Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5620–5624, July 1989
Medical Sciences

Detection of mRNA for the tax_1/rex_1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction

(human retrovirus/trans-activation/gene expression)

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Contributed by Takashi Sugimura, May 2, 1989

ABSTRACT **Expression of human T-cell leukemia virus type I (HTLV-I) is not detectable by immunofluorescence analysis or RNA blot analysis in most fresh peripheral blood mononuclear cells of patients with adult T-cell leukemia or of asymptomatic HTLV-I carriers. However, in this work, mRNA for the HTLV-I tax_1/rex_1 genes was detected in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and asymptomatic HTLV-I carriers by using reverse transcription followed by the polymerase chain reaction. By using fresh peripheral blood mononuclear cells, the expression of tax_1/rex_1 mRNA was detected in five of the six adult T-cell leukemia patients and four of the eight HTLV-I carriers examined. The amounts of tax_1/rex_1 mRNA detected corresponded to $\approx 10^5$ to 10^6 times less than that in the HTLV-I-infected MT-2 cell line. These results indicate that, in some individuals infected with HTLV-I, the provirus in circulating blood cells is transcribed *in vivo*. Thus the expression of viral antigens in circulating blood cells *in vivo* is suggested.**

Adult T-cell leukemia (ATL) is a mature T-cell malignancy that is closely related to human T-cell leukemia virus type I (HTLV-I) (1–3). HTLV-I is a human retrovirus that has been shown to be associated with human malignancy. It has a sequence, *pX*, that is not derived from cellular protooncogenes like other mammalian retrovirus oncogenes (4). This region is expressed as a doubly spliced mRNA, and the mRNA has two overlapping open reading frames, designated tax_1 and rex_1 (5). tax_1 encodes $p40^{tax}$, which was shown to be a trans-activator of the long terminal repeat of HTLV-I (6–10). rex_1 encodes $p27^{rex}$ and $p21^{x\text{-}III}$, and $p27^{rex}$ was found to be a posttranscriptional trans-regulator for HTLV-I gene expression (11, 12). Moreover, $p40^{tax}$ can trans-activate many cellular genes, such as those of interleukin 2 (IL-2), the IL-2 receptor (IL-2R), IL-3, IL-4, granulocyte–macrophage colony stimulating factor, and c-*fos* (13–18). Since many ATL cells have the helper/inducer T-cell phenotype and express Tac antigens, a subunit of IL-2R (19), immortalization of virus-infected cells might occur by means of an autocrine circuit consisting of IL-2 and its own receptor. However, expression of HTLV-I cannot be detected by conventional methods, such as immunofluorescence analysis or RNA blot analysis, in fresh ATL cells or fresh peripheral blood mononuclear cells (PBMCs) of HTLV-I carriers (20–24), with rare exceptions (25). So it is suggested that expression of the viral gene may not be important for leukemogenesis of immortalized cells. However, it is unknown whether the provirus is actually not expressed *in vivo* or expressed at too low a level to be detected by conventional methods.

Recently a procedure for enzymatic amplification of DNA, called the polymerase chain reaction (PCR), was developed whereby a short segment of a genome can be amplified 10^6- to 10^7-fold *in vitro* in a few hours (26–28). As this method is available for amplification of cDNA obtained by reverse transcription (RT) of mRNA (29, 30), it can be used to detect mRNA that is expressed in small amounts (31–34). In this study, we examined the expression of the HTLV-I provirus in fresh PBMCs of ATL patients and HTLV-I carriers by RT followed by the PCR (RT–PCR). Here we report that the HTLV-I provirus is transcribed and tax_1/rex_1 mRNA is expressed in fresh PBMCs of some ATL patients and HTLV-I carriers. The expression of tax_1/rex_1 mRNA per total RNA in these PBMCs was $\approx 10^5$–10^6 times less than that in HTLV-I-infected MT-2 cells.

MATERIALS AND METHODS

Cells. MT-2 cells are a human HTLV-I-infected cell line established by cocultivation of human cord blood lymphocytes of a normal subject with PBMCs of an ATL patient (35). Ra-1 cells are a rabbit HTLV-I-infected cell line established by cocultivation of rabbit PBMCs with MT-2 cells (36). TARS-1 and TART-1 cells are HTLV-I-infected cell lines from rats established by cocultivation of rat lymphocytes with PBMCs of an ATL patient (37). ATL-1K cells are a human HTLV-I-infected cell line established from PBMCs of an ATL patient (38). MT-2, Ra-1, and HL-60 cells were cultured in RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. TARS-1, TART-1, and ATL-1K cells were cultured in RPMI 1640 supplemented with 20% fetal calf serum. PBMCs were separated by Ficoll-Conray gradient centrifugation of heparinized peripheral blood.

RNA Preparation. RNAs from cell lines or PBMCs were isolated as described previously (39). Possible activation of HTLV-I gene expression in these cells after their transfer to *in vitro* conditions was minimized by extracting RNA from the PBMCs as soon as possible. It usually took 30–60 min

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Abbreviations: HTLV-I, human T-cell leukemia virus type I; ATL, adult T-cell leukemia; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RT, reverse transcription; RT-PCR, RT followed by PCR; HAM, HTLV-I-associated myelopathy; IL-*n*, interleukin *n*; IL-2R, IL-2 receptor.

after the blood was collected to lyse PBMCs with guanidine thiocyanate.

Titration of Anti-HTLV-I Antibody. Anti-HTLV-I antibody was titrated by the particle agglutination method (40) and confirmed by immunoblot analysis (41).

Materials. Oligonucleotide primers for PCR and for a probe for Southern blot analysis were synthesized on an Applied Biosystems model 380A. A random hexadeoxynucleotide primer for RT was purchased from Takara Shuzo (Kyoto, Japan). Moloney murine leukemia virus reverse transcriptase was from Bethesda Research Laboratories. *Thermus aquaticus* DNA polymerase (*Taq* polymerase) was from New England Biolabs. T4 polynucleotide kinase was from Toyobo (Osaka, Japan). [γ-^{32}P]ATP was from Amersham.

RT-PCR. RT-PCR was carried out as described (32, 34), with minor modifications. Briefly, 0.5 μg of cellular RNA was annealed with 100 ng of a random hexadeoxynucleotide primer in a total volume of 10 μl and was reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase at 37°C for 1 hr. Then 8.3 μl of 6× PCR buffer [1× PCR buffer consists of 16.6 mM ammonium sulfate, 67 mM Tris·HCl (pH 8.8 at 25°C), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, 6.7 μM EDTA, and bovine serum albumin at 170 μg/ml], 5 μl each of 15 mM dATP, 15 mM dCTP, 15 mM dGTP, and 15 mM TTP, and 50 pmol of each PCR primer were added to the reaction vessel in a final volume of 50 μl. The sequences of the primers for PCR (RPX3 and RPX4) and of a probe for Southern blot analysis (RPXPR1) are shown in Fig. 1. RPX3 and RPX4 are located upstream and downstream, respectively, of the second splice junction site of tax_1/rex_1 mRNA. The amplified DNA fragments obtained with RPX3 and RPX4 after PCR were 145 base pairs. The probe RPXPR1 is a 20-mer of the sequence surrounding the second splice junction site of tax_1/rex_1 mRNA. PCR was carried out in a DNA Thermal Cycler (Perkin–Elmer/Cetus) for 35 cycles as described (28) with 2.5 units of *Taq* polymerase. The reaction cycle was as follows: primer annealing at 55°C for 45 sec, primer extension at 72°C for 2 min, and denaturation at 90°C for 1 min. After the reaction, 10 μl of the 50-μl sample was electrophoresed in a composite gel of 2% Nusieve GTG Agarose (FMC) and 1% standard agarose (Bio-Rad). After alkaline denaturation and neutralization, the gel was transferred to a Nylon membrane filter (Hybond-N; Amersham). After fixation by UV irradiation (1.6 kJ/m^2), the filter was hybridized at 36°C with ^{32}P-end-labeled RPXPR1 (1–1.5 × 10^7 cpm) for 16 hr. Prehybridization and hybridization were carried out as described (43). Then the filter was washed with 0.2× SSC/0.1% $NaDodSO_4$, twice at room temperature for 15 min and twice at 48°C for 15 min (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0), and exposed to Kodak XAR film with a single intensifying screen for 2–66 hr at −80°C.

RESULTS

Detection of HTLV-I tax_1/rex_1 mRNA in HTLV-I-Infected Cell Lines. RNA from HTLV-I-infected cell lines was analyzed to evaluate the sensitivity and specificity of RT-PCR. RNA from MT-2 cells, which contain multiple copies of HTLV-I provirus and express HTLV-I antigens, was analyzed by RT-PCR as described in the *Materials and Methods* (Fig. 2). After RT-PCR, the specific signal derived from tax_1/rex_1 mRNA in MT-2 cells was observed. To determine the sensitivity of RT-PCR, RNA of MT-2 cells was diluted serially with RNA of HeLa cells that were not infected with HTLV-I and tested for tax_1/rex_1 mRNA by RT-PCR. tax_1/rex_1 mRNA in MT-2 cell RNA could be detected in as much as a 10^6-fold dilution (Fig. 2, lane 7). To compare the sensitivity of RT-PCR to that of RNA blot analysis, RNA blot analysis was performed with the RNA of MT-2 cells diluted serially with the RNA of HeLa cells. The viral RNA could be detected when 10 μg of MT-2 RNA was diluted 100-fold with HeLa RNA (data not shown). These results indicate that the sensitivity of RT-PCR is about 10^6 times that of RNA blot analysis, since the amount of RNA required for RT-PCR analysis (0.5 μg) is 20 times less than that required

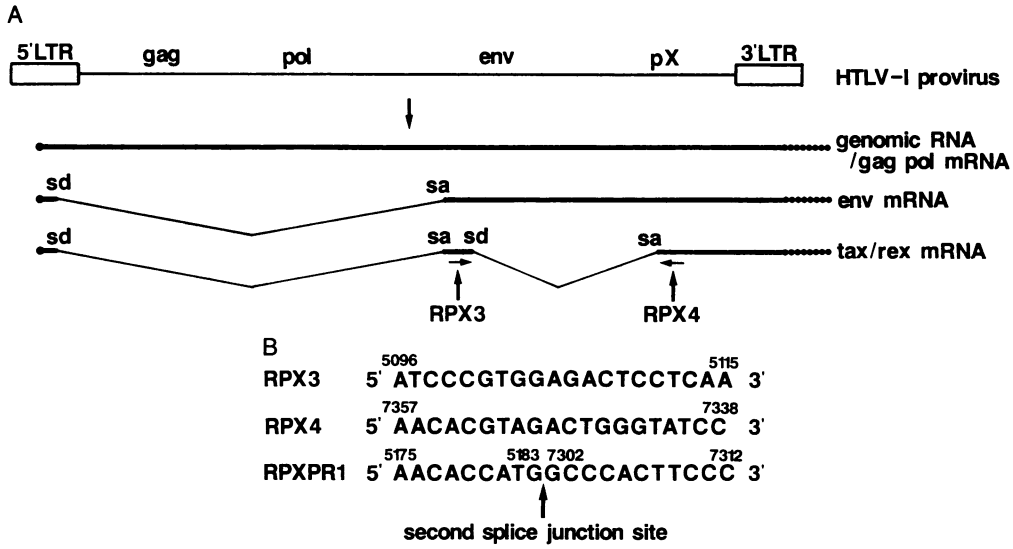

FIG. 1. Region of amplification in virus RNA using RT-PCR and sequences of the primers for PCR and of an oligonucleotide used as a probe for Southern blot analysis. (*A*) Genomic structure of HTLV-I provirus and its three RNA transcripts. sd, Splice donor site; sa, splice acceptor site. The locations of the primers for PCR, RPX3 and RPX4, are shown as horizontal arrows under the tax_1/rex_1 mRNA. The heads of these arrows indicate the 3′ end. The positions of RPX3 and RPX4 are located upstream and downstream, respectively, of the second splice junction site of tax_1/rex_1 mRNA. (*B*) Sequences of primers for PCR and an oligonucleotide probe for Southern blot analysis. Nucleotide numbers corresponding to those in the HTLV-I provirus (4, 42) are shown above each sequence. The locations of the 5′ end and the 3′ end of each primer and the splice junction site of RPXPR1 are indicated. RPXPR1 is a 20-mer that is identical to the sequence surrounding the second splice junction site of tax_1/rex_1 mRNA. The upward arrow below RPXPR1 indicates the second splice junction site of tax_1/rex_1 mRNA. The amplified DNA for tax_1/rex_1 mRNA obtained with these primers is 145 base pairs.

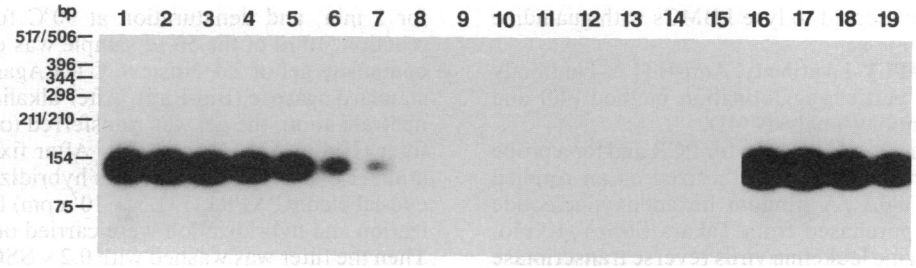

FIG. 2. Detection of an amplified DNA fragment derived from HTLV-I tax_1/rex_1 mRNA in HTLV-I-infected cell lines. Samples of 0.5 μg of each RNA were subjected to RT–PCR and one-fifth of the reaction mixture was analyzed. Lanes: 1, MT-2 cell RNA; 2–9, MT-2 cell RNA serially diluted 10-fold with HeLa cell RNA; 10, HeLa cell RNA; 11, HL-60 cell RNA; 12–14, three normal human PBMC RNAs; 15, MT-2 RNA without the step of RT in RT–PCR; 16, Ra-1 cell RNA; 17, TARS-1 cell RNA; 18, TART-1 cell RNA; 19, ATL-1K cell RNA. The positions of DNA size marker in a *Hin*fI digest of pBR322 are shown on the left. The exposure time was 2 hr for lanes 1–15 and 4 hr for lanes 16–19. bp, Base pairs.

for RNA blot analysis (10 μg), and in this study only one-fifth of the RT–PCR products were subjected to Southern blot analysis. RNA of HeLa cells, HL-60 cells, or three samples of normal human PBMCs, none of which were infected with HTLV-I, were analyzed by RT–PCR, and no signals could be observed (Fig. 2, lanes 10–14). Without the step of RT in RT–PCR, no signal specific for the probe was detected with RNA of MT-2 cells (Fig. 2, lane 15). No specific signal was observed in Fig. 2, lanes 8–15, even after 3 days of exposure (data not shown). Thus the signal observed by RT–PCR is specific for the sequence derived from tax_1/rex_1 mRNA of HTLV-I.

Four HTLV-I-infected cell lines other than MT-2 cells were tested for expression of tax_1/rex_1 mRNA by RT–PCR. Ra-1, TARS-1, and TART-1 are all infected with HTLV-I and express HTLV-I antigens. Transcripts of the virus in these three cell lines were detected by RNA blot analysis, and the amounts of these transcripts were about 1/2–1/50th of that detected in the MT-2 cell line (data not shown). tax_1/rex_1 mRNA was also detected by RT–PCR in RNAs from all three cell lines (Fig. 2, lanes 16–18). ATL-1K cells have been shown by Southern blot analysis to contain a single copy of the full-length HTLV-I provirus (44), but their expression of HTLV-I could not be detected by immunofluorescence analysis of HTLV-I antigens or reverse transcriptase assay of their culture supernatants (22, 38). Moreover, the transcripts of HTLV-I could not be detected in the cells by RNA blot analysis (data not shown). Thus ATL-1K had been considered not to express HTLV-I genes. However, when RNA from ATL-1K cells was subjected to RT–PCR, expression of the viral genes was detected (Fig. 2, lane 19). These results show that RT–PCR is more sensitive than immunofluorescence analysis or RNA blot analysis for detecting viral gene expression and is a reliable method for detecting the specific sequence of tax_1/rex_1 mRNA of HTLV-I.

Detection of HTLV-I tax_1/rex_1 mRNA in Fresh PBMCs of ATL Patients and Asymptomatic HTLV-I Carriers. RT–PCR was used to examine the expression of tax_1/rex_1 mRNA in fresh PBMCs of ATL patients and asymptomatic HTLV-I carriers. PBMCs from six ATL patients (three with the acute type, one with the chronic type, and two with the smoldering type), eight asymptomatic HTLV-I carriers, and one patient with HTLV-I-associated myelopathy (HAM) (45) were tested for expression of tax_1/rex_1 mRNA by RT–PCR. tax_1/rex_1 mRNA was detected in the PBMCs of five of the six ATL patients and four of the eight HTLV-I carriers but not in the patient with HAM (Fig. 3). From the intensities of the bands, the amounts of RNA expressed were concluded to be $\approx 10^5$–10^6 times less than that expressed in MT-2 cells. These results together with clinical data on these subjects are summarized in Table 1. Thus, we conclude that in some HTLV-I-infected individuals HTLV-I provirus in circulating blood cells is transcribed and tax_1/rex_1 mRNA is expressed *in vivo*, although the level of transcripts is very low.

DISCUSSION

As ATL cells contain the genome of the HTLV-I provirus and express large amounts of a subunit of IL-2R, it has been suggested that $p40^{tax}$ plays a key role in the early stage of leukemogenesis of ATL through its trans-activation of the IL-2 system. In other words, in the early stage of leukemogenesis, tax_1 gene expression induces multiclonal expansion of some subpopulations of the virus-infected T cells by an autocrine mechanism [that is, by trans-activation of IL-2 and IL-2R by $p40^{tax}$ (46)]. In fact, normal peripheral blood lymphocytes can be immortalized by HTLV-I by their cocultivation with HTLV-I-producing cells *in vitro*. These cells express IL-2R and proliferate in the presence of IL-2. To the best of our knowledge, until now only one paper has reported the expression of HTLV-I genes in fresh PBMCs of an ATL patient (25), and no expression was observed in fresh PBMCs of HTLV-I carriers (20–24). Therefore, the role of HTLV-I gene expression in leukemogenesis of ATL in HTLV-I carriers *in vivo* has remained uncertain. In this study, we demonstrated substantial expression of virus genes *in vivo*. This finding suggests that virally infected T cells proliferated *in vivo* by means of trans-activation by $p40^{tax}$ of some cellular genes, such as those for IL-2 and IL-2R, and also suggests a role of tax_1/rex_1 gene products at some stage of leukemogenesis.

The amounts of tax_1/rex_1 mRNA in PBMCs from the individuals so far tested correspond to $\approx 10^5$ to 10^6 times less than that in MT-2 cells. MT-2 cells are known to be an HTLV-I-infected cell line, and 100% of these MT-2 cells express larger amounts of HTLV-I antigens than other

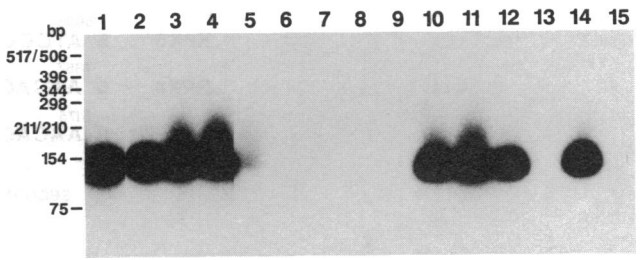

FIG. 3. Detection of HTLV-I tax_1/rex_1 mRNA in fresh PBMCs of ATL patients and HTLV-I carriers. Lanes: 1–3, acute type ATL patients; 4, a chronic type ATL patient; 5 and 6, smoldering type ATL patients; 7–14, HTLV-I carriers; 15, a HAM patient. The exposure time was 66 hr. The intensities of these signals obtained after 2 hr of exposure (data not shown) were estimated to correspond to that of 10^5- to 10^6-fold diluted MT-2 RNA as shown in Fig. 2. bp, Base pairs.

Table 1. Characteristics of ATL patients and HTLV-I carriers

Patient*	Age	Sex	Diagnosis†	Anti-HTLV-I antibody‡	WBC per mm³	% atypical lymphocytes	RT-PCR§
1	61	M	Acute	2048	14,800	39	+
2	53	F	Acute	>8192	51,500	81	+
3	64	M	Acute	>8192	26,700	44	+
4	40	M	Chronic	256	16,100	44	+
5	62	F	Smoldering	128	8,300	15	+
6	72	M	Smoldering	4096	9,900	19	−
7	64	F	Carrier	64	8,600	0	−
8	63	F	Carrier	>8192	4,300	1	−
9	41	M	Carrier	1024	12,000	0	−
10	62	F	Carrier	>8192	4,800	0	+
11	22	F	Carrier	4096	8,500	0	+
12	29	F	Carrier	4096	ND	ND	+
13	30	F	Carrier	4096	ND	ND	−
14	36	F	Carrier	4096	ND	ND	+
15	63	M	HAM	1024	8,900	0	−

M, male; F, female; WBC, white blood cells; ND, not determined.
*The patient numbers correspond to the lane numbers in Fig. 3.
†Acute, acute type ATL; chronic, chronic type ATL; smoldering, smoldering type ATL; carrier, asymptomatic HTLV-I carrier.
‡Anti-HTLV-I antibody was titrated by the particle agglutination method. Reciprocals of end points of plasma dilution are shown.
§The + or − indicates the presence or absence of viral RNA, respectively.

HTLV-I-infected cell lines. Assuming that the amounts of tax_1/rex_1 mRNA in PBMCs of asymptomatic viral carriers, which express viral genes actively, are the same as those in MT-2 cells, about one in 10^5–10^6 PBMCs may express viral genes. With respect to proliferation of cells expressing the virus, it will be important to examine the expression of tax_1/rex_1 mRNA after various periods in the subjects in whom its expression was not detected in this study.

Judging from the copy numbers of other retroviral RNAs in vegetatively growing cells (47), there should be at most 10^3–10^4 copies of HTLV-I transcripts per MT-2 cell. If this is so, there should be less than 0.001–0.1 copy of mRNA of the tax_1/rex_1 gene per PBMC of ATL patients. In other words, 1–100 copies of tax_1/rex_1 mRNA should be expressed in 1 out of 1000 PBMCs of ATL patients. These results suggest that the majority of leukemic cells in these patients do not express tax_1/rex_1 mRNA *in vivo*, but a small number of PBMCs of these ATL patients do. It is unclear how many proliferating cells that are infected with HTLV-I express the viral genes during the cell cycle, since the main organ in which ATL cells proliferate has not been determined and the mode of expression of the tax_1/rex_1 genes during the cell cycle is not fully understood. Thus the expression of HTLV-I genes may be involved in development of ATL, although the level of expression in PBMCs of ATL patients is very low as a whole.

Anti-HTLV-I antibodies persist in HTLV-I-infected subjects for a long period after the first infection, so it seems likely that low levels of some HTLV-I antigens are expressed *in vivo*. In this study we examined only the expression of tax_1/rex_1 mRNA in PBMCs from HTLV-I-infected individuals, but these cells may also express mRNAs for viral structural proteins. In fact, deposition of HTLV-I antigens as immune complexes in the glomeruli of an ATL patient has been reported (48). Moreover, *gag* gene products have also been found in the sera of some ATL patients as immune complexes (49, 50).

In this study, when PCR was performed without RT, only a very faint signal could be detected in a few cases (data not shown). The signals detected by RT–PCR were about 10 times greater than these very faint signals. Thus we conclude that the signals detected by RT–PCR in this study actually reflect the presence of tax_1/rex_1 mRNA. The meaning of the faint signals detected when RT was omitted is unknown. Possibly a cDNA form of the tax_1/rex_1 gene transcript is present in a small percentage of PBMCs from some patients and its sequence is amplified from a trace amount of DNA contaminating the RNA during PCR.

Atypical lymphocytes are observed frequently in the peripheral blood of ATL patients and some healthy HTLV-I carriers and are considered to be a typical feature of ATL. tax_1/rex_1 mRNA was not detected in a patient with smoldering ATL and an HTLV-I carrier who both had atypical lymphocytes (Table 1, patients 6 and 8). On the other hand, tax_1/rex_1 mRNA could be detected in some HTLV-I carriers who were hematologically normal and had no atypical lymphocytes (Table 1, patients 10 and 11). These findings suggest that the level of tax_1/rex_1 mRNA expression is not correlated with the amount of atypical lymphocytes in the peripheral blood. The titer of antibodies against HTLV-I varied in the ATL patients and HTLV-I carriers in this study as shown by the particle agglutination method, and this titer was not correlated with the expression of tax_1/rex_1 mRNA.

In this work, we detected HTLV-I gene expression in fresh PBMCs of five of the six ATL patients and four of the eight asymptomatic viral carriers. The levels of viral transcripts detected were very low, so these results are not inconsistent with previous reports that viral antigens or mRNAs could not be detected in fresh PBMCs of ATL patients or asymptomatic viral carriers by less sensitive methods. Further analysis of viral gene expression *in vivo* should also provide information on the mechanism of leukemogenesis in HTLV-I-infected subjects.

We thank Dr. I. Miyoshi of Kochi Medical School for supplying MT-2 and Ra-1 cells, Dr. T. Yoshiki of Sapporo City General Hospital for supplying TARS-1 and TART-1 cells, Dr. T. Sekiya of the National Cancer Center Research Institute for critical reading of the manuscript, and Dr. M. Miwa of the National Cancer Center Research Institute for helpful advice. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan and by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control.

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