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Novel Interleukin-2 Dependent T-Cell Line Derived from Adult T-Cell Leukemia Not Associated with Human T-Cell Leukemia Virus Type I

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A novel interleukin-2 (IL-2)-dependent T-cell line, WHN2, was established from a patient with adult T-cell leukemia (ATL) not associated with human T-cell leukemia virus type I (HTLV-I). Neither the original leukemic cells nor the WHN2 cells showed proviral integration in their cellular DNAs by Southern blot analysis. The surface phenotype showed that both the original leukemic cells and the WHN2 cells had a common phenotype of ATL, i.e., positive for CD2, CD4, human leukocyte antigen DR (HLA-DR) and CD25, but negative for CD8, a characteristic of helper/inducer T-cells. Most of the chromosomal abnormalities of the original leukemic cells were maintained in the WHN2 cell line. Furthermore, Southern blot analysis of the T-cell receptor β-chain gene rearrangement revealed that the original leukemic cells and WHN2 cell line had identical patterns, suggesting that the WHN2 cell line was truly derived from the original leukemic cells. Dose-dependent growth on IL-2 was demonstrated, and at the maximal stimulation, the number of cells doubled within three days. This IL-2-dependent growth was inhibited by the simultaneous existence of anti-IL-2 receptor α and β chain antibodies. These results indicate that the character of the WHN2 cell line is similar to that of the cell lines derived from ATL associated with HTLV-I. Thus, the HTLV-I-negative ATL cell line, WHN2, should be useful in the comparative study of the pathogenesis of ATL associated with or without HTLV-I.

Key words: ATL — HTLV-I — T-cell line — IL-2

Adult T-cell leukemia (ATL), which is associated with a poor prognosis,[1] is considered to be a good model for the study of human leukemogenesis in that human T-cell leukemia virus type I (HTLV-I) has been identified as a causal agent of ATL.[2] The relationship, however, between viral infection and oncogenesis is still unclear. Shimoyama *et al.* described five cases of ATL that were not associated with HTLV-I and pointed out that there were no differences in clinical, hematological, morphological and immunophenotypic features between patients with and without the anti-HTLV-I antibody.[3] Furthermore, no proviral integration was demonstrated in the leukemic cells of these patients.[4] Thus, characterization of ATL not associated with HTLV-I would be important in studying the common genetic and/or epigenetic mechanism of oncogenesis for ATL with and without HTLV-I.

In attempting to characterize ATL not associated with HTLV-I, we cultured peripheral blood mononuclear cells of an ATL patient not associated with HTLV-I in the presence of interleukin-2 (IL-2) and human plasma. The proliferating cells were maintained for more than two years and named cell line WHN2. In the present study, we characterized WHN2 to show that this cell line is derived from the leukemic cells of the patient by examining surface phenotype, karyotype and T-cell receptor β-chain gene rearrangement.

PATIENTS AND METHODS

Patient A 68-year-old Japanese man was admitted to the National Toyohashi Hospital in June, 1989, for fever and systemic lymphadenopathy. The leukocytosis (19,900/μl) noted eight days before admission was advanced (73,800/μl) on admission, and the lymphadenopathy also progressed rapidly. No hypercalcemia or skin lesions were observed. Examination of peripheral blood smears revealed that 89% of leukocytes were leukemic cells and those cells had polymorphic nuclei, with a "flower cell" morphology (Fig. 1), a characteristic of ATL. Surface marker analysis showed that the phenotype of the patient's leukemic cells was similar to that of helper/inducer T cells, being positive for CD2, CD4, CD25 and human leukocyte antigen DR (HLA DR), but negative for CD8 (Table I). Neither particle agglutination nor immunoblot analysis identified an anti-HTLV-I antibody

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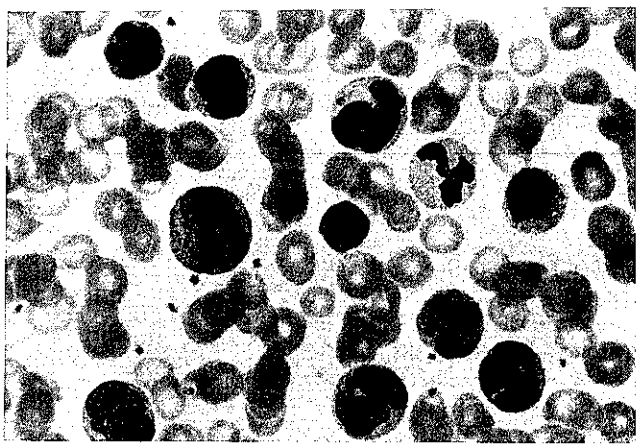

Fig. 1. Flower cell morphology of leukemic cells of the patient on admission. Most of the peripheral mononuclear cells have polymorphic nuclei which are typical of the leukemic cells of ATL (May-Giemsa staining; magnification ×400).

in this patient's serum. The patient received serial courses of combination chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine and prednisolone. One month later, he achieved complete remission, but six months later, he relapsed during consolidation chemotherapy. He died from interstitial pneumonitis in March 1990.

Establishment of a cell line Before initiation of chemotherapy, peripheral blood samples were obtained for various examinations as follows. Leukemic cells were separated from the heparinized peripheral blood by density centrifugation and some parts were frozen in a solution of RPMI1640 with 20% fetal calf serum and 10% dimethyl sulfoxide. In October 1989, a frozen sample was thawed and cultivated in Cosmedium 001 (Cosmo Bio, Tokyo) to which 20% by volume of human plasma and 70 U/ml of recombinant human IL-2 (rIL-2) were added. Within one week of cultivation, leukemic cells began to grow vigorously and have continued to proliferate for over three years. One year after primary cultivation was started, the cell line became capable of proliferating in the absence of human plasma. We named the cell line WHN2.

Surface marker analysis The surface phenotype was analyzed by indirect immunofluorescence assay using flow cytometry (Spectrum III; Ortho Diagnostic Systems, Tokyo). Monoclonal antibodies used in this study were CD2 (9.6), CD3 (OKT3), CD4 (OKT4), CD7 (TP40), CD8 (OKT8), CD20 (B1), CD25 (Tac) and OKI1 for HLA-DR.

Chromosomal analysis Chromosomal analysis was performed by the G-banding method (Special Reference Laboratory, Tokyo). A fresh sample of the patient's bone marrow cells acquired on admission was analyzed to obtain the karyotype of tumor cells. The karyotype of WHN2 cells presented was that after three months' cultivation.

Southern blot analysis High-molecular-weight DNA of original leukemic cells was extracted from the patient's peripheral mononuclear cells on admission, after density centrifugation (these were the cells from which WHN2 was established). The DNA of WHN2 was extracted three months after the beginning of cultivation. For the detection of HTLV-I proviral genome, DNA fragments that cover the HTLV-I genome, except for a small part of *gag*, were radiolabeled and used as probes as described previously.[4] ATL-1K having one copy of intact HTLV-I proviral DNA[5] was used as a positive control. The probe for T-cell receptor β-chain gene (TCR-β) rearrangement was in the constant region of human TCR-β cDNA.[6]

Polymerase chain reaction (PCR) For sensitive detection of the HTLV-I proviral genome, PCR was used for DNA extracted from the patient's peripheral blood mononuclear cells.[7] One μg of the DNA was subjected to 35 cycles of amplification in 50 μl of the reaction mixture with one unit of *Thermus aquaticus* DNA polymerase (New England Biolabs). Other reaction conditions were as described previously.[8] Ten μl of the 50 μl reaction mixture was electrophoresed on agarose gel and analyzed by Southern blot analysis. Primers used in this study were as follows: LTR3 (5′-CTTAGAGCCTCCCAGTGAAA-3′) and LTR6 (5′-AGCCATATGCGTGCCATGAA-3′) for the U3 region of HTLV-I LTR; LTR51 (5′-GATCGAAAGTTCCACCCCTT-3′) and LTR52 (5′-AGTGCTATAGGATGGGCTGT-3′) for the U5 region of LTR; and gag 1 (5′-TTATGCAGACCATCCGGCTT-3′) and gag2 (5′-TATCTAGCTGCTGGTGATGG-3′) for the *gag* region. Oligonucleotide probes used for Southern blot analysis were LTR5 (5′-AGACTAAGGCTCTGACGTCT-3′) for the U3 region; LTR53 (5′-CCCTTTCATTCACGACTGAC-3′) for the U5 region; and gag3 (5′-ACTGCCAAAGACCTCCAAGA-3′) for the *gag* region.

IL-2-dependent growth curve Proliferating cells were washed three times in Cosmedium 001 without rIL-2. Cells at a density of 1×10^4/ml were inoculated with various concentrations of rIL-2. Viable cells that excluded trypan blue dye were counted daily for ten days.

Growth inhibition by anti-IL-2 receptor (IL-2R) antibodies Ta60a and Ta60b were used as the antibodies for IL-2R α chain.[9] The antibody for IL-2R β chain was Mik-β1, kindly supplied by Dr. Miyasaka (Tokyo Metropolitan Institute of Medical Science, Tokyo).[10] Growth inhibition by anti-IL-2R antibodies was examined as follows: 10^4 WHN2 cells were inoculated into 96-well microplates, each well of which contained 100 μl of

Cosmedium 001 with 25 U/ml of rIL-2 and 50 or 100 μg/ml of antibody; three days later, proliferation was measured by the colorimetric MTT (tetrazolium) assay[11]; then the plates were read with a multiwell scanning spectrophotometer (Microelisa reader, Nihon-BioRad, Tokyo) using a test wavelength of 595 nm and a reference wavelength of 655 nm.

RESULTS

Surface marker analysis Table I shows the immunologic phenotypes of the patient's peripheral blood mononuclear cells on admission and the WHN2 cells. The leukemic cells were positive for CD2, CD4, CD25, HLA-DR and were weakly positive for CD3; these findings are characteristic of HTLV-I-associated ATL.[12] WHN2 had an almost identical phenotype to that of the original leukemic cells except for an increased positivity for CD25.

Detection of HTLV-I proviral DNA Southern blot analysis was negative for proviral integration in the original leukemic cells (data not shown), indicating that HTLV-I proviral DNA was not integrated in the cellular DNA of the leukemic cells. For more sensitive detection, PCR analysis using various oligomers was carried out. The DNAs from the ATL-1K cell line having one copy of HTLV-I as well as two asymptomatic HTLV-I carriers were positive for HTLV-I, but the sample from the patient was negative with all of these oligomers (Fig. 2a), indicating that the patient had not been infected with HTLV-I. Southern blot analysis with the WHN2 cell line was negative (Fig. 2b).

Origin of cell line Cytogenetic analysis of the patient's bone marrow cells showed that the original leukemic cells had a complex karyotype. The chromosome abnormalities are summarized in Table II and examples are

Table I. Surface Phenotypes of Peripheral Leukemic Cells and WHN 2

	Peripheral leukemic cells (%)	WHN2 cell line (%)
CD 2	96.8	99.9
CD 3	29.7	27.2
CD 4	96.8	99.7
CD 7	5.3	1.3
CD 8	6.4	1.8
CD 20	5.3	2.6
CD 25	51.4	99.4
HLA DR	94.2	100.0

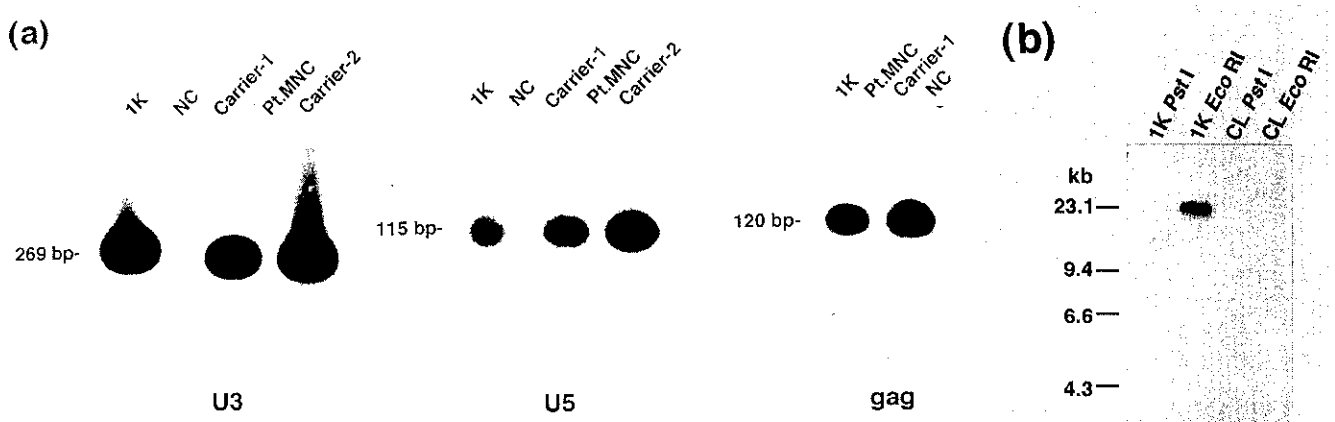

Fig. 2. Detection of HTLV-I proviral DNA. (a) Detection of HTLV-I proviral DNA in peripheral mononuclear cells by PCR analysis. U3, U5, or *gag* regions were amplified and detected by Southern blot analysis with appropriate probes (see "Patients and Methods"). 1K, ATL-1K cell line containing one copy of HTLV-I; NC, a healthy volunteer not infected with HTLV-I; Carriers 1 and 2, asymptomatic HTLV-I carriers; Pt.MNC, the fresh peripheral mononuclear cells of the present patient. (b) Detection of HTLV-I proviral DNA in the WHN2 cell line by Southern blot analysis. Ten μg of DNA from ATL-1K or WHN2 was digested with *Pstl* or *Eco*RI and analyzed by Southern blot analysis. 1K, ATL-1K cell line; CL, WHN2 cell line of the present study. The first and second lanes show the presence of intact provirus of HTLV-I in ATL-1K, while the third and fourth lanes did not show positive signals, indicating the absence of HTLV-I proviral DNA in WHN2.

Table II. Chromosomal Abnormalities in Patient's Bone Marrow Cells and WHN2

	Bone marrow cells			WHN2 cell line		
Cells/total	3*/19	1/19	15/19	6*/19	7/19	6/19
Numbers of chromosome	49	49	46	49	47	46
Abnormality	$3p^+$ $-4, -4$ $6p^+, 6p^+$ -8 $9p^-$	$3p^+$ $-4, -4$ $6p^+, 6p^+$ -8 $9p^-$ -10	none	$3p^+$ $-4, -4$ $6p^+$ -8	$3p^+$ $-4, -4$ $6p^+$ -8	$3p^+$ $-4, -4$ $-6, 6p^+$ -8
	$11p^+$	$11p^+$		$11p^+$	$11p^+$	$11p^+$ -13
	-14	-14		-14	-14	-14 -15
	$17p^+$	$17p^+$		-17 $19p^+$ -20	-17 $19p^+$	-17 $19p^+$
	$+21$ $+22$	$+21$ $+22$		$+21$ $+22$	-22 $-Y$	$+22$ $-Y$
	$+5mar$	$+6mar$		$+7mar$	$+8mar$	7mar

* Karyotypes are shown in Fig. 3.

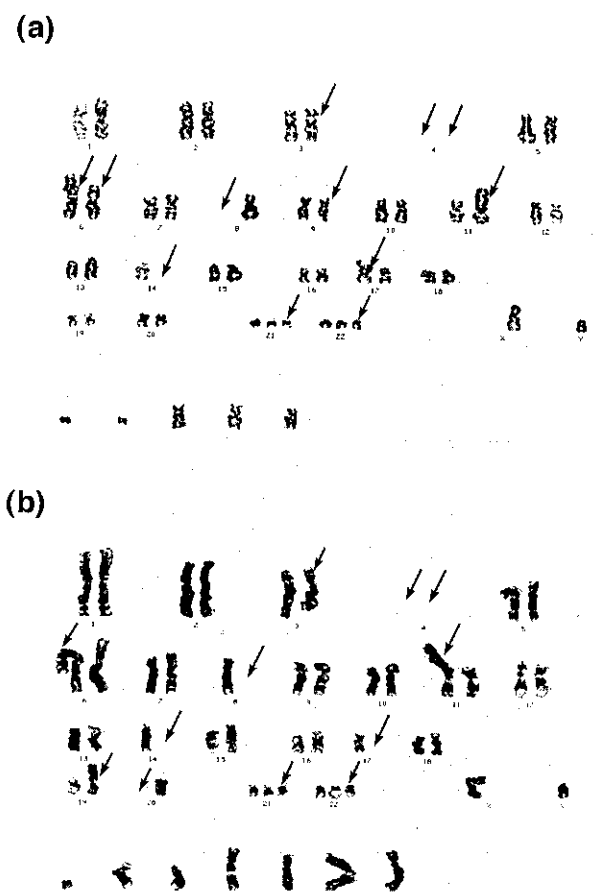

Fig. 3. Karyotypes of fresh leukemic cells from bone marrow (a) and WHN2 (b) analyzed by the G-banding method. The karyotypes marked with asterisks in Table II are shown and are as follows; (a) 49, XY, −4, −4, −8, −14, +21, +22, $3p^+$, $6p^+$, $6p^+$, $9p^-$, $11p^+$, $17p^+$, +5mar, (b) 49, XY, −4, −4, −8, −14, −17, −20, +21, +22, $3p^+$, $6p^+$, $11p^+$, $19p^+$, +7mar. Abnormal chromosomes are indicated by arrows. Marker chromosomes are shown at the bottom.

shown in Fig. 3. In the WHN2 cell line, the majority of those abnormalities found in the original leukemic cells were maintained, except that $9p^-$ and $17p^+$ were deleted. These abnormalities suggested that the WHN2 was derived from the original leukemic cells. To study the clonality more precisely, we examined the TCR-β gene rearrangement. As shown in Fig. 4, the original leukemic cells and WHN2 showed the same pattern of rearrangement in three different enzymes, indicating that the WHN2 is derived from the leukemic cells.

IL-2-dependent growth Fig. 5 shows the growth curves for the WHN2 cells incubated with various concentrations of IL-2. With the highest concentration of IL-2 (380 U/ml), the number of cells doubled within 3 days; the rate of proliferation was slower with lower concentrations of IL-2. In the absence of IL-2, the cells died within a week. Thus, the proliferation of WHN2 cells was shown to be dependent on IL-2.

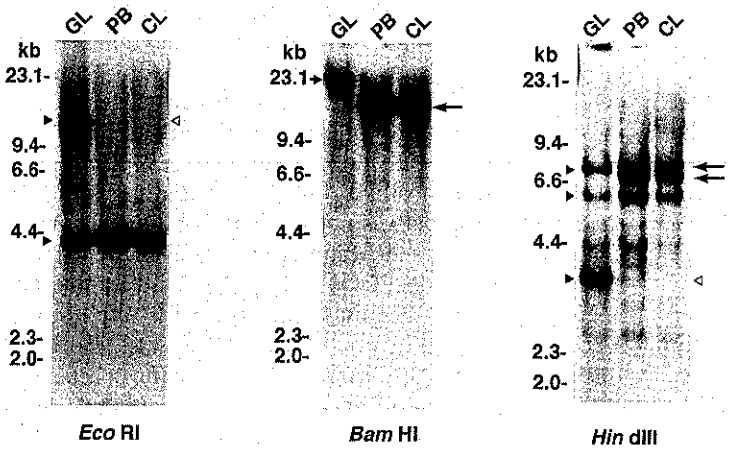

Fig. 4. Southern blot analysis of T-cell receptor β-chain gene rearrangement in fresh leukemic cells and WHN2 cell line. Samples (10 μg) of DNA from the myeloid cell line, HL-60 (GL), the fresh leukemic cells (PB) and the WHN2 cell line (CL) were digested with *Eco*RI, *Bam*HI or *Hin*dIII. The open triangles indicate the 10.2 kb germ line band (*Eco*RI, left panel) and 3.5 kb germ line band (*Hin*dIII, right panel) which were deleted in the patient's peripheral mononuclear cells (PB) and WHN2 (CL). The rearranged bands in *Bam*HI or *Hin*dIII digests are indicated by arrows. The closed triangles on the left of each panel depict the germ line bands.

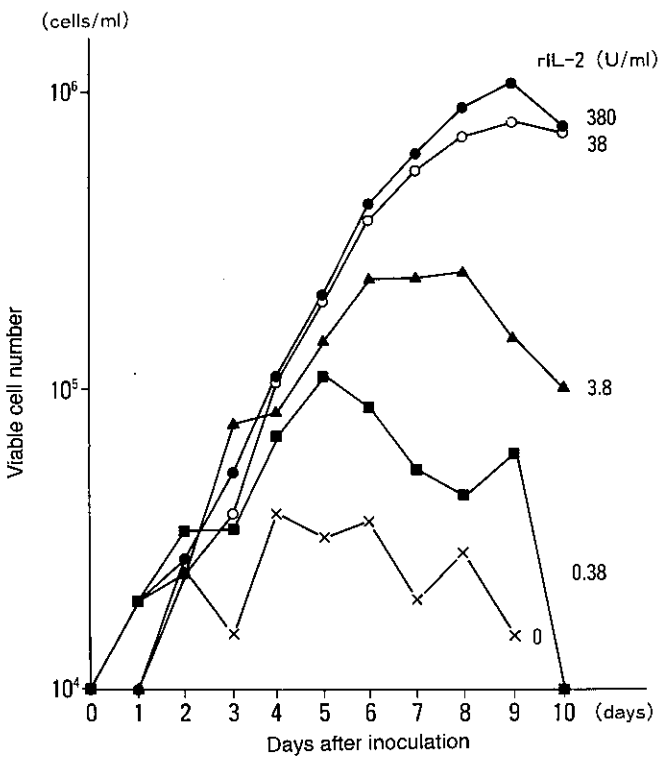

Fig. 5. Growth curves of the cell line with various concentrations of rIL-2. Cells (10^4/ml) inoculated with rIL-2 were plated and the number of viable cells was counted. The concentration of rIL-2 was 380 U/ml (●), 38 U/ml (○), 3.8 U/ml (▲), 0.38 U/ml (■) or 0 U/ml (×).

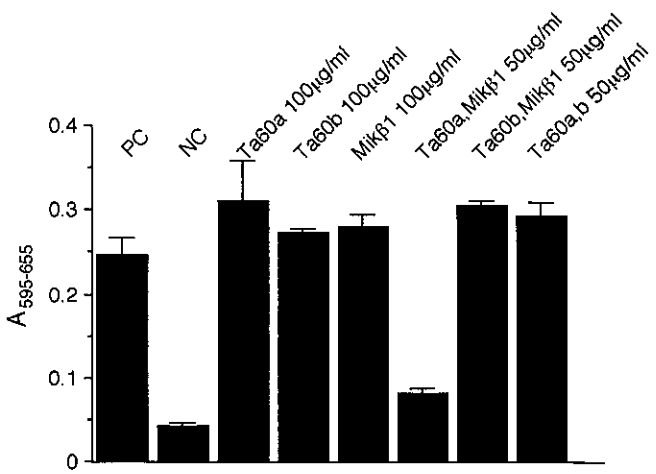

Fig. 6. Growth inhibition by various anti-IL-2R antibodies. The WHN2 cells were cultured in the presence of various combinations of anti-IL-2 receptor antibodies against α chain (Ta60a, Ta60b) or β chain (Mik-β1). Data shown are the average$\pm$SD of triplicate cultures. PC, cells cultured without antibody; NC, cells cultured without rIL-2.

Growth inhibition by anti-IL-2R antibody The IL-2 dependency of WHN2 was further examined by using anti-IL-2R antibodies. Ta60a was shown to have an inhibitory effect on the IL-2 dependent T-cell line, but Ta60b or Mik-β1 alone does not have the inhibitory effect.[9, 10] Fig. 6 demonstrated that the simultaneous addition of Ta60a and Mik-β1 completely inhibited the growth of WHN2, but neither Ta60a, Ta60b, nor Mik-β1 alone had any inhibitory activity. This indicated that the stimulation through IL-2R was necessary for the proliferation of WHN2 although the mode of inhibition by anti-IL-2R antibodies was different from that of other IL-2-dependent cell lines.[9, 10]

DISCUSSION

Cases of ATL were first described by Uchiyama *et al.*[1] and was defined by Takatsuki *et al.*[13] and Shimoyama *et al.*[14] The morphology and surface phenotype of the leukemic cells from the present patient were typical of ATL.[12, 15] An anti-HTLV-I antibody, however, was not detected by the particle agglutination method or by immunoblot assay. Although in some HTLV-I-seronegative patients with mycosis fungoides, deleted HTLV-I provirus was detected in blood and cutaneous lesions,[16] our case showed no proviral integration of HTLV-I by Southern blot analysis. Matutes *et al.* reported a disease entity designated T-prolymphocytic leukemia (T-PLL), another type of mature T-cell leukemia. This disease is similar to ATL in that leukemic cells are positive for CD4 in most cases and that it has a poor prognosis.[17] The leukemic cells of T-PLL, however, consistently show prominent nucleoli and CD7 antigen.[17] On the other hand, the leukemic cells of the present patient showed a flower-like nuclear morphology without prominent nucleoli (Fig. 1), and did not express CD7 antigen (Table I); neither was inv(14), a characteristic karyotype abnormality in T-PLL, found. These results indicated that our patient had not T-PLL but ATL. Thus, our patient was diagnosed as ATL not associated with HTLV-I.

As for the oncogenic process of ATL, Okamoto *et al.* suggested that five serial genetic changes are necessary for leukemogenesis of T-cells infected with HTLV-I.[18] The evidence that not all ATLs have HTLV-I infection suggests that similar accumulation of genetic alterations could take place without HTLV-I. Such ATL cases without HTLV-I infection would be very important for studying the common genetic changes leading to clinical manifestation of the disease. For the etiological study of ATL not associated with HTLV-I, attempts were made to detect possible viral involvement by using the methods utilized for HTLV-I.[2, 19] We tried to detect viral antigen expression in WHN2 using the patient's own serum by indirect immunofluorescence assay. We also measured reverse transcriptase activity. These results, however, were all negative (data not shown), indicating that the present patient did not harbor unknown retroviruses, which should be detected by these assays.

The WHN2 cell line was identified as being derived from the original leukemic clone by cytogenetic analysis and by the presence of the identical rearrangement pattern of the TCR-β chain gene. Generally, in the presence of IL-2, many cell lines are established from cells from ATL patients associated with HTLV-I, but cell lines derived from an original leukemic clone are rare. Many cell lines have been derived from CD4(+) T lymphocytes infected with HTLV-I.[20, 21] Since the WHN2 cell line was shown to be derived from leukemic cells of ATL, it is conceivable that the cell line retains many of the characteristics of *in vivo* leukemic cells of ATL. The ATL cell line, ATL-5T, derived from the pericardial effusion of an ATL patient not associated with HTLV-I was reported.[22] This cell line, however, was reported to be CD4 and CD8 positive, but CD3 negative, indicating that the phenotype was not typical of ATL. The WHN2 cell line of the present study has a helper/inducer phenotype (Table I), i.e., CD3 and CD4 positive, but CD8 negative, which is the most common phenotype in ATL associated with HTLV-I.[12] The WHN2 cell line, therefore, should serve as a useful cell line for the comparative study of the common genetic changes leading to leukemogenesis of ATL with and without HTLV-I involvement.

The chromosomal abnormalities in the present study were complex, as is the case in most ATL patients. Chromosome abnormalities of +3, +7, +21, −X, −Y, translocation or inversion involving 14q32 and 14q11, and deletion of 6q have been reported to be the most common in ATL associated with HTLV-I.[23] The chromosomal abnormalities of ATL not associated with HTLV-I were reported to be complex and some of these abnormalities were also frequently found in HTLV-I-associated ATL.[24] The chromosome abnormalities found in the present study, however, did not demonstrate these common abnormalities except +21 (Table II, Fig. 3A and 3B). It still remains to be elucidated whether a common chromosomal abnormality is important for leukemogenesis of ATL. It is also possible that the common chromosomal abnormality may not be microscopically detectable. Regarding this interesting point, WHN2 would be a useful cell line to study putative common genetic alteration(s) yet to be identified.

It should be noted that CD25 is expressed in WHN2 (Table I) but not in ATL-5T.[22] ATL-5T proliferated only in the presence of human cord serum and did not respond to IL-2,[15] while the WHN2 required IL-2 for proliferation. WHN2 responded to IL-2 in a dose-dependent manner (Fig. 5), and anti-IL-2R antibodies completely blocked its growth (Fig. 6). The CD25 antigen of the WHN2 was, therefore, shown to be functionally important for proliferation. The general growth of IL-2-dependent cell lines was blocked by Ta60a but not by Ta60b[9]; however, Ta60a alone had no inhibitory activity on WHN2, whose blocking needed the simultaneous presence of anti-IL-2R β chain antibodies. This evidence suggests that the affinity between IL-2 and IL-2R molecules on WHN2 was different from that in other IL-2-dependent cell lines.[9, 10] Further analysis of IL-2R molecules is necessary, and this difference may become important for understanding leukemogenesis of ATL not associated with HTLV-I.

The WHN2 cell line, together with other ATL cell lines, should provide a useful experimental system for elucidating the leukemogenesis of ATL with or without HTLV-I.

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