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HTLV-V: A New Human Retrovirus Isolated in a Tac-Negative T Cell Lymphoma/Leukemia

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A new human retrovirus was isolated from a continuous cell line derived from a patient with CD4$^+$ Tac$^-$ cutaneous T cell lymphoma/leukemia. This virus is related to but distinct from human T cell leukemia/lymphoma virus types I and II (HTLV-I and HTLV-II) and human immunodeficiency virus (HIV-1). With the use of a fragment of provirus cloned from one patient with T cell leukemia, closely related sequences were found in DNA of the cell line and of tumor cells from seven other patients with the same disease; these sequences were only distantly related to HTLV-I. The phenotype of the cells and the clinical course of the disease were clearly distinguishable from leukemia associated with HTLV-I. All patients and the wife of one patient showed a weak serological cross-reactivity with both HTLV-I and HIV-1 antigens. None of the patients proved to be at any apparent risk for HIV-1 infection. The name proposed for this virus is HTLV-V, and the date indicate that it may be a primary etiological factor in the major group of cutaneous T cell lymphomas/leukemias, including the sporadic lymphomas known as mycoses fungoides.

PREVIOUS ANALYSES OF SERA FROM patients with mycosis fungoides (*1, 2*) led to the suggestion that a retrovirus related to human T cell leukemia virus types I and II (HTLV-I and HTLV-II) (*3*) might be associated with this form of T cell lymphoma. Mycosis fungoides begins with cutaneous lesions and progresses slowly. There is often no evidence of visceral infiltration for several years, and the disease has a much better prognosis than adult T cell leukemia/lymphoma (ATLL) (*4*).

In recent studies on HTLV-I involvement in T cell malignancies in Italy (*5*), the presence of DNA sequences homologous to, but not identical to, HTLV-I provirus was shown by hybridization in two patients. One patient (G.T.) had T cell leukemia, and the other (G.P.) had cutaneous T cell lymphoma (mycosis fungoides). Both patients appeared to be infected by a variant of HTLV-I (*6, 7*). Here we report on the isolation of a new retrovirus from a cell line derived from a male patient (G.B.) with a cutaneous T cell lymphoma in the leukemic phase. The peripheral blood mononuclear cells of this patient were >90% CD3$^+$, CD4$^+$, and Tac$^-$. Only a few cells expressed antigens recognized by Leu 2b (CD8) or Leu 16 (CD20) monoclonal antibodies (Table 1).

Samples of serum from patient G.B. showed a slight reaction by enzyme-linked immunosorbent assay (ELISA) with both HTLV-I and HIV-1 (*8, 9*) antigens, a very faint p24 by Western blot analysis, and negative immunocompetition with both antigens. The patient did not come from an HTLV-I endemic area and did not appear to be at risk for HIV-1 infection.

Lymphocytes from patient G.B. were maintained in RPMI 1640 medium without the addition of any growth factors. After 6 to 7 weeks in culture, a small number of cells began to proliferate spontaneously and form large clumps. Most of the cells were large and generally mono- or polynucleated. These cells were markedly different from most fresh peripheral blood mononuclear cells in that they lacked T cell markers such as CD3, CD4, and CD8 and expressed surface immunoglobulins and B cell antigens recognized by Leu 12 (CD19), Leu 16 (CD20), and CR2 (CD21) monoclonal antibodies. The cells in culture also expressed activation markers such as transferrin and interleukin-2 receptors (CD25 or Tac) and major histocompatibility class II antigens. The cell phenotype, which was examined every 2 weeks, remained relatively stable for several months (Table 1). Electron microscopic examination revealed typical type C virus budding from cell membranes with most particles present as immature and mature extracellular virions; no particles with eccentric or cylindrically shaped nucleoids were found (Fig. 1).

Supernatants from cultured cells showed reverse transcriptase (RT) activity that increased with passages until it stabilized on a level comparable to that present in culture fluids of the HTLV-I–producing cell line MT2 (*1*).

DNA extracted from fresh and cultured cells from patient G.B. was hybridized with probes derived from cloned HTLV-I, HTLV-II, and HIV-1 genomes. The HTLV-I probe (*10*) hybridized specifically, but only under low stringency conditions [3× standard saline citrate (SSC) at 60°C], with the same bands in the DNA from fresh and cultured cells. This indicates that the provirus is integrated in the same way in both cell populations (Fig. 2D). The

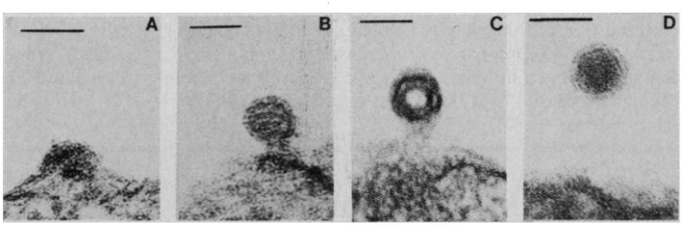

Fig. 1. Electron micrographs of virus particles from patient G.B. Cells were fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in osmium tetroxide, and then embedded in Epon 81. Ultrathin sections were stained with uranyl acetate–lead citrate. Mature virus particles show a centrally located round dense core (scale bar, 100 nm). (**A**) Budding with crescent-shaped nucleoid. (**B**) Virus in formation. (**C**) Extracellular immature virus. (**D**) Extracellular mature virus sectioned perpendicular to the core axis.

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HTLV-II (*11*) and HIV-1 (*8*) probes did not hybridize under the same conditions. Different degrees of hybridization were observed when we used HTLV-I subclones from the *gag-pol*, *pX*, and long terminal repeat (LTR) regions (Fig. 2C) as probes. None of the probes hybridized under more stringent conditions (1× SSC at 65°C). However, hybridization to the same band under high stringency conditions was observed when we used a probe obtained by cloning provirus from the DNA of the T cell leukemia patient G.T. (*12*).

Virus particles purified from cell supernatants by sucrose density gradients were used to study the biochemical characteristics of the virus. When samples of every collected fraction were assayed for RT activity, two peaks were demonstrated at 1.19 and 1.16 g/ml, the densities typical of retrovirus (Fig. 3A). The RT-positive fractions were pooled and concentrated, and a portion of each fraction, together with purified HTLV-I and HIV-1 proteins, was subjected to electrophoresis on a polyacrylamide gel (*13*). The protein pattern of the RT-positive samples indicated a putative major core protein migrating to a band with a molecular weight (equivalent to that of p27) slightly higher than the p24 of HTLV-I and HIV-1 (Fig. 3B).

When purified virus was used to test the divalent cation sensitivity of the RT, we found that like other viruses of the HTLV family, this new virus preferred magnesium over manganese. We then examined 150 other patients with T cell leukemias and lymphomas, and found that DNA from seven of these patients contained exogenous DNA sequences integrated in the genome. These sequences hybridized to the cloned probe from HTLV-I (*9*) at low stringency conditions with the characteristics of the sequences from the DNA of patient G.B. (Fig. 2, A and B). Also, like the DNA from patient G.B., the DNA from these seven patients showed no hybridization with HTLV-II or HIV-1 DNA probes under the same conditions, which indicates the specificity of the hybridization.

Restriction enzyme analysis with Hind III, Sst I, and Pst I revealed common bands in the DNA from the eight patients (approximately 5, 7, and 2.8 kb, respectively); no common bands were found with Eco RI and Bam HI, indicating conserved internal bands but different integration sites of the sequences. RNA extracted from the cell line derived from patient G.B. expressed viral RNA as shown by hybridization with both the HTLV-I probe (Fig. 2E) and a probe obtained by cloning exogenous sequences from patient G.T. (Fig. 2F). Hybridization was stronger in all patients with the specific probe (HTLV-I for MT2 cells, sequences from G.T. for the other patients) both on RNAs and on DNAs.

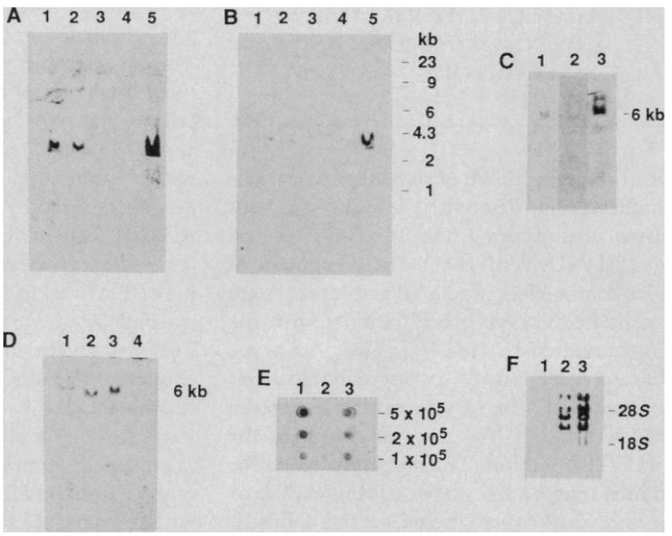

Fig. 2. Hybridization of HTLV-I and HTLV-V probes with DNA and with RNA from HTLV-V–infected cells. DNA extracted from lesion biopsy or peripheral blood lymphocytes and from cultured cells (with proteinase K, phenol, and chloroform) was digested with restriction enzymes, subjected to electrophoresis (0.8 agarose gel), transferred to a GeneScreen membrane (New England Nuclear) by blotting, and hybridized to a HTLV-I 3′ 5.6-kb cloned DNA (*10*) or to subcloned fragments of HTLV-I genome nick-translated with ^{35}S-labeled deoxyadenosine-5′-triphosphate. Hybridization was performed in 3× SSC, 5× Denhardt's solution, 50% formamide, 0.5% SDS, 10% Dextran sulfate at 37°C. Filters were then washed in 3× SSC, 0.5% SDS at 60°C, or in 1× SSC, 0.5% SDS at 65°C. RNA was either extracted with the guanidinium/HCl method, poly(A)-selected on an oligo(dT)-cellulose affinity column, run in a denaturing agarose gel containing formaldehyde, and transblotted on GeneScreen membrane (New England Nuclear), or it was spotted utilizing whole cells in a Hybridot apparatus (Bethesda Research Labs). RNA hybridizations were performed under the same conditions as DNA utilizing as probe either HTLV-I or a 5-kb Hind III–Hind III fragment HTLV-V cloned probe (*12*). (**A**) Digestion of DNA with Pst I. DNA was taken as follows: lane 1, from cells of patient G.T.; lane 2, from cells of patient G.P.; lanes 3 and 4, from normal peripheral blood lymphocytes; lane 5, from HTLV-I–infected MT2 cells washed in 3× SSC at 60°C. (**B**) Same filter washed in 1× SSC at 65°C. (**C**) Hind III digestion of DNA from cells of patient G.B. hybridized to Pst I subcloned fragments of HTLV-I genome: lane 1, *gag-pol* 1.5 kb; lane 2, *pX* 1.3 kb; lane 3, LTR 0.5 kb. (**D**) Hind III digestion of DNA: lane 1, from fresh cells of patient G.B.; lane 2, from cells of patient G.B. 60 days in culture; lane 3, from cells of patient G.B. 120 days in culture; lane 4, from cells of a negative control. (**E**) Dot blot RNA: lane 1, from cells of the MT2 cell line; lane 2, from cells of an uninfected control; lane 3, from cultured cells of patient G.B. (120 days) hybridized with HTLV-I probe. (**F**) RNA: lane 1, K562 RNA; lane 2, HTLV-I–positive MT2 cell line RNA; lane 3, cultured cells from patient G.B. RNA hybridized with HTLV-V probe.

Table 1. Phenotypic pattern of fresh and cultured cells from patient G.B. Cell-surface antigens were identified by indirect immunofluorescence and analyzed by cytofluorometry. The cells (1 × 10^6) were incubated in 100 μl of phosphate buffered saline (PBS) medium at 4°C with appropriate concentrations of mouse monoclonal antibodies: Leu 4 (CD3), Leu 3a (CD4), Leu 2b (CD8), Leu 12 (CD19), Leu 16 (CD20), antibody to CR2 (CD21), antibody to interleukin-2 receptor (IL-2R) (CD25), antibody to transferrin receptor (TR), antibody to human leukocyte antigen (HLA-DR) (Becton Dickinson Corporation) and W6/32 (*16*). After 30 minutes, the samples were washed twice in PBS and further incubated for another 30 minutes at 4°C with 25 μl of a 1:10 dilution of fluorescein isothiocyanate (FITC) conjugate with $F(ab')_2$ sheep antibody to mouse immunoglobulins (IgG) (Cappel, Cooper Biomedical Inc.). Control samples were not exposed to monoclonal antibodies. Surface immunoglobulins were detected by the $F(ab')_2$ fragment of FITC-conjugated goat antiserum to human immunoglobulin (Cappel, Cooper Biomedical Inc.). After the second incubation all samples were washed twice, resuspended in PBS, and analyzed by a fluorescence-activated cell sorter (FACS) analyzer (Becton Dickinson). The percentage positivity is indicated as follows: ND, not determinated; −, <10%; ±, >10%; +, >30%; ++, >50%; and ++++, >90%.

Day of culture	Leu 4	Leu 3a	Leu 2b	Leu 12	Leu 16	CR2	IL-2R	TR	HLA-DR	W6/32	Ig
0	++++	++++	−	−	−	ND	−	−	−	ND	ND
50	−	−	−	−	++	++	±	ND	++++	++++	ND
150	−	−	−	++	++	++	+	++	++++	++++	++

Clinically, all patients except one (G.T.) had cutaneous T cell lymphomas (mycosis fungoides), although G.T. had had leukemia since onset. Neoplastic cells were always $CD4^+$, $CD8^-$, and Tac^-. All the patients were males over 50 years old with apparently no risk factors for infection by HIV-1. All the cutaneous lymphomas were initially not aggressive and showed no bone marrow involvement. In three of the patients, however, the disease evolved to a leukemic phase approximately 2 years after diagnosis, and the patients died within 6 months. The leukemic patients died 8 months after diagnosis (*6*). The other patients are alive to date. All patients came from central and southern Italy, outside any known HTLV-I endemic area, and there was no apparent clustering.

The sera of these patients displayed a borderline positivity against both HTLV-I and HIV-1 antigens by ELISA (*14*). Immunocompetition with HTLV-I–infected cell extracts was negative in all patients, but a faint p24 and some inconsistent additional bands were evident when serum samples from these patients were tested with HTLV-I by the Western blot technique; more intense bands were obtained with HIV-1 antigens in sera from AIDS patients. Serum from the wife (I.V.) of patient M.V. was tested and showed the same reactions as serum from M.V. (Fig. 4); however, I.V. had no lymphatic pathology and her blood lymphocytes showed no integrated proviral sequences, which possibly indicates transmission of this virus occurs through sexual contact as it does for other HTLV infections (*15*).

In conclusion, the clinical aspects of the pathology associated with these patients are different from typical HTLV-I ATLL. Moreover, the neoplastic cells from the patients described here are Tac^-. All HTLV-I–, HTLV-II–, and HIV-1–infected T cells are Tac^+. Because of the homologies and differences between this virus and the other HTLVs, we suggest that it is a new member of the HTLV family and should be named, at least provisionally, HTLV-V. Epidemiological studies are needed to further define its spectrum of infection and its etiological role in T cell malignancies.

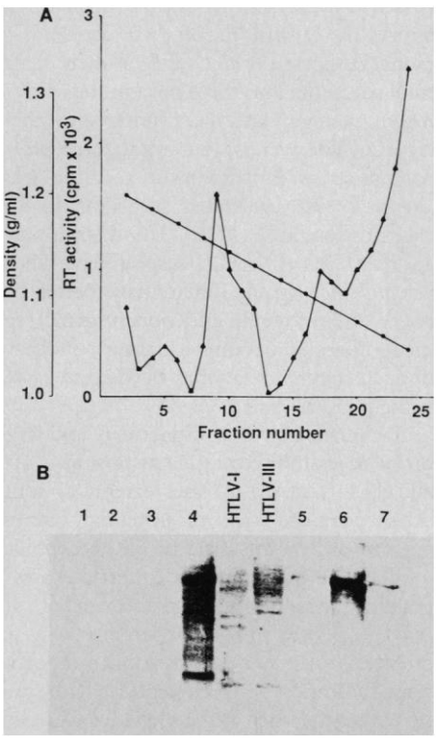

Fig. 3. Reverse transcriptase activity and protein distribution in virus particles. (**A**) RT-activity distribution according to density. Supernatant from a 24-hour culture was clarified and ultracentrifuged 1 hour at 35,000 rev/min in Beckman type 35 rotor. The pellet was resuspended in TNE (tris-HCl 50 m*M*, NaCl 150 m*M*, EDTA 5 m*M*) and layered on a 10 to 50% sucrose gradient in TNE. The gradient was run to equilibrium in a Beckman SW 28 rotor; 26 fractions were collected from the gradient, and a 10-μl aliquot was assayed for RT activity with oligo(dT):poly(A) as primer template in a standard assay. Density (grams per milliliter) is designated by ●; RT activity is designated by ▲. (**B**) Protein electrophoresis of purified virions. Fractions collected from the gradient were pooled in order to get RT activity in the same fraction; proteins were precipitated and run on a 12% slab polyacrylamide gel electrophoresis according to Laemmli (*13*) together with HTLV-I– and HTLV-III (HIV-1)–purified proteins for comparison. Lane 4 corresponds to the RT-activity peak and gradient density 1.17 to 1.15 g/ml. Putative major core protein migrates more slowly than HTLV-I and HIV-1 p24, and it has an approximate molecular size of 27,000 daltons (equivalent to that of p27). Lanes 1 to 7 depict gradient pooled fractions for HTLV-I– and HTLV-III–purified HTLV-I and HIV-1 proteins.

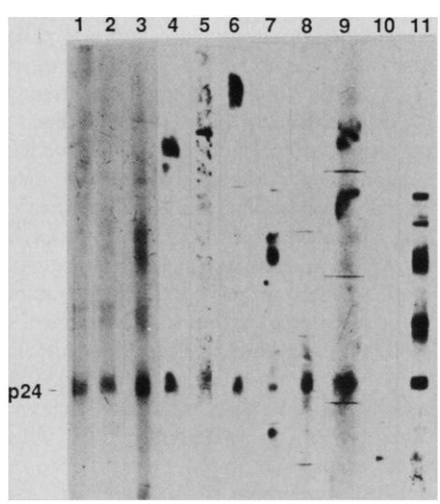

Fig. 4. Serological cross-reactivity between patients' sera and HIV-1 antigens. Western blots were performed with purified HIV-1 proteins run on a polyacrylamide gel electrophoresis and transblotted on nitrocellulose. Sera were used at a 1:50 dilution, and antibodies were detected by ^{125}I-labeled protein A/antihuman immunoglobulin antibodies. Sera in lanes were taken as follows: lane 1, from patient M.V.; lane 2, from patient G.P.; lane 3, from patient G.T.; lane 4, from patient G.R.; lane 5, from patient G.D.; lane 6, from patient A.S.; lane 7, from patient A.G.; lane 8, from patient G.B.; lane 9, from patient I.V.; lane 10, from a normal control; lane 11, from an HIV-1–positive control.

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