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Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease

(RNA tumor virus/nucleic acid hybridization/proviral DNA)

MITSUAKI YOSHIDA*, ISAO MIYOSHI†, AND YORIO HINUMA‡

*Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan; †Department of Internal Medicine, Kochi Medical School, Nankoku, Kochi 785-51, Japan; and ‡Institute for Virus Research, Kyoto University, Kyoto 606, Japan

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ABSTRACT **A retrovirus (ATLV) was unequivocally demonstrated in human adult T-cell leukemia (ATL) cell lines by density (1.152–1.155 g/cm^3) in a sucrose gradient, reverse transcriptase activity insensitive to actinomycin D, RNA labeled with [^{3}H]uridine, and specific proteins with molecular weights of 11,000, 14,000, 17,000, 24,000, and 45,000. Furthermore, cDNA prepared by endogenous reaction with detergent-treated virions hybridized to 35S RNA containing poly(A), which was inducible by IdUrd treatment of a T-cell line derived from leukemic cells of the ATL, and the integrated form of ATLV proviral DNA was detected in T-cell lines derived from ATL. The ATLV proviral DNA was also detected in fresh peripheral lymphocytes from all five patients with ATL tested so far but not in those from healthy adults. On the other hand, ATLV protein of M_r 42,000 was found to be at least one of the ATL-associated antigen(s) that were previously detected in ATL-leukemic cells by all sera from patients with ATL. These findings on the close association of ATLV protein and proviral DNA with ATL are direct evidence for the possible involvement of the retrovirus ATLV in leukemogenesis of human ATL.**

Adult T-cell leukemia (ATL) is a clinical entity of T-cell malignancy proposed by Takatsuki and colleagues (1, 2) on the basis of its characteristic clinical and hematological features. The disease has also been called "endemic adult leukemia/lymphoma (ATLL)" because of its leukemic lymphomatous nature and the peculiar geographic distribution of the birthplaces of patients—clustering in the southwestern part of Japan (3). Recently, Hinuma *et al.* (4) found antigen(s) in a T-cell line, MT-1, derived from peripheral leukemia cells of a patient with ATL. The antigen(s) reacted with sera from all ATL patients tested and also with sera from about 25% of the healthy adults in the endemic area but with very few sera of subjects from nonendemic areas. Virus-like particles with type C appearance were also found by electron microscopy (4). These observations suggested the presence and possible relationship of type C virus with ATL. Similar antigen(s) and type C particles were also detected in another T-cell line, MT-2, established from normal cord lymphocytes cocultivated with leukemia cells from an ATL patient (5) and reverse transcriptase activity was detected in the culture fluid of MT-2 cells (unpublished data).

In this work, we have unequivocally demonstrated and characterized the ATL retrovirus (ATLV) by biochemical techniques in two T-cell lines, MT-1 and MT-2. Furthermore, we obtained data indicating a close association of ATLV proviral DNA with human ATL. These pieces of direct evidence suggest the involvement of ATLV in human leukemogenesis.

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MATERIALS AND METHODS

Cells. Two cell lines, MT-1 and MT-2, were used. The MT-1 cell line (6) was derived from leukemia cells of peripheral blood from a patient with ATL, and the MT-2 cell line (5) was established from cord lymphocytes that had been cocultivated with leukemia cells from a patient with ATL. Both are T-cell lines resembling leukemia T cells of ATL and have been cultured in RPMI-1640 medium supplemented with 10% fetal calf serum.

Virus Purification. Virus particles were concentrated and purified in essentially the same way as avian sarcoma viruses (7, 8). Briefly, virus particles in the culture fluid were precipitated at 50% ammonium sulfate saturation or by centrifugation at 25,000 rpm for 2 hr (Beckman SW 27 rotor) and purified by centrifugation through a 20–60% sucrose gradient in 20 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA at 30,000 rpm for 18 hr (Beckman SW 41 rotor). The gradient was collected as 14–15 fractions and the fractions corresponding to a density of 1.152–1.155 g/cm^3 were pooled and kept at −70°C until use.

DNA Polymerase Assays. DNA polymerase activity Poly(A)-dependent was measured in 50 μl of 50 mM Tris·HCl, pH 7.5/5 mM dithiothreitol/100 mM KCl/10 mM $MgCl_2$/10 μM [^{3}H]dTTP/0.1% Triton X-100 containing 2 μg of poly(A), 0.4 μg of $(dT)_{12-18}$, and an appropriate amount of virus preparation. The reaction mixture was incubated at 37°C for 1 hr, and [^{3}H]dTMP incorporated into polymers was precipitated with 10% trichloroacetic acid, collected on a glass fiber filter, and measured.

The endogenous polymerase reaction was performed similarly but the reaction mixture consisted of 50 mM Tris·HCl, pH 7.5/5 mM dithiothreitol/3 mM $MgCl_2$ or 1 mM $MnCl_2$ containing dATP, dGTP, and dTTP at 0.1 mM each, 10 μCi of [^{3}H]dCTP (30 Ci/mmol), 0.02–0.04% Nonidet P-40, actinomycin D at 50 μg/ml, and an appropriate amount of virions.

Preparation of [^{32}P]cDNA and Cellular DNA and RNA. [^{32}P]DNA complementary to viral RNA ([^{32}P]$cDNA_{ATLV}$) was prepared by the endogenous reaction of detergent-treated virions under these conditions described above with [α-^{32}P]dCTP. The [^{32}P]$cDNA_{ATLV}$ synthesized was treated with 0.1 M NaOH at 37°C for several hours to remove RNA and was separated from shorter products on a Sephadex G-25 column.

Total cellular DNA was extracted from cultured or peripheral blood cells as described (9), and total cellular RNA was prepared by the method of Weiss *et al.* (10).

Nucleic Acid Hybridization. Hybridization of viral or cellular RNA with $cDNA_{ATLV}$ was carried out in 50% formamide/0.6

Abbreviations: ATL, adult T-cell leukemia; ATLV, ATL virus.

M NaCl/1 mM EDTA/20 mM Tris·HCl, pH 7.5, at 42°C and assayed by resistance to single strand-specific nuclease S1.

DNA was digested with restriction endonucleases, separated by agarose gel electrophoresis, and transferred onto a nitrocellulose membrane by a modification (9) of the Southern procedure (11). The membrane filters were hybridized with [^{32}P]cDNA$_{ATLV}$ at 68°C for 18–48 hr in 0.6 M NaCl/0.06 M sodium citrate, pH 7.5/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll 4000 containing 100 μg of sonicated and heat-denatured *Escherichia coli* DNA per ml and 1–3 × 10^6 cpm of [^{32}P]cDNA$_{ATLV}$. After hybridization the filter was washed with 0.075 M NaCl/7.5 mM sodium citrate at 68°C and exposed to x-ray film.

RESULTS

Characterization of the Retrovirus in the MT-2 Cell Line from ATL. The culture fluid of the MT-2 cells was used as a source of virus. Virus particles were concentrated from the culture fluid and separated on a sucrose density gradient. The DNA polymerase activity of each fraction was measured by using poly(rA)-oligo(dT). Activity was detected in fractions with densities of 1.152–1.155 g/cm^3 (Fig. 1*A*). Incorporation activity of [^{3}H]dCTP into polymer was also detected at the same density without addition of the exogenous template. Because this activity was also detected in the presence of actinomycin D, which completely inhibits DNA-dependent polymerase, the polymerase activity seemed to be reverse transcriptase.

If this activity were reverse transcriptase associated with retrovirus particles, it should be associated with RNA and specific protein components. To demonstrate these, we added [^{3}H]uridine and [^{35}S]methionine separately to the MT-2 cell culture and carried out the analysis described above. [^{3}H]Uridine incorporated into polymer was detected in the same fractions as polymerase activity, indicating the presence of RNA in these fractions (Fig. 1*B*). Proteins containing [^{35}S]methionine gave a peak at the same density as polymerase activity (Fig. 1*C*). Polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins gave two prominent bands with M_rs of 24,000 and 45,000 to-

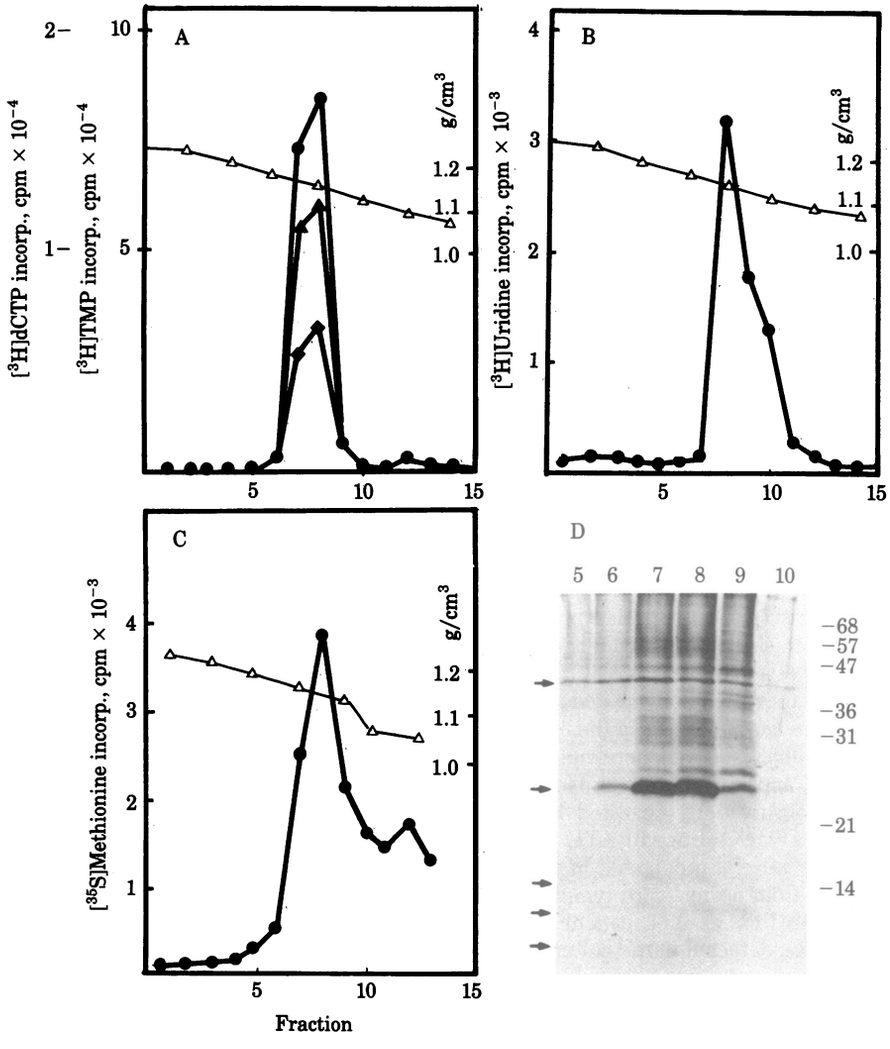

FIG. 1. DNA polymerase activities (*A*), RNA profile (*B*), and protein profiles (*C* and *D*) of concentrated ATLV particles released from the MT-2 cell line. Virus preparations were centrifuged on a 20–60% sucrose gradient in an SW 41 rotor at 30,000 rpm for 18 hr. Then the gradient was collected in 14 or 15 tubes. △, Density determined by refractometry. (*A*) DNA polymerase activity was assayed in the presence of poly(A)-oligo(dT) with 10 mM $MgCl_2$ (●) or in the absence of exogenous template with 3 mM $MgCl_2$ (▲) or with 1 mM $MnCl_2$ (◆). (*B*) MT-2 cells were incubated with [^{3}H]uridine and the labeled virus fractions were precipitated with 10% cold trichloroacetic acid and assayed. (*C*) Virus particles were labeled with [^{35}S]methionine and assayed as in *B*. (*D*) Portions of each fraction in *C* were treated with 0.1% $NaDodSO_4$ and analyzed by polyacrylamide gel electrophoresis. The dried gel was examined by autoradiography. The numbers at the top of lanes correspond to the fraction numbers in *C*; numbers along the side are M_r × 10^{-3}.

gether with faintly labeled bands at 11,000, 14,000, and 17,000 (Fig. 1*D*). These bands were easily distinguished from cellular protein contaminants by their distribution at a density of 1.152–1.155 g/cm^3. Thus, it was concluded that the MT-2 cell line released the retrovirus named ATLV.

Characterization of cDNA Prepared from ATLV. For characterization and further confirmation of ATLV, [^{32}P]cDNA$_{ATLV}$ was prepared by the endogenous reaction with detergent-treated virions and analyzed. The cDNA$_{ATLV}$ hybridized nearly 90% to virion RNA with a $C_rt_{1/2}$ of 0.12 (Fig. 2). This $C_rt_{1/2}$ value and the kinetics of the reaction were similar to those obtained with Rous sarcoma virus cDNA·RNA under the same conditions (unpublished data), indicating that main fraction of cDNA$_{ATLV}$ was made from viral RNA contained in virions in a reasonably homogeneous state. Cellular RNA of MT-1 and MT-2 protected the cDNA$_{ATLV}$ from nuclease S1 digestion to nearly the same extent as virion RNA (Fig. 2), but cellular RNA of human fibroblast cell line A204 (12), unrelated to ATL, did not hybridize significantly. These findings clearly indicated that cDNA$_{ATLV}$ prepared from ATLV did not represent cellular RNA components such as rRNA or tRNA common to human cells.

To characterize the RNA in MT-1 and MT-2 cells, poly(A)-containing RNA was separated by agarose gel electrophoresis and transferred to diazobenzyloxymethyl-paper as described by Alwine *et al.* (13). The main components of MT-1 cells hybridized with cDNA$_{ATLV}$ were a 35S RNA and a ≈26S component, and the levels of these RNAs were increased by treating the cells with IdUrd (Fig. 3). In MT-2 cells, 35S RNA was also detected, but several other smaller RNAs were observed as strong bands. Because the extent of maximal hybridization with MT-2 cellular RNA was almost the same as that with MT-1 and virion RNA (Fig. 2), it is reasonable to assume that these smaller RNA species contained sequences similar to those of 35S and 26S RNA. However, it is still possible that these bands were detected by a contaminant cDNA in the cDNA$_{ATLV}$ preparation. In contrast to MT-1 and MT-2 cells, RNA from human sarcoma cell line A204 did not show any detectable band. The presence of 35S RNA that was hybridizable to cDNA$_{ATLV}$ and was inducible with IdUrd suggested that ATLV is a typical retrovirus containing 35S RNA as its genome and that cDNA$_{ATLV}$ was transcribed from the RNA.

ATLV Proviral DNA Is Integrated into Chromosomal DNA of T-Cell Lines from ATL. Another characteristic property of a retrovirus is that its genome is integrated into the host chromosomal DNA during replication. To demonstrate this, cellular high molecular weight DNA was carefully prepared from MT-1 and MT-2 cells and also from the human cell lines A204 and KB as control. The high molecular weight DNA was digested with *Eco*RI or *Bam*HI, and the digests were separated on agarose gel and analyzed by the Southern blotting technique (11) (Fig. 4). The DNAs of MT-1 and MT-2 cells gave several strong bands, and some of them were larger than 10,000 base pairs, which corresponded to the size of 35S RNA. These findings clearly demonstrated that the provirus DNA of ATLV is integrated into the chromosomal DNA of ATL-derived cell lines, probably as multicopies. In contrast to DNA of these cells, DNA of A204 and KB cells did not show strong bands. Very faint bands were visible after longer exposure of DNA of A204 and KB cells; however, we do not know whether these bands represented shorter fragments of ATLV-related sequences or only partially homologous sequences or ATLV-unrelated sequences detected with possible contaminant cDNA. In any case, it could be concluded that ATLV itself is not an endogenous virus widely distributed in human cells. This conclusion was also supported by the finding that lymphocyte DNA from normal adults did not give any bands hybridizable with cDNA$_{ATLV}$ (see below). Of course, the technique used in these experiments was not sensitive enough to exclude the possibility that small portions of the ATLV genome are contained in normal human DNA as discussed above.

Detection of ATLV Proviral DNA in Fresh Lymphocytes from ATL Patients. To obtain more information on the association of ATLV with ATL, we tested for the presence of ATLV proviral DNA in cellular DNA of fresh peripheral lymphocytes from ATL patients and from healthy adults. DNA was extracted from fresh lymphocytes from the peripheral blood of five patients with ATL and analyzed by the Southern blotting procedure (11). All five ATL patients showed discrete positive bands indicating the presence of ATLV proviral DNA in their cellular DNA (Fig. 5). The presence of only one band of each DNA with different sizes in different patients indicates differences

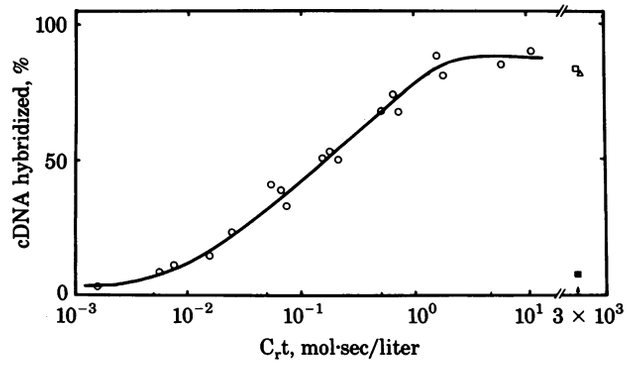

FIG. 2. Hybridization of [^{32}P]cDNA$_{ATLV}$ (5,000 cpm) with various amounts of 30–70S virion RNA (○) or total cellular RNA from MT-1 (□), MT-2 (△), and A204 (■).

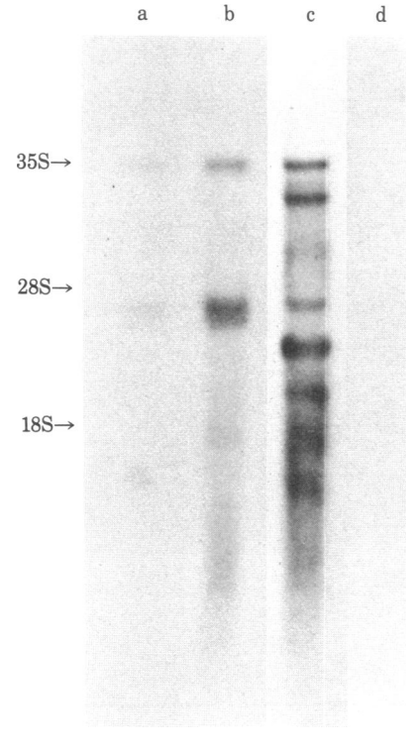

FIG. 3. ATLV-specific RNA in MT-1 and MT-2 cells. Poly(A)-containing RNA was selected with oligo(dT)-cellulose from total RNA of MT-1 cells (lane a), MT-1 cells treated with IdUrd (50 μg/ml) for 24 hr (lane b), MT-2 cells (lane c), and A204 cells (lane d) and analyzed with [^{32}P]cDNA$_{ATLV}$ by the technique of Alwine *et al.* (13).

in cellular sites of integration of ATLV proviral DNA. This finding was in agreement with the above conclusion that ATLV is not a widely distributed endogenous virus but is acquired only locally.

DNA preparations of fresh lymphocytes from three healthy adults in nonendemic area did not contain the proviral DNA sequences. This close association of ATLV proviral DNA with ATL suggested the involvement of ATL in leukemogenesis in human ATL.

DISCUSSION

Immunofluorescence studies have previously demonstrated an ATL-associated antigen in cells in short-term cultures of peripheral lymphocytes and the involvement of some viruses, possibly type C, with ATL have been suggested (4). In this study, the retrovirus was unequivocally demonstrated in leukemic cell lines of ATL. The virus preparation from the MT-2 cell line (5) had the following properties. (*i*) Its density in a sucrose gradient was 1.152–1.155 g/cm^3. (*ii*) It contained reverse transcriptase activity, and the endogenous reaction was insensitive to actinomycin D. (*iii*) It contained RNA labeled by [^{3}H]uridine. (*iv*) It contained specific proteins with M_r of 11,000, 14,000, 17,000, 24,000, and 45,000; those of M_r 24,000 and 45,000 were the main components labeled with [^{35}S]methionine.

Furthermore, cDNA prepared by the endogenous reaction of the detergent-treated virions hybridized to 35S RNA containing poly(A), which was inducible by IdUrd treatment of MT-1 cells derived from leukemia cells of ATL, and DNA sequences homologous with this cDNA were found in chromosomal DNA

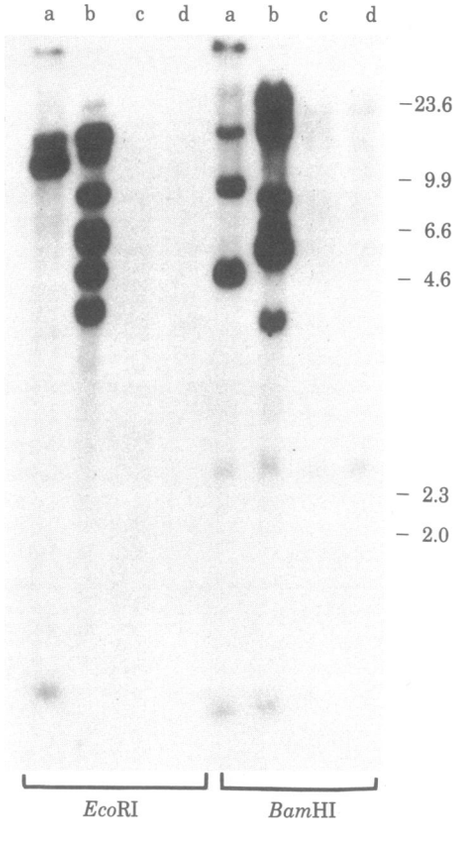

FIG. 4. Detection of proviral DNA sequences of ATLV in MT-1 and MT-2 cell DNA. High molecular weight DNA extracted from MT-1 cells (lane a), MT-2 cells (lane b), A204 (lane c), and KB cells (lane d) was digested with *Eco*RI (*Left*) or *Bam*HI (*Right*) and analyzed by the Southern transfer technique (11). Numbers are size in kilobases.

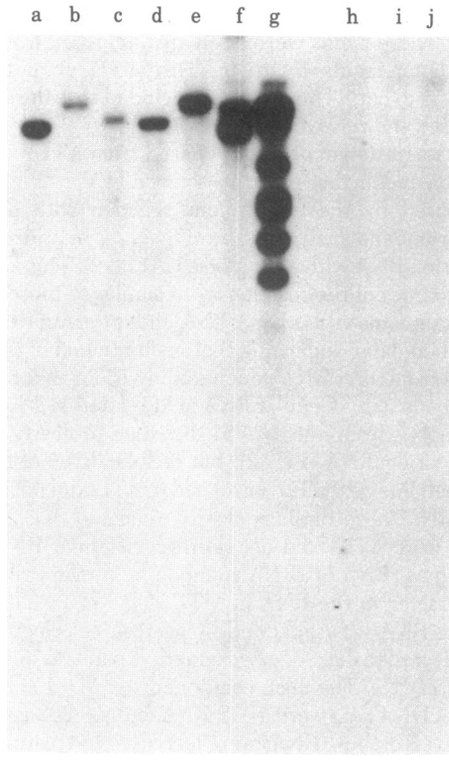

FIG. 5. Detection of proviral ATLV DNA sequences in leukemia cells from ATL patients. Total DNA was extracted from fresh lymphocytes from peripheral blood of ATL patients (lanes a–e) and of healthy adults (lanes h–j) and digested with *Eco*RI. The digests were analyzed as in Fig. 3. As positive controls, MT-1 and MT-2 cell DNAs (lanes f and g) were included.

of MT-1 and MT-2 cell lines. All these observations fitted the requirements for retroviruses. From this study and the previous electron microscopic study (4), this virus is concluded to be a type C retrovirus, ATLV. The presence of 35S RNA containing poly(A), which was hybridizable with cDNA of ATLV and inducible by IdUrd treatment of MT-1 cells, showed that ATLV is a typical retrovirus because all known lymphatic leukemia viruses of animal origin contain 35S RNA as their genome (14).

The effects of divalent cations on the reverse transcriptase activity and the molecular weight of the viral proteins are similar to those of human T-cell leukemia virus from T-cell lymphoblastoid cell lines derived from a cutaneous T-cell lymphoma, which was recently reported by Poiesz *et al.* (15). However, the density of ATLV was slightly less. Although both viruses were from a human leukemia T-cell, the types and distributions of the leukemias are different.

Cell lines MT-1 and MT-2, derived from T cells of ATL, contained the ATLV genome in their chromosomal DNA. However, other human cell lines unrelated to ATL did not contain the ATLV proviral DNA. These observations indicate that ATLV is not a typical endogenous virus widely distributed in human cells. However, DNA preparations from fresh peripheral lymphocytes of all five patients with ATL tested contained the ATLV proviral DNA. This finding strongly suggests that the ATLV is closely associated with human ATL. Furthermore, all three sera from ATL patients tested so far were shown to react with M_r 24,000 viral protein of ATLV (Fig. 6). All sera from ATL patients including these three sera were previously shown to contain antibody against ATL antigen, indicating specific association of the antigen(s) with ATL (4). The finding in this paper clearly demonstrated that at least the M_r 24,000 ATLV protein is one of the ATL antigens. Thus, in this work, association of

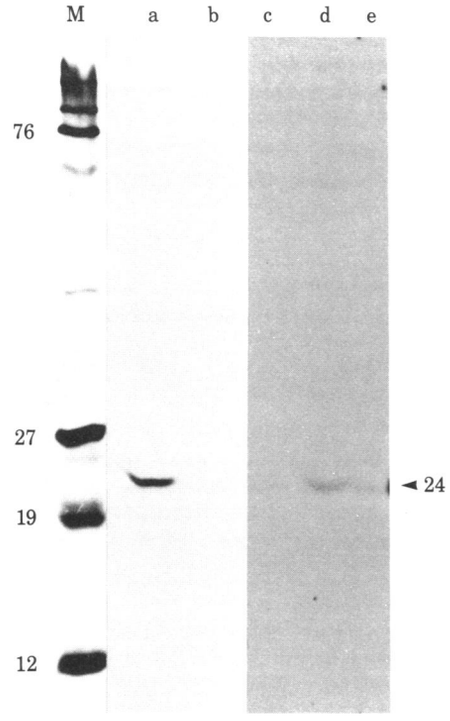

FIG. 6. Immunoprecipitation of M_r 24,000 ATLV protein by sera from patients with ATL. Virus particles labeled with [^{35}S]methionine (fraction 8 in Fig. 1*C*) were disrupted with RIPA buffer (10 mM Tris·HCl, pH 7.5/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$) and treated with sera from patients with ATL (lanes a, d, and e) or from normal human adults (lanes b and c). The immunoprecipitates were isolated as described by Kessler (16) and analyzed on 10% polyacrylamide gel (17). Immunoprecipitates from chicken cells transformed with Y73 sarcoma virus (18) were also subjected to electrophoresis as marker (lane M); the sizes are indicated as $M_r \times 10^{-3}$.

ATLV protein and ATLV proviral DNA with ATL was demonstrated.

It is noteworthy that only a single band of proviral DNA was obtained with each DNA of fresh lymphocytes from ATL patients and that the sizes varied in different patients. These results suggest that the cellular site of integration of ATLV provirus differs, thus implying that ATLV is not a widely distributed endogenous virus but is acquired exogenously. These findings and the endemic distribution of ATL indicate that the mode of transmission of the ATLV genome must be very interesting. More precise studies on the distribution of the ATLV genome in Japanese people, especially in the endemic area, are important for understanding leukemogenesis of ATL.

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