Human T-cell leukemia-lymphoma virus (HTLV) is in T but not B lymphocytes from a patient with cutaneous T-cell lymphoma

(type C retrovirus/molecular hybridization/T-cell growth factor)

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ABSTRACT A human type C retrovirus, designated HTLV, previously was isolated from or identified in some patients with leukemias and lymphomas of mature T lymphocytes. HTLV is genetically and serologically distinct from any known animal retroviruses. The absence of HTLV proviral sequences in DNA from normal humans showed that HTLV is not a ubiquitous endogenous (germ-line transmitted) virus of humans. Antibodies to HTLV core proteins have been identified in some people with T-cell neoplasias and are particularly prevalent in Japanese with adult T-cell leukemia, suggesting that HTLV is acquired horizontally. However, it was possible that HTLV is transmitted through the germ line of some (possibly rare) families and is then expressed in the HTLV-positive malignancies. An opportunity to study this question was provided by the development of several T-cell lines and a B-cell line from one HTLV-positive patient with a cutaneous T-cell lymphoma. Here we report that HTLV proteins or nucleic acids (or both) are found in three independently derived T-cell lines, all shown by HLA typing to have originated from the patient. In contrast, the B-cell line, the identity of which was also ascertained by HLA typing, contained no detectable HTLV protein, RNA, or proviral DNA. Because the sensitivity of the latter assay is more than sufficient to detect one proviral equivalent per haploid genome, the results indicate that HTLV was not transmitted to this patient through the germ line but rather was acquired by infection.

The discovery (1, 2) and purification (3) of T-cell growth factor (TCGF) has made it possible to grow relatively mature human T cells from normal (1, 2) and neoplastic (4) sources routinely in liquid suspension culture for long periods. We have identified a human type C retrovirus in cultured T cells from several patients with leukemia-lymphoma of relatively mature T cells. In some instances, the virus (called HTLV) has been constantly produced by these T cells (5, 6) which have become independent of TCGF and established as permanent cell lines, apparently because of eventual constitutive release of TCGF (7). In two instances, the virus has been characterized in detail (5, 6). HTLV is not an endogenous virus ubiquitous in human germ lines (8) and is readily distinguishable from the known animal retroviruses, including those from subhuman primates (8–10). Antibodies to two HTLV proteins, p24 and p19, have also been found in sera from a few other patients with T-cell malignancies in the United States (11, 12), in a high percentage of patients with adult T-cell leukemias and other leukemias in Japan (13, 14), and, more recently, in T-cell malignancies from many other parts of the world (unpublished data).

Recently, workers in Japan have also shown that a retrovirus is associated with these diseases in southern Japan (15–17), and

we have ascertained that this virus is HTLV (or a closely related strain) (unpublished data). Here we show that HTLV is present in some of the T cells in three independently derived T-cell lines from an infected T-cell lymphoma patient but is not detectable in cultured B cells and other T cells from the same patient.

METHODS

Cell Lines. Several cell lines have been established from a patient (CR) with a highly malignant T-cell lymphoma (diagnosed as mycosis fungoides). HUT-102 is the cell line from which HTLV was first isolated (5). Several clonal sublines of HUT-102 have now been obtained. Two of these (B2 and A9) were used in the current study. All three lines are TCGF-independent. CTCL-3 was established from peripheral blood of patient CR by use of purified TCGF [free of phytohemagglutinin (PHA)] as described (5); it remains dependent on TCGF for growth. CTC-16 was established from peripheral blood cells of patient CR as described (18) with TCGF containing PHA. Because of the presence of PHA it is expected that normal as well as malignant T cells could be present in this culture. This line is composed primarily of T cells with suppressor cell activity as judged by their ability to inhibit immunoglobulin synthesis by pokeweed mitogen-activated B cells in mixed culture. Two previously described (18) sublines (G5 and C6) obtained from CTC-16 were used in this study. All three lines are TCGF-dependent. A B-cell line (CR-B) was established by immortalization of peripheral blood B cells with Epstein-Barr virus strain B-95 (19). All factor-independent cell lines proliferate more rapidly and have higher saturation densities than do the TCGF-dependent cultures.

The T-cell lines and the B-cell line derived from patient CR were cultured as described (4, 5, 18) at a density of 5.0×10^5 /ml in RPMI-1640 medium containing 20% (vol/vol) heat-inactivated fetal calf serum, 4 mM L-glutamine, and 50 units of penicillin and 50 μ g of streptomycin per ml. In order to promote continuous growth of T cells, all TCGF-dependent lines were cultured in the presence of 15–20% (vol/vol) concentrated conditioned medium containing TCGF, obtained from cultures of PHA-stimulated peripheral blood lymphocytes and prepared according to published methods (1, 2). The conditioned medium was concentrated 4-fold by putting polyethylene glycol powder (approximate M_r , 6,000; Sigma) around dialysis tubing containing the conditioned medium. The contents of the tubing were dialyzed against 0.15 M NaCl overnight at 4°C, were passed through a Millipore filter (pore size, 0.45 μ m), and stored at

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Abbreviations: HTLV, human T-cell leukemia-lymphoma virus; TCGF, T-cell growth factor; PHA, phytohemagglutinin; P_i /NaCl, phosphate-buffered saline.

–20°C until used. After 5 or 7 days of culture in the presence of conditioned medium, when the cell concentration had reached about 1×10^6 /ml, the cells were diluted with fresh medium to yield a concentration of 3.0×10^5 /ml. Thereafter, the cultures were fed twice a week and split in a similar manner, usually every 4–7 days.

Clonal derivatives were obtained from HUT-102 and CTC-16 by the limiting dilution method. Cells were pipetted vigorously in order to get a single-cell suspension, and one drop of cell suspension was put into a hemocytometer chamber to confirm the absence of a cell clump consisting of more than two cells. Cell-suspension samples (0.2 ml) at various cell concentrations were put into wells of a flat bottom microtiter plate (Falcon 3040 Microtest II) and cultured in humid 5% CO₂/95% air at 37°C. In order to promote growth, TCGF was used as described above. No cells were used as a feeder layer. Growth of cells was obtained from 10 of 80 wells seeded with an average dose of one cell per well.

Analyses of Viral Nucleic Acids and Proteins. HTLV RNA and proviral DNA sequences were detected by liquid hybridization assays with [³H]cDNA (20×10^6 cpm/ μ g) as described (8). Briefly, cDNA-DNA hybridizations were carried out in 0.6 M NaCl at 65°C to the indicated corrected C₀t with 600 μ g of DNA and 500 cpm of [³H]cDNA in a 20- μ l reaction mixture. RNA-cDNA hybridizations were carried out in 50% (vol/vol) formamide/0.45 M NaCl/0.045 M sodium citrate, pH 7, at 37°C to the indicated C₀t with 400 μ g of RNA in 0.1-ml reaction mixtures. Hybrid formation was monitored by resistance of cDNA to digestion with S1 nuclease as described (8). This probe using these assay conditions can detect one proviral copy or less per eight haploid genomes (unpublished data). Viral core proteins p24 (9) and p19 (20) were assayed by competition radioimmunoassays with homogeneously purified ¹²⁵I-labeled HTLV p24 and rabbit antibody to p24 and by *in situ* immunofluorescence with monoclonal antibody to HTLV p19, respectively, as described.

RESULTS

Characterization of Cell Lines by HLA Typing. It was important to verify the identity of the cell lines used in this study. Consequently, the cultured T- and B-cell lines from patient CR and their clonal derivatives were tested for HLA alloantigens by using the microcytotoxicity technique as described by Amos and Pool (21). One hundred forty antisera were used to detect HLA-A and -B alloantigens. These antisera detect 18 different A-locus specificities and 31 different B-locus specificities. HLA-DR, MB, and MT antigens were analyzed by using 112 antisera. Eight HLA-DR specificities could be detected by these antisera as well as MT1, MB1, MT2, MB2, and MT3. Antisera detecting the HLA-DR, MB1, and MT antigens were absorbed with pooled platelets to remove HLA-A, -B, and -C antibodies. HLA-C antigens were not characterized.

The most common phenotype that could be assigned to these different cell clones was based on the serologic reactions detecting the Aw30, Aw31, B17, and B18 antigens (Table 1). Antisera detecting these four antigenic determinants reacted consistently with all cell lines. Additional reactions were observed with some of the clones. The provisional assignments shown in Table 1 are based on positive reactions with at least two antisera detecting the indicated antigens. Additional antigenic determinants that were detected in these cell lines were A10, A11, A29, Bw35, and Bw50. All five of these antigens appeared in at least three of the cell lines. Other cell lines reacted with antisera in addition to those that detected the primary phenotype and detected the A10, A11, and A29 antigens. Two cell lines

Table 1. HLA typing of cell lines derived from patient CR

Cell line*	HLA-A [†]	HLA-B [†]	HLA-DR and MB [†]
T cells:			
HUT-102	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50)	DR2, DRw6, MB1
B2 (HUT-102)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50), (B12)	DR2, DRw6, MB1
A9 (HUT-102)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50), (B7)	DR2, DRw6, MB1
CTCL-3	Aw30, Aw31 (A10), (A11)	B17, B18 (Bw35)	DR2, DRw6, MB1
G5 (CTC-16)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18	DR2, DRw6, MB1
C6	Aw30, Aw31	B17, B18	DR2, DRw6, MB1
B cells:			
CR-B	Aw30, Aw31	B17, B18	DR2, DRw6, MB1

* Cell lines and derivative clones were established and tested for HLA specificities as described in the text. HUT-102 is a HTLV-producer T-cell line from patient CR (5); B2 and A9 are TCGF-independent cell sublines established from HUT-102; CTCL-3 is a TCGF-dependent cell line established from peripheral blood of patient CR (5); G5 and C6 are TCGF-dependent T-cell sublines established from CTC-16 (18); CR-B is an Epstein-Barr virus-immortalized B-cell line established from the peripheral blood of patient CR. Fresh peripheral blood from patient CR had Aw30 and 31 reactivities for HLA-A and B17 and 18 for HLA-B.

[†] HLA antigens were detected as described in the text. Antigenic subtypes listed in parentheses represent apparent alien histocompatibility antigens. The designations of HLA alloantigens conform to the nomenclature defined by the Eighth International Workshop on Histocompatibility Testing (22).

(T-cell clone C6 and B-cell line CR-B) had no additional reactivity.

Antisera detecting the HLA-DR and MB specificities detected HLA-DR2 and DRw6 and MB1 antigens on all of these cultured T- and B-cell lines, making it extremely probable that all the cell lines tested originated from the same individual. Furthermore, the HLA phenotype of all cell lines used here are consistent with the HLA phenotype of patient CR and with the HLA phenotypes of his parents (unpublished data).

Presence of HTLV in T Cells but Not B Cells. The various T-cell lines, their clonal derivatives, and the B-cell line were analyzed for the presence of HTLV viral core proteins p19 and p24, mRNA, and proviral DNA. These lines were also characterized for surface determinants by using OKT3, -4, and -8 monoclonal antibodies (Ortho). Positive results were observed with p19 immunofluorescence with the HTLV-producing line HUT-102 and its clonal derivatives B2 and A9, both of which also produce HTLV. The majority of the cells in all three cultures expressed p19 and are OKT3⁺, OKT4⁺, OKT8⁺ (Table 2). It has not been possible to grow the T-cell lines CTCL-3 and CTC-16 in sufficient quantities to establish positively that they release extracellular virus. However, they were clearly positive for p19 (15–40% p19-positive cells), indicating that at least some of the cells within these cultures are also infected with HTLV. The majority of the cells in CTC-16 also were OKT3⁺, OKT4⁺, whereas most of the cells in CTCL-3 were OKT3⁺, OKT4⁺. Both cultures were predominantly OKT8⁺. HUT-102 and clones B2 and A9 were also highly positive by radioimmunoas-

Table 2. Distribution of HTLV proteins in cell lines derived from patient CR

Cell line*	p19, [†] % cells positive	p24, [‡] ng/mg protein	OKT3, [§] % cells positive	OKT4, [§] % cells positive	OKT8, [§] % cells positive
HUT-102	+ (60–70)	200	ND	ND	ND
B2 (HUT-102)	+ (90–100)	200	5	90	5
A9 (HUT-102)	+ (90–100)	200	2	70	2
CTCL-3	+ (15–20)	ND	87	94	15
CTC-16	+ (35–40)	ND	29	86	6
C6 (CTC-16)	– (0)	2.7	75	5	2
G5 (CTC-16)	– (0)	2.0	26	5	62
CR-8	– (0)	<0.2	5	5	5
Normal human T-cells	– (0)	<0.2	ND	ND	ND

ND, not done.

* HUT-102, TCGF-independent T-cell line from patient CR producing HTLV (5); B2 and A9, TCGF-independent clonal derivatives of HUT-102; CTCL-3, TCGF-dependent T-cell line from patient CR (5); CTC-16, TCGF-dependent T-cell line from patient CR (18); C6 and G5, clonal derivatives of CTC-16 (18); CR-B, Epstein-Barr virus immortalized B-cell line from patient DR. Normal human T cells of peripheral blood pooled from normal volunteers were purified by nylon chromatography from short-term cultures (72-hr) in the presence of PHA.

[†] A monoclonal antibody to HTLV p19 was used to assay cells for the presence of the p19 antigen by indirect immune fluorescence (20). Briefly, cells fixed with methanol/acetone, 1:1 (vol/vol), were incubated 30 min at room temperature with a 1:400 dilution [in phosphate-buffered saline (P_i/NaCl)] of BALB/c ascites fluid containing the monoclonal anti-p19. After a 30 min wash with P_i/NaCl, an appropriate dilution of fluorescein isothiocyanate-conjugated F(ab')₂ fragment of sheep anti-mouse IgG, which had been passed through a human IgG-Sepharose column to remove crossreacting antibodies, was applied to the cells for 30 min at room temperature. The cells were then washed for 2 hr with P_i/NaCl. In some cases, cells were counterstained with 0.02% Evans blue in P_i/NaCl for 5 min and washed in P_i/NaCl before examination with the fluorescence microscope. Control ascites fluid was obtained from mice inoculated with the IgG-synthesizing BALB/c P3x63 myeloma cell line.

[‡] HTLV-p24 was analyzed by radioimmunoassay as described (9).

[§] Viable cells were incubated with the indicated monoclonal antibody at saturating dilutions for 1 hr at room temperature, washed, and incubated with fluorescein-conjugated goat anti-mouse IgG heavy and light chain for 1 hr at 4°C. The cells were washed and examined for fluorescence on a fluorescence-activated cell sorter (Becton Dickinson) as described (23). The percentage of cells fluorescing was determined on all cells demonstrating a forward light scatter pattern. The OKT monoclonal antibodies and their specificity for subsets of T cells have been described (for a review, see ref. 24).

say for p24 [200 ng/mg of cell protein (Table 2)]. The B-cell line (CR-B), in contrast, expressed no detectable p19 or p24, either with or without treatment with iododeoxyuridine. The CTC-16-derived T-cell sublines C6 and G5 also were negative in the p19 assay. However, low levels of p24 ($\approx 1\%$ of the value for clone B2) were present in these two clones. Most of the cells in these cultures were OKT4⁺. C6 contained mostly OKT3⁺ cells, whereas G5 was predominantly OKT8⁺.

Viral mRNA sequences were readily detectable in both HUT-102 and clone B2. Hybridization kinetics indicate a relatively high viral mRNA content ($\approx 0.2\%$ by weight of total cytoplasmic RNA) (Fig. 1A). The B-cell line from patient CR (CR-B) had no detectable HTLV mRNA, consistent with the protein data. CTC-16 and its clonal derivatives have not yet been tested for viral mRNA because of considerable difficulty in obtaining a sufficient amount of these slow-growing cells.

Proviral DNA was present in both HUT-102 and B2 at two to four copies per haploid genome (Fig. 1B). No proviral sequences were detectable in CR-B, even at C_0t of 1.5×10^5

FIG. 1. HTLV RNA and proviral DNA in cell lines derived from patient CR. (A) HTLV cDNA was hybridized to RNA from B2 (●), CR-B (▲), or PHA-stimulated normal human peripheral blood T lymphocytes (■) prepared as described for Table 2. (B) HTLV cDNA was hybridized to cellular DNA from HUT-102 (○), B2 (●), C6 (□), G5 (Δ), CR-B (▲), or PHA-stimulated normal human peripheral blood T lymphocytes (■).

(corrected), indicating that no proviral sequences were present or they were present at far less than one copy per haploid genome. DNA from CTC-16 derivatives C6 and G5 also were negative for HTLV to a C_0t of 20,000, indicating that these cells contained substantially less than one proviral copy per haploid genome. CTC-16 itself was not tested because of the inability to grow a sufficient amount of cells.

DISCUSSION

Several T-cell lines, some clonal derivatives of these lines, and a B-cell line have been established from a patient (CR) with cutaneous T-cell lymphoma. One of these T-cell lines, as reported (5), produces HTLV. This and the other cell lines described here were identified by HLA typing and analyzed for expression of HTLV antigenic determinants and RNA and the presence of proviral DNA sequences. All the derived cell lines and clones showed identical reactions for two HLA-A and two HLA-B antigens and were identical in this respect to fresh peripheral blood lymphocytes from the patient and consistent with his parental haplotypes. The cell lines were also identical with respect to HLA-DR and MB specificities. It thus is likely that all cell lines used in fact originated from patient CR.

Most of the T-cell lines express a few additional HLA-A and -B antigens. One might ascribe the extra reactions seen with

the antisera detecting the HLA-A and -B antigens to reactivity of undetermined antibodies detecting the DR or associated antigens. This explanation, however, seems unlikely due to the fact that this same pattern was not seen with antisera absorbed with platelets. Platelets do not bear HLA-DR-associated determinants and therefore could not remove antibodies directed toward these antigens. The nature of the antibody detecting these determinants or other cell surface antigens on the cultured cells remains to be determined. It is intriguing to speculate that the cultured cell lines from this patient express an unusual variety of HLA alloantigens. This observation would be in keeping with the reports of additional H-2 antigens found in some murine T-cell lymphomas (25).

Viral proteins (p19 and p24) and mRNA, as well as HTLV proviral DNA, were detected in the TCGF-independent line HUT-102 and in clones B2 and A9. Viral protein was also expressed in CTCL-3, a TCGF-dependent line established from peripheral blood (5), and in CTC-16, an independently derived TCGF-dependent line (18). No viral components were detectable in the B-cell line CR-B. Sublines derived from CTC-16 (G5 and C6) were negative for p19 and proviral DNA, although very low levels of p24 were detected. It therefore is probable that C5 and C6 are not monoclonal and that they contain a small subpopulation of infected cells expressing virus. In keeping with this, the p19 assay is less sensitive than the p24 assay and, if only a few cells were infected, provirus would not be detectable under the conditions used. Thus, the available evidence indicates that not all T cells from patient CR were infected and at least the majority of the B cells appear not to have been infected. Furthermore, the surface phenotype OKT3⁻, OKT4⁺, OKT8⁻ appears to correlate with the presence of HTLV. This may represent a type of T cell that is selectively infected by HTLV. Alternatively, HTLV may be affecting the phenotype of infected T cells. It is worthy of note that the typical phenotype of T cells from Japanese cases of adult T-cell leukemia is OKT4⁺ OKT8⁻ (26).

The presence of HTLV provirus in T but not B lymphocytes and its apparent absence in some of the TCGF-dependent T cells of the same patient indicate that HTLV is not endogenous to the patient of origin—i.e., it is not a hereditary element transmitted in the germ line of his family. Rather, HTLV must have been acquired by some type of infectious event. These results are similar in kind to data of Baxt *et al.* (27) who showed DNA sequences in the leukocytes of a leukemic but not normal member of a pair of identical twins. In that study, however, the [³H]cDNA was prepared from a cellular extract, and the interpretation that the probe represents viral sequences depends primarily on their nonendogenous nature. We have recently identified other HTLV-infected patients whose T cells but not B cells express p19 and p24 (unpublished data). Consistent with our conclusion that HTLV is transmitted by some type of infection rather than through the germ line is the finding of serum antibodies to HTLV core proteins in some patients in the United States with T-cell malignancies (11, 12), in the spouse of patient CR (11), in a vast majority of patients with T-cell leukemias from Japan (13–16), and in other selected cases of T-cell leukemias.

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