Human Lymphotropic Retroviruses Associated With Mycosis Fungoides: Evidence That Human T-Cell Lymphotropic Virus Type II (HTLV-II) as Well as HTLV-I May Play a Role in the Disease

By Dorothea Zucker-Franklin, W. Craig Hooper, and Bruce L. Evatt

The human T-cell lymphotropic virus type I (HTLV-I) is causally associated with adult T-cell leukemia, but its role in mycosis fungoides (MF) has remained enigmatic. The virus is suspect because a small percentage of patients withMF have antibodies to it, the cells of others harbor deleted HTLV-I proviral sequences, and particles resembling HTLV-I emerge in cultured blood lymphocytes obtained from most patients. An alternative possibility is that disparate lymphotropic retroviruses may infect or affect a population of epidermotropic lymphocytes, leading to the same outcome, ie, MF. In studies designed to identify the particles detected in lymphocyte cultures of nine patients with a diagnosis of skin involvement characteristic of MF, this concept has gained support. While the cells of four patients provided evidence of HTLV-I infection, molecular hybridization with HTLV-II-specific probes showed HTLV-II in the cells of another patient. The 103-bp fragment amplified by the HTLV-II-specific probe was sequenced and proved to have greater than 90% homology with the same fragment amplified from cells known to be infected with HTLV-II. A role for HTLV-II in MF has not been suggested heretofore. Therefore, HTLV-I, HTLV-II, and their incomplete forms may be found in cells of MF patients, suggesting new theories regarding the pathogenesis of this disease.

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MATERIALS AND METHODS

Patients. Heparinized peripheral blood (PB) was obtained from nine patients with an unequivocal diagnosis of MF on the basis of clinical manifestations and skin biopsies. Their ages ranged from 35 to 80 years. They were either untreated or received topical applications of steroids or nitrogen mustard. The time from documentation of MF to culture of blood cells varied from 1 month to 12 years. Their circulating Sézary cells, identified and counted by electron microscopy as described, ranged from 12% to 85%. None of the patients had risk factors for HTLV-I/II, with the exception of patient EB. Patient EB is described in greater detail elsewhere.

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because, to our knowledge, she represents the first patient with MF who is infected with HTLV-II. The patient is a 52-year-old black female who had been symptomatic for only 1 year. Physical examination showed widespread lichenification of the skin with fine scales, multiple 2- to 4-mm nodules, and onchodystrophy. There were palpable axillary and inguinal nodes, but no organomegaly. Laboratory data were significant for a white blood cell (WBC) count of 18.9 × 10^9/L with 55% large and small atypical lymphocytes seen on a routine PB smear. Abdominal scan showed no abnormalities. Bone marrow (BM) aspirate as well as biopsy were normal and showed no lymphocyte infiltrates. A skin biopsy was diagnostic for MF (Fig 1). The cells infiltrating the skin were morphologically identical to those circulating in the PB. A lymph node biopsy was considered consistent with "dermatopathic lymphadenopathy."

Immunofluorescence phenotyping of her blood lymphocytes showed the following: 8% of the cells had surface Ig (5.4% light chains, 2.6% λ light chains; CD2, 92%; CD5 (pan T), 80.9%; CD3, 88.9%; CD4, 85.1%; CD8, 7.8%; CD4/CD8 ratio, 10.9; CD1a, 4.5%; CD10, 2.8%; CD20 (pan B), 13.5%; interleukin-2 receptor (IL-2R) (CD25), 4.3%; T-cell receptor, 81.5%; CD7, 6%. Western blot on the patient's serum showed the following antibodies to HTLV-I/II: P15, 19, 24, 26, 28, 32, 36, 42, and Gp 46.

The patient was born in North Carolina. She is a Jehovah's Witness who accepts no blood products. She had been widowed for 2 years and raised four healthy daughters, who have produced 13 healthy grandchildren. No risk factors for either human immunodeficiency virus-I (HIV-I) or HTLV-II infection were elicited, except that her deceased husband used intravenous drugs for 2 years before his death. Two deceased brothers were said to have had Gaucher's disease. Two siblings who were available for testing proved to be serologically negative for antibodies to HTLV-I/II. They stated to have been breast-fed by the same mother as EB. Their lymphocytes were also cultured. On our request, the patient's pregnant daughter was tested for antibodies to HTLV-I/II in North Carolina. She claims to be negative. No documentation could be obtained.

**Cell cultures.** The PB mononuclear cells (PBMC) of the nine MF patients, EB's siblings, and five healthy individuals were isolated by ficoll/hypaque gradient centrifugation, washed twice in Hank's Balanced Saline (GIBCO, Grand Island, NY), and resuspended in RPMI-1640 (GIBCO) containing 10% heat-inactivated fetal calf serum, penicillin, and streptomycin. Then the cells were plated on 35-mm petri dishes at a concentration of 5 × 10^6 cells/mL. All cultures were supplemented with 1,000 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Cetus Corporation, Emeryville, CA) and 10 U/mL IL-2 (Genzyme, Boston, MA). Half of the dishes also received 30 mg phytohemagglutinin (PHA; ICN Immuno Biologies, Lisle, IL) for the first 72 hours of culture. When aggregates consisting of 20,000 to 90,000 cells had formed, GM-CSF was omitted. IL-2 was discontinued when cell proliferation appeared well established, which was within 4 to 6 weeks.
Electron microscopy and ultrastructural immunohistochemistry.
Freshly isolated mononuclear cells, as well as cells cultured for various time periods, were fixed in 3% phosphate-buffered glutaraldehyde, postfixed with osmium, dehydrated, and embedded in Polybed 812 (Polysciences, Inc, Warrington, PA) as routine in this laboratory. Thin sections were stained with uranyl acetate and lead citrate. They were viewed with a Siemens Elmiskop I electron microscope. For ultrastructural identification of the virus, the immunogold technique was used as described. The antibody consisted of either IgG isolated from the patient’s own serum or a monoclonal mouse antibody directed against the envelope epitopes of HTLV-I/II gp46 and gp63 (Genzyme). Briefly, the fixed cells were incubated with 0.1% bovine serum albumin (BSA) for 10 minutes at 4°C to decrease nonspecific binding of the label. The cells were then washed three times with 0.01 mol/L glysine in phosphate-buffered saline (PBS) buffer to quench free aldehyde groups. Subsequently, the cells were incubated with 0.1 mL of monoclonal antibody (MoAb) diluted 1:10 with PBS for 30 minutes at 4°C. After being washed with buffer three times, the cells were incubated for 30 minutes with 10 nm protein A-Colloidal gold particles (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:10 in PBS containing polyethylene glycol and BSA. This was followed by five washes in PBS and fixation with 3% glutaraldehyde in 0.067 mol/L phosphate buffer containing 1% sucrose at pH 7.2. Postfixation and embedding was performed as described above.

Co-culture. Aliquots of EB-cultured lymphocytes were incubated with 5,000 R. Cell death was confirmed by the Trypan blue exclusion test and the failure of the irradiated cells to have incorporated 3H thymidine 72 hours after its addition. The cells were then washed three times with 0.01 mol/L glysine in phosphate-buffered saline (PBS) buffer to quench free aldehyde groups. Subsequently, the cells were incubated with 0.1 mL of monoclonal antibody (MoAb) diluted 1:10 with PBS for 30 minutes at 4°C. After being washed with buffer three times, the cells were incubated for 30 minutes with 10 nm protein A-Colloidal gold particles (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:10 in PBS containing polyethylene glycol and BSA. This was followed by five washes in PBS and fixation with 3% glutaraldehyde in 0.067 mol/L phosphate buffer containing 1% sucrose at pH 7.2. Postfixation and embedding was performed as described above.

Enzymatic amplification of viral DNA. High molecular weight DNA was extracted from the patient’s freshly isolated blood as well as from her cultured cells using the Applied Biosystems 340A DNA extractor (Foster City, CA). Control DNA was prepared from PBMC obtained from five healthy individuals, HL-60 cells, MT-2 cells (a cell line infected with HTLV-I), and MoT (a cell line infected with HTLV-II). The extracted DNA was subsequently resuspended in sterile water. Primer pairs SK110-SK111, corresponding to a conserved region of both HTLV-I and -II; SK58-59, corresponding to a conserved pol region of only HTLV-II; and SK43-SK44, corresponding to a conserved pol region of tax common to both HTLV-I and -II were commercially obtained from Perkin Elmer (Norwalk, CT). The hybridization probes SK112, SK118, SK45, and SK60 were purchased likewise. The primer pair 2P4-2P6, corresponding to another conserved pol region of HTLV-II and the probe 2P5, was kindly provided by Dr Barum De (CDC, Atlanta, GA). The primer pairs and probes are listed in Table 2.

One microgram of DNA was amplified through 35 cycles of polymerase chain reaction (PCR) with the annealing temperature at 55°C for 1 minute and the extension temperature at 72°C for 1 minute. The reaction mixture consisted of 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 2.5 mmol/L MgCl2, 200 mmol/L dNTP, 50 pmol of each primer, and 1.75 U of the Taq polymerase. The reaction volume was 50 mL. To avoid possible contamination, sample preparations and reactions were performed in separate rooms using separate sets of positive displacement pipettors. Furthermore, the PCR reactions were set up in an enclosed plexiglass hood (Oncor, Gaithersburg, MD).

After PCR, the reaction mixture was electrophoresed through a 1% agarose gel and the amplified product was visualized by ethidium bromide staining. The gel was denatured and neutralized, and the amplified products were transferred to Gene Screen Plus (Dupont, Wilmington, DE). The primer pairs and probes are listed in Table 2.

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Nucleotide sequencing of the amplified fragments. After visualization on a 5% polyacrylamide gel, the DNA fragments were excised

Table 2. Position of Primer Pairs and Probes

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<th>Name</th>
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<th>Region</th>
<th>Fragment Size (bp)</th>
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as slices from the gel and electroeluted. Direct sequencing was performed according to Winship with the exception that 200 ng of template was used and dimethylsulfoxide (DMSO) was eliminated from the termination mixture. The reactions were run on an 8% denaturing polyacrylamide gel. The gel was subsequently dried and exposed to film overnight with 1× intensifying screens.

RESULTS

A representative electron micrograph of the lymphocytes (devoid of adherent cells) isolated from EB is shown in Fig 1C. These cells constituted 80% of her lymphocytes and were in no way distinguishable from the cells of other patients with MF or Sézary syndrome. Virus particles were not seen in fresh isolates. A thorough search for ribosomal lamellar complexes, frequently considered a hallmark for HCL, yielded negative results. The long processes characteristic for hairy cells were also absent. Of note was the abundance of cytoplasmic filaments commented on by us in previous reports. Indirect fluorescence staining with a variety of antibodies suggested that these structures consisted of vimentin (data not shown).

Ultrastructural and immunohistochemical analyses. Because we have recently reported on the emergence of virus particles in the lymphocyte cultures of patients with MF who were infected with HTLV-I, only morphologic data relevant to EB will be presented here. Particles were readily found in specimens that had been fixed as early as 2 weeks after the beginning of culture. These measured 100 to 150 nm in diameter, and were usually closely apposed to the plasma membrane. They were indistinguishable from those reported by us in cultured lymphocytes of patients with MF who had no demonstrable antibodies to HTLV-I/II. Many particles were incomplete in that they lacked nucleoids. On the other hand, occasional cells were surrounded by massive numbers of particles, most of which appeared to be complete virions (Fig 2). Viral budding, as illustrated in Fig 2D, was also seen. The immunogold method using either the patient’s own antiserum or monoclonal antisera directed against envelope proteins gp46 and gp63 reacted specifically with the particles as well as with areas of the plasma membrane of cells with which viruses were associated at other sites (Figs 3 and 4). By indirect immunofluorescence microscopy performed when the cultures were 71 days old, 43% of the cells were brightly fluorescent, 33% had faint fluorescence, and 24% were negative for viral epitopes (Fig 5A). At the time these immunofluorescence studies were performed, ie, after 2.5 months in culture, 80% of the cells had remained CD2+, 50% were CD4+, 5% reacted with anti-CD20, and none were IL-2R positive. The cells appear to be immortalized as they continue to proliferate for more than 1 year in the absence of any growth factors. It is noteworthy that the monoclonal cells obtained from EB’s siblings did not show any viral particles.

Coculture. Syncytia formation was seen within 3 to 9 days among Molt-4 cells that had been cocultured with irradiated EB cells (Fig 5B). It may be of interest to mention that syncytia gradually disappeared from these cultures and were no longer seen after 4 weeks. Whether the virus is still detectable by electron microscopy has not yet been determined.

Molecular identification of viruses. The findings on DNA extracted from the cells of all nine patients are summarized in Table 3. The “group specific” primer SK43.44 that amplifies a conserved region of tax, common to both HTLV-I and -II, yielded a positive result only with EB cells. DNA fragments of two patients (CO and AW) hybridized with an HTLV-I–specific pol probe and extracts from cells of four patients hybridized with HTLV-I/II Env probes. The last-named fragments are under further study. Southern blot analyses of PCR products using an HTLV-I–specific pol probe have been published previously. These data are included here only to underline the specificity of results obtained on DNA extracted from EB cells. To further identify the virus with which EB appeared infected, the pol region primer pairs SK110-111 and SK58-59 were used in the PCR performed with DNA from the patient’s freshly isolated cells as well as from her cultured cells. Hybridization with the HTLV-II–specific pol probes, SK60 and SK188, showed that only the DNA fragments extracted from EB-derived cells and those from the HTLV-II–infected cell line, MoT, were positive for HTLV-II (Fig 6).

DISCUSSION

There is little doubt that one of the nine patients with MF studied here is infected with HTLV-II. Although there is considerable immunologic cross-reactivity between HTLV-I and HTLV-II, a 103-bp DNA fragment extracted from the patient’s freshly isolated cell, nor in DNA extracted from the cultured lymphocytes of the other eight patients with MF, five healthy individuals, and HL60 cells examined at the same time. In addition, the virus particles that emerged in cultures of the patient’s mononuclear cells reacted with antisera to HTLV-I/II. EB presented with classical MF. Her symptoms and pathology were indistinguishable from those of other patients with this disease. Only a small number of patients with MF have antibodies to HTLV-I/II when this is tested with commercially available reagents by enzyme-linked immunosorbent assay (ELISA) or Western blot. In our own series of MF patients, this may amount to less than 3%, particularly if black patients or individuals who originate from endemic regions are excluded (unpublished data). The present patient, a middle-aged black female, had no risk factors, except that her husband abused intravenous drugs alledgedly for only 2 years before his death in 1987. Because two of the patient’s siblings, who had been breast-fed by the same mother as EB, as well as
The pathogenic relationship between the patient's viral infection and her neoplastic disease deserves serious consideration. Firstly, the patient's cultured PBMC showed, within the first 2 weeks of growth, an ample number of viral particles that resembled published electron micrographs of HTLV-I/II. Almost 80% of the patients freshly isolated...
cells were typical MF cells and, when subjected to PCR before culture, were shown to harbor the virus. The cells have become immortalized, i.e., as of this writing, they have been maintained for more than 1 year in the absence of any growth factors. Thirdly, while reverse transcriptase was not detected in the culture medium, there was evidence of viral budding (Fig 2). This provides incontrovertible evidence of a productive infection. Moreover, coculture studies of the

**Fig 3.** Details of three different cells taken from a 70-day-old culture showing virus particles stained with the immunogold technique (see Materials and Methods). Magnification ×50,000.

**Fig 4.** (A) Representative illustration of immunogold-stained regions of plasma membranes. The membranes appear thickened at such sites, suggesting integration of viral protein. Magnification ×50,000. (B) In this illustration, the antiserum did not react with the plasma membrane, but specifically with the virus particle (arrow). Magnification ×50,000.
patient's irradiated cells with a noninfected lymphocyte line resulted in the formation of syncytia (Fig 5B) believed to be characteristic for retroviral infections.33 Last, but not least, the PCR performed with DNA extracted from the cells proved to have proviral sequences that hybridized with HTLV-II pol-specific probes. Thus, integration of the proviral genome into the DNA of the patient's cells was established. PCR performed on DNA extracted from mononuclear cell cultures derived from eight other patients with MF as well as from five lymphocyte cultures prepared from the blood of healthy individuals and run in parallel with EB's specimen were negative. At the time these experiments were conducted, no cell lines containing HTLV-II had been maintained in this laboratory. Because the patient's PB cells used to initiate the cultures were identical to those seen in her skin lesions, it seems reasonable to assume that, in this particular individual, HTLV-II infection is related to her cutaneous disease.

Although HTLV-II was first isolated from a patient with atypical HCL, the clinical presentation and pathology of the patient reported here did not warrant the inclusion of HCL in the differential diagnosis. However, it may be important to reiterate some features of the patient's cells, which could turn out to be suggestive of infection with HTLV-II rather than HTLV-I. The cytoplasm of the majority of her cells was replete with filaments, which led us to perform immunofluorescence studies with a variety of antisera directed against cytoskeletal proteins. As expected from their ultrastructural appearance, the most intense cytoplasmic fluorescence was obtained with a monoclonal antiserum directed against vimentin, whereas an antibody directed against keratin was negative (data not shown). We commented on the presence of conspicuous cytoplasmic filaments in Sézary cells of some patients in very early reports on this subject.20,26 This feature has not been described in the cells of patients with HTLV-I-associated adult T-cell leukemia,34 nor have they been observed in ATL or hairy cells in this laboratory. The cells of EB also appear to have more surface villi (Fig 1C) than most Sézary cells studied by us. At no time could these be mistaken for the long processes visible on light microscopy of hairy cells. A patient who developed Sézary syndrome after having been observed for many years with bona fide HCL illustrated this distinction.25 The latter case antedated discovery of HTLV-I/II and the availability of antisera to these viruses. EB's cells also lacked ribosome lamellar complexes, often considered an ultrastructural hallmark of hairy cells.24,25 In the future, it may be useful to note these features to determine whether

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Fig 6. PCR amplification of HTLV-II pol in only one of 9 MF T-cell lines. The primers used were SK58-59. The HTLV-II–specific oligonucleotide probe used for hybridization was SK60. DNA from MoT cells infected with HTLV-II served as a positive control. HL-60 and normal PMBC were negative controls. Patients AW and CO are known to be infected with HTLV-I.
they are indicative of infection with HTLV-II rather than HTLV-I.

What is the significance of these observations in the context of the disease defined as MF? On the basis of our own findings as well as those of others, it seems likely that patients with MF may harbor various types of HTLVs as well as their incomplete forms. This is the first demonstration that HTLV-II may also be associated with this disease. In the light of an increasing prevalence of adult-acquired HTLV-II infection, the observation provides food for thought. The existence of a population of lymphocytes that recirculates primarily to the skin has been recognized. A cutaneous neoplasm consisting of lymphocytes could be the final common pathway of circulating CD4+ cells serving as targets for a variety of retroviruses. Alternatively, cells other than CD4+ lymphocytes may harbor the virus. Even specimens with very high Sézary cell counts are not devoid of a few cells belonging to different cell populations, such as monocytes, B cells, and hematopoietic progenitors. In vitro conditions may favor proliferation and release of virions from such reservoirs and could subsequently infect almost any cell in the culture. To determine the exact cell type from which the particles emerge requires in situ hybridization in conjunction with phenotypic analyses at repeated time intervals. It has also been shown that transactivating genes, such as tax, may have oncogenic potential. The possibility that in vivo “externally” driven transformation of CD4+ lymphocytes could occur as a result of factors released by other cells is supported by the observation that it is not always possible to detect clonality among freshly isolated Sézary cells either by karyotypic analysis or by gene rearrangements of the T-cell receptor-β chain. These considerations, together with the observation that deleted viral sequences of HTLV-II as well as HTLV-I have been found in the mononuclear leukocytes of patients with MF, may throw a new light on the pathogenesis of this disease.

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Human lymphotropic retroviruses associated with mycosis fungoides: evidence that human T-cell lymphotropic virus type II (HTLV-II) as well as HTLV-I may play a role in the disease

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