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Human T-Cell Leukemia Virus Type I Infection of CD4$^+$ or CD8$^+$ Cytotoxic T-Cell Clones Results in Immortalization with Retention of Antigen Specificity

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The human T-cell leukemia virus type I (HTLV-I) is capable of chronically infecting various types of T cells and nonlymphoid cells. The effects of chronic infection on the specific functional activities and growth requirements of mature cytotoxic T lymphocytes (CTL) have remained poorly defined. We have, therefore, investigated the results of HTLV-I infection of both CD4$^+$ and CD8$^+$ human CTL clones. HTLV-I infection resulted in the establishment of functional CTL lines which propagated indefinitely in culture many months longer than the uninfected parental clone. The infected cells became independent of the need for antigen (target cell) stimulation as a requirement for proliferation and growth. Like their uninfected counterparts, however, these HTLV-I-infected clones remained strictly dependent on conditioned medium from mitogen-stimulated T lymphocytes for their growth. This growth factor requirement was not fulfilled by recombinant interleukin-2 alone. Furthermore, the infected lines remained functionally identical to their uninfected parental CTL clones in their ability to specifically recognize and lyse the appropriate target cells. Our findings indicate that the major effects of HTLV-I infection on mature CTL consist of (i) the capacity for proliferation in the absence of antigen stimulation and (ii) a prolonged or immortal survival in vitro, but they also indicate that the fine specificity and cytolytic capacity of these cells remain unaffected.

The human T-cell leukemia virus type I (HTLV-I) is a pathogenic retrovirus which is named for its ability to chronically infect T lymphocytes, but it can also grow in a number of other cell types (26, 29, 40). HTLV-I was first isolated from adult patients with T-cell leukemias or lymphomas, and epidemiologic studies indicate that this virus is the etiologic agent of adult T-cell leukemia-lymphoma (3, 30, 31, 40). Among the hallmarks of the adult T-cell leukemia-lymphoma syndrome is an impairment of immune function which results in a susceptibility to opportunistic infection. Because HTLV-I does not cause lysis of infected T lymphocytes, a number of investigators have studied the effects of a chronic HTLV-I infection on the immune phenotype and function of T cells. Infection of T cells of the CD4$^+$ noncytotoxic subtype has in some cases resulted in a loss of specific antigen reactivity, with proliferation in response to any class II major histocompatibility complex (MHC) antigens (34, 35) or even in the absence of antigen (22, 23, 27, 37). A decrease in the requirement for exogenous interleukin-2 (IL-2) for proliferation of infected lymphocytes has been frequently reported when the starting population of cells is heterogeneous, as in lymphocytes derived from bone marrow, cord blood, or peripheral blood (7, 20, 22, 27, 28). Along with these virus-induced alterations in growth properties, *trans*-activation of certain cellular genes, including the class II MHC genes and the IL-2 receptor genes, has been reported (6, 15, 24, 27).

Cytotoxic T lymphocytes (CTL) are one of the primary immune defenses of the host against viral infections and virus-induced tumors. The consequences of HTLV-I infection of cytotoxic T-cell lines or clones with well-defined antigen specificities, however, are not well understood. In a total of four allospecific, CD4$^+$ CTL clones, HTLV-I infection resulted in a progressive loss of cytotoxic function and specificity, accompanied by the progression of a transformed phenotype (27, 39). A single CD8$^+$ CTL, specific for HTLV-I, was also reported to have lost cytotoxic potential and continued to proliferate in culture without the need for stimulation with the appropriate antigen (23).

To further investigate the impact of HTLV-I infection on human CTL, we have introduced the virus into a number of well-characterized human allospecific CTL clones expressing either the CD4$^+$ or CD8$^+$ phenotype. We report here that the viral infection of CTL clones rapidly resulted in the growth of cells independent of the need for antigen stimulation. These CTL clones proliferated indefinitely in culture but retained their cytotoxic capacity and specificity.

MATERIALS AND METHODS

Cell lines and culture conditions. (i) Culture medium. Cells were grown in culture medium consisting of RPMI 1640 (M.A. Bioproducts, Bethesda, Md.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 U/ml; GIBCO), streptomycin (100 U/ml; GIBCO), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), and 25 μM 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.). Long-term CTL cultures and CTL clones were maintained in culture medium and 10% conditioned medium.

(ii) IL-2-containing human T-cell-conditioned medium. IL-2-containing medium was made as previously described (14). Briefly, peripheral blood leukocytes obtained from three to five different platelet donors were mixed and washed three times. The cells were suspended at a concentration of 3×10^6 cells per ml in RPMI 1640 containing 3% fetal calf serum

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and 0.15% phytohemagglutinin (PHA-P; Difco Laboratories, Detroit, Mich.). Special additives included 2.8 μM indomethacin (Sigma), 3 mM lithium chloride (Sigma), and 50 μM hydroxyurea (Sigma). The cells were cultured for 72 h, and the supernatants were collected. The supernatants were ammonium sulfate precipitated (50% and 75%). After dialysis, the purified, conditioned medium was filter sterilized and stored at −20°C.

(iii) Human tumor cell lines. The human tumor cell lines which served as targets or stimulators were maintained in culture medium and regularly passaged. All of the following lines were typed by the HLA Tissue-Typing Laboratory, Dana-Farber Cancer Institute: JY (HLA-A2, B7, DR4, DR6, DQ1, DQ3, DP2, DP4), K562 (HLA- --, --, --), Bri8 (HLA-A1, A2, B8, B13, DR2, DR4, DQ1, DP+), M14B (HLA-A1, A2, B7, B8, DR2, DR3, DQ1, DQ2, DP3, DP4), PGF (HLA-A3, B7, DR2, DQ1, DQ3, DP+), Priess (HLA-A2, B15, DR4, DQ3, DP3, DP4), WT 49 (HLA-A2, B17, Bw15, DR3, DQw2), M7 (HLA-A2, A3, Bw53, Bw35), DKI (HLA-A2, Aw33, B40, Bw44), KNE (HLA-A2, A1, B8, B27).

(iv) Generation of CTL clones. CTL clones were isolated from a single volunteer donor (HLA-A1, B8, Bw57, Cw6, DQ3). Peripheral blood mononuclear cells were separated on a Ficoll-Hypaque gradient (Lymphocyte Separation Medium; Litton Bionetics, Kensington, Md.). The mononuclear cells, at 2×10^6 cells per ml, were cocultured with irradiated JY, M14B, or Priess cells at 1×10^5 cells per ml in 2-ml wells (Linbro, McLean, Va.) at 37°C in a 5% CO_2 incubator. The bulk culture was maintained in 9.6-cm^2 wells (Nunc, Neptune, N.J.) by stimulation every 1 to 2 weeks with irradiated stimulator cells. After 4 weeks in culture, the cells were again isolated by using a Ficoll-Hypaque gradient and cloned by using limiting dilution in 96-well round-bottom microtiter plates (Linbro, McLean, Va.) with 2×10^4 irradiated JY or M14B cells as a feeder layer. Culture medium containing 10% human conditioned medium was used for cloning; fresh medium was added every 3 days. Clones were obtained at a concentration of 1 cell per 10 wells. Both clones CTL-329 and CTL-342 were subcloned at less than 1 cell per well. The specificity of each clone was determined by using a panel of human leukocyte antigen (HLA)-typed target cells and monoclonal antibody (MAb) blocking as described previously (21).

Blood lymphocyte preparation. Lymphocytes from peripheral blood or from cord blood were purified by banding in Ficoll-Hypaque. Lymphocytes were maintained in RPMI with 10% fetal calf serum and 10% T-cell-conditioned medium. Phytohemagglutinin was added to a concentration of 0.15% to stimulate proliferation prior to cocultivation with virus-producing cell lines.

Infection of lymphocytes with HTLV-I. The HTLV-I producer-cell line MJ (28, 29) was generously provided by M. Essex, Harvard School of Public Health, and was grown in RPMI with 10% fetal calf serum. The producer-cell line SPP was established from cord blood lymphocytes which had been transformed after cocultivation with MJ. The infected lymphocyte population was cloned by limiting dilution, and a clone which expressed HTLV-I_{MJ} at high titers was selected and designated line SPP. Human T-cell-conditioned medium was added to 10% for 24 h before MJ or SPP was used for cocultivations to increase the production of virus. Immediately prior to cocultivation, the HTLV-I-producing cell line was irradiated to 10,000 rad with a gamma ray source. Cocultivations were carried out by using an equal number of irradiated producer cells and uninfected CTL or blood lymphocytes in the presence of 10% human T-cell-conditioned medium and 8 μg of Polybrene (Sigma) per ml. CTL were stimulated with the appropriate stimulator cells 2 days prior to the cocultivations to assure proliferation of the cells during the period of their exposure to the virus.

Lymphokines. Recombinant human interferon gamma and recombinant human T-cell growth factor (IL-2) were the generous gift of Biogen (Cambridge, Mass.).

Cytotoxicity assays. CTL assays were performed in triplicate in V-bottom microtiter wells (Linbro). Effector cells were added in threefold dilutions. Target cells, preincubated with 0.1 mCi of ^{51}Cr ($Na^{51}CrO_4$; New England Nuclear Corp., Boston, Mass.) for 2 h and washed three times, were added at 10^3 cells per well. Microtiter plates were centrifuged and incubated at 37°C for 4 h. After incubation, the plates were again centrifuged and the supernatants were assayed for ^{51}Cr release. Specific cytotoxicity was calculated as percent cytotoxicity = (counts per minute of experimental release − counts per minute of spontaneous release)/(total counts per minute − counts per minute of spontaneous release) × 100. When blocking MAb were used, they were added at the start of the assay. Inhibition of cytotoxicity was expressed as a percent reduction of the specific cytotoxicity. The standard deviation of the triplicate wells rarely exceeded 3 to 5% of the specific lysis. The uninfected CTL clones were stored frozen after cloning because of their limited life span in culture. Aliquots of the original clones CTL 329 and CTL 342 were thawed and stimulated once in culture prior to their inclusion in the cytotoxicity assays of their infected, immortal counterparts.

Proliferation assays. Proliferation of CTL was quantitated by incorporation of [^{3}H]thymidine over a 24-h period. From 1×10^5 to 5×10^5 cells, some samples of which had been exposed to stimulator cells 48 h previously, were plated per well of a 24-well Linbro dish, in growth medium plus 10% human T-cell-conditioned medium. Cells were harvested and assayed for thymidine incorporation as described previously (9). All assays were done in triplicate. In addition, parallel cultures were counted at 24-h intervals to assess the doubling time.

Cell surface immunofluorescence. Cells were washed twice with phosphate-buffered saline containing 2.5% fetal calf serum and 0.02% sodium azide. Approximately 5×10^5 cells were incubated on ice for 30 min with an excess concentration of phycoerythrin-conjugated MAb and/or fluorescein-conjugated MAb. Conjugated Leu3a and Leu2 antibodies were obtained from Becton Dickinson (Mountain View, Calif.). The cells were washed three times and fixed in 1% paraformaldehyde and analyzed on a FACS I analyzer (Becton Dickinson).

Cell cytoplasmic immunofluorescence. Infected or uninfected cells were spun onto gelatin-coated slides by using a cytocentrifuge. The slides were washed twice in a balanced salt solution, drained, and fixed and permeabilized by a 10-min exposure to a 50:50 (vol/vol) mixture of acetone and water at −20°C, followed by a 10-min exposure to pure acetone at −20°C. After evaporation of the acetone, the slides were stained at 4°C with a 1:50 dilution of a goat antiserum directed against HTLV-I p24 (generously provided by F. Wong-Staal), followed by incubation with a fluorescein-conjugated rabbit anti-goat immunoglobulin G antibody preparation (Cooper Biomedical, Malvern, Pa.). Cells incubated with the second antibody alone served as additional controls for specificity. Fluorescent staining was quantitated by using a fluorescent microscope.

MAb. MAb-producing hybridoma cells were grown in BALB/c mouse ascites. The specificities of the HLA MAb

have been previously described (5). MAb recognizing HLA determinants were W6/32 (monomorphic HLA-A, -B, -C), LB3.1 (monomorphic HLA-DR), B7/21 (HLA-DP), and PA2.1 (HLA-A2, A28). The MAb TS1/22, recognizing the lymphocyte function-associated antigen 1, has been described elsewhere (32). Commercially available MAb were also used: OKT3, OKT4, and OKT8 (Ortho, Raritan, N.J.) and Leu2, Leu3a, and anti-IL-2R (Becton Dickinson).

Analysis of HTLV-I transcripts and integrated proviruses. Total cellular RNA was extracted from cells, separated on the basis of size on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled DNA probes as previously described (38). After being stripped, all blots were rehybridized with a β-actin-specific and/or a β-tubulin specific probe(s) to normalize for the total amounts of RNA added per lane. High-molecular-weight genomic DNA was isolated from cells essentially as described by Bell et al. (1). This DNA was digested to completion with restriction endonucleases by using the conditions recommended by the manufacturer (New England BioLabs, Inc., Beverly, Mass.). Digested fragments were separated on the basis of size on agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled DNA probes by the method of Southern (33). All washes of DNA and RNA blots were performed under conditions of high stringency (38). Labeling of DNA probes was performed by using the random primer-labeling technique (10). The β-actin, β-tubulin, and HLA probes were described previously (8, 38). The HTLV-I probe consisted of an entire HTLV-I provirus (isolate MT-2) which had been cloned into pUC-18 via the *Sst*I sites in the long terminal repeat (generously provided by J. Mullins, Harvard University).

RESULTS

Specificity of CTL clones. Human HLA class I- or class II-specific CTL lines were generated by repeated stimulation of peripheral blood lymphocytes from an HLA nonidentical donor with either the JY or the M14B cell line. These lines were cloned and subcloned by limiting dilution, and their phenotypes and functional characteristic specificities were determined by using MAb and HLA-typed target cell panels.

As expected, both CTL clones expressed CD3 (not shown). Clone CTL-329 was typical of the class I-specific CTL clones thus generated. All of the cells in this clone were simultaneously Leu2 (CD8) positive and Leu3 (CD4) negative, as determined by immunofluorescence analysis by two-color sorting (Fig. 1). The lytic activity of this clone against its target (JY) was inhibited by 89% in the presence of CD8-reactive antibodies and by 82% in the presence of a pan-HLA-reactive antibody (W6/32). Antibodies against class II framework antigens or against CD4 had no effect on the cytotoxicity of CTL-329 (see below). Clone CTL-342, conversely, was typical of the class II-specific CTL clones, and its phenotype was simultaneously positive for the CD4 antigen and negative for the CD8 antigen (Fig. 1). The lytic activity of this clone was inhibited by 59% and 65% in the presence of antibodies directed against CD4 or HLA-DP, respectively, while antibodies recognizing CD8 or HLA class I framework antigens had no effect on its lytic activity.

The target specificity of CTL-329 is shown in Tables 1 and 2. Target cell lines homozygous for HLA-A2 were recognized and lysed very efficiently by this clone. Cell lines heterozygous for A2 were lysed less efficiently, and lines negative for A2 expression were lysed poorly (at background levels). Furthermore, the lytic activity of this clone was

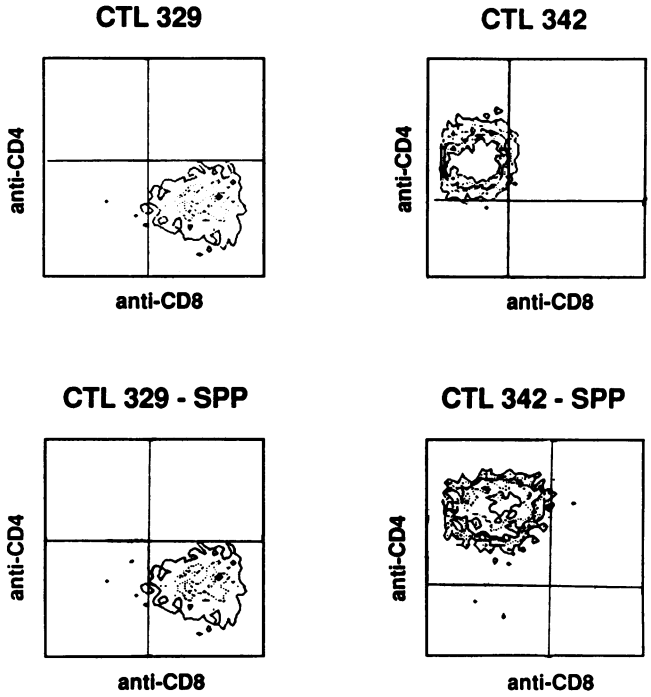

FIG. 1. Cell surface expression of CD4 and CD8 by clones CTL-329 and CTL-342, uninfected or HTLV-I infected, as dual parameter immunofluorescence profiles. Anti-CD4 (Leu3a) was directly conjugated with phycoerythrin, and anti-CD8 (Leu2) was directly conjugated with fluorescein. Profiles are shown for the uninfected clones (CTL-329 and CTL-342) and for their HTLV-I-infected counterparts (CTL-329-SPP and CTL-342-SPP, respectively). Quadrant markers are based on dual parameter immunofluorescence of control cells from peripheral blood.

blocked by an anti-HLA-A2 MAb (PA2.1), thus establishing clone CTL-329 as HLA-A2 specific. Analysis of the HLA-A2 subtype specificity (2) of CTL-329 demonstrated a clear hierarchy of reactivity depending on the HLA-A2 subtype, with HLA-A2.1-expressing cells being better recognized than HLA-A2.2 and HLA-A2.2 being better recognized than HLA-A2.3. The antigen A2.4 is recognized poorly if at all (see below). In contrast, clone CTL-342 recognized and lysed efficiently only those target cells expressing the class II antigen DP3 (Tables 1 and 2) and was inhibited by MAb directed against HLA-DP. CTL-342 was therefore an HLA-DP3 responder expressing the CD4 phenotype.

Infected CTL clones contain HTLV-I genomes and express HTLV-I transcripts. These class I- or class II-specific CTL clones were then infected with HTLV-I by cocultivation with an irradiated, HTLV-I_{MJ}-producing cell line called SPP. Several CTL clones which had been cocultivated were carried in parallel with cells from the same clone which had never been exposed to virus. Those CTL which had been exposed to the HTLV-I continued to grow and proliferate in culture. Two representative clones were selected and passaged for over 8 months, at which time it was elected to stop their continuous passage. CTL clones which had not been exposed to HTLV-I, however, did not proliferate beyond 6 weeks after mock infection, despite weekly stimulation with the original stimulator cell lines. The CD4 and CD8 cell surface phenotype of the CTL clones was unchanged after HTLV-I infection (Fig. 1).

Total cellular RNA was extracted from the virus-producing cell line SPP, from uninfected bulk CTL, and from

TABLE 1. HLA-specific cytotoxicity of HTLV-I-infected CTL clones at 1 month after cloning

Expt no.	Target cell[a]	E:T[b]	Cytotoxicity[c] with CTL clone: CTL-329	CTL-329-SPP	CTL-342	CTL-342-SPP
1	JY (A2)	10:1	97	83	3	5
		3:1	65	61	1	−1
	AS (A2)	10:1	87	79	3	2
		3:1	51	45	0	3
	Priess (DP3)	10:1			43	48
		3:1			16	13
	F2B (A2/A1)	10:1	29	43	1	−2
		3:1	12	14	2	1
	M14B (DP3)	10:1			68	86
		3:1			28	33
2	JY (A2)	10:1	58	61	2	
		3:1	22	25		
	PGF (B7)	10:1	21	23	7	
		3:1	5	6		
	Bri-8 (A2/A1)	10:1	38	36	16	
		3:1	15	11		
	WT 49 (A2, DR3)	10:1	28	19	5	
		3:1	12	11		
	K562	10:1	5	3	−2	

[a] Target cell line is indicated, along with the relevant HLA specificity in parentheses. Complete HLA type of each target cell is listed in Materials and Methods.
[b] E:T, Effector cell:target cell ratio.
[c] Cytotoxicity is expressed as percent specific ^{51}Cr release (see Materials and Methods).

two CTL clones, designated CTL-342-SPP and CTL-329-SPP, which had been cocultivated 1 month before with SPP. RNA blot analysis with a labeled HTLV-I_{MJ} DNA genome as a probe disclosed HTLV-I-specific transcripts in the

TABLE 2. HLA-specific cytotoxicity of HTLV-I-infected CTL clones at 8 months after cloning

Expt no.	Target cell[a]	E:T[b]	Cytotoxicity[c] with CTL clone: CTL-329	CTL-329-SPP	CTL-342	CTL-342-SPP clone 1	CTL-342-SPP clone 2
1	JY (A2)	10:1	62	51			
		3:1	36	31			
	Priess (A2)	10:1	42	42			
		3:1	12	15			
	PGF (B7)	10:1	24	18			
		3:1	10	7			
	DPV01 (DR6)	10:1	3	5			
	K562	10:1	2	1			
2	M14B (A2 DP3)	10:1			59	57	37
		3:1			18	20	14
	Priess (DP3)	10:1			51	48	51
		3:1			20	21	17
	JY (DR4, 6)	10:1			−3	6	−3
	DPV01 (DR6)	10:1			−1	4	1

[a] Target cell line is indicated, along with the relevant HLA specificity in parentheses. Complete HLA type of each target cell is listed in Materials and Methods.
[b] E:T, Effector cell:target cell ratio.
[c] Cytotoxicity is expressed as percent specific ^{51}Cr release (see Materials and Methods).

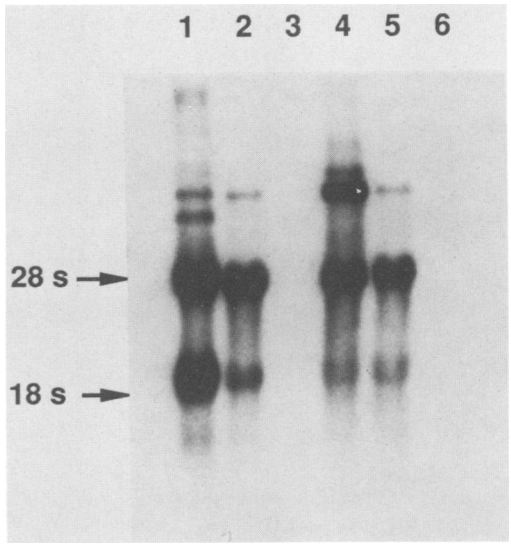

FIG. 2. Detection of HTLV-I-specific RNA transcripts in CTL clones which had been cocultivated with an HTLV-I-producing cell line. Total cellular RNA from the HTLV-I-producing cell line SPP (lane 1), from CTL-329-SPP (lane 2), from the bulk CTL line from which clones 329 and 342 were derived (lane 3), from CTL-342-SPP (lane 4), from a line of cord blood lymphocytes transformed by cocultivation with CTL-329-SPP (lane 5), and from the human T-cell lymphoma PEER (lane 6), were separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a radiolabeled probe consisting of DNA encoding the entire HTLV-I proviral genome. Autoradiography with enhancing screens was carried out for 48 h. The positions of the 28S and 18S rRNA markers are indicated.

HTLV-I producer line SPP and in the cocultivated CTL clones (Fig. 2). The 8.5-kilobase (kb) viral genome, the 4.0-kb *env* mRNA, and the 1.9-kb *pX* transcript were all detected. In contrast, no transcripts were detected in uninfected T-cell lines or in the parental bulk CTL line which had not been cocultivated with SPP (Fig. 2). Analysis of the lines CTL-342-SPP and CTL-329-SPP, after 8 months in culture, demonstrated continued expression of HTLV-I transcripts in a pattern identical to that observed at 1 month in culture (not shown).

Genomic DNA was extracted from the virus-producing cell line used for cocultivation, from uninfected CTL bulk cultures, and from the CTL clones CTL-329-SPP and CTL-342-SPP, which had been cocultivated with the HTLV-I producer-cell line SPP. Digestion of genomic DNA containing an integrated HTLV-I provirus with *Eco*RI, which does not cut within the HTLV-I_{MJ} genome (19, 23), yields one or more fragments containing unique proviral integrations. Hybridization with a labeled DNA probe specific for HTLV-I demonstrated one or more viral integration sites in all of the infected or cocultivated cell lines but no HTLV-I integrations in the parental CTL bulk lines (Fig. 3). There are two cleavage sites for *Bam*HI in the HTLV-I genome, separated by approximately 1.0 kb. Digestion of the genomic DNA specimens with *Bam*HI and hybridization with a labeled HTLV-I-specific probe identified this 1-kilobase-pair internal fragment in all the infected or cocultivated lines, along with multiple junction fragments (Fig. 3).

The lines CTL-329-SPP and CTL-342-SPP were also able to produce infectious virus, as determined by their ability to generate transformed lymphocyte populations after cocultivation with cord blood lymphocytes (not shown). Analysis

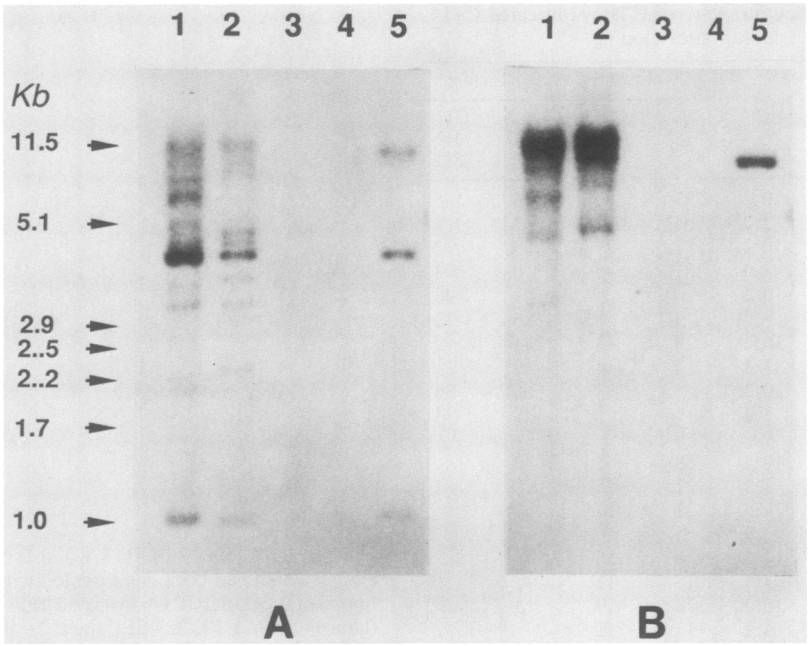

FIG. 3. Identification of integrated HTLV-I proviruses in CTL clones cocultivated with an HTLV-I-producing cell line. High-molecular-weight genomic DNA was digested with *Bam*HI (panel A) or *Eco*RI (panel B), separated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a radiolabeled probe consisting of DNA encoding the entire HTLV-I proviral genome. Autoradiography with intensifying screens was carried out for 4 days. Source of the DNA used in each lane: lane 1, HTLV-I-producer SPP; lane 2, CTL-329-SPP; lane 3, bulk CTL line used to generate clones CTL-329 and CTL-342; lane 4, the T-cell lymphoma PEER; lane 5, CTL-342-SPP. The positions of double-stranded DNA molecular mass markers are indicated.

of the RNA derived from such a transformed cord blood lymphocyte population revealed HTLV-I-specific transcripts (Fig. 2), demonstrating transmission of the virus from the CTL-SPP lines.

HTLV-I-infected clones upregulate expression of class I and class II MHC antigens and of the IL-2 receptor. mRNA and DNA analyses do not establish whether every cell in the population was infected and expressing HTLV-I. Immunoassay for the presence of the viral p24 antigen within the CTL by using a specific antiserum demonstrated low but specific levels of p24 immunoreactivity in 86 and 91% of the cells in the lines CTL-342-SPP and -329-SPP, respectively, when assayed at 8 months after infection (not shown). This same relative level of p24 expression has been noted in other HTLV-I-infected T-cell lines (12, 23), including lines MJ and SPP. The levels of expression of cellular proteins which are known to be induced by HTLV-I infection (or by T-cell activation) were also examined. The levels of class II MHC antigen on the infected clones increased by a factor of 3.5 to 3.9 (Table 3), and more than 90% of the infected cells demonstrated this increase. Similarly, the expression of the IL-2 receptor was upregulated by 1.5- to 2.0-fold on the infected CTL clones. The cell surface levels of a control lymphocyte-specific cell surface antigen, LFA-1, were unchanged after infection. The expression of class I MHC antigens was strongly induced (2.5 to 4-fold) in the HTLV-I-infected clones or cord blood lymphocytes. This increase was of the same magnitude as that found after gamma interferon stimulation of the uninfected clones or cord blood lymphocytes. Treatment of the infected clones or infected cord blood lymphocytes with interferon did not cause a further increase in class I MHC expression. Because the HLA type of the CTL clones was known (HLA-A1, B8), it was possible to determine whether the upregulation of class I MHC expression, as determined by the pan-HLA-reactive antibody W6/32, was due to specific upregulation of the HLA antigens normally expressed on the CTL or, alternatively, whether new MHC-like antigens were being expressed as a result of the HTLV-I infection. Analysis with HLA-A1- and HLA-B8-specific typing antisera demonstrated that the expression of both these endogenous antigens was increased by the same magnitude as the increase demonstrated by the panreactive HLA antibody (not shown). This suggests that the upregulation in cellular HLA

TABLE 3. Expression of cell surface antigens after HTLV-I infection

Cell line	Mean fluorescence intensity[a]				
	IL-2R[b]	Class II	HLA	HLA (IFN)[c]	LFA-1
PBL[d]	100	100	100	246	100
CTL-329	110	111	79	245	103
CTL-329-SPP	160	356	295	289	88
CTL-342	107	92	103	ND[e]	97
CTL-342-SPP	196	291	328	ND	84
SPP	282	370	700	742	69
CBL (co-cult)[e]	280	372	354	350	75

[a] Relative to the antibody labeling of peripheral blood lymphocytes, which was arbitrarily set at 100.

[b] The antibodies used for detection of the IL-2 receptor (IL-2R), all class II MHC (Class II), all class I MHC (HLA), or lymphocyte functional antigen 1 (LFA-1) are described in Materials and Methods.

[c] The cell lines analyzed in this column had been pretreated for 48 h with recombinant gamma interferon at 200 U/ml.

[d] PBL, Peripheral blood lymphocytes, in culture with 10% human T-cell-conditioned medium for 48 h.

[e] CBL (co-cult), Cord blood lymphocytes which had been cocultivated with irradiated CTL-329-SPP 1 month prior to analysis and were expressing HTLV-I transcripts.

TABLE 4. Growth kinetics of CTL clones

CTL clone	Stimulated[a]	[3H]thymidine uptake[b]	Doubling time (h)[c]
CTL-329	−	960 ± 142	>150
CTL-329	+	5,652 ± 578	45
CTL-329-SPP	−	4,111 ± 780	45
CTL-329-SPP	+	5,075 ± 911	40
CTL-342	−	758 ± 263	>150
CTL-342	+	4,956 ± 564	55
CTL-342-SPP	−	4,116 ± 644	50
CTL-342-SPP	+	3,808 ± 750	55

[a] Growth kinetics were assessed either 7 days after the last stimulation with the appropriate target cell (JY or M14B) (−), or 2 days after stimulation (+). All the clones were cultured in medium containing 10% human T-cell-conditioned medium.

[b] Counts per minute of [3H]thymidine taken up by 5×10^5 cells per 24-h period.

[c] Estimated doubling time of clones, based on quantitation at 24-h intervals.

levels found after HTLV-I expression is due to enhancement of the expression of the normally expressed HLA antigens.

HTLV-I-infected CTL clones do not require antigen stimulation for proliferation. Analysis of the growth kinetics of the infected or uninfected clones by using quantitation of cell number or uptake of [3H]thymidine demonstrated that the HTLV-I-infected clones CTL-342-SPP and CTL-329-SPP proliferated at about the same rate as the target cell-stimulated clones from which they were derived. Both the HTLV-I-infected and uninfected clones had a doubling time of approximately 50 h (Table 4). Whereas the uninfected clones remained strictly dependent on exposure to stimulator (target) cells for proliferation, however, the HTLV-I-infected cells continued to proliferate at the same rate, whether or not they were stimulated with target cells at weekly intervals. Both the uninfected and infected CTL clones remained completely dependent on the presence of 10% human T-cell-conditioned medium for growth. Recombinant human IL-2, at concentrations up to 100 U/ml, was unable to substitute for this T-cell-conditioned medium requirement of all CTL lines. Gradual decreases in conditioned-medium concentration did not yield any conditioned-medium-independent CTL lines. Thus, proliferation of the HTLV-I-infected CTL had become antigen independent but not factor independent. Lymphocyte growth factors in addition to IL-2 continued to be required for the growth of these cells.

HTLV-I-infected CTL retain antigen specificity. Despite their lack of a requirement for antigen stimulation, the HTLV-I-infected CTL clones retained their ability to recognize and lyse cells bearing the appropriate MHC antigens. At both 1 month and 8 months after infection, line CTL-342-SPP remained specific for the class II antigen DP3 and lysed cells bearing DP3 at efficiencies similar to the original clone CTL-342 (Tables 1 and 2). Similarly, line CTL-329-SPP retained its specificity for HLA-A2-bearing cells at 1 month and 8 months in culture. Furthermore, CTL-329-SPP preserved its fine HLA-A2 subtype specificity. After 8 months in culture, the infected line continued to recognize and lyse the A2 subtype-bearing target cells in the same hierarchical pattern as the parental clone (A2.1 > A2.2 > A2.3 >> A2.4) (Table 5). The cytotoxicity of this HTLV-I-infected cell line was still blocked appropriately by antibodies directed against monomorphic HLA, HLA-A2, CD8, and CD3 after 8 months of continuous passage (Table 6). These results indicate that the HTLV-I-infected CTL continue to utilize the same antigen-independent cell-cell interaction molecules as their uninfected counterparts. Lytic activity against K562, a classic NK target, was never detected by using either the uninfected or the HTLV-I-infected CTL clones.

TABLE 5. Preservation of fine specificity of HTLV-I-infected CTL-329

Target cell	Haplotype[a]		% Cytotoxicity at E:T[b]	
	A	B	9:1	3:1
JY	A2.1, 2	7	61	49
M7	A2.2, 3	w50, 8	63	33
DKI	A2.3, w33	40, w44	45	26
KNE	A2.4, 1	8, 27	22	15
PGF	A3	7	19	13

[a] Haplotype of the target cell line, including the HLA-A2 subtype.

[b] Cytotoxicity is expressed as percent specific ^{51}Cr release. E:T, Effector-to-target cell ratio.

DISCUSSION

The mechanisms by which HTLV-I, which contains no recognized oncogene, transforms lymphocytes in culture or in vivo remain obscure. Infection of heterogeneous cultures of peripheral, cord blood, or bone marrow lymphocytes results in the outgrowth of a population of rapidly proliferating cells which demonstrate a decreased requirement for IL-2 and are independent of the need for antigen or mitogen stimulation as a requirement for growth. Such a population of cells is usually positive for the expression of the CD4 antigen although there may be some loss of CD3 antigen expression, a pattern similar to that observed on tumor cells derived from patients with adult T-cell leukemia-lymphoma (25, 36, 39). We find no major differences in the susceptibility of CTL clones to HTLV-I infection regardless of whether they express CD4 or CD8. The expression of CD3 was maintained on the infected CTL clones, consistent with their retention of antigen recognition and cytotoxic capacity.

Loss of CTL antigen-specific recognition and cytolytic function has been a universal finding in previous reports of HTLV-I-infected CTL lines and populations. In a total of 8 infected CTL lines described to date (5 allospecific [27, 35, 39] and 3 HTLV-I specific [23]), all lost specific function or died within 2 months after infection. This loss of function was in some cases associated with a loss of CD3 expression on the cell surface. In addition, other investigators have reported the loss of antigen specificity with retention of the

TABLE 6. Blocking of HTLV-I-infected CTL cytotoxic activity with MAb

Blocking antibody[a]	Specificity[b]	% Cytotoxicity[c]	% Inhibition[d]
None		61	
W6/32	Pan-HLA	26	57
PA 2.1	A2	19	69
OKT8	CD8	25	59
OKT4	CD4	65	0
OKT3	CD3	26	57

[a] Antibodies used to block cytotoxicity of CTL-329-SPP were used at a concentration of 20 μg/ml, which was saturating.

[b] Antigen specificity of antibodies used for blocking studies.

[c] Cytotoxicity is expressed as percent specific ^{51}Cr release. Effector-to-target cell ratio in this assay was 8:1.

[d] Inhibition of cytotoxicity in the presence of the antibody, relative to the killing observed in the absence of antibody (61% lysis).

lytic mechanisms (e.g., NK activity) in HTLV-I-infected T cells. In contrast, we have observed no loss of antigen specificity or cytotoxic function in our infected T-cell clones, even after 8 months of continuous culture. Even fine specificity has been preserved. Furthermore, there is no evidence for nonspecific cytotoxic function, as defined by the inability of the infected CTL to lyse traditional NK targets like K562 cells.

All of the CTL clones we have infected with HTLV-I remain dependent on exogenous human T-cell-conditioned medium for growth. This conditioned medium is usually thought of primarily as a source of IL-2. However, recombinant human IL-2 will not substitute for the conditioned medium. This suggests that other, as yet undefined, factors present in the conditioned medium continue to be required for growth of CTL after HTLV-I infection. Why these HTLV-I-infected CTL remain dependent on factors elaborated by other T cells, whereas infected heterogeneous T-lymphocyte populations are able to proliferate in the presence of serum and medium alone (7, 20, 22, 27, 29), is not yet clear. This may reflect intrinsically different growth requirements for cytotoxic versus noncytotoxic T lymphocytes. More likely, it is because infection of mixed populations of lymphocytes allows for the elaboration of T-cell growth factors by various subpopulations of cells within the bulk cultures or because in vitro selection occurs, with the most autonomous clones eventually overgrowing the populations. The use of strictly clonal lymphocyte lines in our studies eliminates the confounding variable of heterogeneous cell populations. Our findings would suggest, then, that growth factor independence is not necessarily an immediate, direct, or universal effect of HTLV-I transformation of T lymphocytes.

A sequence of events in the course of antigen stimulation leading to CTL cell proliferation has been proposed by a number of investigators (see reference 13). In this model, the interaction of antigen with the T-cell receptor leads to upregulation of IL-2 receptor expression on the T-cell surface. Binding of IL-2 to this receptor then leads to the acquisition of cytotoxicity and cellular proliferation and clonal expansion. Independence of the need for antigen or mitogen stimulation occurred in all of our infected clones, regardless of whether they were stimulated at weekly intervals or selected for such autonomy. This loss of an antigen requirement was not accompanied by the loss of antigen receptor expression, as evidenced by the ability of infected CTL to recognize and lyse target cells in a strictly antigen-specific manner—a function that requires the T-cell (antigen) receptor. We have previously observed such a loss of requirement for antigen stimulation, with the retention of functional antigen specificity, in well-defined lymphocyte lines into which acutely transforming murine retroviruses have been introduced (16, 17). We were able to show in these cases that antigen autonomy was not due to autocrine elaboration of lymphokines normally produced by the cells after antigen stimulation, and we have postulated that these retroviruses are able to act within the antigen-growth factor signal transduction pathway itself (9; J. N. Zullo and D. V. Faller, submitted for publication). The present data is consistent with such a mechanism operating in the HTLV-I-infected CTL. The infected CTL behave as if they are chronically activated by antigen, demonstrating increased expression of the IL-2 receptor and class II MHC antigens. HTLV-I may thus be bypassing the need for antigen stimulation by affecting the signal transduction pathway distal to the T-cell receptor, simulating an activated state. Further work will be necessary to fully develop such a model.

In the course of characterizing the expression of lymphoid cell surface antigens on the surface of CTL after infection with HTLV-I, the previously reported increases in IL-2 receptor antigen and class II MHC antigen expression were observed. In addition, a 5- to 10-fold increase in the levels of class I MHC (HLA) antigens were consistently observed after infection. High levels or alterations in HLA expression have been noted on HTLV-I-induced tumors or in populations of T lymphocytes infected with the virus (18). Similarly, we report here that the HTLV-I-infected SPP cell line and HTLV-I-infected cord blood lymphocytes express substantially higher levels of HLA than do peripheral blood lymphocytes. Interpretation of such observations is difficult, however, because the phenotype of the original cell, prior to infection with the virus, could not be ascertained in such studies. By starting with well-characterized, clonal, untransformed T cells, we can demonstrate unequivocal consistent increases in class I MHC expression after HTLV-I infection. Furthermore, because the specific HLA type of these CTL clones is known, we can demonstrate that the increases in HLA expression are due to specific increases in the levels of the endogenous, normal HLA antigens expressed by the CTL, rather than to the expression of new or altered class I MHC-like molecules. Thus, this upregulation of HLA expression appears to be due to an effect of HTLV-I on the infected cell rather than to the cross-reactivity of the HTLV-I *env* antigen with panreactive anti-HLA antibodies (as proposed by Clarke et al. [4]). Other lymphocyte cell surface antigens, including LFA-1, CD4, and CD8, are not upregulated in the infected CTL, indicating that there is a specificity to the upregulation of HLA antigens (and HLA-DR and IL-2R antigens) by the virus. We have previously reported that the murine leukemia retroviruses cause upregulation of class I MHC genes in the cells they infect by a *trans*-acting mechanism at the level of gene transcription (8, 11, 38). The HTLV-I-infected CTL clones reported herein similarly express higher levels of class I MHC-specific mRNA transcripts than do their uninfected counterparts, suggesting a similar mechanism (D. V. Faller, unpublished data). Whether this HLA upregulation could be caused by the HTLV-I *trans*-acting *tat* gene is currently under investigation.

Our use of extensively characterized and stable cytotoxic T-lymphocyte clones, with well-defined functions and antigen specificities, has allowed us to elucidate the general and direct effects of HTLV-I infection on this effector arm of the cellular immune system. Loss of the need for antigen stimulation as a growth requirement occurred universally, as did the limitless potential for proliferation of the infected lymphocytes in culture. Both of these new characteristics could contribute to malignant behavior in vivo. However, a major change in growth factor requirements was not observed, and functional, cytotoxic antigen specificity was completely maintained over many months in culture. Thus, previous reports of growth factor autonomy, loss of CD3 expression and of function, and cell death in HTLV-I-infected T lymphocytes may represent secondary events occurring in culture, rather than direct results of the virus on the infected cells.

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