We previously reported isolation of human T-cell leukemia virus II (HTLV-II) from a second patient (N.R.A.) with atypical 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 hepatic and soft tissue infiltration with malignant T cells by depletion with OKT4 and Blc monoclonal antibodies (Tac, +, tartrate-resistant, acid phosphatase-positive (TRAP +) B cells of clonal origin. 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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**METHODS**

**Cell fractionation.** Approximately 40 mL of peripheral blood was obtained at autopsy, and mononuclear cells were separated on a Ficoll-Hypaque density gradient. Mononuclear cells were further fractionated into a T cell and non–T cell fraction by rosetting with sheep RBC and repeat passage over a Ficoll-Hypaque gradient. By using previously described methods, the rosetting T cell fraction was further subfractionated into two populations: one enriched for CD8 + cells by depletion with OKT4 and B1 monoclonal antibodies (Becton Dickinson, Mountain View, CA) in combination with goat antimouse, Ig-coated, iron-core beads (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and passage over a magnet; and a second enriched for CD4 + cells (OKT4/Leu 3 reactive) by depletion with OKT8 and B1 monoclonal antibodies. The enriched T cell, CD8 +, and CD4 + fractions had <1% of antibody-depleted B cells, and cellular fractions of interest were enriched to approximately 80%. Further enrichment was not possible because of the paucity of available autopsy material.

**Cell surface marker analysis.** The methods for flow cytometric analysis and details of nomenclature have been published previously. Monoclonal antibodies included antibodies directed against Leu 2 (CD8), Leu 3 (CD4), Leu 4 (CD3), interleukin 2 (IL-2) receptor (Tac antigen), HLA-DR (Becton Dickinson), and B1 antigens (Coulter Immunology, Hialeah, FL). The reagents were available from the James H. Harless Fund, Hematopathology Tutorial, Inc. and The Mobil Foundation.

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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**NRA**-transformed T cell lines derived from the patient’s peripheral blood lymphocytes.13 The HTLV-II–infected SHNRA-2P1 cell line was derived by culture of unrelated normal donor lymphocytes with lethally irradiated HTLV-II**NRA**-infected NRA-P cells by previously described methods. The HTLV-II**NRA**-infected Mo-T and JLB-II cell lines2 and the HL-60 human myeloid leukemia cell line and 729-6 human B cell lines have been previously described.2,8,9 Cellular DNA and RNA were prepared as previously described.

**Molecular probes and Southern blot analysis.** A molecular clone of the HTLV-II**NRA** provirus 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).28 For analysis of immunoglobulin gene rearrangements, **BamHI** digests of cellular DNA were hybridized with a JH probe for the human heavy-chain joining region of IgG as previously described.21

**S1 nuclease analysis.** S1 nuclease analysis was performed to assay for viral RNA synthesis as previously described.22,24 The DNA hybridization probe was prepared from the HTLV-II**NRA** clone pHe6, extends from the **Taql** site at nucleotide 50 to the **BamHI** site at nucleotide 361, and spans the HTLV-II cap site located at nucleotide 313. The probe was 5'γ[32P]dATP labeled at the **BamHI** site. A 52-nucleotide fragment protected by this probe from S1 nuclease digestion corresponds to RNA synthesis from the cap site of HTLV-II.

### 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.14 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 λ 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 λ light-chain clonality as determined by immunoperoxidase staining (data not shown).

**Detection of integrated HTLV-II****NRA** in peripheral blood mononuclear cells. A consistent feature of ATL caused by the related HTLV-I virus is the presence of oligoclonally integrated HTLV provirus in DNA from leukemic cells. Southern blot analysis of DNA extracted from fresh peripheral blood mononuclear cells showed the presence of integrated HTLV-II**NRA** (Fig 1). By using a restriction map of HTLV-II**NRA**, we had previously determined that two internal EcoRI sites were conserved at the same locations as in the original HTLV-II**MboI** isolate.14 To determine whether HTLV-II**NRA** provirus was oligoclonally or polyclonally integrated, we digested DNA from peripheral blood mononuclear cells with the EcoRI restriction enzyme and hybridized with a 3' HTLV-II**NRA** 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. As can be seen in Fig 2, two distinct bands measuring 4.7 and 5.7 kb in length and a faint band of 13 kb are detected. The viral integration pattern and relative band intensity appeared constant on two occasions one year apart, although the overall intensity of hybridization changed, which suggests that the same expanded cell clone persisted in the patient's peripheral blood over this period of time. Faint oligoclonal integration of HTLV-II was also detected in DNA extracted from the patient’s bone marrow obtained at autopsy (data not shown) but not in DNA from pleural fluid cells (Fig 1). The presence of oligoclonal HTLV-II integration is similar to the oligoclonal integration pattern seen in various forms of adult T cell leukemia linked to HTLV-I6,38 and the tumor

---

**Table 1. Peripheral Mononuclear Cell Antigenic Phenotype (Percentage Positive) of Patient N.R.A.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>2/85</th>
<th>3/85*</th>
<th>6/85</th>
<th>6/86†</th>
<th>8/86 (Autopsy)</th>
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<tbody>
<tr>
<td>WBC (x 10^3/μL)</td>
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<td>16.4</td>
<td>3.5</td>
<td>7.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>84</td>
<td>90</td>
<td>69</td>
<td>60</td>
<td>69</td>
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<tr>
<td>(unseparated blood)</td>
<td>85</td>
<td>91</td>
<td>92</td>
<td>69</td>
<td>65</td>
</tr>
<tr>
<td>CD3+ (Leu 4)</td>
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<td>30</td>
<td>36</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>CD8+ (Leu 2)</td>
<td>46</td>
<td>63</td>
<td>52</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Tac</td>
<td>&lt;10</td>
<td>1</td>
<td>ND</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>CD20+ (B1)</td>
<td>13</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>35</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Phenotype</th>
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<th>6/86</th>
<th>8/86</th>
<th>8/86 (Bone Marrow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ (Leu 4)</td>
<td>1</td>
<td>9</td>
<td>30</td>
<td>23</td>
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<td>CD8+ (Leu 2)</td>
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<td>6</td>
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<td>11</td>
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<td>CD4+ (Leu 3)</td>
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<td>17</td>
<td>13</td>
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<tr>
<td>Tac+</td>
<td>70</td>
<td>33</td>
<td>70</td>
<td>72</td>
</tr>
<tr>
<td>CD20+ (B1)</td>
<td>71</td>
<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>
</tr>
<tr>
<td>Tac + T cells**</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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.
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 BamH I' fragment of HTLV-IIza as a probe. BPL, peripheral blood mononuclear cells from patient N.R.A.; pleural, pleural effusion cells from June 1986; Mo-B, B cell line infected by HTLV-IIza; 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).

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 CD8 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 λ 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 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 phase of bovine leukemia linked to bovine leukemia virus (BLV). 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. 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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-IIe 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).

Fig 3. Southern blot analysis for immunoglobulin heavy-chain gene rearrangement in B cell-enriched pleural effusion cells and peripheral blood mononuclear cells from patient N.R.A. Heavy-chain rearrangement was detected only in DNA from the TRAP+ B cell-enriched pleural effusion cells. DNA from the peripheral blood mononuclear cells (NRA PBL) from July 1985 and June 1986 and from the HL-60 control cell line shows a germline configuration.

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 harbored 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 mononuclear 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-II and BLV is the lack of viral RNA expression in leukemic cells despite the presence of integrated provirus. Similarly, S1 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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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

JD Rosenblatt, JV Giorgi, DW Golde, JB Ezra, A Wu, CD Winberg, J Glaspy, W Wachsman and IS Chen