Brief Definitive Report

Persistence of Human T Cell Lymphotropic Virus Type 1 (HTLV-1) Sequences in Peripheral Blood Mononuclear Cells from Patients with Mycosis Fungoides

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Summary

Mycosis fungoides (MF) is a rare form of cutaneous T cell lymphoma suspected of having a viral etiology. As in adult T cell leukemia, the virus involved may be human T lymphotropic virus type 1 (HTLV-1). We cultured the peripheral blood mononuclear cells (PBMC) of 29 patients with MF HTLV-1 seronegative by enzyme-linked immunosorbent assay and Western blot. The presence of reverse transcriptase (RT) and p24 antigen was investigated in the concentrate supernatant of the culture. The DNA of all studied patients was submitted to polymerase chain reaction and Southern blot analysis using primers and probes recognizing the *tax* region of HTLV-1/2 and the *pol* region of HTLV-1. 10 of 29 patients were found positive to HTLV-1, whereas they were always negative to RT and p24. The same results were confirmed in double blind after 6 mo. Our findings suggest HTLV-1 may be involved in the etiology of MF, at least in certain cases.

Cutaneous T cell lymphomas (CTCLs) are rare neoplastic disorders of $CD4^+$, $4B4^+$ (helper/memory) T cells, including adult T cell leukemia/lymphoma (ATL), mycosis fungoides (MF) and its leukemic variant, the Sezary syndrome (SS) (1). ATL is an aggresive disorder causally associated with HTLV-1 infection (2–4). High incidences of ATL have been reported in Japan, the Caribbean, and parts of Eastern Europe where HTLV-1 is endemic (5, 6). Most if not all patients with ATL are HTLV-1 seropositive (7). T cells from patients with ATL express receptors for IL-2 (Tac^+) and are predominantly $CD4^+$, whereas the leukemic cells of MF and SS are $CD4^+$ and Tac^-. In the etiology of MF, HTLV-1 is implicated. Hall first described an association of deleted HTLV-1 provirus and MF in seronegative patients (8, 9); Zucker et al. (10) detected human T lymphotropic viruslike particles in cultures of PBL from patients with MF. Sahai et al. (11) found that 4 of 27 patients with MF and SS were infected with HTLV-1, whereas Wittacher and Luzzatto (12) found defective HTLV-1 sequences in only 10% of patients with MF. Moreover, Bazarbachi et al. (13) found no HTLV-1 infection markers in 24 cases of French and Portuguese patients with MF and SS, and more recently Pancake and Zucker (14) detected HTLV-related *tax* sequences in a large number of patients with MF.

In light of these data, we analyzed genomic DNA isolated from mononuclear cells, using the PCR method, with primers flanking HTLV-1/2 *tax* and HTLV-1 *pol* sequences in 29 Italian patients with MF. The patients were HTLV-1 seronegative in both ELISA and Western blot (WB) assay. As control, PCR was used to detect the presence of HTLV-1 sequences in DNA prepared from cultured blood mononuclear cells obtained from 20 healthy blood donors.

Materials and Methods

Patients. We studied 29 patients (Table 1). All patients, except the one who received intravenous chemotherapy, either received topical applications of steroids, UV irradiation (PUVA) or IFN-α at the time the specimens were obtained. Their ages ranged from 28 to 89 yr. All the patients were Italian, from HTLV-1–nonendemic areas, with no family history of neurological disease or personal history of drug abuse, blood transfusion, or contact with people from Asia or Africa. All patients were seronegative to HTLV-1/2 antibodies by ELISA and WB. As a negative control, 20 healthy blood donors were studied.

Cell Culture. PBMC were isolated by Lymphoprep (Ficoll-Hypaque) density gradient centrifugation, washed three times in PBS (Oxoid, Basingstoke, UK), and cultured at a density of 10^6 per ml in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 20% FCS serum (GIBCO BRL), antibiotics, 0.2 mg/ml PHA, and 10% IL-2 (Pan-Data Systems, Inc., Rockville, MD).

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Table 1. *Patients with Mycosis Fungiodes*

Patient	Sex/age	Duration of disease	Therapy	Blood transfusion	TNM stage
Z. B.	M/46	6 yr	PUVA	No	I A (T_1 N0M0)
B. G.	M/79	5 yr	PUVA	No	I A (T_1 N0M0)
M. O.	F/54	6 yr	PUVA + IFN-α	No	I A (T_1 N0M0)
B. B.	F/57	5 yr	PUVA	No	I A (T_1 N0M0)
B. G.	M/65	8 yr	Steriod	No	I A (T_1 N0M0)
V. R.	M/36	3 yr	PUVA	No	I A (T_1 N0M0)
A. G.	F/74	5 yr	PUVA	No	I A (T_1 N0M0)
M. P.	F/58	3 yr	PUVA	No	I A (T_1 N0M0)
C. V.	M/53	1 yr	PUVA	No	I A (T_1 N0M0)
S. M.	M/57	15 yr	PUVA	Yes	I A (T_1 N0M0)
S. A.	M/53	1 yr	PUVA	No	I A (T_1 N0M0)
S. A.	M/55	3 mo	PUVA	No	I A (T_1 N0M0)
D. P.	M/62	1 yr	PUVA	No	I A (T_1 N0M0)
B. M.	F/70	1 yr	PUVA + IFN-α	No	I B (T_2 bN0M0)
P. E.	F/44	2 yr	PUVA + IFN-α	No	I B (T_2 N0M0)
Z. E.	M/53	1 yr	PUVA + IFN-α	No	I B (T_2 N0M0)
M. G.	M/57	2 yr	Radiotherapy	No	I B (T_2 bN1M0)
F. C.	M/45	1 yr	PUVA + IFN-α	No	I B (T_2 N0M0)
B. F.	M/44	1 yr	PUVA + IFN-α	No	I B (T_2 N0M0)
A. N.	M/70	2 yr	PUVA + IFN-α	No	I B (T_2 N0M0)
G. C.	M/28	3 yr	PUVA	No	I B (T_2 N0M0)
B. B.	M/45	7 yr	IFN-α	No	I B (T_2 aN0M0)
F. G.	M/72	1 yr	PUVA	No	I B (T_2 N0M0)
P. F.	F/58	1 yr	PUVA + IFN-α	No	II A (T_2 bN1M0)
B. P.	M/70	2 yr	Chemotherapy	No	II B (T_3 N0M0)
G. B.	M/68	2 yr	PUVA + IFN-α	No	III (T_4 N0M0)
G. B.	M/85	10 yr	PUVA	No	III (T_4 N0M0)
C. R.	M/61	7 yr	PUVA	No	III (T_4 N0M0)
P. B.	M/78	5 yr	PUVA	No	III (T_4 N0M0)

DNA Purification, PCR, and Southern Blot Analysis for HTLV-1. DNA was isolated by overnight incubation of PBMC at 37°C in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, and 0.5% SDS) containing 100 μg/ml proteinase K. After phenol chloroform extraction, the DNA was precipitated with ethanol and redissolved in sterile H_2O. DNA concentration was estimated by absorption at 260 nm.

Amplification was carried out in a total volume of 100 μl using buffer, deoxynucleotides, $MgCl_2$, and *taq* polymerase (Perkin Elmer Cetus, Norwalk, CT). Reaction mixtures contained 25 pmol of each primer, 1 μg genomic DNA, and 2.5 U *taq* polymerase. Samples were denaturated in a DNA thermal cycler (Perkin Elmer Cetus) for 2 min at 95°C and then subjected to 35 cycles of amplification. Each cycle consisted of a denaturating phase of 95°C for 1 min, primer annealing at 58°C for 1 min, and chain elongation at 72°C for 1 min. DNA samples from normal blood donors were used as negative controls. As a positive control, DNA from HTLV-1–infected cells (MT_2) was used (kindly provided by Drs. R.C. Gallo and M.G. Sarngadharan, National Institutes of Health, Bethesda, MD). Oligonucleotide primers used were: SK 43 and SK 44, designed to amplify a 159-bp fragment of DNA from the *tax* region of both the HTLV-1 and HTLV-2 genomes, SK 54 and SK 55, designed to amplify a 119-bp fragment of DNA from the *pol* region of the HTLV-1 genome (Perkin Elmer Cetus).

20 μl of each amplified sample was run on 0.8% agarose gel in TBE (0.089 M Trisborate, 0.089 M boric acid, and 0.002 M EDTA) and blotted into a nylon membrane (Amersham International, Amersham, Bucks, UK) by standard Southern method (Maniatis et al.) (15).

Hybridization was performed with rapid hyb-buffer (Amersham International) for 2 h at 42°C with probes SK 45 or SK 56 (Perkin Elmer Cetus).

20 pmol of probes were labeled at 3′ OH ends with dUTP-biotin in a tailing reaction, using the enzyme terminal transferase.

The membranes were then washed for 20 min in 5× SSC, 0.1% (wt/vol) SDS at room temperature, and twice for 15 min in 0.1 ×

SSC, 0.1% (wt/vol) SDS at 42°C. For the detection of the nucleic acids hybridized with the biotinylated probes, we used the Polar Plex Chemiluminescent blotting Kit® (Millipore Corp., Bedford, MA) according to product specifications.

PCR for the Detection of EBV Genome. The primer pair (primers ebvP1 and ebvP2) specific for the 405-bp region was generated from the oriP region of the EBV genome. These primers have been described by Glukhov et al. (16).

To perform "nested" PCR, two sets of primers were used; both are located in the BamHI-W region of the EBV genome. The BamHI-W region is repeated up to 11 times within the genome. The sequences of the primers were: (*a*) "outer" primers ebvP3 (5'-GGGTGAGGCCCAGCCCCTC-3', positions from 13.822 to 13.841) and ebvP4 (5'-CATTTGTGTGGACTCCTGGC-3', position from 14.411 to 14.392); and (*b*) "inner" primers ebvP5 (5'-GGAAGCGGGTCTATGGTTGG-3', positions from 14.138 to 14.157) and ebvP6 (5'-GTCCCCCTCCCTAGAACTGA-3', positions from 14.339 to 14.320). PCR amplification products were 590 and 202 bp in length for outer and inner primers, respectively.

All oligonucleotides were synthesized on an automated DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA) using the phosphoramidite method.

Nested PCR was carried out in two steps. Both steps were performed in a final volume of 50 μl containing 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, 2.5 U Biotaq polymerase (Biomaster Co., Moscow, Russia), 200 μM of each dNTP, 15 pmol of each primer, and 0.18 μg (DNA from the B95-8 cell line, EBV-positive control DNA) or 0.5 μg genomic DNA. The mixture was subjected to PCR amplification using a DNA thermal cycler (Perkin Elmer Cetus). The first step consisted of 35 cycles of denaturation at 94°C for 1 min (3 min for the first cycle); annealing of primers at 56°C for 1 min (2 min for the first cycle); extension at 72°C for 2 min (3 min for the first cycle and 5 min for the last cycle), and was performed with outer primers ebvP3 and ebvP4. PCR conditions for primers ebvP1 and ebvP2 were identical.

In the second step, 1 μl (1:100 diluted) of the first PCR products was amplified, using the same protocol, with inner primers ebvP5 and ebvP6, for another 25 cycles.

5 μl of PCR reactions was resolved on a 1.5% agarose gel, containing 0.5 μg/ml ethydium bromide and electrophoresed in TBE buffer. The bands were visualized with UV illumination and photographed.

As positive controls for PCR analysis, we chose the B95-8 cell line, which is an EBV-positive Marmoset cell line.

Results and Discussion

PBMC from all patients were cultured as reported in Materials and Methods. After 15 d of culture, the cells were harvested and their DNA extracted. PCR was used to detect HTLV-1 DNA in these samples using the conditions, primers, and probes described in Materials and Methods.

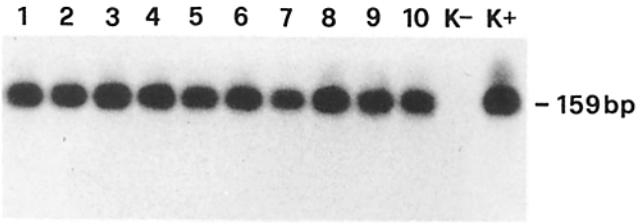

Figure 1. Amplification of HTLV-1/2 target sequences in DNA samples from the cultured lymphocytes of the patients with MF. The DNA samples were amplified by PCR and by Southern blot analyzed with primers SK 43–SK 44 recognizing *tax* region of HTLV-1/2 and hybridyzed with probe SK 45 as described in Materials and Methods. (Lanes *1–10*) Patients with MF. (K^-) Negative control was genomic DNA from lymphocytes of healthy blood donors. (K^+) Positive control was genomic DNA from HTLV-1 producer cell line MT 2.

The results show that 10 of 29 patients with MF were positive to the *tax* region of the HTLV-1/2 genome (Fig. 1) and that the same patients were also positive to the *pol* region of the HTLV-1 genome (Fig. 2).

Five lines proliferated spontaneously and grew in large clumps. Comparative phenotypic analysis showed them to be predominantly B cells. EBV is one of the viruses that is capable of immortalizing B lymphocytes (17, 18). The cells were shown to be EBV infected by PCR analysis with oriP (ebvP1/ebvP2) and BamHI-W (ebvP3/ebvP4 and ebvP5/ebvP6) primers (Fig. 3, *A* and *B*). In addition, one cell line from the patients with MF was not immortalized, but it was shown to be positive to the EBV genome by PCR (Fig. 3, patient 6).

Our results also showed two patients with concomitant sequences of HTLV-1 and EBV in B cells. The detection of HTLV-1 and EBV DNA in cell lines from patients with MF is summarized in Table 2. This last finding is not unusual in patients with ATL (19, 20), or in those with MF as found by Hall et al. (8). Additionally, EBV is expressed in a large number of conditions (21–23).

Our results demonstrate a high incidence of HTLV-1 *tax* and *pol* genes integrated into the DNA of peripheral lymphocytes of MF patients. No significant level of Mg^+- or Mn^+-dependent RT was detected in the concentrate supernatant fluid of cultures, so no evidence was found of infec-

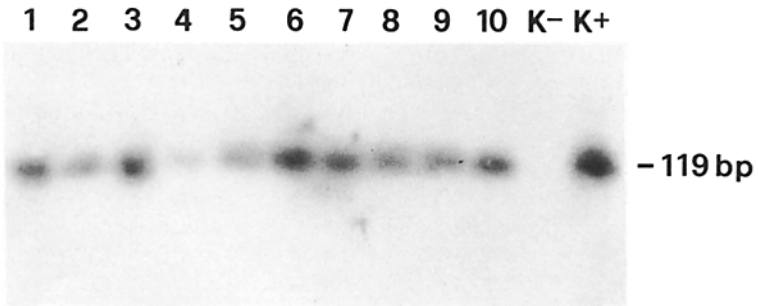

Figure 2. Amplification of HTLV-1 target sequences in DNA samples from the cultured lymphocytes of the patients with MF. The DNA samples were by PCR and by Southern blot analyzed with primers SK 54 and SK 55 recognizing the *pol* region of HTLV-1 and hybridzed with probe SK 56 as described in Materials and Methods. (Lanes *1–10*) Patients with MF. (K^-) Negative control was genomic DNA from lymphocytes of healthy blood donors. (K^+) Positive control was genomic DNA from HTLV-1 producer cell line MT 2.

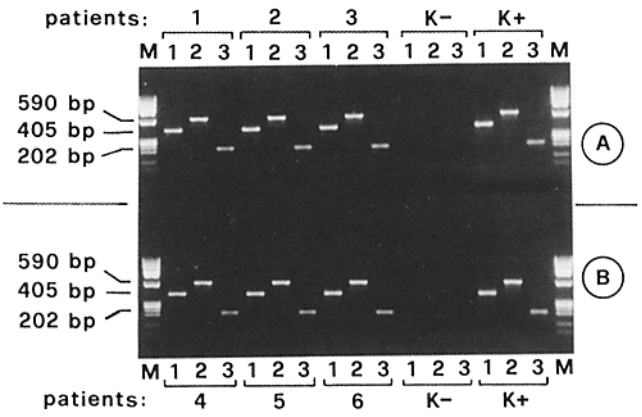

Figure 3. Amplification of EBV target sequences in DNA samples from the cultured lymphocytes of the patients with MF (*A* and *B*). (*A* and *B*) Yield of PCR products after amplification of strongly EBV-positive DNA samples. The DNA samples were amplified by PCR (as described in Materials and Methods) with EBV primer pairs: ebvP1/P2 (lanes *1*, *A* and *B*) specific for the ori P-region of EBV, nested primer pairs: ebvP3/ebvP4 (lanes *2*, *A* and *B*) and ebvP5/ebvP6 (lanes *3*, *A* and *B*) specific for the Bam HI-W region of EBV. Positive control was genomic DNA from ~27,000 cells) from the EBV-producer cell line B95-8 (K^+ in *A* and *B*). Negative controls (genomic DNA from the H9 cell line was also used (K^- in *A* and *B*).

tion due to a retrovirus of such cells lines. The p24 capture antigen test for HTLV-1 was also negative as well (data not shown).

As the incidence of HTLV-1 in northern Italy, where HTLV-1 is not considered endemic, was found to be very high, after 6 mo we took blood from the same patients and cultivated lymphocytes in double-blind experiments to re-

Table 2. *HTLV-1/EBV Detection in Patients with Mycosis Fungoides*

Patient	Sex/age	Duration of disease	orlP	BamHI-W (590 bp)	BamHI-W (202 bp)	PCR Tax HTLV1-2	PCR Pol HTLV1	TNM stage
Z. B.	M/46	6 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I A (T_1 N0M0)
B. G.	M/79	5 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I A (T_1 N0M0)
M. O.	F/54	6 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I A (T_1 N0M0)
B. B.	F/57	5 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I A (T_1 N0M0)
B. G.	M/65	8 yr	Pos.	Pos.	Pos.	Pos.	Pos.	I A (T_1 N0M0)
V. R.	M/36	3 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I A (T_1 N0M0)
A. G.	F/74	5 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I A (T_1 N0M0)
M. P.	F/58	3 yr	Pos.	Pos.	Pos.	Neg.	Neg.	I A (T_1 N0M0)
C. V.	M/53	1 yr	Pos.	Pos.	Pos.	Neg.	Neg.	I A (T_1 N0M0)
S. M.	M/57	15 yr	Pos.	Pos.	Pos.	Neg.	Neg.	I A (T_1 N0M0)
S. A.	M/53	1 yr	Pos.	Pos.	Pos.	Neg.	Neg.	I A (T_1 N0M0)
S. A.	M/55	3 mo	Pos.	Pos.	Pos.	Pos.	Pos.	I A (T_1 N0M0)
D. P.	M/62	1 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I A (T_1 N0M0)
B. M.	F/70	1 yr	Pos.	Pos.	Pos.	Neg.	Neg.	I B (T_2 bN0M0)
P. E.	F/44	2 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
Z. E.	M/53	1 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I B (T_2 N0M0)
M. G.	M/57	2 yr	Neg.	Neg.	Neg.	Pos.	Pos.	I B (T_2 bN1M0)
F. C.	M/45	1 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
B. F.	M/44	1 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
A. N.	M/70	2 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
G. C.	M/28	3 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
B. B.	M/45	7 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 aN0M0)
F. G.	M/72	1 yr	Neg.	Neg.	Neg.	Neg.	Neg.	I B (T_2 N0M0)
P. F.	F/58	1 yr	Neg.	Neg.	Neg.	Pos.	Pos.	II A (T_2 bN1M0)
B. P.	M/70	2 yr	Neg.	Neg.	Neg.	Neg.	Neg.	II B (T_3 N0M0)
G. B.	M/68	2 yr	Pos.	Pos.	Pos.	Neg.	Neg.	III (T_4 N0M0)
G. B.	M/85	10 yr	Neg.	Neg.	Neg.	Neg.	Neg.	III (T_4 N0M0)
C. R.	M/61	7 yr	Pos.	Pos.	Pos.	Neg.	Neg.	III (T_4 N0M0)
P. B.	M/78	5 yr	Neg.	Neg.	Neg.	Pos.	Pos.	III (T_4 N0M0)

search, in concentrate supernatant, RT and p24 of HTLV-1 and to detect specific antibodies by ELISA and WB. HTLV-1 sequences were researched using the same primers and probes and the previous results were confirmed. Our data are in line with Hall's findings (8) and show a higher rate of HTLV-1 than that reported by Wittacher and Luzzatto, (12). Our data also show a lower incidence of HTLV *tax* gene sequences than reported by Pancake and Zucker (14). However, our data differ from those of Bazarbachi et al. (13) who found no specific HTLV-1 sequences in the cells of patients with MF and SS. In addition, even under the same cell culture conditions and using PCR, we never found any HTLV-1 sequences in the lymphocytes of the 20 healthy blood donors. It is interesting to note that the seronegativity described by other authors (8, 12, 14), persisted in all our patients, although HTLV-1 sequences were documented. Our findings may be explained by the fact that the virus in patients with MF has one or more deletions, as clearly documented by Hall (8), which may limit or prevent virus replication, thus avoiding or reducing antibody response.

This last hypothesis requires further documentation, as 6 mo later no patient showed signs of serum conversion but all remained seronegative. Virus deletion may explain the absence of RT and of p24 antigen in the supernatant of all the samples of cultured lymphocytes, although their search was performed every week for 50–60 d and the presence of *tax* and *pol* gene sequences was always positive in the same cell cultures, thus indicating that the virus was not in its replicative phase.

In conclusion, our results show a high incidence of the HTLV-1 sequences in a large number of patients with MF.

This finding seems to be significant because it was confirmed 6 mo later in double-blind experiments in the same patients whereas no HTLV-1–positive culture was present in the laboratory, thus, ruling out the possibility of contamination as a cause of our positive results.

Another element which confirms the high significance of our data is that all the patients studied came from countries where HTLV-1 is not endemic, and they were not at risk for HTLV-1 infection.

This last fact suggests the possible role of HTLV-1 (or deleted HTLV-1) or of a related virus as the cause of certain cases of MF. It would be useful, from the onset of the first lesions, to monitor the patients with serological reaction (generally patients with MF are seronegative with commercially available reagents) but also to grow lymphocytes, for the detection of RT and p24 in the supernatant and especially of HTLV gene sequences in DNA so as to understand more clearly the relationship between HTLV-1 and MF.

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