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Search for human T-cell leukemia virus type I (HTLV-I) proviral sequences by polymerase chain reaction in the central nervous system tissue of HTLV-I-associated myelopathy

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Summary. Using the polymerase chain reaction (PCR), proviral DNA sequences of the *pol* and *env* regions of human T-cell leukemia virus type I (HTLV-I) were directly amplified in paraffin-embedded and frozen tissue sections of active inflammatory central nervous system (CNS) lesions in three autopsy cases of HTLV-I-associated myelopathy (HAM) with serological confirmation. In parallel, the enumeration of UCHL-1 (monoclonal antibody reactive to T-cells) positive cells in the tissue sections subjected to PCR were carried out. Although the control DNA sequence of parathyroid hormone-like peptide gene was definitely amplified, no signals for HTLV-I proviral sequences were detected in these specimens. The number of UCHL-1 positive cell nuclei was almost on the border line of our PCR sensitivity in formalin-fixed tissue, which was estimated to be 20–200 copies. Therefore, it is unlikely that the central nervous system tissue damage in HAM/TSP is a consequence of productive infection of HTLV-I in the CNS tissue.

Introduction

A close association of human T-cell leukemia virus type I (HTLV-I), an aetiologic retrovirus of adult T-cell leukemia (ATL) [3, 17, 27], with certain cases of chronic inflammatory myelopathy has been shown by two groups of researchers [2, 14, 18]; i.e., with tropical spastic paraparesis (TSP) prevalent in the Caribbean and other tropical countries and with HTLV-I-associated myelo-
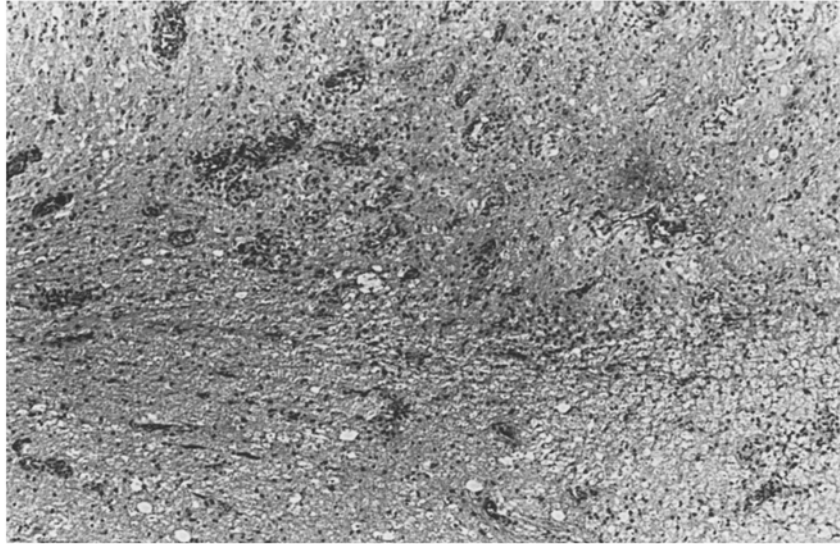
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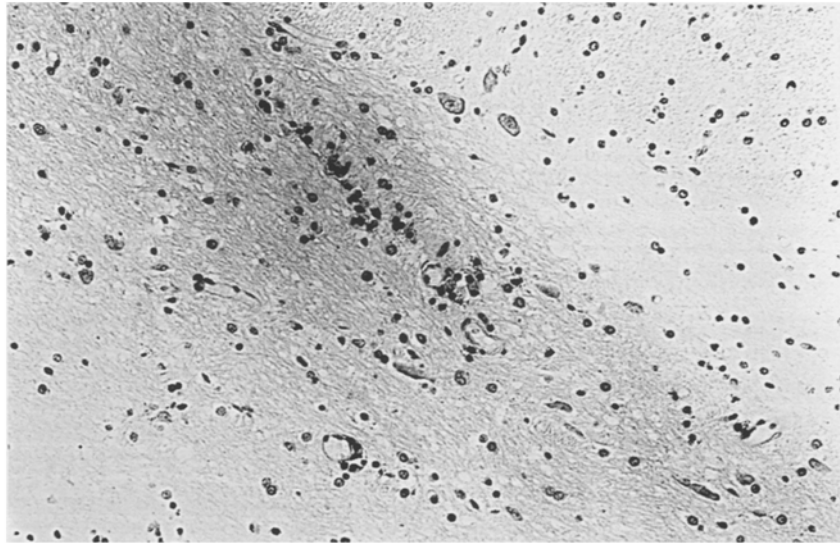
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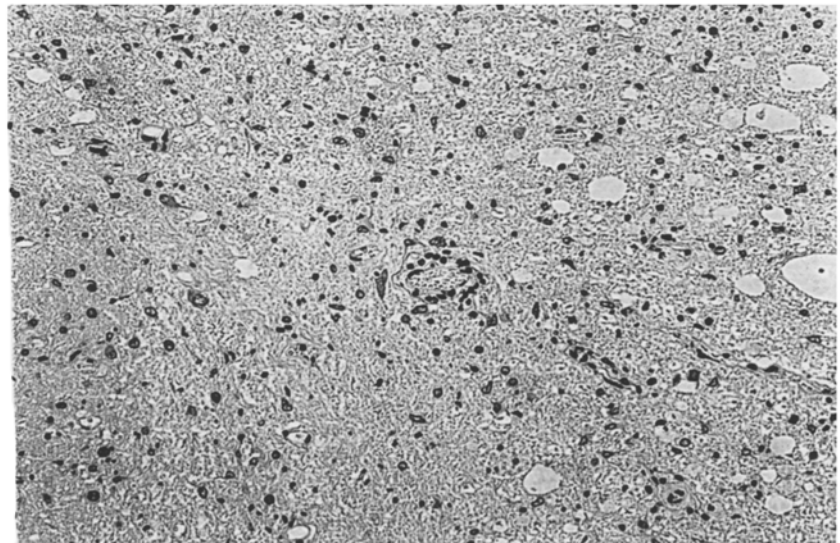
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pathy (HAM) in certain districts of the island of Kyushu, Japan. Both occur in the endemic areas of HTLV-I infection but the occurrence of sporadic cases in non-endemic areas has also been reported [5, 12, 15, 19, 24].

The virus has been repeatedly isolated from the cerebrospinal fluid (CSF) lymphocytes of HAM/TSP patients [4, 8, 13] but no convincing evidence for the presence of HTLV-I antigens or mRNA in the parenchymal cells of diseased central nervous system (CNS) tissues has yet been provided [16]. Thus, a key issue which still remains unsettled in elucidating the mechanism of tissue damage in HAM/TSP is whether it is a direct consequence of the virus infection to CNS tissues or an indirect insult on CNS tissues exerted by the infected lymphocytes.

To clarify the role of HTLV-I infection in the pathogenesis of HAM/TSP, we attempted to quantitate the amount of HTLV-I proviral DNA sequences in the CNS tissues of three autopsy cases of HAM by the polymerase chain reaction (PCR), the most sensitive technique for amplifying the target nucleic acid at present [20], in parallel with the enumeration of T-cell infiltrates in the tissues.

Patients and methods

Patients

Case 1

A detailed clinical history and neuropathological findings have been reported elsewhere [6, 7]. A 75-year-old man without a history of blood transfusion developed difficulty in urination when he was 72 years and a half, followed by gait disturbance and dysesthesia of the lower extremities. Neurological symptoms progressed gradually in spite of temporary amelioration with steroid therapy. He died from acute embolisms of the leg. The anti-HTLV-I antibody titers were × 2,048 in serum and × 32 in CSF by the particle agglutination (PA) method. Autopsy disclosed an active inflammatory process involving the entire spinal cord and symmetrical degeneration of the lateral funiculi. The middle thoracic cord with severe parenchymal cell infiltration was used in the present study (Fig. 1 A).

Case 2

This case was also reported elsewhere [6, 10]. A 63-year-old woman, with a history of blood transfusion noticed difficulties in walking and urination when she was 59 years old. Weakness of both lower extremities worsened gradually and the numbness and dysesthesia also developed. She died of the rupture of the esophageal varix due to liver cirrhosis. The anti-HTLV-I antibody titers were × 256 in serum and × 128 in CSF by the PA method.

Fig. 1. Active inflammatory lesions of three HAM cases subjected to the PCR. A The middle thoracic cord of Case 1, H-E stain, × 25. B Pons of Case 2, immunostain with UCHL-1 monoclonal antibody, × 50. C The middle thoracic cord of Case 3, immunostain with UCHL-1 monoclonal antibody, × 50

At autopsy, in addition to the degenerative spinal cord lesions compatible with HAM, lymphocytic cell infiltrates in the parenchyma were found in the brainstem. Part of the pons was used in the present study (Fig. 1 B).

Case 3

A 69-year-old woman, who had been born and raised in a non-epidemic area for ATL and had no history of blood transfusion or the family history of ATL, complained of gait disturbance. The patient was referred to Department of Neurology, Kanazawa Medical University from an orthopedics for the examination. The neurological examination revealed spastic paraparesis of both lower extremities with sensory and urinary disturbances. Gait and urinary disturbances progressed gradually, and no effect of prednisolone administration (60 mg/day) was observed. She died of disseminated consumption coagulopathy during the treatment for fever of unknown origin. The anti-HTLV-I antibody titers were $\times 5{,}120$ in serum and $\times 256$ in CSF by the fluorescent antibody test. The middle thoracic cord, in which symmetrical degeneration of the lateral funiculi and active inflammatory cell infiltration are observed, was used in the present study (Fig. 1 C).

DNA preparation

A peripheral lymphocyte fraction of an ATL patient, or MT-2 cell in which HTLV-I sequences are integrated [11] was treated with proteinase K (200 µg/ml) (Merk, Darmstadt) and sodium dodecylsulfate (1%) at 37 °C overnight, followed by three-time phenol extraction. Ethanol-precipitated DNA was then dissolved in distilled water.

Tissue preparations

The brain and spinal cord of HAM cases or the spleen of an ATL case, which was kindly supplied by Dr. Mitsuaki Yoshida, Institute of Medical Science, University of Tokyo, were fixed in 10% formalin for less than 2 weeks after autopsy and then embedded in paraffin. In Case 3, a part of unfixed spinal cord was embedded in Tissue-Tek O.T.C. compound (Miles Inc., Elkhart). Serial 3 µm thick sections for the paraffin-embedded tissues or serial 6 µm thick sections for the frozen tissue were prepared with a great caution to avoid cross contamination of DNAs among tissue sections. The sections adjacent to those used for PCR were stained by hematoxylin and eosin (H-E) and by the avidin-biotin complex technique using a UCHL-1 monoclonal antibody (Dakopatts, Denmark), which is known to be reactive to T-cells [23].

PCR and DNA hybridization procedures

After deparaffinization with xylene in the case of paraffin-embedded tissue or removal of O.T.C. compound with H_2O in the case of frozen tissue of Case 3, a single cut (about 12 mm^2) from tissue sections was heated at 100 °C for 10 min [22], after which the reaction mixture was added to the samples. Both boiled tissues and extracted DNA were subjected to 50 cycles of PCR as previously described [26]. In brief, the reaction mixture, in a total volume of 100 µl for boiled tissues and 50 µl for the extracted DNA, was composed of 2.5 units of Taq polymerase (Biotech, WA), 200 µM of dNTP, and 0.5 µM and 1 µM of each primer for extracted DNA and boiled tissues, respectively, and the buffer (67 mM Tris hydrochloride pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1 mM 2-mercaptoethanol, 6.7 µM ethylene-diaminetetraacetic acid, 0.15% Triton-X 100, 200 µg/ml gelatin, supplemented with 1.0 mM

for extracted DNA and 1.5 mM for boiled tissues of $MgCl_2$). One cycle of PCR was denaturation at 95 °C for 1 min, reannealing at 55 °C for 2 min, and polymerization at 70 °C for 1.5 min.

For the amplification of HTLV-I sequences, primers derived from two different regions (*pol, env*) [21] were used. The *pol* primers were

5'-CTGGAGGCAGGCCATATCGAACCC-3' and
5'-AAACCCTTGGGGTAGTACTTTCCA-3',

corresponding to 2638–2661 nucleotide position (np) and 2944–2967 np. The *env* primers were

5'-CTAGTCGACGCTCCAGGATATGACC-3' and
5'-CAGACCGCCACCGGTACCGCTCGGC-3',

corresponding to 5669–5693 np and 6111–6135 np. For parathyroid hormone-like peptide (PTHLP) sequences,

5'-GAGGGCAGATACCTAACTCAG-3' (601–621 np) and
5'-GAGCTCCAGCGACGTTGTGGA-3' (799–819 np)

were used [25]. The DNA sequences of PTHLP gene are unrelated to that of HTLV-I and are considered to be a single copy per haploid genome [25]. This sequence was used as an internal control for PCR.

After repeating 50 cycles of PCR, one-fifth of the amplified DNA was subjected to electrophoresis in 2% agarose gel. Then the DNAs were alkaliblotted to Biodyne A (Pall Biosupport, NY), followed by analysis by hybridization with probes. The probes were

5'-CTTTTTAACTGGGAATACTGGGTT-3' (2680–2703 np)

for the *pol* region, and

5'-GCCTCTCCACTTGGCACGTCCTATA-3' (5877–5901 np)

for the *env* region. And

5'-AGACAGGTGGTCCCCTTCTAG-3' (772–792 np)

was used for PTHLP sequence. These probes were nonradioactively labeled at the 3'-end by terminal transferase and digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim). Hybridization was performed at 42 °C in 5 × SSC for 12 h. The filter was washed at 37 °C in 5 × SSC and the hybridized probes were visualized by an enzyme-linked immunoadsorbant assay.

Results

Effect of formalin fixation on the sensitivity of the PCR

As formalin is known to break DNA into small fragments after converting to formic acid, the effect of formalin fixation on the sensitivity to detect HTLV-I sequences by PCR was examined. DNA was extracted from MT-2 cell fixed in 10% formalin for the various time, and was subjected to PCR after a serial 10-fold dilution. In the PCR using the *pol* primers, the intensity of signals of specific bands decreased after the fixation. The signals decreased 10-fold after 10-day fixation although the signals are still detectable up to 10^{-5} dilution after 4-day fixation (Fig. 2). The same result was obtained in the *env* region (data not shown). Comparing with the fixation of cell suspension in this experiment, the fixation of the tissue is thought to be extremely mild. Therefore,
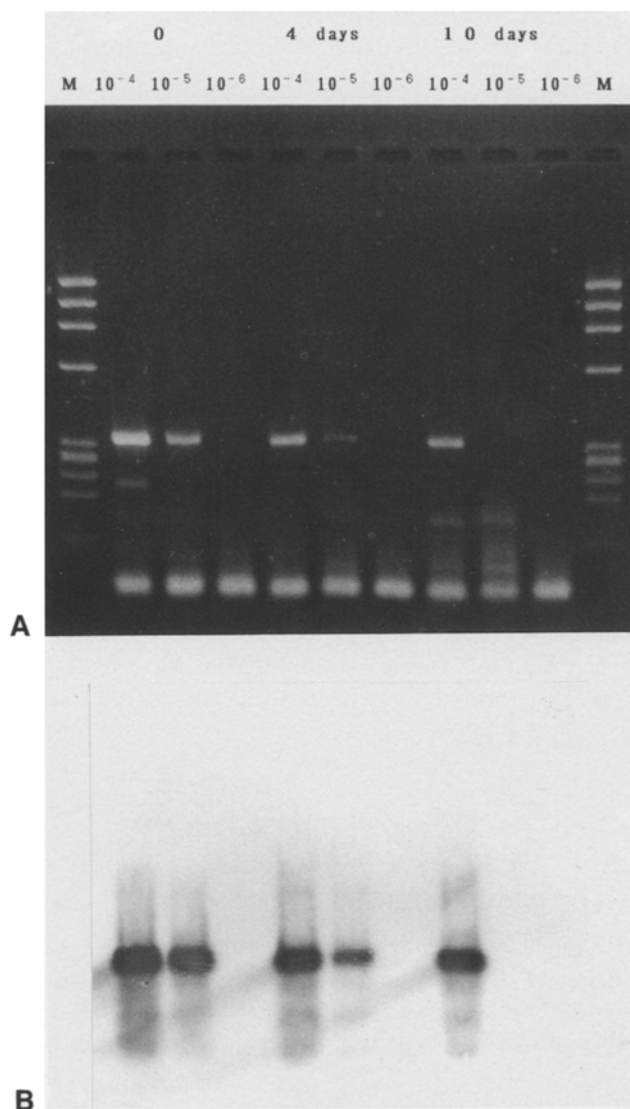
Fig. 2. The effect of formalin fixation on the sensitivity of PCR with *pol* region primers. HTLV-I sequences are detected up to 10^{-5} dilution of DNA extracted from unfixed MT-2 cell (*0*) and 4 day-fixed MT-2 cell (*4 days*) although 10-fold decrease of the sensitivity is observed in 10-day fixation (*10 days*). A 2% agarose gel stained by ethidium bromide, and **B** hybridization with nonradioactively-labeled probe with digoxigenin-11-dUTP. *M* Phi X DNA digested by Hae III as a size marker

DNA in those tissue specimes is presumably preserved for PCR although the possible decrease of sensitivity should be taken into consideration.

Integrity of DNA sequences in paraffin sections

To further ascertain the integrity of the DNA sequences in the paraffin sections, a DNA sequence of PTHLP in the same paraffin section used for the detection of the HTLV-I proviral sequence was amplified. Since, in a preliminary experiment, the addition of primers from PTHLP DNA sequences to the same reaction mixture substantially suppressed the amplification of HTLV-I-specific sequences, the PTHLP sequences were amplified in a sample taken from the area next to the tissue used for the amplification of HTLV-I sequences, and the PCR was run in parallel. As shown in Fig. 3, a specific band for PTHLP was amplified in the CNS tissue of all three HAM cases and the spleen tissue
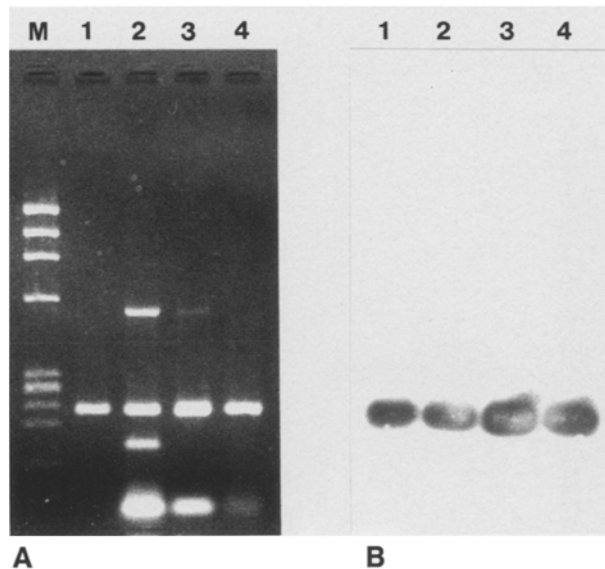
Fig. 3. The amplified PCR products using PTHLP-derived primers. PTHLP-specific sequences are amplified in all cases. **A** 2% agarose gel stained with ethidium bromide, and **B** hybridization. *1* Case 1, *2* Case 2, *3* Case 3, *4* the spleen tissue of an ATL patient, and *M* PhiX DNA digested by Hae III as a size marker

of the ATL patient. Therefore, it was safely assumed that the DNA sequences in those paraffin sections of formalin fixed CNS tissues were reasonably well preserved, and were applicable for PCR.

Sensitivity of the PCR

With a serial dilution of 1 μg of the DNA extracted from a peripheral lymphocyte fraction of the ATL patient, the present PCR technique with nonradioactive-hybridization detected distinct HTLV-I signals at dilutions up to 10^{-5} (Fig. 4, lanes 1–4). Since 1 μg of genomic DNA is composed of 3×10^5 copies, the amount of DNA at 10^{-5} dilution is equivalent to the genomic DNA of 3 infected cells if all peripheral lymphocytes were infected. However, the infection ratio in peripheral lymphocytes of ATL patients is assumed to be lower than 100%. The number of HTLV-I provirus DNA copies detected by this method was, therefore, roughly estimated to be 2 copies, at the most, 3 copies.

To examine the possible interference of PCR amplification of DNA sequences with co-existing paraffin embedded tissues, small fragments of normal CNS tissues in paraffin sections (about 12 mm^2 each) were added to the reaction mixtures containing serially-diluted DNA from the ATL patient. The addition of tissue fragments decreased the sensitivity of PCR 10-fold (Fig. 4, lanes 5–8). Therefore, our PCR system can detect about 20 copies under the interference with the co-existing tissue fragment.

In the next experiment, 3 μm thick serial paraffin sections were prepared from the formalin-fixed spleen tissue from the ATL patient, and a various size of tissue fragments taken from a serial sections were subjected to PCR and the number of nuclear profiles in the adjacent area sections were counted. As shown in Fig. 5, even the smallest tissue specimen suitable for manipulation gave
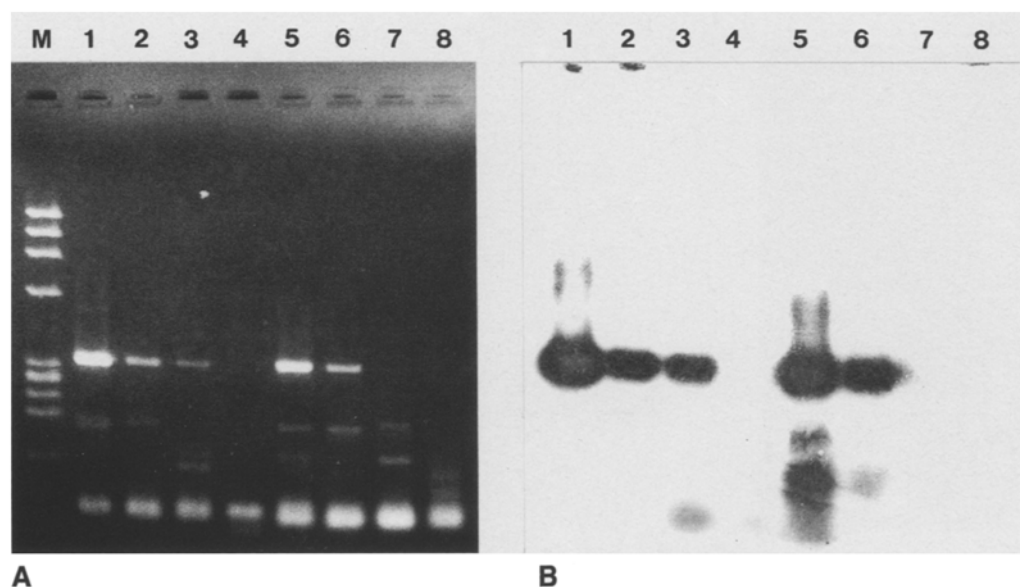
Fig. 4. The PCR products of serially-diluted of 1 μg of DNA extracted from a peripheral lymphocyte fraction of an ATL patient (10^{-3}–10^{-6}) without (*1–4*) or with normal brain tissue (*5–8*). HTLV-I-specific sequence of the *pol* region is amplified up to 10^{-5} (*3*) in the PCR without the brain tissue, whereas they are amplified up to 10^{-4} (*6*) in the PCR with the brain tissue. **A** 2% agarose gel stained with ethidium bromide, and **B** hybridization

positive PCR reaction, and it contained about 400 hundred nuclear profiles of cells. As the diameter of cell nuclei is approximately 6 μm, 400 nuclear profiles in 3 μm sections could be equivalent to 200 nuclei. Assuming that 100% of those cells contained in the corresponding area were infected by HTLV-I, the copy number which our PCR system detects could be 200, or less than 200. This number is almost equivalent to the number deduced from both the data of the decrease of sensitivity caused by formalin fixation and the data of the interference with a co-existing tissue fragment. The sensitivity of our PCR system is, therefore, estimated to be 20–200 copies of HTLV-I sequences in the formalin-fixed tissue.

PCR of the CNS tissue of HAM patients

In the CNS tissue sections of the HAM patients, no signals for *pol*- and *env*-specific sequences were detected by PCR (Fig. 6), although the PTHLP sequence was clearly amplified in the CNS tissues of three patients (Fig. 3). In addition, distinct bands for the *pol* and *env* regions of HTLV-I proviral sequences were definitely detected in the spleen tissue of the ATL patient by both ethidium bromide staining and hybridization (Fig. 6). Even in the case of the frozen section of Case 3, no signals for *pol*- and *env*-signals were detected (data not shown).
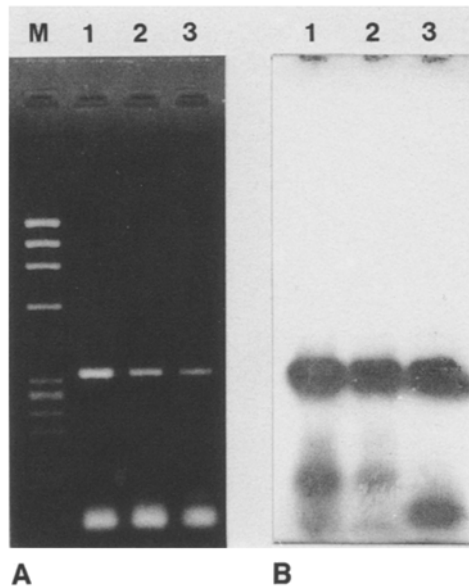
Fig. 5. The amplified PCR products in the spleen tissue of an ATL patient. The 3 μm-thick section was cut out smaller and smaller and subjected to PCR using primers derived from *pol* region of HTLV-I sequence. The number of cell nuclei contained in the corresponding area were counted in the adjacent serial section stained by H-E. **A** 2% agarose gel electrophoresis stained with ethidium bromide, and **B** hybridization. *1* More than 5,000 nuclear profiles, which is equivalent to more than 2.500 nuclei, *2* 2,000–3,000 nuclear profiles (1,000–1,500 nuclei), *3* 400 nuclear profiles (200 nuclei), and *M* Phi X DNA digested by Hae III as a size marker

Enumeration of UCHL-1 positive cells contained in the sections

The numbers of nuclear profiles of UCHL-1 positive cells in the areas corresponding to the tissue fragments subjected to PCR ($12 \, mm^2$) were enumerated in the adjacent paraffin sections (Table 1). The numbers of nuclear profiles of those cells in Case 1, Case 2, and Case 3 were 356, 289, and 733, respectively. The numbers of nuclear profiles in 3 μm thick sections were, therefore, estimated to be equivalent to 178, 145, and 387 nuclei, respectively.

Discussion

Direct amplification of the target sequences in paraffin embedded tissue specimen [22] instead of amplification of DNAs extracted from tissues allows for examination of the histopathological features of the tissue where the target sequences were present. Using this technique, the present study clearly showed a low prevalence of HTLV-I provirus sequences in active inflammatory CNS lesions in HAM/TSP.

At present, PCR is the most sensitive method for amplifying a minute amount of DNA sequences in tissues, and it can amplify target sequences in an exponential manner [20]. Both the experiment to examine the effect of formalin fixation on the PCR sensitivity and the experiment using primers derived from the PTHLP sequence assured that the preservation of DNAs in paraffin sections of those formalin-fixed tissues was satisfactory for amplification by PCR. As one might expect, the efficiency of DNA amplification in the formalin-fixed paraffin-embedded tissue was 10- to 100-fold lower than that of extracted DNA.
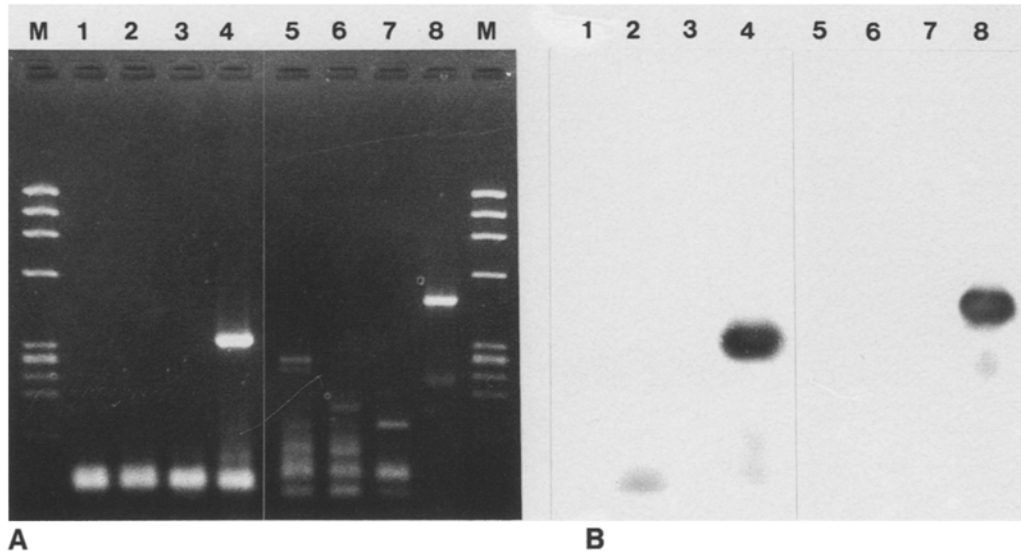
Fig. 6. The amplified PCR products using HTLV-I-specific primers derived from *pol* and *env* regions. No HTLV-I-specific sequences are detected in Case 1 (*1, 5*), Case 2 (*2, 6*), or Case 3 (*3, 7*), whereas those are clearly amplified in the spleen tissue of the ATL patient as a positive control (*4, 8*). **A** 2% agarose gel stained by ethidium bromide, and **B** hybridization. *1–4 pol* region, and *5–8 env* region. *M* Phi X DNA digested by Hae III as a size marker

Table 1. The enumeration of UCHL-1 positive cells in the sections

	Number of nuclear profiles[a]	Number of nuclei[b]
Case 1	356	178
Case 2	289	145
Case 3	773	387

[a] The number of nuclear profiles of UCHL-1 positive cells in the areas corresponding to the tissue fragments (3 µm thick) subjected to PCR were enumerated in the adjacent sections

[b] The number of nuclei of UCHL-1 positive cells could be equivalent to a half of the number of nuclear profiles in 3 µm thick sections assuming the diameter of cell nuclei is about 6 µm

It is reported that the replication of HTLV-I is increased in mononuclear peripheral blood cells of the patients with HAM [9, 28] and that those proviral DNAs are 10- to 100-fold higher than in seropositive carriers [9]. Although there is no evidence that there is no discrimination in the influx of inflammatory cells into the CNS, the estimated number of infected inflammatory cells in those CNS tissues is below the lower limit or on the border line of our PCR sensitivity, even if almost all of those UCHL-1 positive cells were assumed to be infected

(Table 1). The results of the present study do not deny the presence of HTLV-I infected lymphocytes in the CNS tissue, but these results strongly suggest the productive infection is highly unlikely to be occurring in the diseased tissues. The tissue damage in HAM/TSP, therefore, may not be a direct consequence of virus replication in the tissue.

In this context, it is noteworthy that HTLV-I isolation from CSF lymphocytes has been possible only after in vitro propagation [4, 8, 13] and even with PCR, HTLV-I sequences in the CSF cells of TSP patients have been detected in only 3 out of 11 cases [1], suggesting that the copy number of HTLV-I is extremely low in the CNS. Therefore, our present data, together with the above reports, suggest that the CNS tissue damage in HAM/TSP is quite likely to be caused by mechanisms other than the replication of HTLV-I in the diseased tissues.

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