Isolation of Human T-Cell Leukemia Virus Type I From a Transformed T-Cell Line Derived Spontaneously From Lymphocytes of a Seronegative Egyptian Patient With Mycosis Fungoides

By Mohamed A. El-Farrash, Hanan A. Salem, Marcelo J. Kuroda, Kouki Morizono, Mari Kannagi, and Shinji Harada

Mycosis fungoides (MF) is a rare form of cutaneous T-cell lymphoma that may be associated with human T-cell leukemia virus type I (HTLV-I) infection. Using the polymerase chain reaction, the HTLV-I pX region was constantly detected in the genomic DNA extracted from peripheral blood mononuclear cells (PBMCs) of an HTLV-I antibody-seronegative Egyptian MF patient enrolled in a study to isolate HTLV-I from North Africa. A CD4+ and interleukin-2 (IL-2) receptor-positive T-cell line was established when the phytohemagglutinin-stimulated PBMCs of that patient were maintained in IL-2-containing culture medium. The cell line (EMF) was initially IL-2 dependent and then became IL-2 independent after gradual withdrawal of the IL-2.

The human T-cell leukemia virus type I (HTLV-I) is etiologically associated with adult T-cell leukemia (ATL) and tropical spastic paraparesis or HTLV-I-associated myelopathy (TSP/HAM). The association between HTLVs (HTLV-I and HTLV-II) and the cutaneous T-cell neoplasms mycosis fungoides (MF) and its leukemic variant called Sezary syndrome (SS) has remained controversial. SS is characterized by circulating clonal neoplastic T cells with cerebriform nuclei and a surface phenotype that is typically CD3+, CD4+, and CD45Ro+. MF is clinically distinct from SS and characterized by cutaneous lesions of five HTLV-I-seronegative MF patients. Also, Manca et al documented the presence of tax and pol regions of HTLV-I in the PBMCs of 10 of 29 patients diagnosed as MF, all of them seronegative for the HTLV-I antibodies. Meanwhile, other investigators did not find HTLV-I infection markers in patients with MF and SS, although they used immunologic tests, electron microscopy of cultured lymphocytes, and polymerase chain reaction (PCR) analysis of blood or cutaneous lesions.

We report here the presence of HTLV-I provirus in the PBMCs of a seronegative Egyptian patient with MF. A T-cell line harboring an HTLV-I provirus and expressing the major HTLV-I proteins could be established from this patient.

MATERIALS AND METHODS

Patients. In a trial to isolate HTLV-I from North Africa, we examined different groups of Egyptian patients diagnosed as having leukemia, cutaneous T-cell lymphoma (CTCL), or spastic paraparesis. Among those individuals, 7 cases of MF were examined. The diagnosis and staging of MF was performed according to the tumor node metastases (TNM) classification using clinical and histopathologic examination of the skin lesion, a blood picture for detection of atypical cells, a chest x-ray, and an abdominal ultrasonography to detect metastatic lesions.

HTLV-I detection. Blood samples were obtained from each patient between September 1992 and March 1993. The sera were examined for HTLV-I antibodies using the particle agglutination (PA) test (serodia-ATLA; Fujirebio Inc, Tokyo, Japan). The indirect immunofluorescence (IF) test and the Western blot (WB) assay as described previously.

PBMCs were separated from heparinized blood using the Ficoll-Hypaque method and were stimulated with 1% phytohemagglutinin (PHA) and maintained in 5 ng/mL of interleukin-2 (IL-2) in complete medium (RPMI 1640 [GIBCO, Grand Island, NY] supplemented with 10% fetal calf serum [FCS], 100 IU/mL of penicillin, and 100IU/mL of streptomycin).
HTLV-I IN A SERONEGATIVE MF PATIENT

µg/mL of streptomycin). After 1 week, genomic DNA was extracted from 1 × 10⁶ cells using the Insta Gene Purification kit (Bio-Rad laboratories, Richmond, CA) as described by the manufacturer. Ten microliters of DNA was used as a template to amplify 233 bp between sites 7324 and 7556 in the pX region using the primers pX-1 (7324-7353; 5' CCCAATCTCCAGGGTTTGACAGATCTTC 3') and the antisense pX-4 (7556-7527; 5' GAGGAAAGGGAGTGCGAGGATAGGAA 3'). Amplification reaction was performed for 25 cycles, as described previously.¹ The amplified DNA was subjected to another 35 cycles to amplify the nested 159 bp products, which were cleaved with HindIII and restriction endonucleases and examined using Southern blot hybridization analysis. The probe used was an 8.2-kb fragment of the HTLV-I env region in the EMF cell line.

Establishment of the cell line. Stimulated PBMCs from a PCR-positive case of MF were assianinated in IL-2 (5 ng/mL) in complete medium with renewal of the medium twice a week. After 3 weeks in culture, the PCR analysis was repeated also using primers to amplify the gag or env regions of HTLV-I. Cells were also monitored for HTLV-I-positive cells after staining with HTLV-I antibody-positive sera or the monoclonal antibodies (MoAbs) REY-7 and GIN-14.²³ Specific for the HTLV-I envelope gp 46 and the matrix protein M, respectively. The cells were stained with ethidium bromide and examined for the presence of the specific band 159 bp in size.

Immunologic characterization of the cell line. The cell line (EMF) and its clone (EMF-1) were stained using MoAbs against CD4, CD8, CD25 (L-2 receptor Tac), and CD19 and examined using FACScan (Becton Dickinson, Mountain View, CA), as described previously.²⁴ For comparison, the MT-2 cell line, which is an HTLV-I–transformed and producer cell line,²⁵ was used as a positive control in this examination and in all of the following experiments.

DNA extraction and Southern blot hybridization analysis. Total cellular DNA from 1 × 10⁶ cells (EMF, EMF-1, or MT-2) was extracted as described elsewhere.²⁴ Twenty micrograms of each DNA was digested using Sac I, Kpn I, HindIII, BamHI, Pst I, and Xho I restriction endonucleases and examined using Southern blot hybridization analysis. The probe used was an 8.2 kb fragment of clone A23-3 (PMT-2).²⁴

Sequence of HTLV-I env region in the EMF cell line. To confirm that the virus detected in the EMF cells is HTLV-I, we amplified the entire env region and sequenced it. The primers used were env-F (5’ GGTAAWITCTCGCCACTIT 3’) between 5183 and 5202 and env-R (5’ GTCTCTGCAAGGATATTGAAG 3’) spanning the sites between 6560 and 6540 (with the induced Pst I cleavage site underlined). Genomic DNAs from either EMF or MT-2 cells (1 to 2 µg/100 µl) were amplified for 35 cycles as described above. The amplified DNA products, which were cleaved with HindIII and Pst I, were inserted in the equivalent sites of the Bluescript vector and molecularly cloned. The plasmid DNA of the positive recombinant clones was extracted, purified, and sequenced from both sides as described previously.²⁵

HTLV-I transmission from the EMF cells. Washed EMF cells were cocultured with MOLT-4 cells or PHA-stimulated PBMCs from a healthy individual at a ratio of 1:1 and examined daily to detect any cell fusion or syncytium formation, as described previously.²⁶ Also, EMF cells were treated with mitomycin-C (50 µg/mL) and cocultured with the PHA-stimulated PBMCs from a healthy individual to test the transforming activity of the HTLV-I provirus.

Expression of HTLV-I proteins in the EMF cell line. The EMF cells were also examined for expression of HTLV-I antigens using the WB analysis, as described earlier.²⁷ Different HTLV-I–positive sera from Japanese ATL patients, Egyptian HAM patients, or asymptomatic carriers were used for the detection of HTLV-I antigens.

RESULTS

Diagnosis of MF. The case mentioned henceforth is a 59-year-old Egyptian woman examined in September 1992. She has been living in a small village in Northern Egypt without any contact with other non-Egyptian people. Her mother was also Egyptian, but no further documentation of the origin or purity of her family could be obtained because of the low educational level in this family. She has been clinically diagnosed as MF stage I-B. No involvement of lymph nodes or other metastatic lesions could be detected by careful examination and investigations. The dermatologic lesions were in the form of eczematous, indurated plaques and psoriasisform lesions involving more than 10% of the body surface. The histopathologic features of the plaques showed marked epidermotropism with many scattered single mononuclear cells in the epidermis. Those cells were aggregated within the epidermis forming many Pautrier's microabscesses. In the upper dermis, there was a dense band-like infiltrate consisting of mononuclear cells, many of which have hyperchromatic irregular nuclei surrounded with a halo, giving them the pleomorphic appearance of the mycosis cells. The blood picture did not show any leukemic or atypical cells and the ATL-characteristic cells could not be found in 6 blood films taken at different occasions.

HTLV-I antibody detection. The patient's serum was initially examined for presence of HTLV-I–specific antibodies using the particle agglutination method. This patient's serum repeatedly tested negative, although we could identify other positive sera by the same test under the same conditions. For further confirmation, two other samples were collected after 3 and 6 months and were re-examined using the PA test, IF, and WB assays. All methods failed to detect any antibody (IgG or IgM) specific for the HTLV-I antigens. The patient did not show signs of generalized hypoglobulinemia and her plasma proteins profile was almost normal (only marginal hypoaalbuminemia and altered A/G ratio explained by the Bilharzial infestation that is endemic in this region).

HTLV-I pX region detection by PCR. Figure 1A shows that the patient's stimulated PBMCs contained the 159 bp of pX region when examined using PCR. Samples from 6 other patients with MF and 3 normal controls from the same province did not give any signal when examined using the PCR under the same conditions. The patient's stimulated PBMCs were maintained in complete medium containing 5 ng/mL of IL-2. After 3 weeks in culture, genomic DNA was extracted again and examined for the presence of pX sequence using the same PCR method as above. The same positive signal was again detected in
this patient sample but not in negative controls. Also, other HTLV-I–specific sequences could be detected in the cultured PBMCs of this patient using other primers. Figure 1B and C show, respectively, the env and gag regions of HTLV-I amplified from this patient’s cells but not from the negative controls.

Establishment of the cell line EMF. PBMCs from this patient were maintained in IL-2–containing medium and were growing exponentially. About $5 \times 10^6$ cells were frozen (1 $\times$ $10^7$ cells per tube) in liquid nitrogen. After 3 weeks of culture, cells were smeared on slides and stained by the IF method for detection of the HTLV-I antigen-positive cells using an HTLV-I antibody-positive serum. Very few IF-positive cells (about 0.1% of total cells) could be detected in the PBMCs. Slides containing cells obtained by punch biopsy from the skin lesions of the same group of patients were also examined using the IF method for HTLV-I antigen detection. Again, few IF-positive cells (1 to 2 positive cells/high-power field) could be detected in this patient’s cutaneous lesion but not in samples from the other 6 MF patients when examined using the same method under the same conditions.

These findings encouraged us to keep the culture for a longer time, although replication of the majority of cells seemed stagnant after about 6 weeks. At week 8 of culture, when most cells stopped replicating, one tube of the frozen cells was thawed and added to the culture. Afterwards, cells began to multiply again for about 4 to 5 weeks before becoming stagnant. Another tube of the frozen cells was thawed and added to the culture at week 14. From that time on, better cell growth and replication was noticed, especially when the culture bottles were kept in an oblique position to allow more cell aggregation. At week 18, cells were stained using the MoAbs GIN-14 and REY-7 against HTLV-I Gag and Env proteins, respectively, and were examined using the FACScan. Figure 2 shows that about 50% of cells were positive for HTLV-I Env or Gag proteins. At this stage, we tried to clone the cells using the limiting dilution method.

Cells were distributed in a 96-well plate (10 cells/well) in 200 $\mu$L of complete medium containing 5 ng/mL of IL-2. Once a week, half of the supernatant was replaced with fresh medium containing IL-2. Of 96 wells, in only 4 wells did the cells keep replicating. Those clones were expanded in bigger plates and then in bottles. Only two clones (EMF-1 and EMF-2) maintained replication after expansion. When examined for HTLV-I antigen expression, almost 100% of the cells were positive.

The parent cell line (EMF) and the clones (EMF-1 and EMF-2) were growing well in IL-2–containing medium. After establishment of the cell lines, gradual withdrawal of IL-2 was performed by decreasing the concentration of IL-2 to 3, 2, 1, and, finally, 0 ng/mL. The parent EMF and EMF-1 cells kept growing after IL-2 withdrawal, whereas EMF-2 cells stopped growing.

The EMF and EMF-1 cells grow as floating cells and some are multinucleated. Their doubling time is about 36 to 48 hours, and they have a tendency to form clumps especially in the late stage of culture. FACS analyses showed that 95.6% of cells were CD4$^+$ and that 95.9% were CD25 (IL-
HTLV-1 IN A SERONEGATIVE MF PATIENT

Fig 3. Immunophenotypic characters of the EMF cells. Cells were stained with the MoAb OKT4 for CD4 expression or with the MoAb anti-CD25 for the IL-2 receptor (Tac) and were examined using FACScan. For comparison, the HTLV-I-transformed MT-2 cells were stained with the same MoAbs under the same conditions. Negative controls show cells incubated with the fluorescein-conjugated second antibodies only.

2 receptor Tac)$^+$ (Fig 3). Almost all of the cells were CD19$^-$ and 6.8% of cells were CD8$^+$. The parent EMF and the cloned EMF-1 were identical in both the immunophenotypic and morphologic characters, implying that both cell lines were derived from the same type of cells.

Detection of HTLV-I genome in EMF cells. Genomic DNAs extracted from EMF, EMF-1, or MT-2 cells were digested using $\text{Sac I}$, $\text{Kpn I}$, $\text{HindIII}$, $\text{BamHI}$, $\text{Psr I}$, and $\text{Xho I}$ enzymes and examined by the Southern blot hybridization method using a $^{32}$P-labeled HTLV-I probe. Figure 4 shows that the EMF and EMF-1 genomic DNA contained HTLV-I proviruses, some of which have a molecular size similar to that of the complete HTLV-I genome. The endonucleases’ cleavage sites in EMF and EMF-1 provirus DNA were exactly the same but were slightly different from those for HTLV-I DNA extracted from MT-2 cells.

Sequence analysis of the EMF provirus. To confirm that the virus detected in EMF cells is HTLV-I, we amplified the highly conserved HTLV-I env region from the genomic DNA of the cells and sequenced it. Figure 5 shows the nucleotide sequences of 300 bases from the 5’ side of the env region compared with the same region previously published for known HTLV-I isolates.$^{27}$ The provirus integrated in EMF cells contained env sequences with 95% to 98% homology to the known HTLV-I isolates. Another 300 bases from the 3’ side were also sequenced and compared with some of the known HTLV-I isolates. The homology with ATK-1 or MT-2 was 98% or 97%, respectively. This finding showed that the EMF cells contained an HTLV-I retrovirus but not another known or new retrovirus. Despite the expected very low genetic variability in the env region among different HTLV-I isolates, the EMF virus appeared to be closer to the Japanese and US (cosmopolitan) isolates than to those viruses isolated from sub-Saharan Africans. The complete nucleotide sequence of the EMF isolate is to be completed soon.

HTLV-I transmission from the EMF cell line. After detection of HTLV-I provirus in the genomic DNA of EMF cells, we examined the virus transmission from those cells. The EMF, EMF-1, or MT-2 cells were cocultured with MOLT-4 cells and their syncytium-forming activities were examined. Only coculture of MT-2 with MOLT-4 cells resulted in syncytium formation after 3 to 4 days, whereas coculture of EMF or EMF-1 with MOLT-4 cells showed no fusion activity even after keeping the culture for longer times. On the other hand, coculture of EMF or EMF-1 cells with PHA-stimulated PBMCs from a healthy individual resulted in some fusion activities. Also, the mitomycin-treated EMF or EMF-1 cells could induce fusion in PBMCs from

\[\text{Sac I}\]
\[\text{Kpn I}\]
\[\text{Hind III}\]

Fig 4. Southern blot hybridization analysis of EMF, EMF-1, or MT-2 cellular DNA probed using the HTLV-I probe. Genomic DNA (20 μg) of each cell was digested with the indicated restriction endonuclease, transferred to nylon filters, and hybridized with $^{32}$P-labeled HTLV-I probe. EMF DNA is shown in lanes 1, EMF-1 DNA in lanes 2, and MT-2 DNA in lanes 3.
Fig 5. The nucleotide sequence of 300 bases from the env region of the EMF provirus compared with the same region from different HTLV-I isolates. The sequences of 2 American (US-1 and US-2), 6 African (AF-1 through AF-6), and 4 Japanese (JA-1, JA-2, JA-3, and ATK-1) isolates are cited from De et al. 77
In a seronegative MF patient, we identified a patient with MF having the HTLV-I sequences in her peripheral blood cells when examined by PCR (Fig 1) despite being seronegative. Also, the cutaneous lesion of this patient had a few HTLV-I-positive cells when examined using the IF method. Previously, different groups of investigators using the PCR technique reported the presence of sequences belonging to different regions of HTLV-I provirus in the blood lymphocytes and/or cutaneous lesions of seronegative patients with MF.8,13,28

To further extend our finding, we maintained the stimulated PBMCs of that patient in culture medium containing IL-2, with the aim of checking the transforming activity of the detected HTLV-I sequence. A T-cell line that is CD4+ and IL-2 receptor positive (Fig 3) could be derived spontaneously from those stimulated PBMCs. This cell line (EMF) was initially IL-2 dependent, but could grow also exponentially after IL-2 withdrawal. The cells are HTLV-I antigen positive (Fig 2), contain the HTLV-I provirus (Fig 4), and express the major structural HTLV-I proteins (Fig 6). The nucleotides sequence of the conserved env region of the provirus integrated in the EMF cells showed that it is an HTLV-I having only minor genetic variability in the env gene when compared with other known HTLV-I isolates (Fig 5). Meanwhile, viral transmission from EMF to other PBMCs was not efficient because the cocultivation experiments did not yield newly transformed cell lines. The absence of p28 and p21 in EMF (Fig 6) may render the provirus in EMF noninfectious or nonreplicating. Complete sequencing of the EMF provirus to explore if it has some minor genomic defects and decide its phylogenetic position among other known HTLV-I isolates is now in progress.

The data presented here may not be enough to clarify the relation between HTLV-I infection and MF as 6 other patients with MF from the same province were HTLV-I antibody and antigen negative under the same conditions. Nevertheless, our data support the previous reports that some of the HTLV-I-seronegative MF patients may have HTLV-I provirus in their PBMCs or cutaneous lesions.8,13,28 Furthermore, these data show that an HTLV-I could be isolated from a T-cell line (most probably transformed by this virus) and derived from the PBMCs of a seronegative MF patient.

A difficult question is why this infected patient remained seronegative. It has been previously reported that HTLV-I-infected persons may not have detectable antibodies to HTLV-I structural proteins as measured using commercially available kits.29 We used three different tests (PA, IF, and WB), performed in two laboratories, alongside positive and negative controls. All tests gave unequivocally negative results for this patient, whereas other positive sera could be detected under the same conditions. In this patient, neither anti-HTLV-I IgG nor IgM could be detected, whereas the plasma proteins profile excluded the possibility of hypoglobulinemia as a cause. The difference in immunogenetic background (eg, in the HLA haplotype) has been shown to affect the immune response and the manifestation of the disease among patients infected with HTLV-I in Japan.30 Zucker-Franklin and Pancake31 reported a case of a child with MF who had HTLV-tax proviral sequences while being seronegative. Because the mother of that child was HTLV-I seropos-
itive, they postulated that the child might have been infected very early in his life so that he developed some tolerance to the virus. A similar possibility for our case may be postulated. Also, Daenke et al reported a patient with spastic paraparesis who carries HTLV-I sequences in his DNA but lacks humoral or cytotoxic T-cell responses to HTLV-I. They supposed that their patient may have developed an effective immune response to HTLV-I that has now waned to a level below the limit of detection with little or no expression of viral antigen since then to maintain the immunologic memory. It is also possible that the virus-containing cells in our patient did not express sufficient amounts of HTLV-I antigens in vivo because of some defect in one of the regulatory genes (eg, rex) and were therefore not recognized by the immune system. In vitro stimulation with PHA and IL-2 might have induced the completely latent HTLV-I in the cells to express some of the HTLV-I structural proteins and the transformation of the EMF cell line.

Our finding may raise more concern in the checking cases of MF using PCR even if those patients are HTLV-I antibody negative. Also, these data may support a role for HTLV-I in the pathogenesis of at least some cases of MF.

ACKNOWLEDGMENT

We thank Drs M. Abou Hashem, Z. Abd El-Azziem, W.K. Mouafi, A. Sultan, and S.E. Abd El-Naby (Mansoura Faculty of Medicine, Mansoura, Egypt) for their help in clinical, histopathologic, and radiologic diagnosis. We also thank Dr H. Akari for his help in some PCR analyses and M. Fujii for helping in the preparation of this manuscript.

REFERENCES

26. Kannagi M, Kuroda MJ, Mueda Y, Harada S: Coexistence of
fusion receptors for human T-cell leukemia virus type-I (HTLV-I) and human immunodeficiency virus type-I (HIV-1) on MOLT-4 cells. Microbiol Immunol 35:729, 1991


Isolation of human T-cell leukemia virus type I from a transformed T-cell line derived spontaneously from lymphocytes of a seronegative Egyptian patient with mycosis fungoides

MA el-Farrash, HA Salem, MJ Kuroda, K Morizono, M Kannagi and S Harada