Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6061–6065, October 1983
Medical Sciences

Establishment and characterization of 10 cell lines derived from patients with adult T-cell leukemia

(human retrovirus/T-cell growth factor/surface marker/immunofluorescence/Southern blot hybridization)

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ABSTRACT **By using human T-cell growth factor (TCGF), 10 cell lines were established from tissue samples of 10 patients with adult T-cell leukemia (ATL). Three cell lines were adapted to growth in medium lacking TCGF. The surface markers of all cell lines were characteristic of inducer/helper T cells, i.e., $OKT3^+$, $OKT4^+$, $OKT6^-$, $OKT8^-$, $OKIa1^+$, and human $Lyt2^+$ and $Lyt3^+$, except that one cell line was $OKT3^-$. The expression of the viral antigen was examined during establishment of 8 of the 10 cell lines. The viral antigen was not expressed in leukemic cells before cultivation. In 5 lines, the viral antigen was detected by immunofluorescent staining after a short period of cultivation. However, 3 cell lines, ATL-6A, ATL-9Y, and ATL-1K did not express the viral antigen during short-term culture: the ATL-6A and ATL-9Y cell lines became positive for the viral antigen after 5 and 2 months of cultivation, respectively; the ATL-1K cell line remained antigen-negative throughout a culture period of 13 months. Southern blot hybridization assay showed that all of the cell lines, including the viral antigen-negative ATL-1K cell line, contained the viral genome. Thus, the retrovirus was associated with all 10 cell lines established from ATL patients, but there was a heterogeneity in the expression time of the retroviral antigen in leukemic cells maintained *in vitro*. Our findings suggested that the expression of the viral antigen was not required for maintenance of the leukemic state *in vivo* and for growth of leukemic cells *in vitro*.**

Adult T-cell leukemia (ATL) was proposed to be a new entity by Takatsuki and co-workers (1, 2). Patients with ATL have been found mainly in southwestern Japan (2, 3), and patients with a T-cell malignancy indistinguishable from Japanese ATL have been found in the West Indies (4, 5). Poiesz *et al.* noticed that cell lines established from patients with cutaneous T-cell lymphoma/leukemia shed a retrovirus named human T-cell leukemia virus (HTLV) (6, 7). Hinuma *et al.* and Yoshida *et al.* found that a retrovirus (adult T-cell leukemia virus, ATLV) is closely associated with ATL (8, 9). Furthermore, almost all ATL patients carry antibodies against the ATL-associated viral antigen (ATLA) (8–11) or antibodies against viral proteins P24 and P19 of HTLV (5). Recent immunological and molecular biological analyses showed that HTLV and ATLV are closely related or identical (12–14).

Leukemic cells of ATL patients have surface markers for T-cells, especially inducer/helper T cells (3, 15–17). So far, cell lines related with leukemic cells of ATL patients have been established by cocultivation of leukemic cells and human cord leukocytes (18, 19); the established cell lines showed properties of either leukemic cells or cord leukocytes. Poiesz *et al.* (20) reported establishment of mature T-cell lines from tissue samples of patients with T-cell lymphoma/leukemia by using T-cell growth factor (TCGF). Therefore, we tried to cultivate cells derived from ATL patients by using TCGF, and in this way we established cell lines efficiently. Then we investigated the expression of the retroviral genome in these cell lines.

MATERIALS AND METHODS

Patients and Leukemic Cells. Ten patients (Table 1) were diagnosed as having ATL because of the presence of typical pleomorphic cells that had markedly deformed nuclei and inducer/helper T-cell markers (data not shown). Five patients (nos. 1–3, 9, and 10) were born and raised in Kyushu, where infection by ATL virus is endemic. Atypical cells were detected in peripheral blood of all 10 patients (Table 1). The peripheral blood of nine ATL patients (nos. 1–6 and 8–10) was collected by venipuncture with heparin-treated syringes. Lymph node cells were aspirated from a lymph node of one ATL patient (no. 7), and about 80% of these cells were found to be atypical cells. Lymphocytes were harvested by a Ficoll-Paque gradient method. Cells were cultivated at densities of $2–5 \times 10^6$ cells per ml in culture medium consisting of autoclavable RPMI 1640 medium (Nissui Seiyaku, Tokyo) and heat-inactivated fetal calf serum, 8:2 (vol/vol), and an appropriate amount of a TCGF solution prepared as described below. Half of the medium was changed twice a week.

Cell Lines. MT-1, MT-2, HUT102, HUT78, XPL-15, and YAC-1 cell lines were maintained in culture medium consisting of RPMI 1640 medium and heat-inactivated fetal calf serum, 9:1 (vol/vol). MT-1 and MT-2 cells were obtained by coculture of peripheral blood cells of an ATL patient and human cord lymphocytes (18, 19). HUT102 and HUT78 cells were from patients with cutaneous T-cell lymphoma (20, 21). XPL-15 cells were an Epstein–Barr virus-transformed lymphoblastoid cell line derived from a patient with xeroderma pigmentosum (22). YAC-1 cells were derived from a lymphoma induced by Moloney murine leukemia virus (Mo-MuLV) (23).

Preparation of TCGF. Human lymphocyte suspension was prepared from spleens removed surgically from patients with gastric cancer, aplastic anemia, or autoimmune hemolytic anemia. Lymphocytes were cultured, and TCGF was partially purified as described by Poiesz *et al.* (20).

Detection of Surface Markers of Lymphocytes. Surface markers of cells were determined by testing rosette formation and by indirect immunofluorescence with monoclonal antibodies against human lymphocytes or goat antibodies against hu-

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Abbreviations: ATL, adult T-cell leukemia; E, sheep erythrocyte; En, neuraminidase-treated E; HTLV, human T-cell leukemia virus; kb, kilobase pair(s); Mo-MuLV, Moloney murine leukemia virus; TCGF, T-cell growth factor.

man immunoglobulin light (κ and λ) and heavy (μ, α, and γ) chains as described (16, 17). Receptors for sheep erythrocytes (E) and neuraminidase-treated E (En) were detected by rosette formation. OK series monoclonal antibodies were purchased from Ortho Diagnostic Systems (Westwood, MA). Anti-human Lyt2 (clone 10.2) and anti-human Lyt3 (clone 9.6) monoclonal antibodies were from New England Nuclear. Anti-B1 and J-5 monoclonal antibodies were kindly supplied by Drs. E. L. Reinherz and S. F. Schlossman (Sidney Farber Cancer Institute, Boston, MA).

Detection of Retroviral Antigen. The expression of the retroviral antigen associated with ATL cells was investigated essentially as described by Hinuma *et al.* (8). Cells were treated with a battery of six reference sera simultaneously—sera of three ATL patients [nos. 1 and 3 (Table 1) and another ATL patient] and sera of two healthy laboratory workers and a patient with B-cell leukemia—at a dilution of 1:10 as the first antibody. Fluorescent isothiocyanate-conjugated rabbit anti-human immunoglobulin serum (Cappel Laboratories, Cochranville, PA) was used as the second antibody.

Reverse Transcriptase Assay. Reverse transcriptase activities of spent culture medium were determined essentially as described by Poiesz *et al.* (6). Briefly, culture medium (7.5 ml) was mixed with polyethylene glycol and then centrifuged. The precipitate was dissolved in 300 μl of virus suspension buffer. Virus suspension (10 μl) was mixed with 90 μl of reaction mixture consisting of 40 mM Tris·HCl (pH 7.8), 4 mM dithiothreitol, 45 mM KCl, 9 μg of poly(rA), 1.8 μg of oligo(dT)$_{12-18}$, 15 μM[^{3}H]TTP (5 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) (Radiochemical Centre), and 10 mM $MgCl_2$ or 0.25 mM $MnCl_2$ and incubated for 1 hr at 37°C. Incorporation of radioactivity into cold trichloroacetic acid-insoluble fractions was assayed in a liquid scintillation counter. Assays were carried out in duplicate and repeated three to five times.

Electron Microscopy. Cells were fixed with 2% glutaraldehyde and 1% osmium tetraoxide and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope.

Southern Blot Hybridization. High molecular weight cellular DNA was assayed as described by Yoshida *et al.* (9). The mixture of ^{32}P-labeled, nick-translated probes that represented more than 80% of the retroviral genome was kindly supplied by Dr. M. Yoshida (Cancer Institute, Tokyo) (24).

RESULTS

Titration of Antibodies Against the Retroviral Antigen. The antibody titers against the retroviral antigen (8) of the sera of the 10 ATL patients were measured by indirect immunofluorescence assays with viral antigen-positive ATL-2M cells and antigen-negative ATL-1K and ATL-6A cells as targets (Table 1). All patients had antibodies against the viral antigen, although the antibody titer of patient no. 6 was quite low. Undiluted serum of this patient reacted with ATL-2M, ATL-3K, ATL-4K, ATL-5S, and ATL-7S cells but not with ATL-1K or ATL-6A cells (see below for the cell lines).

Establishment of Cell Lines Derived from ATL Patients. Peripheral blood lymphocytes or lymph node cells from ATL patients were cultured in medium supplemented with TCGF. After a lag time of about 1–6 weeks, cell proliferation became evident, and cells could be passaged at 3- to 4-day intervals at split ratios of 1:2–4. The established cell lines were named ATL-1K, ATL-2M, ATL-3I, ATL-4K, ATL-5S, ATL-6A, ATL-7S, ATL-8K, ATL-9Y, and ATL-10Y (Table 1). They grew as single-cell suspensions containing many cell aggregates. Usually during 15–30 cell passages, cell growth decreased transiently. The cells were transferred periodically to culture medium without TCGF. ATL-1K, ATL-3I, and ATL-5S cells could grow without TCGF from passage levels of 10, 40, and 6, respectively, but the other cell lines required TCGF for growth.

Surface Markers of Cell Lines. The surface markers of peripheral blood lymphocytes or lymph node cells of eight ATL patients (nos. 2 and 4–10) were investigated before cultivation. These cells had E and En rosette receptors and reacted with the monoclonal antibodies OKT3, OKT4, anti-human Lyt2, and anti-human Lyt3 (data not shown). The established cell lines had E and En rosette receptors and were OKT3$^+$, OKT4$^+$, OKT6$^-$, OKT8$^-$, and OKIa1$^+$ (Table 2). Only ATL-1K cells were OKT3$^-$. More than 80% of the cells reacted with anti-human Lyt2 and Lyt3 monoclonal antibodies. None of the cell lines had surface immunoglobulins (δ, μ, γ, α, κ, and λ). No cell lines reacted with OKM1, anti-B1, or J-5 monoclonal antibodies. Thus, the established cell lines almost all retained surface markers for an inducer/helper subset of T cells as expressed in uncultured leukemic cells isolated from ATL patients.

Detection of Retroviral Antigen in the Established Cell Lines. The 10 cell lines were tested for expression of the retroviral antigen at various passage levels by indirect immunofluorescent staining (Table 3, Fig. 1). Sera of three ATL patients reacted with ATL-2K, ATL-3I, ATL-4K, ATL-5S, ATL-7S, ATL-8K, ATL-10Y, MT-1, MT-2, and HUT102 cells but not with ATL-1K, HUT78, XPL-15, or YAC-1 cells (Table 3). Sera of two normal adults and a patient with B-cell lymphoma did not react with any of these cell lines. The viral antigens in ATL-6A and

Table 1. Patients with ATL and cell lines derived from them

Patient no.	Age/sex	Antibody titer*	White blood cell count	Atypical cell, %	Survival, mo	Cell line: Code	Origin†	Culture period, mo	TCGF dependency‡
1 MK	82/M	×80	50,500	95	4	ATL-1K	PB	13	−
2 MM	56/M	×160	13,300	65	8	ATL-2M	PB	13	+
3 KI	71/M	×160	63,600	80	8	ATL-3I	PB	13	−
4 SK	62/M	×80	48,000	82	>9	ATL-4K	PB	11	+
5 YS	34/M	×320	78,700	92	>19	ATL-5S	PB	9	−
6 TA	33/F	×1	13,600	68	12	ATL-6A	PB	7	+
7 IS	68/M	×160	8,200	1	9	ATL-7S	LN	7	+
8 FK	63/F	×40	164,700	76	>9	ATL-8K	PB	4	+
9 SY	59/M	×20	15,800	57	>15	ATL-9Y	PB	4	+
10 KY	47/M	×320	26,100	85	>72	ATL-10Y	PB	4	+

*Antibody titers of plasmas against the viral antigen determined by indirect immunofluorescent staining using ATL-2M cells.
†PB, peripheral blood; LN, lymph node.
‡−, Cells that could be maintained without TCGF; +, cells that required TCGF for growth.

Table 2. The surface markers of the cell lines established from ATL patients*

Cells	Rosettes		OK monoclonal antibodies				
	E	En	T3	T4	T6	T8	Ia1
ATL-1K	5	27	0	95	0	0	80
ATL-2M	35	31	80	95	0	0	95
ATL-3I	23	36	60	80	0	0	90
ATL-4K	25	41	80	95	0	10	90
ATL-5S	28	61	80	80	0	0	95
ATL-6A	71	74	72	85	0	0	80
ATL-7S	9	35	76	94	0	2	86
ATL-8K	40	59	64	82	0	0	100
ATL-9Y	87	NT	95	90	0	10	NT
ATL-10Y	75	NT	76	70	0	10	100

NT, not tested.
* Positive cells, %.

ATL-9Y cells were first detected after 5 and 2 months of cultivation, respectively (Fig. 1).

Reverse Transcriptase Activity of Spent Culture Medium. The reverse trancriptase activity of the cell lines that we established were assayed (Table 3). When the spent culture media of ATL-2M, ATL-3I, ATL-4K, ATL-5S, ATL-7S, ATL-8K, and ATL-10Y cells were used as enzyme sources, significant radioactivity was incorporated into acid-insoluble fractions. Mg^{2+} was preferred to Mn^{2+} as reported by Poiesz *et al.* (6) and Yoshida *et al.* (9). The culture media of ATL-1K, ATL-6A, and ATL-9Y cells did not show any activity. Reverse transcriptase of Mo-MuLV produced by YAC-1 cells greatly preferred Mn^{2+} to Mg^{2+} for the reaction. These results indicated that the viral antigen-positive cell lines established from the ATL patients shed the retrovirus, although in much smaller amounts than that produced by YAC-1 cells.

Electron Microscopic Findings. ATL-1K, ATL-2M, ATL-3I, and ATL-6A cells were examined by electron microscopy. Type C virus was readily detected in the extracellular space of antigen-positive ATL-2M and ATL-3I cells (Fig. 2) but not of antigen-negative ATL-1K and ATL-6A cells.

Detection of Retroviral Antigen During Short-Term Culture. Peripheral blood cells from six ATL patients (nos. 4–6 and 8–10) and lymph node cells of one ATL patient (no. 7) were

Table 3. Expression of the retroviral genome in the established cell lines

Cells	Antigen-positive cells, %	Reverse transcriptase activity, cpm	
		Mg^{2+}	Mn^{2+}
ATL-1K	<0.1	<400	<400
ATL-2M	60–90	13,100	5,800
ATL-3I	15–35	2,500	1,700
ATL-4K	40–80	3,700	1,700
ATL-5S	40–80	2,300	600
ATL-6A	<0.1	<400	<400
ATL-7S	45–70	3,600	400
ATL-8K	15–30	1,200	<400
ATL-9Y	2–15	<400	<400
ATL-10Y	55–80	4,600	1,100
MT-1	0.7–2	500	<400
YAC-1	<0.1	2,900	171,500

Viral antigen-positive cells were detected by indirect immunofluorescence with three reference sera of ATL patients as the first antibody. Reverse transcriptase activities of spent culture media were assayed in the presence of Mg^{2+} or Mn^{2+} as the divalent cation. One thousand counts per minute corresponds to the incorporation of [^{3}H]TMP (0.8 pmol/ml of culture medium) into acid-insoluble fractions.

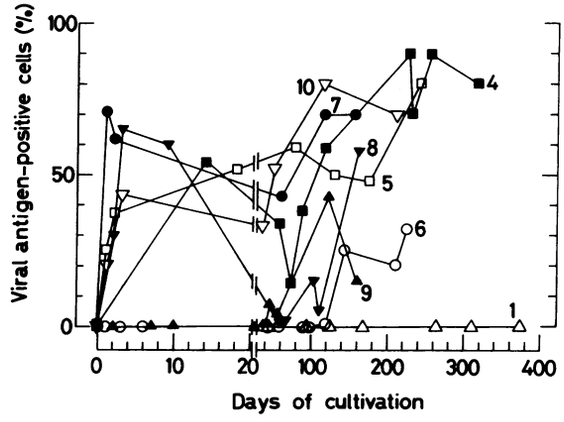

FIG. 1. Expression of the viral antigen during establishment of cell lines. Acetone-fixed cell smears were tested by indirect immunofluorescence assay with serum of patient no. 3 as the first antibody. Percentages of fluorescent cells are shown. Peripheral blood lymphocytes were obtained from patients nos. 4 (■), 5 (□), 6 (○), 8 (▼), 9 (▲), and 10 (▽). Lymph node cells were obtained from patient no. 7 (●).

cultured for a few days and more and then tested for expression of the viral antigen (Fig. 1). Before cultivation the peripheral lymphocytes did not express the antigen, but after 1 or 2 days of cultivation, the lymphocytes obtained from four patients became antigen-positive as reported by Hinuma *et al.* (8, 25). However, peripheral blood lymphocytes of patients no. 6 and no. 9 did not express the viral antigen during short-term culture, although about 70% and 60% of the nucleated cells, respectively, in the peripheral blood of these patients were atypical cells when cultures were started (Table 1). Thus, the original leukemic cells of patients no. 6 and no. 9 did not express the viral antigen even after *in vitro* cultivation. The viral antigen also was not expressed by lymph node cells of patient no. 7 before cultivation, although about 80% of these cells were atypical cells. These cells became positive for viral antigen upon *in vitro* cultivation for a few days (Fig. 1).

Southern Blot Hybridization. The retrovirus-associated with ATL contains no *Eco*RI site in the proviral genome (9). Fig. 3 shows the results of Southern blot-hybridization experiments of *Eco*RI-digested DNA of the established cell lines with nick-translated probes specific for this virus (24). All cell lines derived from ATL patients contained the viral genome (Fig. 3). Interestingly, even the viral antigen-negative ATL-1K cells gave

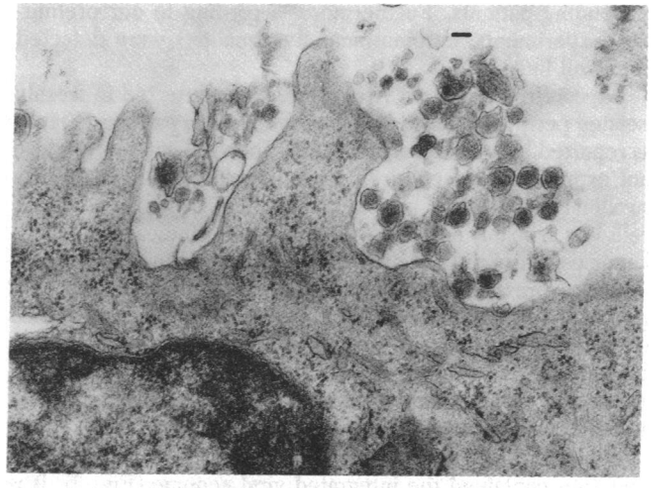

FIG. 2. Electron microscope observation of ATL-2M cells. Numerous type C virus particles were observed. (Bar, 100 nm; ×24,000.)

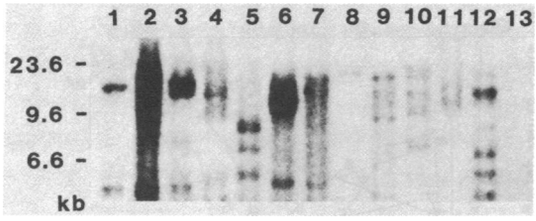

FIG. 3. Detection of the retroviral genome in various cell lines by Southern blot hybridization assay. Cellular DNA (5 μg) from each cell line was assayed as described by Yoshida *et al.* (9). Lanes: 1, ATL-1K; 2, ATL-2M; 3, ATL-3I; 4, ATL-4K; 5, ATL-5S; 6, ATL-6A; 7, ATL-7S; 8, ATL-8K; 9, ATL-9Y; 10, ATL-10Y; 11, MT-1; 12, MT-2; 13, XPL-15.

two different bands [about 20 and 5.6 kilobase pairs (kb)]. Identical bands were detected when DNA isolated from uncultured peripheral lymphocytes of patient no. 1 was assayed (data not shown). Fig. 3 shows that most cells contained several viral genomes, and some of them seemed to be smaller than the whole proviral genome (8.7 kb) (9, 24). Epstein–Barr virus-transformed XPL-15 cells did not contain the viral genome.

DISCUSSION

We established 10 T-cell lines from 11 patients with ATL by using TCGF. Previously, two cell lines, MT-1 and MT-2, were established by cocultivating leukemic cells of ATL patients with cord leukocytes (18, 19). There is also a recent report of establishment of a cell line KH-2 without use of TCGF or coculture (26). However, isolation of a cell line like KH-2 is probably a rare event because we were unable to establish any T-cell line without TCGF. We showed that use of TCGF provides an efficient method for isolation of T-cell lines derived from ATL patients. Quite recently, Popovic *et al.* reported that they isolated several T-cell lines from patients with mature T-cell malignancies, including one Japanese ATL patient, by using TCGF (27). Three of our 10 cell lines, ATL-1K, ATL-3I, and ATL-5S, could grow without TCGF, whereas the other 7 lines that expressed the viral antigen required TCGF for growth (Table 1). Thus, viral infection did not always make the lymphocytes able to grow without TCGF.

Leukemic cells in peripheral blood were reported previously to have surface markers for inducer/helper T cells (3, 15, 17). Table 2 shows that the surface markers of all established cells were characteristic of inducer/helper subsets of T cells and were quite similar to those of uncultured leukemic cells from the corresponding patients. Furthermore, according to our preliminary experiments, chromosomal abnormalities were detected in all cell lines.

We confirmed that no viral antigen was detected in freshly isolated peripheral blood lymphocytes of ATL patients (Fig. 1) as reported by Hinuma *et al.* (8, 25). The viral antigen was also not expressed in uncultured leukemic cells aspirated from a swollen lymph node of patient no. 7, where growth of leukemic cells was probably occurring. These findings indicated that leukemic cells also did not express the viral antigen *in vivo*. Continuous expression of the viral antigen, i.e., the *gag* or *env* gene product (5, 8, 9), did not seem to be required for maintainance of malignant phenotypes of leukemic cells. It still remains to be determined whether a viral gene product that is not immunogenic to humans or that is present in a very minute amount in leukemic cells is responsible for the leukemic state.

Southern blot hybridization experiments showed that all 10 cell lines contained the integrated viral genome (Fig. 3). It is interesting that even the DNA of the antigen-negative ATL-1K cells contained the viral genome. However, the viral antigen was not induced in ATL-1K cells by treatment with 5-iododeoxyuridine or 5-azacytidine (unpublished data). *Eco*RI digests of DNA of ATL cell lines gave several bands on Southern blot hybridization. Because the retrovirus associated with ATL contains no *Eco*RI site in the proviral genome and the proviral genome is 8.7 kb long (9, 24), it is probable that there were many sites of integration of the virus and that some provial genomes were subgenomic. Manzari *et al.* proposed that subgenomic proviral DNA (4–5 kb) was present in HUT102 cells (28).

Nucleated peripheral blood cells from patients no. 6 and no. 9 contained leukemic cells (about 70% and 60%, respectively) at the beginning of cultivation (Table 1). But the viral antigen was not detected in these leukemic cells during short-term culture (Fig. 2). ATL-1K cells did not express the viral antigen after >1 year of cultivation. Therefore, the established cell lines described in this paper were classified into three types according to the time course of their expression of the viral antigen: lines in which viral antigen was expressed (*i*) after short-term cultivation (ATL-4K, ATL-5S, ATL-7S, ATL-8K, and ATL-10Y cells), (*ii*) after a few months of cultivation (ATL-6A and ATL-9Y cells), or (*iii*) not after >1 year of cultivation (ATL-1K cells). This heterogeneity in the expression time suggested that the retroviral antigen was not required for growth of leukemic cells *in vitro*.

ATL is a disease entity recently recognized from clinical symptoms and hematological and immunological findings (1, 2). In some cases, ATL is difficult to distinguish from related diseases, such as chronic lymphocytic leukemia of T-cell origin, Sézary syndrome, mycosis fungoides, or Hodgkin disease. Information on the presence or absence of antibodies against the retroviral antigen in each patient and of the retroviral antigen in cultured leukemic cells of each patient will be helpful in its differential diagnosis. However, these antibodies or antigens should be detected carefully because some patients like patient no. 6 may have a very low antibody titer, and their leukemic cells may not express the viral antigen during short-term culture. For establishment of ATL as a disease entity, it will be necessary to know whether leukemic cells of patients with ATL or other T-cell malignancies harbor whole or partial retroviral genomes and, if so, how it is harbored.

We showed that the retroviral antigen was not expressed in leukemic cells *in vivo* and that it was expressed in different ways in leukemic cells of different ATL patients *in vitro*. In ATL-1K cells, the viral antigen was not expressed in spite of the presence of the viral genomes. In lymphomas induced by avian leukosis virus (ALV), the expression of the avian leukosis virus genome is not required for maintenance of the tumor state (29), and a cellular oncogene is activated by promoter insertion or virus integration (30–32). It is probable that ATL virus and avian leukosis virus induce leukemia by a similar mechanism. The cell lines described here will be useful for analyzing molecular mechanisms by which ATL virus exerts oncogenic effects on human lymphocytes.

We thank Drs. G. Fujii, M. Sekiguchi, and I. Osada for supplying TCGF, Dr. I. Miyoshi for supplying MT-2 cells, and Dr. M. Yoshida for supplying nick-translated probes specific for ATL virus and for discussion. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and from the Ministry of Health and Welfare of Japan.

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