-1 transformants, on the other hand, proliferated only in response to IL-2 and showed no proliferative responses to IL-4 or IL-7. Clearly, HTLV-1 T-lymphocyte transformation can occur in the presence of cytokines other than IL-2.

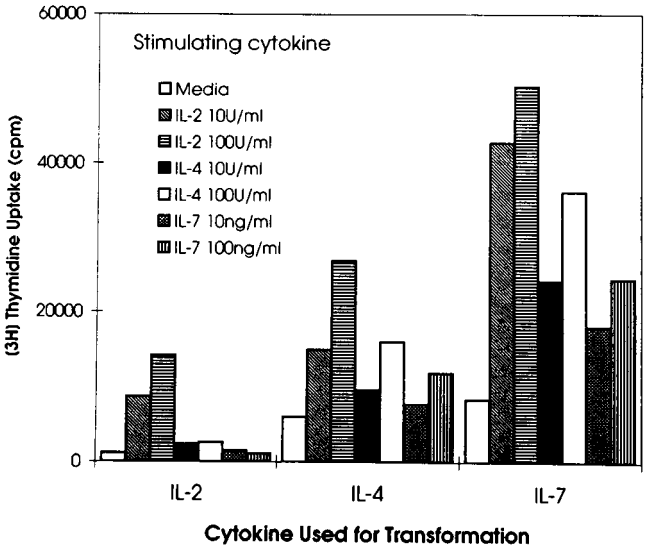

FIG. 4. Proliferative responses of three different HTLV-1-infected lymphocyte cultures to two different concentrations of IL-2 (10 and 100 U/ml), IL-4 (10 and 100 U/ml), or IL-7 (10 and 100 ng/ml). Transformed lymphocytes were recovered from cocultivation of C91/PL cells, as described in the text, in the presence of IL-2 (10 U/ml), IL-4 (10 U/ml), or IL-7 (10 ng/ml). Cultures were used within 15 to 20 weeks after cocultivation.

DISCUSSION

In this report, a reproducible method for transforming human T-lymphocytes in vitro with limiting numbers of HTLV-1-producing cells is described. The present studies are the first quantitative investigations of the in vitro transformation of T lymphocytes by HTLV-1. The limiting-dilution T-lymphocyte transformation studies were very reproducible and revealed consistent transformation of $CD4^+$ and $CD8^+$ T lymphocytes but only in the presence of exogenous cytokines. On the basis of the results presented here, HTLV-1 T-lymphocyte transformation may be defined as unlimited cytokine-driven T-lymphocyte mitogenesis in T lymphocytes productively infected with HTLV-1.

Past definitions of HTLV-1 T-lymphocyte transformation have implied IL-2 independence (2); however, several lines of evidence suggest that such a definition of HTLV-1 T-lymphocyte transformation is overly restrictive. First, in the absence of exogenous IL-2 or the two other cytokines studied (IL-4 and IL-7), HTLV-1 T-lymphocyte transformation was not observed. As we have shown, cytokine-dependent T-lymphocyte proliferation is the more likely outcome of HTLV-1 transformation in vitro and mimics the cytokine requirements of cells obtained from patients with acute and chronic ATL, which also require IL-2 for continued maintenance in culture (15). IL-2 independence may therefore represent a late event that occurs after a prolonged period of cytokine dependence. Second, cytokine independence has been equated with HTLV-1 T-lymphocyte transformation in a number of systems (2). In our

system, the increased growth potential and the duration of cellular proliferation in response to IL-2 of T lymphocytes exposed to HTLV-1 qualitatively exceeded the proliferative potential of T lymphocytes exposed to nonspecific T-cell mitogens. Cytokine independence should therefore not be used as a criterion for the transformation of T cells by HTLV-1. Furthermore, the ability to recover HTLV-1 transformants that are dependent on cytokines other than IL-2, IL-4, and IL-7 for in vitro maintenance certainly makes IL-2 dependence an insufficient definition for HTLV-1 T-lymphocyte transformation.

Recently, the receptors for IL-2, IL-4, and IL-7 have been shown to share γ_c of IL-2R (14, 20, 26). Whether γ_c of IL-2R, IL-4R, and IL-7R is the critical link in T-lymphocyte transformation by HTLV-1 is not clear. The recent demonstration that γ_c is a component of IL-9R (25) and IL-15R (10) indicates that these lymphokines may also support HTLV-1 transformation. The β-γ_c heterodimer of IL-2R is considered to be the minimal signaling component necessary for T-lymphocyte mitogenesis in response to IL-2 (19). That all of the HTLV-1 transformants tested (IL-2, IL-4, and IL-7) exhibited vigorous proliferative responses to IL-2 supports the constitutive expression of not only the alpha chain of IL-2R but of all three components of IL-2R in HTLV-1 transformants. The ability of the IL-4 and IL-7 HTLV-1 transformants to proliferate in response to IL-4 and IL-7 suggests that IL-4R and IL-7R are also constitutively expressed in these transformants.

The restrictive proliferative responses observed for the IL-2-dependent HTLV-1 transformants suggest that a more differentiated cell is transformed by HTLV-1 in the presence of IL-2. Likewise, a less differentiated T lymphocyte may be susceptible to HTLV-1 T-lymphocyte transformation in the presence of cytokines other than IL-2. Alternatively, IL-4R and IL-7R may share other receptor elements, in addition to γ_c necessary for cell signaling. The exact role of γ_c in the intracellular signaling pathways involved in IL-2- and other cytokine-mediated T-lymphocyte systems requires further study. The system described in this report may provide a useful tool for studying the roles of the various components of the cytokine receptors in T-lymphocyte mitogenesis and in HTLV-1 T-lymphocyte transformation.

To date, we have not observed latent infection of T-lymphocytes with HTLV-1 as an outcome of our transformation assay. In vitro transformation of T lymphocytes by HTLV-1 is associated with high levels of viral expression analogous to the high virus levels observed in patients with HTLV-1-associated myelopathy (8). This is in contrast to the observation of patients with ATL, in whom viral expression may be undetectable. ATL cells in vivo are characteristically HTLV-1 p24 antigen negative (6) and may represent nonproducing HTLV-1-infected T lymphocytes that have escaped immune surveillance. When placed in vitro, however, ATL cells readily express HTLV-1 antigens, supporting a role for immune suppression of cells exhibiting high levels of viral gene expression. The assay described in this report, in addition to providing a method for reproducibly generating multiple phenotypically diverse, cytokine-dependent HTLV-1 transformants, provides a model for elucidating the cellular mechanisms involved in HTLV-1-induced T-lymphocyte mitogenesis.

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