Human T-Cell Leukemia-Lymphoma Virus Type I (HTLV-I) Expression in Fresh Peripheral Blood Mononuclear Cells from Patients with Tropical Spastic Paraparesis/HTLV-I-Associated Myelopathy

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Tropical spastic paraparesis/human T-cell leukemia-lymphoma virus type I (HTLV-I)-associated myelopathy (TSP/HAM) is a chronic neurological illness epidemiologically associated with HTLV-I infection. We investigated the role of HTLV-I in the pathogenesis of this disease by studying viral expression in fresh uncultured peripheral blood mononuclear cells (PBMCs) of six patients of Caribbean origin with TSP/HAM. The PBMC genomic DNA of all the patients studied carried HTLV-I provirus, but viral expression was not detected by Northern (RNA) blot analysis of total cellular PBMC RNA. When the reverse transcriptase polymerase chain reaction technique was used with primers specific for the tax-rex mRNA, all of the samples were positive for this viral mRNA species, regardless of the duration of the illness (range, 2 to 13 years). The splice junctions for the tax-rex mRNA described in cases of HTLV-I-induced adult T-cell leukemia (position 5183 of the envelope and position 7302 of the pX region) were identical in three TSP/HAM cases studied. To ascertain whether viral expression occurred at a low level in many cells or at a high level in a few permissive cells, we performed in situ hybridization on fresh PBMCs from two patients (2 and 7 years after clinical diagnosis), seeking HTLV-I RNA sequences. Our finding indicated that in vivo HTLV-I expression occurred at a high level in a few cells (1 of every 5,000 PBMCs) in both cases studied. The fact that cells of all six patients with TSP/HAM were positive for viral expression, regardless of the time lag from diagnosis, suggests that persistent expression of a viral product(s) may be pivotal in the pathogenesis of TSP/HAM.

The human T-cell leukemia-lymphoma virus type I (HTLV-I), discovered in 1980 (32), is the causative agent of adult T-cell leukemia (ATL) (23, 29, 33, 42–44), a malignant disorder affecting CD4+ lymphocytes (for a review, see reference 12). HTLV-I is highly endemic in some areas of Japan (23, 44), the Caribbean basin (3), and some parts of Africa (10). In 1985, HTLV-I infection was also associated with a subgroup of chronic progressive myelopathies common in tropical areas (13, 14, 34) and in Japan (31), and is now referred to as tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) (35).

ATL is a neoplastic disease caused by the proliferation of a single-cell clone, and usually one copy of the HTLV-I genome is clonally integrated into the leukemic cell population (12, 43). However, this clonal leukemic population does not appear to express viral RNAs or viral antigens (11, 19, 41) when standard methods for detection are used. However, a recent report indicated the discovery of low-level viral RNAs in the peripheral blood mononuclear cells (PBMCs) of a small number of leukemic patients (25) by the sensitive technique of reverse transcriptase polymerase chain DNA amplification reaction (RT PCR). It is unclear whether viral mRNAs are expressed in a small subpopulation of leukemic cells or in the normal infected PBMCs of patients with ATL. Whichever is the case, in the majority of the leukemic cells the virus appears to be quiescent, and some other mechanism may be involved in maintaining the leukemic state.

In TSP/HAM, viral replication appears to be more directly related with disease development than in ATL because of (i) rapid onset of disease in a few well documented cases of transfusion-associated TSP/HAM (16, 36); (ii) the presence of immunoglobulin G and immunoglobulin M oligoclonal bands in serum and cerebrospinal fluid, some of which are directed against HTLV-I antigens (6, 14); (iii) higher titers of serum and cerebrospinal fluid antiviral antibodies (7, 14); and (iv) a higher number of circulating HTLV-I-infected cells compared with the number in healthy HTLV-I carriers (15, 45). To gain some insight into the pathogenesis of TSP/HAM, we investigated qualitative and quantitative viral expression in fresh uncultured PBMCs from patients with TSP/HAM.

The origin, age, sex, and duration and clinical severity of illness of the patients with TSP/HAM who were studied are summarized in Table 1. The range of duration of illness was 2 to 13 years. All of the patients with TSP/HAM who were studied had lymphoid cells with abnormally shaped nuclei (10% to 30% of the total lymphoid cells). Furthermore, typical ATL-like cells with hyperconvoluted nuclei were found in the patients and, on the average, represented 3% of the total lymphocytes (range, 1 to 4%) (Fig. 1). Phenotypic analysis revealed that the majority of the PBMCs were T cells (mean CD2, 77%; mean CD3, 71%) and that the majority of T cells were CD4+ (mean, 52%), with a slight elevation of the CD4/CD8 ratio (range, 1.8 to 5.2; mean, 3.5). High percentages of DR* and DP* cells (means, 33 and 32%, respectively) were detected in most of the patients, indicating the presence of in vivo activated T cells. This percentage

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<table>
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<th>Patient</th>
<th>Age (yr)</th>
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<th>Duration of illness (yr)</th>
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<td>3</td>
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<td>M</td>
<td>Martinique</td>
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<td>39</td>
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<td>Martinique</td>
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These six patients were referred to the Salpetrière Hospital, Paris, France. TSP/HAM was diagnosed according to previously described clinical and biological criteria (17).

F, Female; M, male.

Number of years between onset of clinical features and date of blood sample.

2, Able to walk with help; 3, bedridden.

Upon EcoRI cleavage, DNA extracted from fresh PBMCs of the six patients with TSP/HAM revealed the presence of a smear of DNA that had hybridized with the pMT2 probe (4), indicating a polyclonal integration of HTLV-I (data not shown). After PstI digestion, a distinct band of 2.3 kb was clearly identified in all of the patients’ DNAs, as well as in the chronically infected FCL62 and 8166/45 cell lines (1, 37), when these DNAs were hybridized with the corresponding HTLV-I envelope fragment of 2.3 kb (Fig.2A). These data demonstrated that all of the patients in the study carried polyclonally integrated HTLV-I sequences in their PBMC DNA.

The total cellular RNAs of the chronically HTLV-I-infected cell lines 8166/45, C91/PL, and FCL62 and the TSP/HAM PBMCs werehybridized with the genomic HTLV-I probe pMT2. Several viral transcripts were easily detectable...
in the three HTLV-I-infected cell lines (Fig. 2B). The cellular RNA of the infected nonproducer cell line 8166/45 contained several sizes of transcripts ranging between 2 and 2.5 kb (probably the tax-rex mRNA); in the C91/PL cell line we detected two 8.5- and 7.0-kb genomic RNAs, known to be present in this cell line, that correspond to a complete and a deleted provirus, respectively, and 2.7- and 2.0-kb tax-rex mRNAs, as previously described (1). The RNAs of the five patients with TSP/HAM analyzed yielded no detectable hybridization to the pMT2 genomic HTLV-I probe, nor did the negative control RNA obtained from the uninfected HUT 78 human neoplastic T-cell line. To rule out the possibility that the lack of detection of specific viral RNAs was due to RNA degradation, the same filters were stripped and rehybridized with an HLA class I gene probe (pDPO-1). The expected 1.6-kb HLA mRNA was readily detected in all five TSP/HAM RNAs (Fig. 2C).

RT PCR on viral mRNAs was carried out as described previously (24, 25) with some modifications using the primers RPX3 (5'-TTTGGAGATCCATCCGTGGAAGAGCCCT CAA-3') and RPX4 (5'-ACTTAAATTCACACGTA GACTGGGTACH-3'), which contained EcoRI and BamHI sites, respectively, at the 5' end. These primers were derived from the HTLV-I sequence by Seiki et al. (38) at positions 5096 to 5115 and 7357 to 7338, respectively. The PCR was carried out in a DNA thermal cycler (Perkin Elmer Cetus) for 30 cycles. Each reaction cycle consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min. After the reaction, 15 μl of each sample was electrophoresed in a 1.5% agarose gel and transferred to a nitrocellulose sheet. The filters were hybridized at 37°C with a 32P-end-labeled oligonucleotide probe, RPX-PR1 (5'-AACACCATGCCCCCTCCC-3' [25]), encompassing the splice junction for tax-rex mRNA or a nick-translated HTLV-I genomic probe (pMT2) for 16 h. Prehybridization and hybridization were carried out as previously described (26). The filters hybridized with the labeled oligonucleotides were washed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C for 15 min and at 45°C for 15 min, whereas when the HTLV-I genomic probe was employed, more stringent washing conditions were used (0.5× SSC and 0.5% sodium dodecyl sulfate twice for 5 min each time at room temperature and once for 30 min at 65°C). The filters were then exposed to Kodak XAR film for 2 to 48 h at −80°C.

DNA fragments of 169 bp were detected in all six TSP/HAM samples (upper part of Fig. 3). When the RT was omitted before the PCR, no amplified DNA was detected, indicating that the PCR product was derived from an RNA template rather than a DNA template (data not shown). Specific hybridization of the DNA fragments was detected with both the HTLV-I genomic probe (pMT2) and the oligonucleotide probe (RPX-PR1) encompassing the splice junction site between the second and third exon of the HTLV-I tax-rex mRNA (lower part of Fig. 3). The HTLV-I probe did not yield any other DNA fragments (lower part of Fig. 3), suggesting that this might be the main splice junction used in vivo to generate the tax-rex mRNA in the TSP/HAM samples studied. A similarly sized band specifically hybridizing with the HTLV-I probes was found in the samples from cell lines chronically infected with HTLV-I (Fig. 3). An additional smaller band was detected in the C91/PL cell line by ethidium bromide staining, but it failed to hybridize specifically with the HTLV-I probes. The intensity of the hybridization was comparable in four of the six TSP/HAM samples, while in two cases, the amounts of tax-rex mRNA amplified transcripts were smaller (Fig. 3). The highest intensity of hybridization was detected in the infected cell lines; this result is compatible with a higher level of viral mRNA expression in these chronically infected cell lines. To
FIG. 4. In situ hybridization of TSP/HAM PBMCs and HTLV-I-infected cell lines. LAF and BAR stand for TSP/HAM patient PBMCs (patients 1 and 4, respectively, of Table 1); HUT 102 is a chronically HTLV-I-infected cell line (32). The HIV-1 probe corresponded to all HIV-1 genomic DNAs.
characterize the cDNA products, we cloned some of them and compared their nucleotide sequences. In the three TSP/HAM cases studied, the nucleotide sequence was indistinguishable from the sequences of the HTLV-I ATK clone (38) and the viral isolates from the C91/PL and 8166/45 cell lines, indicating a high degree of conservation of the DNA nucleotide sequence of this particular region of the HTLV-I genome. The splice junction (positions 5183 to 7302) in the tax-rex transcript, which was previously detected in HTLV-I-infected cell lines (1, 39), is used in the generation of the tax-rex mRNA in the three TSP/HAM cases that we studied. Thus, the same acceptor and donor splice sites are used in vivo to generate a mature mRNA for the Tax-Rex proteins.

In situ hybridization was performed as previously described (18) on the fresh PBMCs of two patients with TSP/HAM. One patient (patient 1) had a clinical manifestation of disease for 7 years, while the other (patient 4) had a clinical manifestation for only 2 years when the samples were obtained. Strongly positive cells were readily detected among the PBMCs of both patients studied when the HTLV-I pMT2 probe was used (Fig. 4, upper panel). We analyzed 6 × 10^4 cells in each case and found 14 and 11 positive cells, respectively. This corresponded to a frequency of approximately one positive cell for every 5 × 10^3 PBMCs. When the same cells were reacted with a human immunodeficiency virus type 1 (HIV-1) probe, no positive signal was detected, either with the patient’s cells or with the chronically HTLV-I-infected cell line HUT 102, which, as expected, showed high positivity when an HTLV-I pMT2 probe was used. PBMCs from healthy uninfected donors were also negative for both HTLV-I and HIV-1 sequences (data not shown).

A considerable amount of information on the regulation of HTLV-I gene expression has been gathered. Studies of the other group of human retroviruses, HIV, have also greatly stimulated interest in possible pathways of regulation common to HTLVs and HIVs. Both viruses have a transactivator protein (Tax and Tat, respectively) which exerts its effect both on the viral long terminal repeat (9) and on cellular genes (the latter is more frequently the case for HTLV-I than for HIV-1). The HTLV-I Tax protein induces the expression in infected cells of interleukin 2 (27, 40) and interleukin 2Ra (2, 5, 21), which help to trigger cell proliferation. This may be a predisposing condition to the development of ATL. The HIV-1 Tat protein turns on viral expression intracellularly and is released into the medium of infected cells (8). Recombinant Tat protein acts as a stimulator of cell proliferation in vitro (8), and similarly, the purified Tax protein induces growth of human PBMCs in culture (3a). Such findings indicate that the effect of viral proteins at a distance is a potential novel mechanism of viral pathogenesis that needs to be considered.

The pathogenesis of TSP/HAM is not currently understood, but HTLV-I infection appears to be a prerequisite. In fact, in TSP/HAM the percentage of PBMCs polyclonally infected by HTLV-I proviral DNA is much higher than that in PBMCs of healthy carriers (15, 45), as are the titer of anti-HTLV-I antibodies in the serum and cerebrospinal fluid (7, 14) and the percentage of activated T cells and ATL-like cells (7, 15, 22, 28). Release of a viral product(s) from these activated T lymphocytes, also found in the cerebrospinal fluid of patients with TSP/HAM (15, 20, 28) and in infected endothelial cells, might cause the pathological lesions in TSP/HAM either directly in the microenvironment of these infected cells or at a distance. A possible role of the Tax protein in the nervous system is further indicated by the facts that HTLV-I tax-transgenic mice develop neurofibromas (30) and that the HTLV-I Tax protein can activate in trans the nerve growth factor promoter (17a).

In this study, using the sensitive techniques of RT PCR and in situ hybridization, we have demonstrated HTLV-I expression in vivo in patients with TSP/HAM regardless of the clinical severity and the duration of the illness. Maintenance of viral expression in vivo, especially of tax-rex regulatory genes, might play an important role in the slowly progressive clinical course of TSP/HAM.

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REFERENCES
4. Brady, L. Personal communication.


17a. Green, J. Personal communication.


