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Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease

(integrated provirus/primary tumor cells/endemic area/Southern blot)

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ABSTRACT **The genome of human T-cell leukemia virus (HTLV) was surveyed in fresh tumor cells of 163 patients with lymphoma and leukemia from the southwest part of Japan where adult T-cell leukemia (ATL) is endemic. Leukemic cells of all 88 cases of ATL tested so far were found to contain the provirus genome and also found to be monoclonal with respect to the integration site of provirus genome. In most cases of ATL, leukemic cells contained one or two copies of the complete HTLV provirus genome, and it was shown that the single species of HTLV with a fully determined sequence is typical in ATL. Some cases of T-cell malignancies, diagnosed as chronic lymphocytic leukemia or non-Hodgkin lymphoma, also had the provirus genome in their tumor cells, whereas some cases with the same diagnosis did not. No cases of other types of lymphoma or leukemia contained the provirus genome in their tumor cells. Monoclonal integration of the HTLV provirus genome in all primary tumor cells of ATL not only indicates that HTLV directly interacts with target cells, which become leukemic, and that integration of the provirus genome is a prerequisite for development of ATL and possibly other related diseases but also indicates that the virus is not associated with other types of lymphoma or leukemia.**

Human retroviruses HTLV (human T-cell leukemia virus) (1–3) and ATLV (adult T-cell leukemia virus) (4, 5) were independently isolated from cases of cutaneous T-cell lymphoma (CTCL) and adult T-cell leukemia (ATL) (6), respectively. Subsequently, HTLV and ATLV were shown to be similar in immunological crossreactivities (7, 8) and nucleic acid hybridization (9). Recently, we determined the total nucleotide sequence of the ATLV provirus genome (10) and showed that ATLV and HTLV type I are identical with respect to the locations of gene-specific sequences and the sites of some restriction enzymes (11). We use the term ATK strain of HTLV ($HTLV_{ATK}$) for the virus previously cloned in ATK-1 DNA and reported as ATLV (5, 10). The retrovirus HTLV is exogenous for humans (2, 5) and distinct from known animal retroviruses with respect to the structure of its provirus genome (10, 12). Furthermore, HTLV was shown to be closely associated with a unique T-cell malignancy, ATL, by extensive surveys of antibodies against the viral proteins (4, 7, 8, 13–15). The association of the virus with ATL was also shown by detecting the provirus genome in leukemic cells of ATL patients (5, 16). Some patients with other types of lymphoma or leukemia, such as CTCL, chronic lymphocytic leukemia (T-CLL), and non-Hodgkin lymphoma of T-cell origin, were also reported to have antibodies against the viral proteins (4, 17). The presence of antibodies to viral proteins is evidence for infection with HTLV but does not provide any information on the mode of involvement of the virus in leukemogenesis.

To understand whether HTLV is directly involved in leukemogenesis of ATL and whether the virus is associated with any other types of lymphomas or leukemias, we surveyed the provirus genome integrated in fresh tumor cells of 163 cases of lymphoma or leukemia from endemic areas in Japan. The results showed that the HTLV provirus genome was integrated in tumor cells of all 88 cases of ATL tested and also in those of some cases of T-cell malignancies that were very similar to ATL but was not integrated in those of other types of lymphoma or leukemia, even in tumor cells from antibody-positive patients. The monoclonality of tumor cells was also shown in all cases of ATL.

MATERIALS AND METHODS

Preparation of Cellular DNA. Patients with typical ATL or ATL-like clinical features were from the Kyushu area, where ATL is endemic. Some other samples with lymphoma or leukemia in Table 1 came mainly from the Kyushu area. Lymphocytes were prepared from fresh peripheral blood of leukemic patients by centrifugation on a Ficoll–Conray gradient (5). To prepare DNA for several blotting analyses, lymphocytes or lymph nodes from lymphoma patients containing more than 10^7 cells were used. High molecular weight DNA was extracted by treating the cells overnight with NaDodSO_4/proteinase K, followed by phenol extraction.

Detection of Provirus DNA Integrated in Cellular DNA. Cellular DNA was digested with *Eco*RI, *Sst* I, or *Pst* I, and the digest was separated by electrophoresis in an agarose gel. The DNAs in the gel were blotted onto a nitrocellulose filter and hybridized with cloned viral [^{32}P]DNAs as shown in Fig. 1, under relatively stringent conditions (10) in buffer containing 4× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7.5), 5× Denhardt solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), sonicated and heat denatured *Escherichia coli* DNA (100 μg/ml), and poly(A) (40 μg/ml) at 65°C for 24–40 hr. The filter was washed several times with 0.5× NaCl/Cit at 65°C and then was exposed to x-ray film at −70°C.

Detection of Antibodies Against Viral Components. Antibodies against ATL-associated antigens (4) were examined by indirect immunofluorescence with MT-1 cells, as described by Hinuma *et al.* (4). Briefly, cultured MT-1 cells were smeared onto a glass slide, dried at room temperature for more than 1 hr, and then fixed with acetone at room temperature for 10 min. The smears were treated with human serum diluted 1:10 in phosphate-buffered saline (P_i/NaCl) at

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Abbreviations: ATL, adult T-cell leukemia; HTLV, human T-cell leukemia virus; ATLV, adult T-cell leukemia virus; CTCL, cutaneous T-cell lymphoma; T-CLL, chronic lymphocytic leukemia of T-cell origin; kb, kilobase(s).

Table 1. Association of the HTLV genome with ATL and related diseases

Group	Viral genome*	Anti-ATLA†	Diagnosis	Cell type	Case no.	Sum
i	+	+	ATL	T	88	
			Non-Hodgkin	T	7	100
			CLL	T	5	
ii	−	+	CLL	T	1	
			Hodgkin‡	ND	2	7
			Non-Hodgkin	ND	1	
			AML	ND	3	
iii	−	−	CLL	T	6	
			CLL	B&N	4	
			Sezary	T	1	
			ALL	TBN	16	56
			Non-Hodgkin	TBN	17	
			Hodgkin‡	ND	1	
			Myeloma	ND	4	
			CML	ND	3	
			AML	ND	4	
			Total			163

Non-Hodgkin, non-Hodgkin lymphoma; Hodgkin, Hodgkin disease; CLL, chronic lymphocytic leukemia; AML, acute myeloblastic leukemia; Sezary, Sezary syndrome; CML, chronic myelogenous leukemia; ND, not determined.
*Provirus genome in tumor cells.
†Serum antibodies against ATL-associated antigens (ATLA).
‡The amount of tumor cells in the specimen might not be sufficient to detect the provirus sequence.

37°C for 30 min, washed with P_i/NaCl, and treated with fluorescein-conjugated rabbit anti-human IgG at 37°C for 30 min. They were then washed with P_i/NaCl, covered with a cover glass, and examined under a Zeiss fluorescence microscope.

RESULTS

Survey of Provirus DNA in Tumor Cells. In this work, only fresh tumor cells were used for the following reasons: HTLV produced from only a few nonleukemic cells can be transmitted into T cells in culture, and the infected cells can reproduce as transformed T cells (18–20). Thus, cell lines or cells maintained in culture do not necessarily represent primary tumor cells. In fact, the available cell lines contained numerous copies of the integrated proviruses (11), whereas fresh leukemic cells contained only one or two copies (5).

For detection of the provirus genome, several different parts of the cloned HTLV genome (ATK strain of HTLV or $HTLV_{ATK}$) were mixed (Fig. 1) and used as representative probes in the blot-hybridization assay. Cellular DNA samples were digested with *Eco*RI or, in a few cases, with *Sst* I, both of which do not cut the proviral genome. By this assay, the provirus sequence integrated at a certain locus of chromosomal DNA was detected as a discrete band (Fig. 1), but sequences integrated into multiple sites were distributed as random fragments and, thus, could not be detected as discrete bands. Table 1 summarizes the results of 163 cases of lymphoma and leukemia. Most of the cases were from the endemic area in Japan. They were divided into three groups according to the presence or absence of the provirus genome in their leukemic cells or antibodies to viral proteins: (*i*) patients with antibodies and the provirus genome in their tumor cells, (*ii*) patients with antibodies but with no detectable provirus genome in their leukemic cell DNAs, and (*iii*) patients who had neither provirus nor antibodies.

All 88 patients with ATL were included in group *i*. Judging from the number of bands containing the provirus sequence, one or two or, in one case, three copies of the provirus genome were detected in their leukemic cells. The intensities of the bands were less than the equivalent amount of λATK-1 DNA [a clone from DNA of leukemia cells of an ATL patient (10)] corresponding to one copy per haploid. Thus the provirus bands in the samples did not represent more than two copies per cell (data not shown). Fig. 1 shows that these provirus sequences were detected as one or two discrete bands in digests with *Eco*RI, which does not cut typical provirus DNA. The intensities of the bands varied in different patients but were found to correlate with the proportion of tumor cells in the lymphocyte preparations. These data clearly indicate that the leukemic cells were monoclonal, originating from a single cell infected with HTLV because integration of HTLV genome in chromosomal DNA of nonleukemic cells was random (21, 22).

The monoclonal expansion of infected cells as tumor cells in all 88 cases strongly suggests that HTLV directly infects the target cell, which becomes leukemic, suggesting that HTLV is at least one of the causative agents in ATL. In only few cases, however, blot-hybridization assays gave a faint band in addition to the main band, as in a patient AI4 (Fig. 1). This faint band could represent a small population of leukemic cells that diverged from the main population of leukemic cells by deletion or rearrangement of cellular DNA or by a second infection of HTLV into a particular leukemic cell. Otherwise, it represents a subpopulation of originally biclonal tumor cells. In any case, these observations do not affect the above conclusion because the frequency and quantity of such additional bands are low.

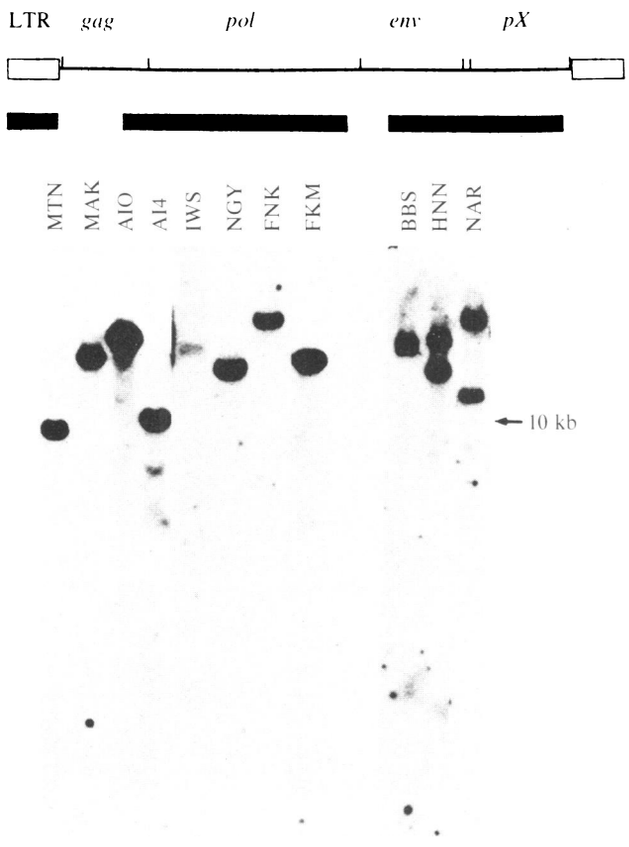

FIG. 1. Detection of the provirus sequence in fresh tumor cells. Cellular DNAs extracted from fresh leukemic cells of 11 ATL patients were digested with *Eco*RI, which does not cut the provirus genome, separated on agarose gel, and analyzed by blot-hybridization (*Lower*) by using the described representative probes as illustrated (*Upper*). The thick bars under the genome represent regions subcloned separately into pBR322. These three subcloned fragments were mixed in equal amounts, labeled with [α-^{32}P]dCTP by nick translation, and used as probes. Molecular sizes were determined with reference to phage λ DNA digested with *Hin*dIII.

In addition to ATL patients, group *i* included some cases of T-cell malignancies diagnosed as non-Hodgkin lymphoma or T-CLL. However, these cases were all clinically and pathologically similar to ATL (23).

No cases in groups *ii* and *iii* were directly associated with HTLV because the provirus genome was not detected in their leukemic cells; that is, their leukemic cells were not infected with the virus. Patients in group *ii* had antibodies against the viral proteins but no provirus genome was detected in their leukemic cells. This failure in detecting the proviruses was confirmed by digesting the DNAs with *Pst* I, which cuts the proviral DNA at multiple sites and produces three large internal fragments. Therefore, the possibility that viral genomes were randomly integrated into leukemic cells and, thus, not detected in *Eco*RI or *Sst* I digests by blotting analysis was excluded. Thus, no cases of lymphoma or leukemia in groups *ii* and *iii* were associated with HTLV.

Single Species of Provirus Genome Integrated in ATL Cells. Nucleotide sequence analysis of HTLV showed that the virus has no typical transforming gene (10). However, this structural information was obtained from analysis of only one provirus genome; thus, it was necessary to show that the provirus with the fully determined sequence is typical in ATL. To demonstrate this, the proviruses integrated in ATL cells were digested with *Pst* I, and the pattern of the viral internal fragments was compared with that of the provirus genome of known sequence (10). From the total nucleotide sequence (10) of one provirus clone, we know that three large internal fragments of 1.2, 1.8, and 2.5 kilobases (kb) will be formed by *Pst* I digestion (Fig. 1). The lengths of the fragments from the two ends containing the cellular flanking sequence will vary depending on the site of integration. In 79 of 88 cases of ATL, the three expected sizes of internal fragments were obtained. Some examples are shown in Fig. 2. The fact that most cases also gave two other bands in addition to those of the three internal fragments suggested but did not prove the presence of two LTRs in the provirus integrated in tumor cells. These findings suggest that a typical and complete HTLV was involved in most cases of ATL in Japan and that this typical provirus is the same provirus whose sequence we determined previously (10). About 10% (9 cases) of the ATL patients did not give the three internal fragments (Fig. 2), indicating that these cases contained either defective provirus or different but related proviruses. Preliminary analysis of these exceptional proviruses indicated that these were defective proviral sequences derived from typical HTLV (data not shown).

DISCUSSION

In this work, we screened the HTLV genome in 163 patients with lymphoma or leukemia, mostly from the endemic area in Japan. Leukemia cells from all 88 cases of ATL were found to contain the viral genome. Furthermore, the leukemic cells were all monoclonal with respect to the integration site of the provirus genome. These results strongly suggest that HTLV interacts directly with the target cells, which then grow as malignant transformed cells, although the infection does not necessarily induce ATL. If viral involvement were indirect—for example, mediated by a factor(s) released by infected cells—or just a coincidental infection in leukemic cells after leukemogenesis, some cases should have leukemic cells in which the provirus genome is absent or is integrated at multiple sites. Therefore, these indirect ways cannot explain the monoclonal origin of leukemic cells in all ATL cases. Alternatively, if most of the T-cell population were infected with HTLV in a preleukemic state, prolifera-

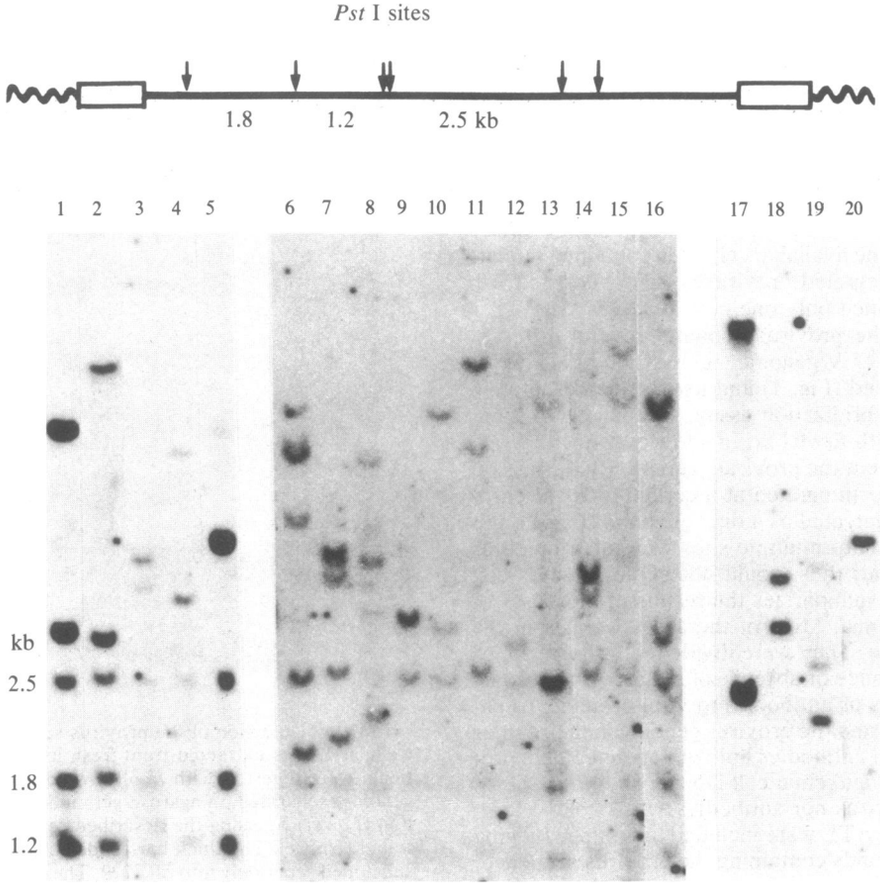

FIG. 2. Analysis of the provirus genome integrated in fresh leukemic cells. (*Lower*) Cell DNA was digested with *Pst* I and analyzed as in Fig. 1. The numbers above each lane, 1–20, represent individual ATL patients. (*Upper*) The *Pst* I sites in the provirus genome.

tion of any T cells induced by a factor(s) other than the virus would give a cell population with monoclonally integrated HTLV provirus. This possibility was excluded, however, because most of the T-cell population of healthy carriers or ATL patients in remission was not infected with the virus (data not shown).

In some cases whose clinical features were similar to those of ATL, the results did not clarify the question of whether HTLV is directly involved in the development of tumors: some cases diagnosed as T-CLL or non-Hodgkin lymphoma were found to have antibodies to HTLV proteins and also provirus genome in their tumor cells (group *i*); however, others with the same diagnosis but no detectable provirus genome in their tumor cells were classified in groups *ii* and *iii*. Therefore, these cases in group *i* may be a result of malignant transformation of HTLV-infected cells by some other factor(s) as discussed above or could be, in fact, ATL. A different diagnosis of these marginal cases of T-cell malignancies is possible by using a classical diagnosis citing the atypical clinical and pathological features of ATL (23). Further analysis is required to determine which possibility is most likely.

Patients in group *ii* had antibodies against HTLV proteins but no detectable provirus genome in their tumor cells. These findings suggest either that the patients developed leukemia as a result of some factors other than the virus or that the patients were infected after developing leukemia. Because 10–20% of healthy adults in the endemic area in Japan have antibodies against the viral proteins (4, 15), the existence of such cases is expected. These data indicate that detection of the provirus in primary tumor cells is crucial in judging the association of the virus with ATL-related diseases. Thus, all lymphoma and leukemia cases listed in groups *ii* and *iii* were shown not to be directly associated with HTLV infection. This conclusion was compatible with the finding that the distribution of T-, B-, and Null-cell lymphoma and leukemia in Kumamoto prefecture in Kyushu, where HTLV is endemic, is almost identical to that in nonendemic areas of Japan, excluding ATL cases (unpublished data).

In avian lymphoma induced by avian leukemia virus (24–26), defective proviruses are found mostly in lymphoma cells. But, in nearly 90% of ATL patients, the leukemic cells contained the complete provirus genome, that is, the genome we sequenced previously (10). This result might be explained by an inefficient expression of the viral protein on the infected cell surface because, in the case of the avian system, cells expressing the viral antigens coded by the complete viral genome were rejected by the host immune response (24).

From the present results, it is suggested that HTLV directly infects the target cell and that integration of the proviral genome is a prerequisite for development of ATL. The virus might be associated with some cases of T-cell malignancies that are very similar to ATL but not with the other types of lymphoma and leukemia.

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