Integrated Human T-Cell Leukemia Virus II Genome in CD8+ T Cells From a Patient With “Atypical” Hairy Cell Leukemia: Evidence for Distinct T and B Cell Lymphoproliferative Disorders


We previously reported isolation of human T-cell leukemia virus II (HTLV-II) from a second patient (N.R.A.) with atypical hairy cell leukemia. Follow-up analysis of the characteristics of the patient's HTLV-II infection over a 2-year period has revealed that the patient had two coexistent lymphoproliferative disorders. Oligoclonally integrated HTLV-II was detected in DNA extracted from the patient's peripheral blood mononuclear cells on separate occasions greater than 1 year apart, similar to integration of HTLV-I seen in adult T cell leukemia/lymphoma. Although integrated provirus was readily detected, no HTLV-II viral RNA expression was seen in fresh peripheral blood lymphoid cells. Although the patient's peripheral blood consistently contained a majority of atypical lymphoid cells with a T cell antigenic phenotype, he ultimately developed extensive pleural, hepatic and soft tissue infiltration with malignant Tac+ T cell lymphoproliferative syndrome consistent with hairy cell leukemia. To further characterize the role of HTLV-II, the patient's peripheral blood mononuclear cells were fractionated into four enriched subpopulations at autopsy. Oligoclonally integrated HTLV-II was detected in DNA from a T cell-enriched fraction and a CD8+ T cell-enriched fraction, but not in a CD4+ T cell-enriched fraction, a non-T cell fraction, or in B cells obtained from the malignant pleural effusion. We conclude that the patient harbored two distinct lymphoproliferative disorders, a TRAP+, Tac+B cell malignancy consistent with hairy cell leukemia that did not contain HTLV-II and a Tac+, CD8+ lymphoproliferative syndrome with oligoclonally integrated HTLV-II.

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labeled with fluorescein isothiocyanate (FITC), except for dual-color staining where anti-Tac antibody labeled with phycoerythrin (PE) was used in combination with FITC–Leu 4 or FITC–Leu 16 (B1).

**Cell lines and nucleic acid preparation.** The NRA and NRA-P cell lines are HTLV-II*NA*-transformed T cell lines derived from the patient’s peripheral blood lymphocytes. The HTLV-II–infected SHNRA-2P1 cell line was derived by culture of unrelated normal donor lymphocytes with lethally irradiated HTLV-II*-NA*-infected NRA-P cells by previously described methods. The HTLV-II*-NA*-infected Mo-T and JLB-II cell lines and the HL-60 human myeloid leukemia cell line and 729-6 human B cell lines have been previously described. Cellular DNA and RNA were prepared as previously described.

**Molecular probes and Southern blot analysis.** A molecular clone of the HTLV-II*NA* provirus was isolated in our laboratory from a genomic library prepared from NRA-P cellular DNA (J. Rosenblatt, in preparation). A 3.5-kilobase (kb) internal BamHI site at position 364 of the HTLV-II provirus (Fig 1) was isolated for use as a molecular probe.

For Southern hybridization analysis, 10 μg of cellular DNA was digested with the indicated restriction endonucleases (International Biotechnologies, Inc, New Haven, CT).

For analysis of immunoglobulin gene rearrangements, BamHI digests of cellular DNA were hybridized with a JH internal BamHI probe isolated from the HTLV-II*NA* clone of the HTLV-II*NA* provirus. DNA fragments containing proviral DNA extending from the conserved 3′ site of HTLV-II provirus was oligoclonally or polyclonally integrated, as determined by Southern blot analysis. DNA fragments containing proviral DNA extending from the conserved 3′ EcoRI site isolated from the original HTLV-II*NA* isolate. To determine whether HTLV-II*NA* provirus oligoclonally or polyclonally integrated, we digested DNA from peripheral blood mononuclear cells with the EcoRI restriction enzyme and hybridized with a 3′ HTLV-II*NA* probe. DNA fragments containing proviral DNA extending from the conserved 3′ EcoRI site into flanking cellular DNA are detected by this method, and each detected fragment represents a single integrated provirus.

**RESULTS**

**Clinical evolution of patient N.R.A.** Patient N.R.A., a 74-year-old white male, presented in 1979 with clinical features consistent with HCL. The percentage of T lymphocytes in the patient’s blood remained elevated until shortly before the patient’s death in August 1986. For most of his course, the patient had a predominance of T cells with CD8+ (OKT8 or Leu 2) markers (Table 1). Several months before death, the patient developed pleural effusions that contained a monomorphic population of TRAP+ cells with B cell markers, as ascertained by flow cytometric analysis (Table 2), and a light-chain clonality, as determined by immunoperoxidase staining. Despite therapy with α- and subsequently β-interferon, the patient developed extensive pleural and hepatic infiltration with TRAP+ cells and died of pulmonary and hepatic failure. Flow cytometric studies of pleural fluid cells on three separate occasions showed that 70% of the cells were CD20+ (Leu 16 or B1 antibody identifying a pan-B cell marker) B cells (Table 2). Autopsy sections of pleura, liver, and bone marrow demonstrated diffuse infiltration by a monomorphic B cell population with light-chain clonality as determined by immunoperoxidase staining (data not shown).

| Table 1. Peripheral Mononuclear Cell Antigenic Phenotype (Percentage Positive) of Patient N.R.A. |
|---------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Phenotype                       | 2/85              | 3/85*             | 6/85              | 6/86†             | 8/86 (Autopsy)    |
|                                 |                   |                   |                   |                   |                   |
| WBC (x 10³/μL)                  | 20.8              | 16.4              | 3.5               | 7.0               | 7.3               |
| Lympocyte (%)                   |                   |                   |                   |                   |                   |
| (unseparated blood)             | 84                | 90                | 51                | 60                | 69                |
| CD3+ (Leu 4)                    | 85                | 91                | 92                | 69                | 65                |
| CD4+ (Leu 3)                    | 44                | 30                | 36                | 37                | 43                |
| CD8+ (Leu 2)                    | 46                | 63                | 52                | 34                | 22                |
| Tac                             | 10                | 1                 | ND                | 0                 | 7                 |
| CD20+ (B1)                      | 13                | 3                 | 2                 | 7                 | 35                |

**Abbreviation**: ND, not determined.

*Start of α-interferon therapy.
†Start of β-interferon therapy.

**Table 2. Pleural Fluid Cells (Percentage Positive) of Patient N.R.A.**

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<th>Phenotype</th>
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<th>8/86 (Bone Marrow)</th>
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<td>30</td>
<td>23</td>
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<td>Tac+</td>
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<td>72</td>
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<tr>
<td>CD20+ (B1)</td>
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<td>78</td>
<td>70</td>
<td>72</td>
</tr>
<tr>
<td>Tac+ B cells*</td>
<td>ND</td>
<td>ND</td>
<td>70</td>
<td>72</td>
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<tr>
<td>Tac+ T cells**</td>
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*Determined by dual-color fluorescence analysis using FITC–Leu 16 to mark B cells and PE–anti-IL-2 receptor antibody.
†As except FITC–Leu 4 is used to mark T cells.
Lack of viral RNA expression in fresh peripheral blood mononuclear cells. An unusual characteristic of malignancies induced by HTLV-I and the related BLV is that, although tumor cells contain provirus, viral RNA expression is not detected in fresh leukemic cells. To determine whether circulating HTLV-II-infected cells expressed HTLV-II in the patient, we assayed for viral RNA expression by using S1 nuclease analysis with a probe for the cap site of HTLV-II (Fig 2). A 52-base pair-protected fragment corresponding to messenger RNA synthesized from the HTLV-II cap site was evident both in HTLV-II infected JLB-II and Mo-T cells and in HTLV-II infected NRA and NRA-P cells grown in vitro. In contrast to the RNA expression seen in the cultured cell lines, no expression of viral RNA was detected in fresh peripheral blood mononuclear cells, even with autoradiographic exposures as long as 3 weeks (a 24-hour exposure is shown). Thus, circulating HTLV-II-infected cells in this patient do not appear to transcribe the provirus, similar to HTLV-I-infected ATL cells and BLV-infected tumors.

Preferential integration of HTLV-II into the CD8 + T cell fraction of peripheral blood: evidence for distinct T and B cell lymphoproliferative disorders. The peripheral blood of patient N.R.A. repeatedly contained a predominance of circulating atypical lymphocytes of T cell antigenic phenotype. A minority of circulating cells in the blood stained positively for TRAP and had classic hairy cell morphology; however, the majority of circulating lymphoid cells were morphologically atypical, with an abnormal nuclear/cytoplasmic ratio and additional abnormalities in nuclear morphology. As opposed to the peripheral blood, the pleural effusions contained a majority (>70%) of TRAP + CD20+ B cells with hairy cell morphology (Table 2). Many of these B cells expressed Tac antigen (Table 2), as is typically seen in HCL. Southern blot analysis of DNA extracted from pleural effusion cells showed clonal immunoglobulin heavy-chain rearrangements (Fig 3) and light-chain gene rearrangements (data not shown). In contrast, analysis of DNA from peripheral blood mononuclear cells obtained July 1985 and June 1986 consistently showed a 16.9-kb germline pattern only, likely due to the small proportion of circulating B cells in the peripheral blood. The pleural effusion results demonstrated that the TRAP + hairy cells were of clonal B cell origin and represented a distinct population from the majority of atypical T cells present in the peripheral blood. The patient's peripheral blood was reanalyzed at autopsy in August 1986, and the percentage of CD20+ B cells had risen to 35% (Table 1). Most of these CD20+ B cells also expressed the Tac antigen. In addition, at autopsy approximately 65% of circulating lymphoid cells in the peripheral blood were T cells (CD3+) that did not express detectable Tac antigen, in contrast to the B cells. Analysis of a bone marrow mononuclear cell suspension at autopsy demonstrated that approximately 72% of lymphoid cells were Tac+, CD20+ B cells (Table 2). These results show that although the majority of atypical lymphoid cells in the

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**Fig 1.** HTLV-II DNA sequences in peripheral blood mononuclear cells. Ten micrograms of DNA was digested with the indicated restriction enzyme and Southern blot analysis performed by using the internal 3.5-kb BamHI III fragment of HTLV-II as a probe. PBL, peripheral blood mononuclear cells from patient N.R.A.; pleural, pleural effusion cells from June 1986; Mo-B, B cell line infected by HTLV-II; 729-6, an Epstein-Barr virus-positive (EBV +) human B cell line not infected with HTLV-II. DNA extracted from N.R.A. PBL in both July 1985 and June 1986 and digested with EcoRI shows persistent oligoclonal integration of HTLV-II (indicated by bands at 4.7 and 5.7 kb).
Fig 2. S1 nuclease analysis of total cellular RNA from HTLV-II-infected cells. A 52-bp protected fragment corresponds to mRNA synthesized from the HTLV-II cap site (see Methods). HTLV-II mRNA is detected in vitro infected cell lines Mo-T, JLB-II (HTLV-II infected), and NRA and NRA-P (HTLV-IIa infected). No HTLV-II mRNA is detected in peripheral blood cells (patient N.R.A.) despite the presence of integrated HTLV-II provirus, even with 3-week exposures (a 24-hour exposure is shown).

Peripheral blood were of T cell origin, the circulating TRAP+ hairy cells were, in fact, of B cell origin.

The presence of HTLV-II in DNA extracted from the patient's peripheral blood mononuclear cells but not in DNA from pleural fluid B cells (Fig 1) implied the coexistence of two distinct lymphoproliferative disorders in patient N.R.A. To define the nature of HTLV-II integration in the patient's peripheral blood, we fractionated peripheral blood lymphoid cells obtained at autopsy into four enriched cellular compartments. These compartments included an SRBC-rosetting T cell fraction, a CD8+ enriched T cell fraction (OKT4/B1 depleted), a CD4+ enriched T cell fraction (OKT8/B1 depleted), and a non-T cell fraction. Integrated HTLV-II was readily detected in the T cell and CD8+, T cell-enriched (OKT4/B1-depleted) fractions, was faintly detected in the CD4+, T cell-enriched (OKT8/B1-depleted) fraction, and was absent in the non-T cell fraction or in the predominantly B cell pleural fluid cells (Fig 4). These results demonstrate that the T cell subpopulation containing oligoclonally integrated HTLV-II was of the CD8+ phenotype and suggest an explanation for the unusual predominance of CD8+ cells in the patient's peripheral blood.

DISCUSSION

The etiologic role of HTLV-I in human T cell malignancy has been established from seroepidemiological studies as well as molecular studies of the virus in patients with ATL. In contrast, the role of HTLV-II in human malignancy is not clear. Recently, we identified a second patient with HCL who also had a predominance of circulating atypical lymphoid cells with a T cell antigenic phenotype. This patient's clinical syndrome was similar to the original patient Mo who harbored HTLV-II, thereby implicating the virus in a possible etiologic role. In contrast to patient Mo, we have studied patient N.R.A. in depth and have determined that he had two distinct lymphoproliferative disorders, one of T cell and the other of B cell origin. We have observed a similar pattern of HTLV-II infection in patient N.R.A. to that seen in ATL.
linked to HTLV-I and in tumors linked to BLV. In the case of ATL, studies of fresh tumor cells demonstrate monoclonal integration of HTLV-I in tumor cells from patients, with a rare patient having two integration sites. Similarly, in the tumor phase of BLV infection, one to three integrated proviruses are generally found. Our molecular analysis demonstrated the presence of persistent oligoclonally integrated provirus in peripheral blood lymphoid cells from patient N.R.A. on several occasions. The viral integration pattern remained constant over time, similar to HTLV-I- and BLV-associated malignancies. Such oligoclonal integration indicates that an HTLV-II–infected cell clone gave rise to the T cell lymphoproliferative disorder in this patient.

Another characteristic of malignancies induced by HTLV-I and BLV is the lack of viral RNA expression in leukemic cells despite the presence of integrated provirus. Similarly, S, nuclease analysis did not demonstrate expression of HTLV-II RNA in this patient. This characteristic lack of detectable viral replication does not preclude active viral replication occurring elsewhere in the patient or exceedingly low levels of replication but does demonstrate that the majority of circulating tumor cells are not productively infected in vivo despite the presence of integrated provirus. By analogy with the malignancies associated with HTLV-I and BLV, it is likely that active ongoing viral replication is not required for maintenance of the lymphoproliferative syndrome.

At the time of initial presentation and analysis, this patient carried a long-standing diagnosis of HCL and had a predominance of morphologically abnormal lymphoid cells of a T cell antigenic phenotype in the peripheral blood and only a small minority (<5%) of circulating B cells. He was therefore initially thought to have a single malignant process of T cell origin. As our studies indicate, however, at the time of death the patient clearly had a B cell malignancy with characteristics of HCL that had an unusually aggressive course. DNA extracted from cell populations enriched for the malignant B cells did not contain integrated HTLV-II, in contrast to the repeated detection of HTLV-II in T cells from the peripheral blood. At autopsy, oligoclonally integrated HTLV-II was localized to the CD8+ T cell fraction of the peripheral blood. These findings demonstrate that the patient had two distinct lymphoproliferative disorders, a CD8+, T-suppressor cell lymphoproliferative syndrome bearing oligoclonally integrated HTLV-II virus and a clonal B cell HCL without evidence of HTLV-II infection.

The preferential association of HTLV-II with the CD8+ (cytotoxic suppressor) T lymphocyte fraction of the patient's peripheral blood mononuclear cells was an unexpected finding. The predominant immunologic phenotype in ATL is CD4+ T cells that express Tac antigen, although leukemias with CD8+ antigenic phenotype have been described. In addition to the CD8+ phenotype, the HTLV-II–infected peripheral blood cells of patient N.R.A. did not express Tac antigen in vivo. This finding suggests that HTLV infection may not necessarily lead to overexpression of the IL-2 receptor in vivo, although it has been frequently observed for HTLV-I–linked malignancies.

The isolation of HTLV-II in two patients with a similar presentation of HCL is intriguing. Patient Mo was originally characterized as a T cell HCL on the basis of similar surface marker analysis of peripheral blood lymphoid cells and on the basis of the derivation of TRAP+ T cell lines from the spleen of the patient. As HTLV-II transforms normal T cells in vitro, cell lines derived from the blood of either patient Mo or patient N.R.A. likely represent in vitro-transformed normal T lymphocytes and may not necessarily derive from the predominant clones in the peripheral blood as has been demonstrated for cell lines derived from ATL patients. Furthermore, as our data indicate, the TRAP+ hairy cells in patient N.R.A. were of B cell origin. Although most HCL patients are seronegative for HTLV-II, the similar presentation of HCL in association with a peripheral T cell lymphocytosis raises the possibility that HTLV-II infection may predispose to the development of HCL. We did not detect EBV sequences in the patient's pleural fluid B cells, which rules out an opportunistic malignancy secondary to HTLV II infection (data not shown). B cell abnormalities including B cell chronic lymphocytic leukemias, monoclonal gammopathy, and hypergammaglobulinemia have been described in association with HTLV-I infection.
lines infected in vitro with either HTLV-I and/or HTLV-II are noted to elaborate a variety of lymphokines including interleukin-2 and granulocyte-macrophage colony-stimulating factor. Therefore, it is possible that cellular factors that affect B cell proliferation and/or differentiation may be elaborated by HTLV-infected T cells in vivo, thereby providing a suitable setting for the development of unusual B cell malignancies. Alternatively, B cell malignancies may evolve in response to chronic antigenic stimulation by HTLV or impaired immunity secondary to HTLV-II infection. Studies of additional patients infected with HTLV-II will be necessary to better understand the potential role of this virus in human malignancy.

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