Human T-cell lymphotropic virus type I (HTLV-I) proviral integration status was examined by Southern blot analysis in peripheral blood mononuclear cell (PBMC) DNA from patients presenting a tropical spastic paraparesis (TSP) and serological evidence of HTLV-I infection. Surface phenotype and morphological aspects of PBMC were also studied. A polyclonal HTLV-I proviral integration was found in the PBMC of the 10 patients studied irrespective of their geographical origin (French West Indies, French Guiana, and Africa), the duration of their clinical illness, or the HTLV-I antibody titer. Furthermore, by dilution experiments and hypothesizing that only one copy of HTLV-I proviral DNA is present in one cell, we estimated that this HTLV-I integration is present in 3% to 15% of their PBMC.

All 10 TSP/HTLV-I patients studied had an average of 10% of their lymphocytes abnormal, presenting either a misshapen nucleus or an adult T-cell leukemia/lymphoma (ATL)-like feature. Moreover, an elevated CD4/CD8 ratio associated with the presence of activated T cells with a high level of DR expression was observed in most patients. The significant frequency of viral-positive PBMC and the important load of HTLV-I proviral DNA that we observed in TSP/HTLV-I patients might play an important role in the pathogenesis of this recently identified clinico-virological entity.

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MATERIALS AND METHODS

Patients and controls. Three groups of patients were studied (Table 1). The first group comprised 10 patients (cases 1 to 10) who were diagnosed at Pitie-Salpetriere Hospital in Paris as suffering from TSP/HTLV-I based on the following clinical and biological criteria: (1) spastic paraparesis or paraplegia with spastic disturbance and minimal sensory loss; (2) chronic and slowly progressive course with neither remission nor attack; (3) no personal or familial neurological antecedent; (4) nonspecific abnormalities according to myography or spinal imaging by computed tomography or nuclear magnetic resonance; and (5) presence of HTLV-I-specific antibodies in serum and CSF.

The second group comprised three patients who were diagnosed as having acute ATL based on clinical, cytologic, and biological criteria (cases 11, 12, 13).

The third and control group included five healthy HTLV-I sero-negative and three HTLV-I sero-positive women, one healthy and two with a smoldering ATL with specific cutaneous lesions and...
with a very low number (less than 1%) of detectable leukemic cells in the peripheral blood.

Three tests were used to detect HTLV-I antibodies: (1) enzyme-linked immunosorbent assay (ELISA; Dupont, Wilmington, DE); (2) indirect immunofluorescence assay (IFA) using the HUT 102 cell line as source of HTLV-I-antigen; and (3) particle agglutination linked immunosorbent assay (ELISA, Dupont, Wilmington, DE) of the peripheral blood.

Genomes per human cell ranging from 2:1 to 1:64. Assuming that with a very low number (less than 1%) of detectable leukemic cells in the peripheral blood, we used serial twofold dilutions of the test serum by ELISA and PA, we used serial twofold dilutions of the test serum or CSF. For Western blot (WB) confirmation, we used the Dupont subcloned BamHI-BamHI fragment of the pATK-1 clone, consisting of a ClaI-ClaI fragment of 1.4 kb and a PstI-PstI fragment of 1.4 kb corresponding to the third exon of human c myc.

**Probes.** Two different HTLV-I probes (kindly provided by M. Yoshida, Cancer Institute, Tokyo, Japan) were used: the pATK-06 subcloned BamHI-BamHI fragment of the pATK-1 clone, consisting of a part of the env region; and the pLTR-111 HindIII-Xho fragment of the pATK-1 clone, consisting of a part of the 3'LTR. The c myc probe (kindly provided by D. Stehelin, Lille, France) was a ClaI-ClaI fragment of 1.4 kb corresponding to the third exon of human c myc.

**Limits of sensitivity and quantification.** Aliquots of 9 μg of uninfected human DNA were mixed with various amounts of HTLV-I viral DNA from an ATL patient (case 13) in ratio of viral genomes per human cell ranging from 2:1 to 1:64. Assuming that 150 x 10^6 cells represent 1 μg of DNA, this dilution corresponds to a range from 2.7 x 10^6 to 2 x 10^5 viral copies per sample of 9 μg. To quantify the HTLV-I DNA viral copies in the TSP/HTLV-I samples, the mixed DNA were treated as the DNA of TSP/HTLV-I and put on the same blot.

**Study of PBMC surface markers.** The surface phenotype of the cells was determined by a standard method of indirect immunofluorescence and cytofluorograph reading (model epic Profile, Coulter, Hialeah, FL) using a large panel of monoclonal antibodies (MoAbs) that recognize T-associated or T-restricted antigens (CD2, CD3, CD4, CD7, CD8) and B-restricted antigens (CD19, CD20). Other MoAbs were also used: CD25 (kindly provided by T. Waldman, Metabolism Branch, NCI, NIH, Bethesda, MD), CD33, CD41, anti-major histocompatibility complex (MHC) class II molecule DR, and WT31 (an antibody that recognizes a monomorphic part of the CD3 Ti αβ-heterodimer). In all cases, the background fluorescence determined using irrelevant isotype mouse antibodies was lower than 5%.

**Morphological studies.** Cell morphology was analyzed by light microscopy on May-Grünewald-Giemsa-stained PBMC. A total of 200 lymphocytes were assessed on each preparation and classified into four groups: normal lymphocytes, lymphocytes with an abnormally shaped nucleus (lobulated or grooved), typical ATL cells with 10 to 8 days.

**Integrity of HTLV-I provirus in PBMC was analyzed by Southern blotting using an HTLV-I env probe. As six PstI restriction endonuclease sites are located inside the provirus (Fig 1), PstI digestion can be used to detect either polyclonal or monoclonal HTLV-I integration. In all the TSP/HTLV-I and the ATL cases, a clear band of the expected 2.3 kb size was observed (Fig 1). To assess the sensitivity of the Southern method, dilutions of ATL DNA in an HTLV-I-free DNA were performed and digested in parallel with TSP/HTLV-I DNA and included in the same gel. Hypothesizing that only one copy of HTLV-I proviral DNA is integrated in a cell, we can estimate that the number of
HTLV-I genes present in 9 μg of TSP/HTLV-I PBMC DNA ranges from 4 x 10^4 to 20 x 10^4. This corresponds to 3% to 15% of HTLV-I infected cells in all the PBMC. The detection of HTLV-I genes in TSP/HTLV-I samples was not due to an overload of the corresponding lanes, as shown by hybridization with a human exon 3 c myc gene (Fig 1).

To determine if the mode of the integration of the HTLV-I provirus was clonal or polyclonal, EcoRI digests were analyzed. As no EcoRI sites are present inside the proviral genome, only clonal integration should be detected using this endonuclease (Fig 1, ATL cases 1 and 2). In TSP/HTLV-I DNA, no band was observed on the blot showing that proviral integration was polyclonal. This was confirmed using an LTR probe on PstI-digested DNAs (data not shown).

It is important to note that this random integration of the provirus in the PBMC DNA was found in all our patients, regardless of their geographical origin (French West Indies, French Guiana, or Africa), the duration of their clinical illnesses (mean = 6 years after onset; range, 1 to 12 years), or the titer of HTLV-I antibodies as determined by ELISA or PA tests (Table 1).

Phenotypic analysis of the PBMC from nine of the TSP/HTLV-I patients is shown in Table 2. As deduced from the results obtained with anti-CD2 and anti-CD3 MoAbs, the percentage of T cells ranged from 65% to 80% of

**Table 2. TSP Associated With HTLV-I: Cytological Analysis and Surface Phenotype Markers of PBMC Using a Panel of MoAbs**

<table>
<thead>
<tr>
<th>Case</th>
<th>WBC (10^9/L)</th>
<th>Lympho (%)</th>
<th>% Abnormal Lymphoid Cells</th>
<th>% Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Irregular Nuclei</td>
<td>ATL-Like Cells</td>
</tr>
<tr>
<td>1</td>
<td>9.3</td>
<td>29</td>
<td>10 3</td>
<td>18 13 8 0</td>
</tr>
<tr>
<td>2</td>
<td>6.9</td>
<td>30</td>
<td>7 2</td>
<td>29 5 2 0</td>
</tr>
<tr>
<td>3</td>
<td>4.9</td>
<td>37</td>
<td>9 2</td>
<td>29 13 7 0</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>57</td>
<td>2 0</td>
<td>28 8 5 0</td>
</tr>
<tr>
<td>6</td>
<td>7.2</td>
<td>26</td>
<td>5 1</td>
<td>20 16 9 2</td>
</tr>
<tr>
<td>7</td>
<td>27.5</td>
<td>41</td>
<td>16 2</td>
<td>37 1 0 0</td>
</tr>
<tr>
<td>8</td>
<td>6.1</td>
<td>41</td>
<td>10 1</td>
<td>32 21 12 5</td>
</tr>
<tr>
<td>9</td>
<td>5.7</td>
<td>33</td>
<td>5 1</td>
<td>17 1 3 0</td>
</tr>
<tr>
<td>10</td>
<td>6.8</td>
<td>28</td>
<td>9 3</td>
<td>38 4 2 1</td>
</tr>
</tbody>
</table>

Abbreviations: WBC, white blood cell count; Lympho, lymphocytes.
mononuclear cells. Almost all CD3 positive cells were also reactive with the WT31 MoAb, indicating that these cells were mature CD3 TCRαβ expressing cells. The majority of T cells were CD4+ and CD4/CD8 ratios were elevated in all but one patient (case 10). The percentage of CD20+ cells (ie, B cells) was below 12%, and of CD33+ cells (representative of the monocyte population) was below 5%. A major finding was the high percentage of DR+ cells, ranging from 17% to 38%. This percentage largely exceeds those of B cells and monocytes in all patients, indicating that DR-expressing T cells are present in these samples. CD25 positive cells were detected in seven of nine patients, but the percentage was low (maximum 12%; range, 1 to 12).

Concerning the cytological analysis (Table 2), lymphoid cells with an abnormally shaped nucleus were detected in 5% to 15% (median, 9%) of the total lymphoid cells in all the patients. Furthermore, typical ATL-like cells with hyperconvoluted nuclei (flower cells) were seen in all but one case (case 4), representing 2% of the total lymphocytes. Neither lymphoid cells with abnormal nuclei nor typical ATL cells were seen in the HTLV-I negative controls. Large hyperbasophilic cells (1% to 3%) were seen in the TSP patients and controls.

**DISCUSSION**

Using a Southern blot analysis, we show here that a polyclonal integration of HTLV-I proviral DNA can be detected in PBMC of patients with TSP/HTLV-I, regardless of their geographical origin, the duration of their clinical illnesses, or their HTLV-I antibody titers. Moreover, we estimated by dilution experiments that this HTLV-I polyclonal integration should be present in 3% to 15% of their PBMC.

In a recent work, Bhagavati et al have failed to detect by Southern blot such HTLV-I proviral DNA in PBMC of 11 patients with TSP/HTLV-I living in the United States, but were able to detect HTLV-I sequences by in vitro gene amplification. The conclusion of their study was that only a small proviral DNA load exists in TSP patients. The discrepancy between these observations and our results could be due to a difference in the Southern blot technique used for HTLV-I detection. In our study by dilution experiments, we were able to detect 4 x 10^4 viral copies in 9 μg DNA (3% of HTLV-I positive cells). A difference in the HTLV-I probe used may also be involved. Finally, differences in geographical origin, duration of illness, or other genetic, environmental, or therapeutic factors may have modified the number of cells infected by HTLV-I in Bhagavati et al's patient population.

Our molecular results on TSP/HTLV-I are comparable to those briefly reported by Yoshida et al who found a polyclonal integration of HTLV-I proviral DNA in peripheral blood lymphocytes of eight of nine Japanese patients with HAM. These data and ours are consistent with the recent proposition that TSP/HTLV-I and HAM are the same clinical entity. Thus, our results from 10 TSP/HTLV-I cases originating from different geographical areas extends this similarity to the intracellular status of the provirus.

In Japanese or Caribbean HTLV-I sero-positive carriers, a small percentage (0.1% to 2%) of lymphoid cells with abnormal convoluted nuclei are found in their peripheral blood films. Most of these cells have been shown to be CD25+. However, it is important to note that in these healthy HTLV-I sero-positive subjects, viral integration is generally not detectable by Southern blot technique, probably due to the very low number of infected cells. All our 10 TSP/HTLV-I patients were found to have a mean value of 10% of abnormal lymphocytes. Most patients show a significant number of circulating DR-expressing T cells. It is worthwhile to note that some of these presumably activated T cells are CD25+. Similar features have recently been found in Japanese HAM patients.

The nature of the cells infected by HTLV-I in our patients was not definitively assessed in this study. Although monocyte infection by the virus is not excluded, the concordance between the total number of abnormal lymphoid cells (range, 2% to 18%; mean, 10%) and the detection of HTLV-I viral copies in 3% to 15% of the patients' studied PBMC strengthens the hypothesis that the majority of the morphologically abnormal lymphoid cells of TSP/HAM may carry the HTLV-I proviral DNA randomly integrated in their DNA, and that these cells belong to DR-expressing T cell population. This hypothesis is also sustained by the fact that HTLV-I antigens detected by immunofluorescence in cultures derived from TSP/HTLV-I PBMC appear rapidly. On the contrary, cultures derived from PBMC of healthy sero-positive carriers become positive less frequently and much later. It is unknown whether the infected cells are T4+ lymphocytes, as suggested by the slight excess of T4+ lymphocytes (Table 2). The situation may be more complex. Recently, we have observed by double labeling that in two of our patients (cases 7 and 10) about half of the DR+ T cells were T8+, and half T4+ (A. Gessain, F. Siguax, unpublished data, May 1989). It is possible that these T8+ cells were induced to express DR by HTLV-I infection. Alternatively, these T8 activated cells may represent cytotoxic T lymphocytes. Whatever the explanation, the results obtained in our study are of interest with respect to the relationship between TSP/HAM and the other polyclonal states induced by HTLV-I.

A polyclonal integration of HTLV-I proviral DNA associated with few abnormal cells and a small increase of CD25+ cells have recently been described by Yamaguchi et al as an intermediate state between healthy carriers and smoldering ATL in HTLV-I sero-positive Japanese subjects. This blood picture is quite similar to that observed in our patients, although the clinical features are clearly distinct. Nearly all the Japanese patients had minor disease, such as Strongyloides stercoralis infection, but no neurological signs, while TSP/HTLV-I patients suffer from a chronic progressive myelopathy. Furthermore, Yamaguchi suggests that patients with intermediate state disease might be at risk to develop ATL. In this context, it is important to point out that none of our patients have developed ATL despite a mean follow-up of 6 years, and that, in general, the concomitant occurrence of ATL and TSP/HAM is an exceptional event.
Such a large proviral DNA load seems a necessary stage in the evolution to an HTLV-I–associated disease (either ATL or TSP/HAM) in an HTLV-I–infected individual. To date, we do not know if the incidences of ATL and TSP/HAM are similar in patients with such intermediate states. The mechanisms by which HTLV-I can induce neurological disorders are unknown, but this high proviral load may increase the probability of the molecular events that lead to emergence of different viral strains, some of them neurotropic. Furthermore, the high number of circulating HTLV-I–infected cells seen in TSP/HAM patients may allow HTLV-I to reach certain central nervous system sites more easily, leading directly or indirectly to neurological lesions.

NOTE ADDED IN PROOF

Since submission of this paper, similar results concerning the polyclonal integration of HTLV-1 proviral DNA in the PBMC of seven patients with TSP have been reported (Greenberg SJ, Jacobson S, Waldman TA, McFarlin DE: Molecular analysis of HTLV-1 proviral integration and T-cell receptor arrangement indicates that T-cells in tropical spastic paraparesis are polyclonal. J Infect Dis 159:741, 1989.

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