Adult T-cell leukemia/lymphoma not associated with human T-cell leukemia virus type I
(Southern blot/retrovirus/surface marker)

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ABSTRACT We describe five patients with adult T-cell leukemia/lymphoma (ATL) with neither integration of human T-cell leukemia virus type I (HTLV-I) nor anti-HTLV-I antibodies in their sera. These findings indicate that ATL-I may not have been involved in leukemogenesis in these patients. The clinicohematological, cytopathological, and immunological features of HTLV-I-negative ATL were exactly the same as those of HTLV-I-associated ATL. Leukemia cells with pleomorphic nuclei, generalized lymphadenopathy, hepatosplenomegaly, skin lesions, hypercalcemia, and elevated lactate dehydrogenase levels, all of which are characteristic features of typical ATL, were also seen in these patients with HTLV-I-negative ATL. Leukemia cells expressed T3, T4, and pan-T-cell antigens in three cases, and T3 and pan-T-cell antigens in two. All five patients had lived in ATL-nonendemic areas. The finding of HTLV-I-negative ATL suggests that factor(s) other than HTLV-I infection may be involved in ATL leukemogenesis.

Adult T-cell leukemia/lymphoma (ATL) was first described as a new disease having unique clinicohematological, morphological, immunophenotypic, and epidemiological features (1). Since 1981, it has been reported that nearly 100% of all patients with ATL have sera that react with the proteins of human T-cell leukemia virus type I (HTLV-I) (2-6). In 1983, however, Shimoyama et al. (7) found that a few patients with ATL have typical clinicohematological, morphological, and immunophenotypic features but give a negative reaction for antibody to HTLV-I. Expression of HTLV-I protein in primary cultures of leukemia cells from those patients was also found to be negative (7). Family members of the patients showing no anti-HTLV-I antibody were also negative for anti-HTLV-I antibody (7). These results suggested that HTLV-I may not be involved in leukemogenesis in some patients with ATL. To determine whether HTLV-I is associated with leukemogenesis in these ATL patients, we examined the DNA of their leukemia cells by Southern blot analysis using probes of the HTLV-I proviral genome. No integration of the HTLV-I proviral genome into cellular DNA of leukemia cells could be detected in these patients who lack anti-HTLV-I antibody, suggesting that factor(s) other than HTLV-I may be involved in ATL leukemogenesis.

MATERIALS AND METHODS

Surface Markers. Surface markers of leukemia cells were analyzed by use of a panel of 20 well-characterized monoclonal antibodies, described previously (8). The markers examined were OKT3, Leu-4, OKT4, Leu-3a, OKT8, Leu-2a, OKT6, NA1/34, OKT11, 9.6, Leu-5, Leu-1, 10.2, 4A, Ta60b (for interleukin-2 receptor), OKIa1, HLA-DR, B1, J5, and BA-3. The leukemia cells were also cultured in the presence of crude T-cell growth factor (9) for induction of HTLV-I antigen in vitro. The expression of HTLV-I antigen in cultured leukemia cells was examined with reference sera that had high titers of anti-HTLV-I antibody and with two monoclonal antibodies (GIN-2 and GIN-14) against P19 and P28 (10).

Southern Blot Analysis. Mononuclear cells were prepared from fresh peripheral blood, ascites, or pericardial effusions of the patients, at the indicated clinical stages described under case reports, by centrifugation on a Ficoll-Conray gradient. For preparation of DNA for various blotting analyses, more than 10^7 lymphoid cells were used. High molecular weight DNA was extracted by treating the cells overnight with NaDodSO4/proteinase K and then extracting the mixture with phenol. Cellular DNA was digested with EcoRI, Pst I, or Msp I, and the fragments were separated by electrophoresis in 10% agarose gel. The DNAs in the gel were blotted onto a nitrocellulose membrane filter or nylon membrane filter and hybridized with nick-translated 32P-labeled probes. The probes used were pCAT-G (corresponding to nucleotides 874-2515 in the proviral genome (11)), covering the gag region; pATK32 (nucleotides 1930-5093), covering the gag-pol region (11); pHT-I(M)3.9 (nucleotides 4990-8309), covering the env-pX region (12); and pHT-I(M)0.7 (nucleotides 8323-9003), covering the long terminal repeat (LTR) region (12) of cloned HTLV-I provirus (Fig. 1). Plasmid pATK32 was kindly supplied by M. Yoshida (Cancer Institute, Tokyo). pHT-I(M)3.9, pHT-I(M)0.7, and pCAT-G were isolated in our laboratory. Hybridization was in 6x standard saline citrate (SSC)/5x Denhardt's solution/0.1% NaDodSO4/5mMEDTA/Escherichia coli DNA (100 µg/ml)/yeast RNA (40 µg/ml) at 65°C. The filter was washed twice for 15 min at room temperature and once for 30 min at 55°C with 0.5x SSC/0.1% NaDodSO4 and then exposed to x-ray film at -70°C. In another experiment, the filter was heated for 10 min at 95°C and rehybridized with nick-translated c-myc. (1X SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0; 1X Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.)

Patients. Between October 1980 and the end of 1984, sera of 69 ATL patients were examined for anti-HTLV-I antibody. Seven of these sera were found to give a negative reaction for the antibody. Five of these seven cases could be used further for DNA analysis because enough tumor cells had been kept frozen. First, as it is important to show that these five ATL patients giving negative reaction for anti-HTLV-I antibody

Abbreviations: ATL, adult T-cell leukemia/lymphoma; HTLV-I, human T-cell leukemia virus type I; LDH, lactate dehydrogenase; LTR, long terminal repeat; PBMC, peripheral blood mononuclear cell; kb, kilobase(s).

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had the same clinicohematological, morphological, and immunophenotypic features as patients with HTLV-I-associated ATL, their clinical courses are summarized below.

**Patient 1.** A 71-year-old man born in Yokohama, Kanagawa in 1910. He had severe anorexia, hoarseness, a productive cough, palpitation, and generalized lymphadenopathy with a subfever level of 37°C in December 1981. He was found to have hepatosplenomegaly, recurrent nerve palsy due to hilar and mediastinal lymph node swelling, left pleural effusion, a high lactate dehydrogenase (LDH) level (2610 units/dl), and leukocytosis (56,800 per μl) with abnormal T lymphocytes (64%). The T lymphocytes, which showed nuclear polymorphism (Fig. 2a), have been called flower cells (13) and are a characteristic of ATL. The leukocyte count reached 67,200 per μl with 70% abnormal lymphocytes and 5% normal lymphocytes on January 21, when peripheral blood mononuclear cells (PBMCs) were prepared and stored at −80°C for analysis of HTLV-I proviral DNA (Fig. 3A). A lymph node biopsy disclosed non-Hodgkin lymphoma of diffuse pleomorphic type. VEPA therapy (14), consisting of vincristine, cyclophosphamide, prednisolone, and adriamycin (doxorubicin), was given, with marked reduction of the tumor mass and of the leukemia cells. One month later, however, hypercalcemia (6.4 milliequivalents/liter) developed, with pulmonary infiltration of leukemia cells and severe dyspnea, and the patient died of the disease on March 28.

**Patient 2.** A 43-year-old woman, born in Shimodate, Chiba in 1940. She suffered from cervical lymph node swelling and edematous itchy erythromelalgia of the face in early June 1983. On July 12, she was found to have marked leukocytosis (74,600 per μl) with 87% abnormal T lymphocytes of flower-cell morphology (Fig. 2b). She had subfever in the range of 37°C, generalized lymphadenopathy, and a high LDH level (1163 units/dl). Histological examination of her swollen inguinal lymph node revealed non-Hodgkin lymphoma of diffuse pleomorphic type. Despite VEPA therapy, interstitial pulmonary infiltration of leukemia cells, progressive leukocytosis, splenomegaly, and aggravation of erythrodermia due to massive infiltration of leukemia cells into the dermis were observed. Methotrexate was added to VEPA therapy from February 3, 1984, which resulted in complete remission. However the disease recurred in early April, and her leukocyte count reached 368,000 per μl with 97% abnormal T lymphocytes on May 10, when PBMCs were prepared and stored at −80°C for HTLV-I proviral analysis (Fig. 3A). The patient died of the disease on May 21.

**Patient 3.** A 47-year-old man, born in Kanzaki, Hyogo in 1936. He had been anemic since 1978. In July 1982, he was found to have leukocytosis (17,300 per μl) with marked abnormal T lymphocytosis (72%) with typical flower cells. This condition remained unchanged for about 2 years without any specific therapy. He was diagnosed as having chronic ATL (15). In March 1984, he first noted a small induration of preauricular skin that developed into a large skin tumor causing hearing loss and lockjaw by May 10. A skin-biopsy specimen showed malignant lymphoma of diffuse medium-sized cell type. Several small skin tumors were observed on the head and trunk; erythrodermia, in pectoral and lumbar regions; and swelling of small lymph nodes, in the left cervical region and both groins. Laboratory examinations on June 1 revealed leukocytosis (17,200 per μl) with T lymphocytosis (79%) having 36% typical flower cells (Fig. 2c) and a high LDH level (1717 units/dl). He also had hepatosplenomegaly and multiple bone lesions detected by 99Tc bone scintigraphy. PBMCs were prepared and stored at −80°C for HTLV-I proviral analysis (Fig. 3A). The patient received CHOP therapy, consisting of cyclophosphamide, Adriamycin, vinblastine, and prednisolone (16), as well as methotrexate and leuphraxis therapy, with complete regression of tumors by August, but about 10% of peripheral blood lymphocytes still showed flower-cell morphology. Meantime, he had herpes zoster. In December, he was found to have bilateral pleural effusion, malignant pericardial effusion, hepatomegaly, leukocytosis (24,900 per μl), and a high LDH level (17,310 units/dl). He underwent pericardial drainage; intrapericardial installation of 1-β-D-arabinofuranosylcytosine, methotrexate, and prednisolone; and systemic CHOP, mitoxantrone, and etoposide therapies without any beneficial effect. On February 27, more than 200 ml of pericardial effusion with 85% leukemia cells having flower-cell morphology was removed as an emergency treatment of cardiac tamponade. Mononuclear cells were separated from the pericardial effusion for HTLV-I proviral analysis (Fig. 3A). Hypercalcemia also developed (6.5 milliequivalents/liter). The patient died of cardiac insufficiency on May 20.

**Patient 4.** A 60-year-old man, born in Ibaraki, Fukushima. He suffered from anorexia, fatigue, loss of weight, pretibial edema, and abdominal distension in early July 1983. He was found to have an advanced gastric tumor of Borrmann type
IV, bilateral pleural effusion, ascites, and leukocytosis (15,000–23,700 per μl). He received mitomycin C and 5-fluorouracil intravenously without beneficial effect. On October 17, he first noted a left cervical lymph node swelling. Histological examination of the lymph node revealed malignant lymphoma of diffuse mixed cell type. On November 8, ascites was removed for immunological marker study and cytological examination. Many abnormal T lymphocytes were found, with 62% typical flower cells and 15% normal lymphocytes (Fig. 2d). Mononuclear cells were separated from the ascites and stored at −80°C for HTLV-I proviral analysis (Fig. 3A). Biopsy specimens of the gastric tumor disclosed lymphomatous infiltration. A few large abnormal lymphocytes with flower-cell morphology were seen in a peripheral blood smear. From these findings, a diagnosis of a lymphoma type of ATL was made (13). On November 16, he was treated by VEP A therapy. However, several skin tumors in the lower abdomen and severe interstitial pneumonitis developed. He died of pneumonitis on March 11, 1984.

**Patient 5.** A 72-year-old man, born in Ise, Mie. He had suffered from numbness and weakness of the lower extremities for 3 years. He visited us on June 9, 1982, with complaints of a markedly ataxic gait, numbness and weakness of the lower extremities, malaise, and general fatigue. Physical examination revealed that he had hepatosplenomegaly, generalized lymphadenopathy, and skin rashes on the back and pectoral regions. Laboratory examinations on June 10 revealed marked leukocytosis (88,000 per μl) with 95% abnormal T lymphocytes with 80% typical flower cells (Fig. 2e), a high LDH level (1622 units/dl) and sarcomatous meningitis. Mononuclear cells were separated from the peripheral blood and stored at −80°C for analysis of HTLV-I provirus (Fig. 3A). Histological examination of the right inguinal lymph node revealed malignant lymphoma of diffuse pleomorphic cell type. He was diagnosed as having ATL. His condition deteriorated abruptly on July 18, and he died of acute heart failure.

**RESULTS**

**Anti-HTLV-I Antibody in Sera and HTLV-I Protein in Cells.***

Sera were obtained before therapy and several times after the start of therapy. The sera were diluted 1:5 and screened for anti-HTLV-I antibody by the indirect immunofluorescence method (2). In all five patients, negative results were consistently obtained. Moreover, no reactivity against HTLV-I antigens was detected in undiluted sera from any of the five patients. No antibody was detected in sera obtained from nine members of two families (patients 2 and 4), including the mother of one patient. In addition, expression of HTLV-I antigen was examined by the indirect immunofluorescence method in primary leukemia cells from the five patients, cultured in the presence of crude T-cell growth factor (9), but in all cases negative results were obtained.

**Immunological Phenotype of Tumor Cells.*** As shown in Table 1, leukemia cells from patients 2, 3, and 4 gave positive reactions for OKT3 or Leu-4, OKT4 or Leu-3a, OKT11 or 9.6, Leu-1 or 10.2, and OKIa1 or HLA-DR, but negative reactions for OKT8 or Leu-2a, indicating that the leukemia cells expressed the activated inducer/helper T-cell phenotype. Leukemia cells from two of these patients (nos. 2 and 3) were reexamined at the time of relapse. In patient 2, the immunological phenotype of leukemia cells at this time was the same as that before therapy. However, the leukemia cells of patient 3 at the time of relapse had gained reactivity with Leu-2a and become double-labeled cells (Leu2a+, 3a+). They did not react with T606b, which indicated that they did not express interleukin-2 receptor. Leukemia cells from two patients (nos. 1 and 5) reacted with OKT3, 9.6, and OKIa1, but not with OKT4 or OKT8, which indicated that they retained the characteristics of peripheral T cells but did not express their specific subset marker.

**Southern Blot Analysis for the HTLV-I Provirus Genome.*** The HTLV-I provirus was detectable in the ATL-1K cell line (9) as a 20-kb fragment when cellular DNA was digested with EcoRI, which does not cut the HTLV-I provirus sequence, and as 12.8- 2.4-, 1.7-, and 1.3-kb fragments when DNA was digested with PsI I, which cuts the provirus internally (Fig. 3A). As expected from the nucleotide sequence of the provirus, when probes covering gag-pol (pATK32), env-pX [pHT-10(M3.9)], and the LTR [pHT-10(M0.7)] were used, a 12.8-kb band was obtained from the flanking cellular DNA fragments containing the 3′ LTR, and a 2.4-kb band was obtained from the flanking DNA fragments containing the 5′ LTR (data not shown). However, there was no detectable proviral band in the digests of DNA from PBMCs, ascites or pericardial effusion cells of patients 1–5. The absence of a proviral band was not due to extensive degradation of DNA, because when the same filter was washed and rehybridized with c-myc probe, the band of the c-myc gene could be clearly detected in leukemic cellular DNA from all five patients, as well as in control DNA from ATL-1K cells or from HL-60 cells, which showed the well-known amplification of c-myc (Fig. 3B).

When the probe covering the whole gag region was used, DNA from ATL-1K cells showed the proviral band of 20 kb in an EcoRI digest and bands of 2.4 and 1.7 kb in a PsI I digest (Fig. 3C). However, no band was detected in digests of DNA of leukemia cells from any of the five patients. When the LTR probe was used, the proviral band of 20 kb was observed in an EcoRI digest of ATL-1K DNA (Fig. 3D), but no band was seen in digests of leukemia-cell DNA from any of the five patients. Msp I, which is a restriction enzyme that cuts within the LTR sequence, was used to see whether the LTR sequence was integrated randomly. The expected bands of 0.1–0.2 kb were obtained with ATL-1K cells, but not with leukemia cells from any of the five patients.

Table 1. Surface markers of leukemia cells

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<th>Marker</th>
<th>No. 1</th>
<th>No. 2</th>
<th>No. 3</th>
<th>No. 4</th>
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<td>80</td>
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<td>5</td>
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<td>2</td>
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Peripheral blood (PB), lymph node (LN), pericardial effusion (PE), or ascites (A) cells (% leukemia cells in parentheses) from patients 1–5 were examined for reaction with a panel of monoclonal antibodies (see Materials and Methods) as well as for sheep erythrocyte-rosette-forming capacity (ERFC) and surface immunoglobulin (slg) expression. Underlined numbers were regarded as positive reactions.

*Surface markers were examined at the time of relapse.*
promoter transcription activate HTLV-I proviral (11). Therefore, has been reported ATL of patients, and this has been shown impossible to exclude the possibility that HTLV-I proviral DNA could not be detected by the present hybridization technique or the possible deletion of once integrated HTLV-I provirus. The clinicohematological, cytological, and patho-

cell) in leukemia-cell DNA of patient 2, examined as a representative case, or in the cellular DNA from Jurkat, a T-cell line not infected with HTLV-I, used as a negative control. Even with 3-fold more DNA of patient 2, no proviral band was detected (data not shown). The above results indicate that the HTLV-I provirus was not integrated into the leukemic cells of any of the five patients.

**DISCUSSION**

Monoclonal integration of the HTLV-I proviral genome has been reported to be observable in all primary leukemia cells of ATL patients, and this integration of the proviral genome has been thought to be a prerequisite for development of ATL (11). Therefore, ATL has been defined as an HTLV-I-induced T-cell malignancy with an inducer/helper T-cell phenotype. However, no oncogene has been identified in the HTLV-I proviral genome (17). A cis-acting mechanism—i.e., promoter or enhancer sequences of the viral LTR that activate transcription of specific cellular genes—is not responsible for the transformation, because the site of integration of the HTLV-I proviral genome into chromosomal DNA of leukemic cells has been shown to differ from case to case (18). The possibility of a trans-acting mechanism by the HTLV-I LTR-linked gene or HTLV-I pX gene product has been examined (19-21), but there is no evidence that most primary leukemia cells express detectable levels of viral mRNA, including pX sequences.

Here we have presented five cases of ATL that did not show either integration of the HTLV-I proviral genome into chromosomal DNA of leukemia cells or anti-HTLV-I antibody in the serum. Moreover, no anti-HTLV-I antibody was detectable in any family members of the patients examined, including the mother of one patient. These facts indicate that HTLV-I was not associated with leukemogenesis in any of these cases of ATL, although we cannot exclude the possible presence of a very small fragment of the HTLV-I provirus that could not be detected by the present hybridization technique or the possible deletion of once integrated HTLV-I provirus.
logical features of these cases were exactly the same as those of cases of HTLV-I-associated ATL (13, 15, 22, 23). As in HTLV-I-associated ATL, the clinical course of HTLV-I-negative ATL shows diversity. From their clinical features, cases 1, 2, and 5 were diagnosed as of an acute type; case 3 was of a chronic type, and case 4 was of a lymphoma type of ATL. Recently, a group from Italy reported HTLV-I-related and -unrelated cases of chronic T-helper lymphocytic leukemia and suggested this be regarded as one disease arising from the same subpopulation of mature T lymphocytes (24). However, their classification of T-cell malignancies was criticized as inaccurate (25). Only three cases with pleomorphic convoluted lymphocytes were exactly ATL and were associated with HTLV-I. Other HTLV-I-unrelated cases were not ATL but more accurately diagnosed as T prolymphocytic leukemia or chronic T-lymphocytic leukemia, because leukemia cells of these cases did not show flower-cell morphology at all (25).

Studies of surface markers showed that cases 2, 3, and 4 had the inducer/helper T-cell phenotype, whereas cases 1 and 5 expressed only the peripheral T-cell phenotype and their subset marker was not specified. The leukemia cells of some ATL patients have been found to express only the peripheral T-cell phenotype and not the inducer/helper T-cell phenotype (8). In case 3 of our study, the leukemia cells gained Leu 2a antigen during the clinical course of the disease and became Leu2a+3a+. Similar changes of cell surface antigens have been observed in some ATL cases (26), especially at the time of relapse or exacerbation.

These results indicate that there were no detectable differences between the clinicohematological, cytopathological, or immunophenotypic features of cases of HTLV-I-positive and HTLV-I-negative ATL. The presence of HTLV-I-negative ATL suggests the existence of unknown cellular oncogenes closely related to the transformation of inducer/helper T cells. The absence of interleukin-2 receptor antigen in leukemia cells of patient 3 indicates that the interleukin-2 receptor may not be necessary for maintaining tumor growth in HTLV-I-negative ATL. We think that the role of HTLV-I in the etiology of ATL may be very similar to that of Epstein–Barr virus in Burkitt lymphoma. Namely, the causative agent of ATL may not always be HTLV-I, and hence, ATL may not always be defined as an HTLV-I-induced T-cell malignancy.

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