Common Human T Cell Leukemia Virus Type 1 (HTLV-1) Integration Sites in Cerebrospinal Fluid and Blood Lymphocytes of Patients with HTLV-1–Associated Myelopathy/Tropical Spastic Paraparesis Indicate that HTLV-1 Crosses the Blood-Brain Barrier via Clonal HTLV-1–Infected Cells

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In the spinal cord of patients with human T cell leukemia virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP), infiltrating CD4+ lymphocytes seem to be the major reservoir for the virus. Little, however, is known about the mechanisms by which HTLV-1 crosses the blood-brain barrier. An oligoclonal proliferation of HTLV-1–infected CD4 lymphoid T cells is present in the peripheral blood of all HTLV-1–infected individuals. Here, such oligoclonal distribution of HTLV-1–infected cells is evidenced in the cerebrospinal fluid (CSF) derived from 5 patients with HAM/TSP. Furthermore, clonal populations of HTLV-1–infected lymphocytes sharing the same HTLV-1 proviral flanking sequences (i.e., integration sites in the cellular DNA), and thus derived from a single HTLV-1–infected progenitor, were found, for a given patient, in both the CSF and the peripheral blood. These data demonstrate that HTLV-1 crosses the blood-brain barrier by migration of HTLV-1–infected lymphocytes in vivo.

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The 5 patients in this study gave their informed consent before the collection of blood and cerebrospinal fluid samples.

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Human T cell leukemia virus type 1 (HTLV-1) causes adult T cell leukemia/lymphoma (ATLL) [1, 2; reviewed in 3], a CD4 malignant lymphoproliferation, and HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic neuromyelopathy [4, 5]. In addition, HTLV-1 infection is associated, to a lesser extend, with the development of a variety of inflammatory diseases, including uveitis [6], arthritis [7], polymyositis [8], Gougerot-Sjögren syndrome [9], alveolitis [10], and infective dermatitis [11]. Dissemination of the virus in numerous body compartments is the hallmark of HTLV-1–associated diseases. Indeed, HTLV-1 sequences or gene products can be detected in the aqueous humor [12], the cerebrospinal fluid (CSF) [13], the synovial fluid [14], and the alveolar liquid [15] of patients with uveitis, HAM/TSP, arthropathy, and alveolitis, respectively.

ATLL is usually monoclonal for HTLV-1 integration, although some patients may harbor 2–3 proviruses [16]. As in other lymphoid malignancies, malignant ATLL cells frequently infiltrate skin, bone, bone marrow, and other organs in vivo [17, 18]. This results from the cellular transport of the integrated provirus to the corresponding sites, as recently confirmed by molecular studies [19].

In contrast to ATLL, HAM/TSP is characterized by a polyclonal proliferation of HTLV-1–bearing lymphoid T cells [20, 21]. This reconciles the high proviral loads seen in the peripheral blood from HAM/TSP patients [22, 23] with an exceptionally low degree of genetic variability [24, 25]. The main pathological feature of the disease is a chronic inflammatory process that predominates in the lateral and posterior columns of the spinal cord in the thoracic region [reviewed in 26]. Tax protein, which is coded by the 3’ region of the provirus, is the main regulatory protein of the virus [reviewed in 27]. Tax mRNA was previously detected in a few glial cells of the spinal cord, whereas infiltrating CD4 mononuclear cells appear to be the major reservoir sites of the provirus within the central nervous system (CNS) [28–31]. In addition, Tax protein can be detected in CSF cells from patients with HAM/TSP [32]. Because sheep choroid plexus cells can be productively infected with HTLV-1 via in vitro cell-to-cell contact [33], it was recently proposed that the
virus may invade the CNS by infecting choroid plexus cells. Such a route of CNS infection had previously been shown for human immunodeficiency virus [34], Moloney murine leukemia virus [35], and visna virus in sheep [34].

Traffic of malignant proliferating lymphoid cells across the blood-brain barrier (BBB) is frequently observed in ATLL [36]. The present study was conducted to determine whether such a mechanism could be identified in HAM/TSP. Lymphoid cells sharing the same HTLV-1 proviral integration sites, as determined by a sensitive polymerase chain reaction (PCR) method, were found in both the peripheral blood and the CSF of patients with HAM/TSP. This shows, for the first time, that there is a cellular-associated transport of HTLV-1 through the BBB in this virus-mediated neurological disorder.

### Materials and Methods

**Samples studied.** CSF mononuclear cells and peripheral blood mononuclear cells (PBMC) were collected from 5 patients with HAM/TSP. Pertinent clinical data are given in table 1. To avoid CSF contamination by PBMC, samples were selected on the basis of atraumatic lumbar puncture, which harvested <1 red blood cell/mm³. PBMC and CSF cells were obtained on the same day from the 5 patients.

### Amplification of HTLV-1 integration sites.

Mononuclear cells from 1–3 mL of CSF were diluted in 5 × 10⁷ cells from the Jurkat cell line. DNA was phenol/chloroform extracted and ethanol precipitated. Ligation-mediated PCR (LMPCR) experiments were performed, as described elsewhere [21, 25, 38, 39]. In brief, DNA was digested with NlaIII restriction enzyme (New England Biolabs, Montigny-le-Bretonneux, France) in 10⁻³ NlaIII buffer for 3 h at 37°C. Digested DNA was ligated with 10 pmol of a synthetic oligonucleotide (BIO1) [25] with 20 U of T4 DNA ligase (New England Biolabs). This was followed by a phenol/chloroform extraction and precipitation. Ligated DNA was submitted to 100 cycles of linear extension by use of the HTLV-1–specific BIO2 primer alone [25]. Ten microliters of this linear PCR reaction was used in a standard PCR amplification, using primers complementary to the HTLV-1 3' end (BIO3, position 8898–8921) [25] and to the BIO1 cassette (BIO4) [25]. Sequences of these oligonucleotides have al-

### Table 1. Clinical status of the 5 patients with human T cell leukemia virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis at the time of sample collection.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)/sex</td>
<td>38/F</td>
<td>61/M</td>
<td>44/F</td>
<td>50/F</td>
<td>45/F</td>
</tr>
<tr>
<td>Geographic origin</td>
<td>Chad</td>
<td>France</td>
<td>Martinique</td>
<td>Martinique</td>
<td>Martinique</td>
</tr>
<tr>
<td>Time elapsed (years) from diagnosis to study</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>EDSS status at time of sample collection</td>
<td>6.5</td>
<td>6</td>
<td>4.5</td>
<td>4.5</td>
<td>1</td>
</tr>
<tr>
<td>CSF antibody titer</td>
<td>1/512</td>
<td>1/4</td>
<td>1/256</td>
<td>1/64</td>
<td>1/4</td>
</tr>
<tr>
<td>Blood antibody titer</td>
<td>1/10,240</td>
<td>1/640</td>
<td>1/10,240</td>
<td>1/64</td>
<td>1/1280</td>
</tr>
<tr>
<td>CSF mononuclear cells per μL</td>
<td>9</td>
<td>4</td>
<td>18</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE.** Neurologic impairment was evaluated by using the Expanded Disability Status Scale (EDSS) [37]. HTLV-1–specific antibody titers were determined by using an immunofluorescence assay on MT2 HTLV-1–infected cells. F, female; M, male; CSF, cerebrospinal fluid.

**Figure 1.** Position of oligonucleotides along the flanking sequences. The last 6 bases of the long terminal repeat (LTR) are represented, together with that of the hexameric repeat derived from each flanking sequence of the human T cell leukemia virus type 1 (HTLV-1)–integrated provirus. Position 0 corresponds to that of the first base of the BIO3 HTLV-1–specific oligonucleotide. P1–P5 indicate patients 1–5, respectively.
ready been published [25]. Amplified products were analyzed by making a runoff, which consists of 8–10 additional cycles of linear PCR amplification, using a 3’ HTLV-1–specific oligonucleotide, BIO5 (position 8995–9019) [25]. DNA extraction, NlaIII diges-
tions, ligations, and first-round PCR were carried out in laboratories that had never handled an HTLV-1 plasmid. Second-round and radiolabeled amplifications were performed in yet another laboratory. Water and DNA from the Jurkat cell line were used as negative controls in all PCR experiments. No contamination was detected. The DNA of the MT4 HTLV-1–infected cell line and the p4.39 HTLV-1 plasmid, which were derived from the HTLV-1 cell line 2060 [40], served as positive controls in quadruplicate experiments.

Cloning and sequencing. LMPCR products >150 bp were first purified on a low-melting-point agarose gel and then blunt-end cloned into M13mp18 (New England Biolabs), as described elsewhere [25, 39]. Clones >150 bp were selected to avoid the selection of the “TG” clone, which results from the integration of virus 5’ to any TG dinucleotide [25, 39]. Moreover, such long flanking sequences were more suitable for the selection of integration site–specific oligonucleotides needed for subsequent PCR experi-
ments. Clones were screened in situ with a 32P-labeled long terminal repeat (LTR)–specific oligonucleotide probe [25, 39]. Hybridization was carried out in 5’ standard saline citrate (SSC) at 42°C, and filters were washed in 2’ SSC, 0.1% SDS, at 42°C (room temperature). Single-stranded templates were sequenced by using a Se-
quenase 2.1 kit (Amersham/Pharmacia Biotech, Orsay, France).

**Figure 2.** Integration site–specific oligonucleotides used for the detection of circulating clonal populations of human T cell leukemia virus type 1 (HTLV-1)–infected cells. These oligonucleotides are complementary to the HTLV-1 flanking sequences of isolated cerebrospinal fluid mononuclear cells (see Materials and Methods). P1–P5 indicate patients 1–5, respectively. In the column labeled “Primers,” the number of the corresponding molecular clone identifies oligonucleotides: e, external primer; i, internal primer. “Positions” are related to the first base of the HTLV-1 proviral flanking sequence.
Results

Clonal expansion of HTLV-1–infected CSF lymphocytes in vivo. The presence of oligoclonal populations of HTLV-1–infected PBMC was first checked through LMPCR amplification of HTLV-1 proviral integration sites in the cellular DNA of infected cells. Clonal expansion, with a range of 24–42 clones, was evidenced in all 5 patients (not shown). To assess the mode of viral spread in the CSF in patients with HAM/TSP, the integration pattern of HTLV-1–bearing lymphocytes was investigated in the CSF of the same 5 patients, and the DNA from CSF mononuclear cells was also analyzed by LMPCR. Typical LMPCR analyses are shown in figure 3. After runoff analysis of amplified products, 2 (patient 3) to 9 (patient 1) bands were obtained. Because LMPCR has a detection threshold of 20 copies [41], these findings indicate extensive clonal expansion of HTLV-1–bearing cells within the CSF.

Common HTLV-1 integration sites in the CSF and blood lymphocytes of patients with HAM/TSP. Because there is no preferential target site for HTLV-1 integration in vivo, the characterization of an HTLV-1 integration site can be used as the specific and unique signature of a given clone of HTLV-1–infected cells. Indeed, the molecular characterization of specific HTLV-1 integration sites shared by both blood and CSF lymphocytes would clearly demonstrate viral trafficking between the two, such as an HTLV-1 provirus. Such clonal populations of HTLV-1–infected cells were, therefore, sought through the amplification of proviral flanking sequences in PBMC and CSF samples derived from each of the 5 patients with HAM/TSP. Eighty-eight CSF flanking sequences corresponding to 24 distinct clones were obtained from the 5 patients. No integration site–specific primer matching LTR-specific oligonucleotides could be found for 9 sequences. The remaining 15 were suitable for clonotypic nested PCR amplification, as detailed in Ma-

Table 2. Distribution of the CSF clonal populations of human T cell leukemia virus type 1 (HTLV-1)–infected cells in the peripheral blood mononuclear cells (PBMC) of the 5 patients with HTLV-1–associated myelopathy/tropical spastic paraparesis.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CSF clone^d</th>
<th>LMPCR results^d (frequency of detection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C277</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>C285</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>C286</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>C298</td>
<td>1/4</td>
</tr>
<tr>
<td>2</td>
<td>C317</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>C320</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>C322</td>
<td>0/4</td>
</tr>
<tr>
<td>3</td>
<td>C307</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>C362</td>
<td>0/4</td>
</tr>
<tr>
<td>4</td>
<td>C1</td>
<td>+^c</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>0/4</td>
</tr>
<tr>
<td>5</td>
<td>C49</td>
<td>+^c</td>
</tr>
<tr>
<td></td>
<td>C61</td>
<td>+^c</td>
</tr>
</tbody>
</table>

NOTE. CSF, cerebrospinal fluid; LMPCR, ligation-mediated polymerase chain reaction.

^a CSF-specific integration sites from which specific oligonucleotides were synthesized. Overall, 8 of these clones were detected in the corresponding PBMC.

^b Quadruplicate LMPCR result products from PBMC were analyzed with internal CSF integration site–specific primers. The frequency of detection of CSF clones in PBMC DNA ranged from 0/4 to 2/4, meaning that CSF clones do not correspond to major clones present in the PBMC of the same patient.

^c Positive signal for circulating CSF clones not analyzed by quadruplicate experiments.

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Figure 3. Linker-mediated polymerase chain reaction (PCR) analysis of human T cell leukemia virus type 1 (HTLV-1) integration sites in cerebrospinal fluid (CSF) samples. Mononuclear cells from 1–3 mL of CSF were diluted with 5 × 10^6 noninfected cells from the Jurkat cell line. DNA was subjected to ligation-mediated PCR (LMPCR). Lanes 1 and 8, Molecular weight marker (size in base pairs is represented on the right). Lane 7, Negative control (¬C^") corresponds to DNA from the Jurkat cell line. Lanes 2–6, Clonality pattern of the HTLV-1–infected cells in the CSF of the 5 patients (P1–P5) with HTLV-1–associated myelopathy/tropical spastic paraparesis. Each sample has been identified by a number below the gel. Because the detection threshold of the LMPCR is 20 copies [41], these findings indicate extensive clonal expansion of HTLV-1–bearing cells within the CSF.
Figure 4. Detection of human T cell leukemia virus type 1 (HTLV-1)-positive cerebrospinal fluid (CSF) clones in the peripheral blood mononuclear cells (PBMC) of 2 patients with HTLV-1–associated myelopathy/tropical spastic paraparesis. The DNA from PBMC was amplified as described in Materials and Methods. Left. Four sets of CSF HTLV-1 integration site–specific oligonucleotides were used to detect the corresponding clonal populations in the PBMC from patient 1. Each CSF clone is represented by its corresponding number at the bottom. Corresponding oligonucleotide sequences and predicted sizes are shown in figures 2 and 1, respectively. Lanes 1 and 14, Molecular weight marker (size in base pairs is represented on the left). The amplification of CSF lymphocyte DNA, which was used as a positive control, gave a positive signal for each clone. The amplification of the DNA from corresponding PBMC (PB) gave a positive signal with oligonucleotides derived from CSF clones C277 and C298, whereas primers corresponding to clones C285 and C286 failed to detect corresponding circulating forms. “C” corresponds to DNA from negative controls (Jurkat cells). Right. Two sets of CSF HTLV-1 integration site–specific oligonucleotides were used to detect the corresponding clonal populations in the PBMC from patient 3. Using the same strategy as for patient 1, 1 positive signal (C307) was obtained with the PBMC sample derived from patient 3.

Materials and Methods. Twenty-eight oligonucleotides corresponding to these 15 CSF integration sites were synthesized to amplify corresponding clonal populations of HTLV-1–infected lymphocytes in PBMC samples with an LTR-specific primer, as described in Materials and Methods (figures 1 and 2). The sensitivity of this clonotypic nested PCR assay was estimated by amplifying serial dilutions of 4 cloned proviral integration sites in Jurkat cell line DNA. The sensitivity threshold of the method was thus found to range from 1 to 10 copies, diluted in 1 μg of noninfected DNA. On the assumption that HTLV-1–infected PBMC contain 1 provirus per cell, the strategy allowed the detection of 1/15,000–1/150,000 infected PBMC (not shown). Typical results from clonotypic PCR experiments are shown in figure 4, and table 2 summarizes final findings. By use of this method, 2 of 3, 2 of 4, 1 of 2, 1 of 3, and 2 of 3 clonal populations of HTLV-1–infected cells present in the CSF could also be detected in the corresponding PBMC DNA samples of the 5 patients with TSP/HAM. Clonotypic PCR failed to detect the remaining 7 CSF clones in the periphery. Nested PCR products were cloned and sequenced as described above. Each positive signal was found to harbor the same sequence as the corresponding CSF integration site, which indicates that the HTLV-1–infected lymphocytes belonging to the same clonal population (and thus derived from a single infected progenitor) were present in both CSF and blood. This demonstrated the existence of a cellular transport of HTLV-1 through the BBB.

Semiquantitative analysis of circulating HTLV-1–infected CSF clones. Previous studies have shown a wide variation in the abundance of circulating HTLV-1–bearing clones between infected individuals [19, 21]. The number and the abundance of these clonal populations of circulating HTLV-1–infected cells are significantly higher in patients with HAM/TSP or ATLL than in asymptomatic carriers [19, 21]. In addition, considerable variations in both the number and the abundance of these clones have been observed between individuals belonging to a given clinical category [21, 41]. Now that it has been established that the virus may cross the BBB as a provirus, the frequencies of the common clones (i.e., those harboring identical integration sites in both compartments) were sought in the periphery. There is a stochastic element to the amplification of low-frequency HTLV-1 integration sites [38]. Quadruplicate LMPCR analysis of NlaIII-digested p4.39 DNA bearing an integrated HTLV-1 provirus showed that, at >500 copies, detection was 4 of 4. At 100, 50, and <10 copies, however, detection was 2 of 4, 1 of 4, and 0 of 4, respectively [38]. Accordingly, the DNA from PBMC derived from patients 1, 2, and 3 was first amplified using quadruplicate LMPCR. Amplified products were then subjected to 8 cycles of linear extension, with 53P-radiolabeled CSF/PBMC integration site–specific oligonucleotides used as primers. As described in detail in table 2, the frequency of detection of all CSF clones in the corresponding PBMC was ≤2 of 4 after quadruplicate experiments, which indicates that their frequencies were <1/1500 PBMC. Accordingly, their frequency was not higher than that of other circulating clones.
Discussion

Using a sensitive PCR-based method, we have demonstrated the presence of oligoclonal populations of HTLV-1-infected lymphoid cells in the CSF of all 5 of the 5 patients with HAM/TSP whom we studied. Furthermore, common HTLV-1 integration sites were found in both the CSF and blood lymphocytes of these patients, thus demonstrating that HTLV-1 crosses the BBB via clonal HTLV-1–infected cells. This was evidenced in the 5 patients studied, which suggests that this kind of viral transport might be the current norm for HAM/TSP. Whether it corresponds to traffic from blood to CSF or from CSF to blood, or from a combination of both, remains to be elucidated.

The failure to detect 7 of the 15 CSF clones in the corresponding PBMC may be due to the sensitivity of our method. The disappearance of these forms from the blood before collection appears to be improbable, because the persistent oligoclonal expansion of HTLV-1–infected cells over a number of years has been demonstrated in HAM/TSP [41, 42]. A third possibility may correspond to clones generated in the CSF that do not cross the BBB from the CSF to the blood. Because HTLV-1 can infect sheep choroid plexus in vitro [33], cell-free traffic across the BBB may finally coexist with cellular transport.

Many features of HTLV-1 infection are consistent with the idea that the virus can cross the BBB in a cell-associated form. First, it has been suggested that T cell activation may facilitate the transport across the BBB [43]. PBMC of patients with HAM/TSP are characterized by a high percentage of such activated large cells bearing activation markers [44]. In addition, the activation of HTLV-1–infected T cells has been found to be independent of B7 costimulation [45]. Second, the adherence of infected cells to the basement membrane of the endothelial wall, cytokine production results in a lowering of the BBB via clonal HTLV-1–infected cells. This was evidenced in patients with HAM/TSP [46]. This results from the increased expression of adhesion molecules and cytokines, such as interleukin-1, interferons, and tumor necrosis factor-α [47]. In addition to the adhesion of infected cells to the basement membrane of the endothelial wall, cytokine production results in a lowering of the BBB, which leads, in turn, to the penetration of infected cells. Third, CSF from patients with HAM/TSP contains high levels of matrix metalloproteinases [48], which may facilitate the disruption of the BBB via the degradation of the endothelial cell matrix [49].

The present study provides some evidence that HTLV-1 can cross the BBB by migration of infected lymphocytes in vivo. HTLV-1 infection in the CSF was found to be associated with the clonal expansion of HTLV-1–infected cells. As in the peripheral blood, the proliferation of infected cells within the CSF is presumably held in check by the intense anti–HTLV-1 cellular immunity present in the CSF of patients with HAM/TSP [50]. It seems unlikely that the sole presence of the virus in the CSF can trigger the inflammatory process in the spinal cord. In addition to the CSF clonal expansion of infected cells, subsequent defects are needed to allow CNS lesions that result in HAM/TSP.

In conclusion, the transport of malignant cells across the BBB by proliferating cells is a frequent event in lymphoblastic leukemia [51] and malignant lymphomas [52], including ATLL [17]. Present results show, for the first time, that such traffic also appears to pertain to the nonmalignant oligoclonal proliferation of HTLV-1–bearing lymphoid cells in HAM/TSP.

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References


