Detection and Characterization of Human T-Cell Lymphotropic Virus Type I (HTLV-I) Associated T-Cell Neoplasms in an HTLV-I Nonendemic Region by Polymerase Chain Reaction

By Amy Chadburn, Eleni Athan, Rosemary Wieczorek, and Daniel M. Knowles

Human T-cell lymphotropic virus type I (HTLV-I) associated adult T-cell leukemia/lymphoma (ATLL) occurs endemically in southwestern Japan, the Caribbean, and West Africa, but occurs sporadically in most of the rest of the world. However, because ATLL and non-HTLV-I associated T-cell neoplasms share overlapping clinicopathologic features, the prevalence of ATLL in nonendemic regions is unknown. In this study, 75 T-cell neoplasms randomly procured from the metropolitan New York City area were examined by polymerase chain reaction (PCR) for the presence of integrated HTLV-I proviral sequences. HTLV-I genomic sequences were detected by PCR in 8 of the 75 cases (8%); this result was confirmed by Southern blot hybridization. The clinicopathologic features of the HTLV-I positive and HTLV-I negative T-cell neoplasms were then compared. Although the clinicopathologic features of patients from these two groups overlapped, some findings were more commonly associated with HTLV-I positive neoplasms. Five of the six patients with HTLV-I positive ATLL are elderly white men. The incidence of hypercalcemia and lytic bone lesions was significantly more common among patients with HTLV-I positive T-cell neoplasms (P < .001 and P = .004, respectively). The immunophenotypes of the HTLV-I positive and negative tumors were similar; however, all HTLV-I positive neoplasms were CD7 negative (P < .001). In summary, our findings: (1) demonstrate the special clinicopathologic and immunophenotypic features of HTLV-I positive T-cell neoplasms, (2) suggest that most of the rare cases of HTLV-I-associated T-cell neoplasms occurring in HTLV-I nonendemic areas are actually endemic cases; and (3) that PCR is a sensitive, clinically useful technique for identifying HTLV-I associated T-cell neoplasms.

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A DULT T-CELL leukemia/lymphoma (ATLL) is an aggressive post-thymic T-cell neoplasm with distinct clinicopathologic features that is pathogenetically associated with the human T-cell lymphotropic virus type I (HTLV-I). An important etiologic role for HTLV-I in the development of ATLL has been suggested by (1) the significantly higher incidence of ATLL in HTLV-I endemic regions, i.e., southwestern Japan, the Caribbean basin, and West Africa; (2) the presence of serum antibodies to HTLV-I in more than 90% of patients with a T-cell neoplasm having the clinical and morphologic features of ATLL; (3) the transformation of normal T cells in vitro by HTLV-I isolates from cultured ATLL tumor cells; and (4) the monoclonal or oligoclonal integration of HTLV-I in neoplastic T cells isolated from individuals with ATLL.

ATLL is characterized by generalized lymph node, peripheral blood and/or skin involvement by pleomorphic tumor cells with hyperlobated nuclei, lytic bone lesions, hypercalcemia, a rapidly progressive course, and a relatively short survival. However, some post-thymic T-cell neoplasms unassociated with HTLV-I infection exhibit some of these same characteristics. Therefore, the clinicopathologic features of HTLV-I positive and negative T-cell neoplasms may be sufficient to overlapping to hamper the diagnosis of ATLL in an individual case, particularly in regions where HTLV-I is not endemic and HTLV-I seropositive individuals are infrequently detected. Furthermore, HTLV-I infected individuals who remain asymptomatic or have malignancies other than ATLL may be HTLV-I seropositive while some individuals with ATLL may be HTLV-I seronegative. Therefore, the true incidence of ATLL, especially in HTLV-I nonendemic regions such as the continental United States, is presently unknown. However, the diagnosis of ATLL can be definitively established by detecting integrated HTLV-I proviral sequences in the malignant T cells by Southern blot hybridization analysis. In addition, it has been recently shown that specific HTLV-I DNA sequences are detectable in the tumor tissues of both HTLV-I seropositive and seronegative patients with the clinical and morphologic features of ATLL using the highly sensitive enzymatic in vitro gene amplification technique, polymerase chain reaction (PCR).

We investigated T-cell malignancies randomly collected from metropolitan New York City for the presence of specific HTLV-I sequences by PCR to unequivocally identify cases of HTLV-I associated T-cell neoplasia occurring in an HTLV-I nonendemic region. We identified six cases of HTLV-I associated T-cell neoplasia from among a diverse panel of 75 clinicopathologically and immunophenotypically well characterized T-cell malignancies by PCR and demonstrated clonal integration of HTLV-I DNA in the tumor cells in all six cases by Southern blotting. We subsequently performed a comparative analysis of the epidemiologic, serologic, clinical, histopathologic, and immunophenotypic characteristics of the HTLV-I positive and HTLV-I negative T-cell neoplasms. The results allowed us to determine the approximate incidence of HTLV-I
associated T-cell neoplasia in an HTLV-I nonendemic region of the continental United States and better define the clinicopathologic features of T-cell neoplasms unequivocally associated with HTLV-I infection.

MATERIALS AND METHODS

Patients and pathologic specimens. A panel of 75 well-characterized T-cell neoplasms, 7 precursor T-cell acute lymphoblastic leukemias and lymphomas (ALL/LBL), 13 T-cell chronic lymphocytic leukemias (CLL), 27 cutaneous T-cell lymphomas (CTCL) (20 mycosis fungoides [MF] and 7 non-MF), and 28 peripheral T-cell lymphomas (PTCL), was selected from among cases routinely processed in the surgical pathology laboratories of the Columbia Presbyterian and New York University Medical Centers between 1982 and 1989. The cases were selected without prior knowledge of the clinical, histopathologic, immunophenotypic, or genotypic findings, on the basis of having adequate numbers of cryopreserved cells or tissue blocks to perform the studies described below. After the molecular genetic investigations were performed, clinical information, including age, sex, race, birthplace, clinical presentation, physical findings, laboratory data, and outcome, was obtained from medical records and/or referring physicians.

Histopathology. Portions of each pathologic specimen studied by PCR were routinely fixed in formalin, B5 or Bouin's, embedded in paraffin, and hematoxylin and eosin (H&E) stained sections and rendered diagnoses according to the working formulation of non-Hodgkin's lymphoma. All additional relevant neoplasms had been determined at the time of diagnosis by biopsy specimens were also reviewed even though these tissues might not have been subjected to molecular genetic analysis by PCR.

Immunophenotypic analysis. The immunophenotype of the T-cell neoplasms had been determined at the time of diagnosis by immunohistochemical staining of cryostat tissue sections using a three step avidin-biotin immunoperoxidase technique or an immunoenzyme phosphorylated anti-alkaline phosphatase method, and/or by direct and indirect immunofluorescent flow cytometry of isolated cells in suspension using the FACSfluorescent activated cell sorter (Becton Dickinson, Mountain View, CA). Indirect immunofluorescent staining for terminal deoxynucleotidyl transferase (TdT) was performed according to the manufacturer's recommendations (Supertechs, Bethesda, MD). Monoclonal antibodies (MoAbs) used to immunophenotype the T-cell neoplasms included OKT3 (CD3), OKT4 (CD4), OKT6 (CD7), OKT8 (CD8), OKT9 (CD26), and OKT11 (CD2). The HUT-102 and HMO cell lines were used as positive and negative sources of genomic DNA, respectively. Positive and negative DNA controls were run for each amplification; the reagent control included all reaction compounds with the exception of template DNA. All of the samples were also screened with primers prepared from the first exon of the c-myc oncogene (5′ primer [sense]: GCACCTGGAATACATCATACGACC; 3′ primer [antisense]: GGTGCTTTACGCTTACATTTCA), which amplified the 135-bp fragment spanning the sequences 2738 to 2871. All DNAs giving a positive control amplification with primers specific for c-myc were examined for Southern blot hybridization analysis. One microgram of DNA (equivalent to approximately 150,000 cells) and 20 pmol (at a concentration of 0.4 μmol/L) of each of the primer pairs were added to a 50-μL reaction volume consisting of 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 2.5 mmol/L MgCl2, 100 μmol/L each dNTP (dATP, dCTP, dGTP, and dTTP), gelatin 0.2% (wt/vol), and 1.5 units of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Cetus Corporation, Norwalk, CT) for each PCR as previously described. Thirty-five cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 60 seconds, extension at 72°C for 90 seconds, and, after the last cycle, extension at 72°C for 7 minutes were performed in a Perkin-Elmer Cetus Thermal Cycler (Perkin-Elmer Cetus Corporation). Thereafter, PCR products were extracted with chloroform. All reaction products were fractionated by agarose gel electrophoresis, visualized under UV by ethidium bromide fluorescence, and then assayed by slot-blot or Southern blot hybridization analysis with specific probes spanning the region between the primer pairs. Five-microliter aliquots of the PCR mixtures were used for slot blot analysis. Briefly, the PCR product was denatured in NaOH, transferred to a nylon filter with a slot-blot manifold (Schleicher and Schuell, Keene, NH), and hybridized to an oligomer probe. The 5-μL aliquots of each PCR product analyzed by Southern blotting were electrophoresed on a 3% NuSieve-1% Seakem agarose composite gel in Tris acetate (TEA) buffer, denatured, neutralized, transferred to a nylon filter (Genescreen Plus, New England Nuclear, Wilmington, DE) and hybridized to an oligomer probe. Southern blot or slot-blot filters were prehybridized at 55°C for 30 minutes and hybridized with a 32P end-labeled probe for 1 hour in a solution consisting of 3 mol/L tetramethyl ammonium chloride salt (TMAC), 1% sodium dodecyl sulfate (SDS), 50 mmol/L Tris (pH 7.5), 2 mmol/L EDTA (pH 8.0), 1X Denhardt's solution, and 10 mg/mL salmon sperm DNA. Filters were washed in 2X standard sodium citrate (SSC)/1% SDS for 10 minutes, in 3 mol/L TMAC/1% SDS for 5 minutes at room temperature, once in 3 mol/L TMAC/1% SDS for 1 hour at 60°C and rinsed briefly with 2X SSC. Autoradiography was performed overnight at −70°C with double intensifying screens.

The oligonucleotide probes used for hybridization were end-labeled with γ-32P ATP (specific activity, 3000 Ci/mmol) by means of T4-polydeoxyribonuclease kinase (New England Bio Labs, Boston, MA), and purified through a Bio-Gel P4 fine column (Bio-Rad Laboratories, Richmond, CA). The specific activities of the kinased probes were between 1.5 and 3 Ci/pmol.

The oligonucleotide primers and probes (Table 1) were synthesized on published DNA sequences and have been previously shown to be HTLV-I type-specific (oligonucleotides of the long terminal repeat [LTR], gag, pol, and env regions) or HTLV-I group specific (oligonucleotide probes for the tax region) or HTLV-I terminal repeat [LTR], gag, pol, and env regions) or HTLV-I group specific (oligonucleotide probes for the tax region) or HTLV-I terminal repeat [LTR], gag, pol, and env regions) or HTLV-I group specific (oligonucleotide probes for the tax region). The HUT-102 and HL60 cell lines were used as positive and negative sources of HTLV-I, respectively. Positive and negative DNA controls were run for each amplification; the reagent control included all reaction compounds with the exception of template DNA. All of the samples were also screened with primers prepared from the first exon of the c-myc oncogene (5′ primer [sense]: GCACCTGGAATACATCATACGACC; 3′ primer [antisense]: GGTGCTTTACGCTTACATTTCA), which amplified the 135-bp fragment spanning the sequences 2738 to 2871. All DNAs giving a positive control amplification with primers specific for c-myc were examined for each of the five HTLV-I regions individually.

Southern blot hybridization analysis. Fifteen-microliter aliquots of genomic DNA were digested with the appropriate restriction endonucleases according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN), electrophoresed in 0.8% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern. The filters were hybridized in 50% formamide/3X SSC at 37°C, washed in 0.2X...
Table 1. Specific and Generic Primer Pairs and Probes for Amplification and Detection of HTLV-I

<table>
<thead>
<tr>
<th>Primer Type Orientation</th>
<th>Primer Type and Orientation</th>
<th>Probe Type and Orientation</th>
<th>Region</th>
<th>Position</th>
<th>Size of Amplified Product</th>
<th>Sequence (5' → 3')</th>
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<td>SG 160</td>
<td>Specific -</td>
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<td>415-376 I</td>
<td></td>
<td>TCAAGGTAGGCGGCGGGCCTGAAAGGAGATACGAGCCC</td>
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<tr>
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<td>LTR</td>
<td>768-745 I</td>
<td>569 bp</td>
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<tr>
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<td>Specific -</td>
<td>gag</td>
<td>1398-1411 I</td>
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<td>CTCGCACTACCTTGCTCCTCCTC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>gag</td>
<td>1640-1601 I</td>
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<td>GCTCTCAGAGCCTTGAGAGAGAGGAGCCCAGAAGGTC</td>
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<tr>
<td>Specific -</td>
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<tr>
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<td>Specific -</td>
<td>pol</td>
<td>3365-3384 I</td>
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<td>3465-3483 I</td>
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<tr>
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<td>Specific -</td>
<td>env</td>
<td>5796-5818 I</td>
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<tr>
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<td></td>
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SSC/0.5% SDS at 60°C for 2 hours, and autoradiographed at -70°C for 16 to 24 hours, as previously described. The presence of HTLV-I sequences was determined by hybridization of Pst I-digested DNAs to a 3-kb DNA fragment containing sequences of the pol and env region. The T-cell receptor β chain (TCRβ) gene was investigated by hybridization of EcoRI- and BamHI-digested DNAs to a DNA probe that hybridizes to the constant region of the TCRβ gene. DNA fragments were labeled with α-32P dCTP by the random priming technique for use as probes.

Immunodiagnostic assays. The presence of serum antibodies to HTLV-I was determined by a modified enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plate wells (Nunc, Kamstrup, Denmark) were coated with purified HTLV-I virus (Pan Data System, Rockville, MD) at a concentration of 1.25 μg/mL in 0.1 M bicarbonate buffer (pH 9.6) overnight at room temperature. After washing the plates three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the plates were incubated for 2 hours at room temperature and washed with Tween three times as before. One hundred microliters of affinity purified peroxidase conjugated F(ab')2 goat antihuman IgG (Cappel, Durham, NC; 1:1,000) was added to each well and incubated for 1 hour at room temperature. After washing, 100 μL of substrate solution containing 0.1 mg/mL of O-phenylenediamine (Sigma) was added. The color reaction was stopped with hydrogen sulfate and the optical density was read at 490 nm. Serum from a known HTLV-I seropositive patient was used as a positive control. The presence of HTLV-I antibodies was confirmed by Western blot analysis.

RESULTS

PCR amplification analysis. DNA prepared from each of the 75 patient specimens was subjected to PCR analysis. The oligonucleotides used to direct amplification and detection of HTLV-I genomic sequences and the lengths of the amplified products are summarized in Table 1. Oligonucleotides that recognize unique HTLV-I sequences from the LTR, gag, pol, and env gene regions have been repeatedly shown to be HTLV-I 'type-specific' and can be used to unambiguously identify HTLV-I genomic sequences in patients who are simultaneously infected with two or more human lymphotropic retroviruses. The DNA fragments were labeled with α-32P dCTP by the random priming technique for use as probes.

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neoplasms was amplified with primer pairs of the env, gag, tax, and 5’ LTR regions of the HTLV-I genome. Hybridization analysis of Southern blots (data not shown) and slot blots (Fig 2) with the appropriate end-labeled oligonucleotides showed that sequences specific to each of these viral genes were present. Therefore, each of these major regions, comprising approximately 30% of the HTLV-I proviral genome, was present in each of the six HTLV-I positive T-cell neoplasms. This finding suggests that the entire HTLV-I genome was probably integrated into each of these six tumors. None of the DNAs from the remaining 69 cases showed hybridization signals to any of these five HTLV-I gene regions. These results indicate the absence of defective or partial HTLV-I genomes or any other retroviruses distinct from, but related to, HTLV-I or HTLV-II among the T-cell neoplasms studied here.

Southern blot hybridization analysis. Unamplified DNA from each of the six T-cell neoplasms found to be HTLV-I positive by PCR was also subjected to Southern blot hybridization analysis to examine the clonal integration pattern of HTLV-I proviral sequences in the cases. The DNAs were digested with restriction endonuclease Pst I, which cuts within the proviral genome, and blot hybridized with a 32P-labeled HTLV-I pol-env probe that detects a 2.6-kb internal viral fragment. As shown in Fig 3, the viral Pst I fragment was present in each of the six DNAs, indicating that all six cases contained the HTLV-I proviral genome, confirming the results obtained by PCR. The additional bands present in each of these samples represent junction fragments of viral and cellular flanking sequences arising from the integration of a proviral copy at a particular integration site. In addition, each of these six HTLV-I positive cases exhibited clear and distinct clonal TCRβ gene rearrangements (Fig 4), indicating the presence of a large clonal T-cell population.

Immunodiagnostic assays. Serum samples from three of the six patients positive for HTLV-I by PCR were available for testing. All three serum samples were found to contain antibodies to HTLV-I by ELISA and these results were confirmed by Western blotting. Serum samples obtained from five of the 69 patients negative for HTLV-I by PCR analysis were available for testing. None of these five samples contained evidence of serum antibodies to HTLV-I by ELISA or Western blotting.

Clinical characteristics. Complete clinical information was available on five of the six patients with HTLV-I positive T-cell neoplasms (Table 2). The sixth patient was a black woman of unknown origin, approximately 40 years of age, who was discovered comatose on the street and died without regaining consciousness 17 days later. Five of the six patients, all women, were 33 to 45 years old; the only man was 88 years old. The median age was 43 years. Four women and the one man were black and one woman was Hispanic. The five patients whose birthplace was known were born in HTLV-I endemic regions, ie, Jamaica, Trinidad, Santa Domingo, Ghana, and Guyana. None had been born in the United States.

Five of the six patients with HTLV-I positive T-cell neoplasms presented with stage III disease; the sixth
patient presented with stage IV disease. All six patients developed generalized lymphadenopathy during the course of their illness. Four patients (67%) had peripheral blood involvement by leukemic cells with white blood cell counts ranging from 25,000 to 68,600 mm$^{-3}$; two patients had bone marrow and skin involvement; three and two patients had liver and spleen involvement, respectively; four had hypercalcemia, with serum calcium levels up to 21.2 mg/dL; and two had lytic bone lesions. Five of the six HTLV-I positive patients died between 15 days and 17 months (median survival 6 months) following diagnosis. The sixth patient is alive, with pulmonary lymphoma, 15 months after presentation.

Clinical information for 55 of the 69 patients with HTLV-I negative T-cell neoplasms was available. Comparison of the epidemiologic features of the patients with HTLV-I positive and negative T-cell neoplasms highlighted several differences (Table 3). The median age of the HTLV-I positive and negative patients was 43 and 60 years, respectively. If children are excluded, the median age of the HTLV-I negative patients was 64, a difference of approximately 20 years. The male to female ratio of the HTLV-I positive and negative patients was 1 to 5 and more than 2 to 1, respectively. All six patients with HTLV-I positive, but only one-third of those with HTLV-I negative, T-cell neoplasms were black or Hispanic. All HTLV-I positive patients came from HTLV-I endemic regions while only eight (16%) of the HTLV-I negative patients were born in HTLV-I endemic regions. These eight patients had emigrated to New York from Cuba (two), Trinidad (one), Jamaica (one), the Ivory Coast (one), and the southeastern United States (North Carolina, two). Of the remaining
HTLV-I negative patients, 33 were born in the continental United States, three in Russia, two in Italy, and one in Mexico.

Comparisons of the clinical features of the patients with HTLV-I positive and negative T-cell neoplasms failed to show any significant differences between these two groups with respect to the extent of disease (Table 3). However, the difference in the incidence of hypercalcemia and lytic bone lesions was statistically significant. Sixty-seven percent of the HTLV-I negative patients had hypercalcemia (two patients; 4%) and/or lytic bone lesions (2 patients; 4%) and 40% of the HTLV-I positive patients, but only three of the HTLV-I negative patients had hypercalcemia (two patients; 4%) and/or lytic bone lesions (2 patients; 4%) (P < .001 and P = .004, respectively; Table 3). A 34-year-old black man from West Africa was the only HTLV-I negative individual with both hypercalcemia (21.5 mg/dL) and lytic bone lesions.

Five of the six (83%) HTLV-I positive patients have died with a median survival of only 6 months. In contrast, 28 of 46 patients (61%) with HTLV-I negative T-cell neoplasms (with adequate follow-up) have died with a median survival of 13 months. Eighteen HTLV-I negative patients are alive, 11 (24%) without evidence of disease, at a median of 17 months after diagnosis.

The extent of disease of the HTLV-I positive patients was also compared with that of the HTLV-I negative patients with PTCLs because all the HTLV-I positive cases had been classified as PTCLs, and because the only HTLV-I negative patients with hypercalcemia and lytic bone lesions had PTCL. All six patients with HTLV-I positive neoplasms and 15 of the 19 (79%) patients with HTLV-I negative PTCLs presented with stage III or IV disease. Comparison of the extent of disease during the course of their illness showed no significant differences between the two groups with respect to malignant involvement of lymph nodes, bone marrow, skin, liver, or spleen. However, four of the six (67%) patients with HTLV-I positive neoplasms compared with only two of 19 (11%) patients with HTLV-I negative PTCLs had circulating malignant cells (P = .01). Similar percentages of HTLV-I positive (83%) and HTLV-I negative (79%) patients with PTCLs have died. However, the median survival of the patients with HTLV-I negative PTCLs was 11 months, almost twice the 6-month median survival of patients with HTLV-I positive neoplasms.

Histopathology. The histopathology of the lymph node biopsies obtained from five patients with HTLV-I positive T-cell neoplasms showed diffuse effacement by neoplastic lymphoid cells. In one case the malignant cells had scant cytoplasm and variably sized, primarily round nuclei containing one or more small nucleoli. Numerous mitotic figures were present. This case was classified as a diffuse large cell lymphoma. One patient had two lymph node biopsies, 1 year apart. The first biopsy showed clusters of large atypical lymphoid cells infiltrating fibroconnective tissue. Many of the cells had irregular nuclei containing dispersed chromatin and small nucleoli. This patient's second biopsy and the lymph node biopsies from three other patients were morphologically similar. The neoplastic cells had relatively large nuclei with prominent nucleoli, some centrally placed and some adjacent to the nuclear membrane, and moderately abundant clear cytoplasm. Numerous mitoses were identified. Two lymph nodes had a starry sky pattern. These four lymph nodes were classified as diffuse large cell immunoblastic lymphoma. The sixth patient had only a lung biopsy. The lymphoma had a multifocal distribution and consisted of a
polymorphous population of large atypical cells and small irregularly shaped lymphocytes surrounding and infiltrating blood vessels, reminiscent of lymphomatoid granulomatosis. This case was classified as an angiocentric diffuse, mixed small and large lymphoma.

Immunophenotypic analysis. All six HTLV-I positive T-cell neoplasms expressed CD5, five expressed CD2, and three expressed CD3. Interestingly, none expressed CD7 (Table 4). Four of the cases (67%) expressed the helper subset phenotype, CD4+CD8−, and the other two (33%) coexpressed CD4 and CD8. They also expressed a variety of T-cell activation-associated antigens, including CD71 (T9; 100%), CD25 (IL-2R; 83%), CD38 (T10; 80%), HLA-DR (60%), CD30 (BerH-2; 40%), and CD11c (LeuM5; 20%) (Table 4).

Comparison of the immunophenotypes of the HTLV-I positive and HTLV-I negative T-cell neoplasms showed no striking differences between the two groups in CD2, CD5, or CD8 expression. However, 75% of the HTLV-I negative, but none of the HTLV-I positive, T-cell neoplasms expressed CD7 (Table 4). In addition, CD3 was expressed by nearly 90% of the HTLV-I negative, but by only 50% of the HTLV-I positive T-cell neoplasms (P = .01). Furthermore, while not statistically significant, all HTLV-I positive, but only 67% of the HTLV-I negative, T-cell neoplasms expressed CD4. The T-cell activation associated antigen profiles of the HTLV-I positive and HTLV-I negative neoplasms were similar, except for HLA-DR, which was expressed by only two of six (33%) HTLV-I positive cases compared with 37 of 55 (67%) HTLV-I negative neoplasms (Table 4).

Only one patient with an HTLV-I negative T-cell neoplasm exhibited epidemiologic, clinical, histologic, and immunophenotypic characteristics similar to those of the HTLV-I positive cases. This was a 34-year-old black man from West Africa who presented with hypercalcemia, increased osteoclasts on bone marrow biopsy, diffuse lymphadenopathy, a peripheral white blood cell count of 110,000/mm³, neoplastic cells in the bone marrow, and hepatomegaly. He had a rapidly downhill course and died 8 days after admission. Immunophenotypic analysis demonstrated that the malignant cells expressed CD2, CD3, CD4, CD5, CD71, CD38, CD25, CD30, and HLA-DR, but not CD7, CD8, nor CD11c. However, his malignant T cells were negative for HTLV-I by Southern blotting using unamplified DNA extracted from both his peripheral blood and lymph node and by slot-blot analysis of PCR-amplified products using all five sets of primers and probes.

DISCUSSION

The human retrovirus, HTLV-I, has been linked pathogenetically to the development of postthymic T-cell neoplasms termed ATLL.15 Patients with ATLL frequently exhibit certain clinicopathologic features, including hypercalcemia, lytic bone lesions, hyperlobated leukemic cells, and widely disseminated disease, which are said to be characteristic.9,10,14-16 However, these features cannot always be used as unequivocal diagnostic criteria for HTLV-I associated T-cell neoplasms, especially in nonendemic areas, because patients with T-cell neoplasms unassociated with HTLV-I infection may exhibit some or all of these same features.1,17-20,45 Serologic studies are capable of identifying individuals infected with HTLV-I.18-12 However, a T-cell neoplasm arising in an HTLV-I seropositive individual is not necessarily pathogenetically related to HTLV-I infection.4,45 Furthermore, HTLV-I seronegativity does not unequivocally eliminate HTLV-I as the etiologic agent of a T-cell neoplasm because integrated HTLV-I proviral sequences have been identified in the neoplastic T cells of some HTLV-I seronegative patients.22,23,25 Therefore, clinical and serologic criteria may not be sufficiently sensitive or specific to identify and separate those T-cell neoplasms associated from those unassociated with HTLV-I.

The detection of proviral DNA integrated in the host tumor cell genome and identification of virally encoded proteins are the most rigorous methods currently available for establishing a pathogenetic relationship between viral infection and malignancy.24,25 The detection of integrated HTLV-I proviral genome in the DNA of the malignant cells of patients with ATLL is strong presumptive evidence of a pathogenic role for HTLV-I in the development of ATLL.24,25,26 Therefore, while the clinical expression of the disease is diverse, the molecular definition is precise. Two molecular analytical approaches that can be used to detect integrated HTLV-I proviral sequences are Southern blot hybridization analysis and PCR.

In this study we used PCR to identify HTLV-I associated T-cell malignancies in a nonendemic area of the continental United States. We found that six of the 75 T-cell neoplasms (8%) examined contained HTLV-I genomic sequences detectable by PCR. This result was confirmed by Southern blot hybridization analysis, which detected clonal integration of the HTLV-I proviral genome in the neoplastic T cells (Figs 1 and 3). All six cases had clonal TCRβ rearrangements, establishing them as T-cell neoplasms.

Using the presence of integrated HTLV-I proviral sequences within tumor cell DNA as the definition of an HTLV-I associated T-cell neoplasm, the epidemiologic, clinical, serologic, and immunophenotypic characteristics

**Table 4. Antigen Expression by the HTLV-I Positive and Negative T-Cell Neoplasms**

<table>
<thead>
<tr>
<th>CD No.</th>
<th>Antibody</th>
<th>HTLV-I Positive</th>
<th></th>
<th>HTLV-I Negative</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>T11</td>
<td>5/6</td>
<td>83</td>
<td>49/53</td>
<td>92</td>
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<td>T4</td>
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<td>100</td>
<td>38/56</td>
<td>68</td>
</tr>
<tr>
<td>CD5</td>
<td>Leu1</td>
<td>6/6</td>
<td>100</td>
<td>50/55</td>
<td>91</td>
</tr>
<tr>
<td>CD7</td>
<td>Leu9</td>
<td>0/6</td>
<td>0</td>
<td>40/53</td>
<td>75</td>
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<tr>
<td>CD8</td>
<td>T8</td>
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<td>33</td>
<td>15/56</td>
<td>34</td>
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<tr>
<td>CD71</td>
<td>T9</td>
<td>6/6</td>
<td>100</td>
<td>42/52</td>
<td>81</td>
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<tr>
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<td>T10</td>
<td>4/5</td>
<td>80</td>
<td>33/53</td>
<td>62</td>
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<tr>
<td>CD25</td>
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<td>4/6</td>
<td>67</td>
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<tr>
<td>CD30</td>
<td>Ki-1</td>
<td>1/5</td>
<td>20</td>
<td>18/50</td>
<td>36</td>
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<tr>
<td>CD11</td>
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<tr>
<td>HLA-DR</td>
<td>2/6</td>
<td>33</td>
<td>37/55</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>
of the HTLV-I positive and negative T-cell neoplasms were compared and contrasted. Some epidemiologic and clinical features, while not diagnostic, were closely associated with the diagnosis of HTLV-I positive T-cell malignancy (Table 2). Five of the six patients with HTLV-I positive T-cell neoplasms were female, five were less than 45 years of age, five were black, and five were known to have been born in HTLV-I endemic areas. In addition, a significantly higher percentage of patients with HTLV-I positive than negative T-cell neoplasms had lytic bone lesions ($P = .004$). In contrast, nearly twice as many men as women, 64% of whom were white and 86% of whom were born in HTLV-I nonendemic regions, developed HTLV-I negative T-cell malignancies. In addition, four of the six patients (67%) with HTLV-I positive but only two of 47 (4%) patients with HTLV-I negative T-cell neoplasms developed hypercalcemia ($P < .001$). These results confirm some findings already reported in the literature concerning clinical and epidemiologic differences between HTLV-I associated and unassociated T-cell neoplasms. For example, (1) if Japanese patients are excluded, 90% of the literature patients with ATLL are black, as in this study; (2) other studies place the median age of patients with ATLL at 49 years, which, as in this report, is less than that of individuals with other T-cell malignancies; (3) many of the previously described patients with HTLV-I associated T-cell neoplasms developed hypercalcemia (45%) and lytic bone lesions (24%), in this study, and 24%, as in this study. Patients with T-cell neoplasms unassociated with HTLV-I only rarely have hypercalcemia (3%) or lytic bone lesions (3%).

The extent of disease in the patients with HTLV-I positive and negative T-cell neoplasms in this series was not significantly different (Table 3). While the percentages of our six patients with HTLV-I associated T-cell neoplasms who had circulating malignant cells, lymphadenopathy, skin lesions, and liver and spleen involvement mirrored those described in the literature for other ATLL patients, fewer of our patients (17%) than those described in the literature (40%) had malignant skin infiltrates. Furthermore, approximately 90% of the literature cases of ATLL have been reported to have stage IV disease. In contrast, five of our six patients presented with stage III disease, although three eventually developed stage IV disease.

All three patients with HTLV-I positive T-cell neoplasms who had been tested for serum anti-HTLV-I antibodies were seropositive. Two more patients were suspected of having ATLL but serologic studies were not performed. The remaining patient, the 88-year-old black man, was never suspected of having an HTLV-I associated T-cell neoplasm and, consequently, his serum was not examined. Thus, based on epidemiologic, clinical, and serologic findings, one of our six HTLV-I positive T-cell neoplasms was missed originally.

Four of the six patients with HTLV-I positive T-cell neoplasms had diffuse large cell immunoblastic lymphomas, one had a diffuse large cell lymphoma, and one had a diffuse mixed small and large cell lymphoma with angiocentric features. A variety of histologic patterns have been described in ATLL. However, the histologic features of HTLV-I positive and HTLV-I negative T-cell lymphomas overlap and, therefore, cannot be used to reliably separate ATLL from other T-cell neoplasms.

Immunophenotypic analysis showed that the majority of the HTLV-I positive and negative T-cell neoplasms studied here expressed CD2, CD3, CD4, and CD5 and that about one-third expressed CD8. However, none of the HTLV-I positive but 75% of the HTLV-I negative T-cell neoplasms expressed CD7 ($P < .001$). Similarly, none of the literature cases of ATLL expressed CD7 while approximately 50% of the literature HTLV-I negative T-cell neoplasms express CD7. The reason for the consistent absence of CD7 from malignant ATLL cells is unknown. Even though HTLV-I infects many cell types, it appears that only T lymphocytes undergo malignant transformation. Perhaps HTLV-I selectively infects and transforms only CD7 antigen-negative T lymphocytes. Interestingly, chromosome 17, which carries the genetic material for the CD7 antigen, also codes for the receptor used by HTLV-I to enter a cell. It is possible that deletion and/or mutation of the region of the CD7 genome allows for the production of the HTLV-I receptor. Alternatively, HTLV-I infection may induce loss of the CD7 antigen, because very weak CD7 expression has been detected on HTLV-I positive T-cell lines after 3 to 12 months in culture. This relationship between CD7 and HTLV-I may also explain the well-known suppressor function of HTLV-I infected CD4-positive malignant T cells, because CD7-negative, CD4-positive normal T cells also function as suppressor cells. However, additional studies are necessary to delineate the relationships between CD7, the HTLV-I receptor, and HTLV-I infection.

Activation associated antigen expression by the HTLV-I positive and negative T-cell neoplasms identified here was not significantly different, except for the expression of HLA-DR. Nearly twice as many HTLV-I negative as HTLV-I positive T-cell neoplasms expressed HLA-DR. This finding parallels those previously described, where over 70% of the HTLV-I negative cases expressed HLA-DR compared with only approximately 30% of the ATLLs. CD25 (IL-2R) was expressed by more than 60% of the HTLV-I associated T-cell malignancies in this study and in previous reports. However, CD25 expression by HTLV-I negative T-cell neoplasms here and in other studies was somewhat less, 44% and 23%, respectively. One patient in this study with an HTLV-I negative T-cell neoplasm by both Southern blot hybridization analysis and PCR displayed clinical, epidemiologic, histologic, and immunophenotypic features closely paralleling those of the six HTLV-I positive cases. Other patients who have had the clinical and epidemiologic features of ATLL, but who were seronegative for anti-HTLV-I antibodies and/or did not have integrated HTLV-I viral sequences in the malignant T cells by Southern blotting, have been described in the literature. It has been suggested that the tumor DNAs from these HTLV-I negative patients with ATLL-like
neoplasms contain only very small fragments of the HTLV-I provirus that are not detectable by Southern blot hybridization. However, it is unlikely, using PCR to amplify five regions of the HTLV-I proviral genome as well as Southern blotting, that small fragments or small copy numbers of HTLV-I would not be detected. It is possible that our HTLV-I negative patient with an ATLL-like syndrome had been infected with HTLV-I in the past, but, like approximately 25% of cases of feline leukemia, deleted the proviral genome at some point in time, or that infection with another virus distinct from HTLV-I induced clinical and pathologic features identical to that of ATLL. Alternatively, ATLL may be analogous to Burkitt’s lymphoma. Endemic (African) Burkitt’s lymphoma is related to Epstein-Barr virus (EBV) infection, with many of the patients having high titer of serum antibodies to EBV and EBV viral genome integrated within the DNA of the malignant cells, while the less common sporadic (American) Burkitt’s lymphoma, in most cases, is not. Therefore, it is possible that there are two types of ATLL, clinically and pathologically indistinct, differing in that one is associated with infection by HTLV-I and the other is not. Obviously, further studies are necessary to investigate these hypotheses.

Historically, the most definitive way to prove that an infectious agent is pathogenetically related to a specific disease is isolation, purification, and identification of that pathogen from patient tissue. However, this can be extremely difficult, especially with retroviruses such as HTLV-I. An alternative method of associating retroviral infection with a disease is by identifying serum anti-viral antibodies. However, seropositivity does not always prove or disprove a pathogenetic relationship between an infectious agent and a specific disease process. Furthermore, ELISA tests fail to identify 10% to 30% of all HTLV-I infected patients with ATLL, possibly due to the immunodeficient state evoked by the retroviral infection itself. ELISA tests may also lack specificity because only 10 of 68 patients (15%) seropositive for HTLV-I by ELISA were confirmed to be positive by other methods. Furthermore, unless there is clinical suspicion for HTLV-I infection, the serum of patients with T-cell malignancies usually is not examined for anti-HTLV-I antibodies, as in one of our patients.

However, molecular techniques can be used to unequivocally establish a pathogenetic relationship between an infectious agent, such as HTLV-I, and a disease, such as ATLL, by identifying integrated proviral sequences within the DNA from diseased patient tissue. Furthermore, clonal integration of the HTLV-I provirus indicates the malignant nature of the T-cell population. Because only one or two copies of the proviral genome is present within each infected cell and the number of infected cells may be small, molecular analysis by PCR-amplified DNA can greatly facilitate the diagnosis of HTLV-I infection. Hybridization with probes specific for the PCR-amplified regions increases the specificity of this extremely sensitive method. In addition, PCR is ideal as a screening technique, because many patient samples can be rapidly examined for integrated HTLV-I sequences at one time once the optimal reaction conditions are established. PCR can also amplify more than one genomic sequence simultaneously because several sets of primers can be used in unison. Furthermore, PCR results are independent of the immune response. However, like all diagnostic methods, potential problems exist with PCR. False positive results, due primarily to amplification of DNA carried over from true positive cases and positive controls, can occur because of the exquisite sensitivity of the technique. Therefore, extreme care must be taken not to contaminate the system and other specimens. In addition, all PCR-positive cases should be confirmed by Southern blotting of unamplified DNA or by amplification and detection with other primers and probes. Amplification of retroviral-like endogenous human sequences is an additional potential problem with PCR. However, these amplification products do not hybridize with probes specific for the desired proviral sequence. Therefore, with fastidious technique, molecular analysis by the combination of DNA sequence amplification by PCR and specific probe hybridization by Southern blotting and/or dot blot analysis is a sensitive and specific method for identifying HTLV-I infection.

The incidence of HTLV-I associated T-cell malignancies, as determined by a variety of clinical findings and laboratory tests, varies according to geographic location but parallels the incidence of HTLV-I infection. However, because neither clinicopathologic findings nor serologic tests definitively prove a pathogenetic relationship between an infectious agent and a disease, an accurate incidence of HTLV-I associated T-cell neoplasms, especially in nonendemic areas, has not been clearly established. In some endemic regions, ATLL is thought to account for over 90% of all diagnosed T-cell neoplasms. In nonendemic areas, such as most of the continental United States and Europe, the percentage of HTLV-I associated T-cell neoplasms has been estimated to be less than 5%. In this study we determined the incidence of HTLV-I associated T-cell neoplasms in a nonendemic region of the United States to be 8% by detecting clonal integration of HTLV-I provirus in tumor cell DNA by molecular techniques. This higher incidence may be due to the fact that the metropolitan New York City area harbors a large immigrant population, reflecting the phenomenon that all of our patients with HTLV-I positive T-cell neoplasms were actually born in HTLV-I endemic regions outside of the United States. Alternatively, this higher incidence may be secondary to our detection method, because molecular screening and analysis by the combination of PCR and Southern blotting is both highly sensitive and specific. However, it is entirely possible that 8% of our cases were found to be HTLV-I positive simply because we looked for the virus. Other investigators have suggested that all patients with T-cell malignancies, but especially those in nonendemic areas, should be screened for evidence of HTLV-I infection. Nonetheless, this incidence of 8% can only be considered a preliminary and approximate figure at this time due to the obvious limitations in the population base and/or unrecognized biases in the selection of the cases studied here.
Interestingly, because all of our patients with HTLV-I positive T-cell neoplasms were born outside of the United States in HTLV-I endemic areas, our findings may also indicate that cases that were previously reported in nonendemic areas as sporadic cases may actually be “endemic cases” if medical, family, and sexual history is closely examined. Because it is well known that HTLV-I is transmitted from mother to child, between sexual partners, and by blood transfusions and that only approximately 5% of all positive T-cell neoplasms were born outside of the United States in HTLV-I endemic areas, our findings may also indicate that distant family members or distant family members of sexual partners of patients with ATLL may have been from an HTLV-I endemic region and the original source of the infection. Further epidemiologic studies and screening for HTLV-I infection by molecular analytic methods such as PCR may help answer this question.

ACKNOWLEDGMENT

The authors thank Dr Bernard Poiesz for primer and probe sequences and technical advice; Drs Jory G. Magidson, Morristown Memorial Hospital, Morristown, NJ, Christine Honig, White Plains Hospital, White Plains, NY, Anthony Marchand and Stephen Halperin, Overlook Hospital, Summit, NJ, Steven Mc Cormack, New York Eye and Ear Infirmary, New York, NY, Bernard Bernhardt, New Rochelle, NY; Ellen Gold, Thomas Lowe, Thomas Moulton, and Akim Ogundipe, Columbia Presbyterian Medical Center, New York, NY, for clinical information; Dr Bang Ying Zhu for technical assistance; and Nilda Inghirami for secretarial assistance.

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Detection and characterization of human T-cell lymphotropic virus type I (HTLV-I) associated T-cell neoplasms in an HTLV-I nonendemic region by polymerase chain reaction

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