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No Evidence of HTLV-1 Infection in Japanese Multiple Sclerosis Patients in Polymerase Chain Reaction

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To test possible association of human T cell leukemia virus type 1 (HTLV-1) with multiple sclerosis (MS), polymerase chain reaction was applied to detect HTLV-1 DNA integrated into peripheral blood mononuclear cell DNA. Nine Japanese MS patients all gave a negative result, indicating no HTLV-1 infection in these MS patients.

Key words: HTLV-1 — Multiple sclerosis — Polymerase chain reaction

Association of human retrovirus infection with multiple sclerosis (MS), the major demyelinating disease, was proposed by Koprowski *et al.*[1] based on the detection of antibodies cross-reactive to human T cell leukemia virus type 1 (HTLV-1). These antibodies suggested the presence of a retrovirus related to, but not identical to HTLV-1, which is an etiologic agent of adult T cell leukemia (ATL).[2–5] This proposal by Koprowski *et al.*[1] was supported by one group,[6] but not by others.[7–10] Independently, a close association of HTLV-1 infection with HAM/TSP (HTLV-1-associated myelopathy or tropical spastic paraparesis) has been established.[11–17]

Very recently, Reddy *et al.*[18] and Greenberg *et al.*[19] independently reported detection of HTLV-1-related sequences in some MS patients using the polymerase chain reaction (PCR).[20] Reddy *et al.*[18] found that the amplified sequences were identical to that of HTLV-1 itself, although a few base substitutions were detected, whereas Greenberg *et al.*[19] suggested infection of a retrovirus related to, but not identical to HTLV-1. In order to confirm HTLV-1 infection in Japanese MS patients, we have established a reliable and sensitive PCR assay with an *env* sequence to detect HTLV-1 DNA in cellular DNA of peripheral blood mononuclear cells (PBMC). Here, we report the absence of evidence for HTLV-1 infection in nine MS patients in Japan, using our quantitatively defined assay system.

Nine patients analyzed here were all diagnosed as typical multiple sclerosis according to a guideline proposed by Poser *et al.*[21] All patients were sero-negative in antibodies to HTLV-1 tested by indirect immunofluorescence assay. The patients were selected from non-endemic areas of HTLV-1, because PCR results in endemic areas of HTLV-1 would be very difficult to interpret because of coincidental overlaps of HTLV-1 infection and MS: five cases from Sapporo, three from Sendai and one from Tokyo. Mononuclear cells were isolated from peripheral blood by centrifugation on a Ficoll-Hypaque layer and total cellular DNA was isolated by the standard procedure with proteinase K treatment followed by phenol extraction.[4] Each DNA was subjected to PCR to amplify the envelope sequence of HTLV-1. The *env* sequence was used because it was thought to be more specific to HTLV-1 than other sequences. The reaction mixture contained 1 μg of sample DNA, 2.5 units of Taq polymerase, 100 μM each of dATP, dCTP, dTTP and dGTP, and 1 μM of each primer. 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. The primers were 25-mers corresponding to two sites in the *env* gene region[22]: CTAGTCGACGCTCCAGGATATGACC (corresponding to nucleotide positions (np) 5669-5693 of the HTLV-1 sequence in λATK-1; see ref. 22) and CAGACCGCCACCGGTACCGCTCGGC (np 6111–6135), and the expected size of the PCR product was 467 bp. After repeating 50 cycles of PCR, one-fifth of the amplified DNAs was subjected to electrophoresis in 2% agarose gel. In addition to MS samples, four other samples were simultaneously analyzed: an ATL patient as a positive control, an un-infected cell line as a negative control, and two antibody-positive carriers of HTLV-1 whose DNAs were negative for HTLV-1 DNA in our standard Southern blot analysis.

On staining the gel with ethidium bromide, a specific band of the expected size was detected in DNA from the ATL patient and HTLV-1 carriers (Fig. 1A). However, no specific band was detected in the lanes of MS patients or un-infected cell line, although several non-specific bands were observed. These results suggest that the spe-

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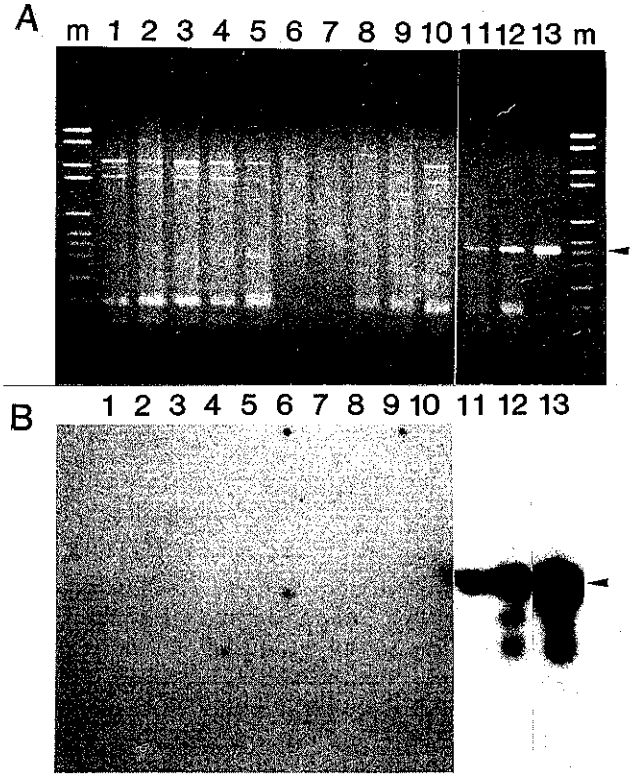

Fig. 1. (A) Stained gel of the amplified DNA of HTLV-1 *env* sequence. DNA was isolated from peripheral blood mononuclear cells of MS patients (lanes 1 to 9), an HTLV-1-negative T cell line CEM (lane 10), antibody-positive carriers (lanes 11 and 12) and an ATL patient (lane 13). The molecular markers (lane m) are 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, and 154 bp, from the top. The arrowhead indicates the position of the specifically amplified band. (B) Hybridization of the amplified DNA with a probe of 25-mer corresponding to the sequence between two primers in the *env* gene (see the text). Exposure was 48 h for lanes 1 to 10, and 3 h for lanes 11 to 13.

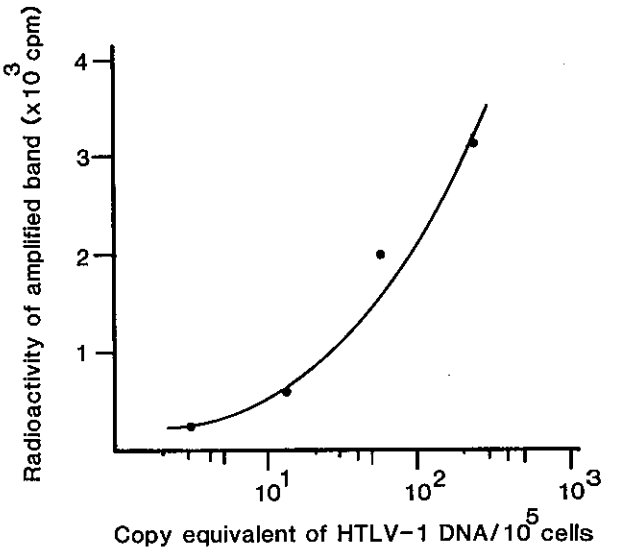

Fig. 2. Effect of dilution of HTLV-1 DNA on PCR. A cloned DNA of HTLV-1 was serially diluted four-fold against DNA from a T cell line, CEM, and the same amount of diluted DNA (1 μg each) was used for PCR. The amplified DNA was hybridized with the same probe as used in Fig. 1B and the radioactivity of the specific band was measured.

cific band is the DNA fragment amplified from the envelope sequence of the integrated HTLV-1 proviral DNA.

After transfer of the DNA onto a membrane filter, the sequence related to the *env* gene was detected by hybridization with a synthetic oligonucleotide, GCCTCTCCACTTGGCACGTCCTATA (np 5877–5901) labeled at the 5′ terminus, which corresponds to the *env* sequence between two primers. The hybridization was performed at 55°C in 4×SSC for 12 h and the filter was washed at 55°C in 2×SSC. As shown in Fig. 1B, DNAs from the ATL patient and HTLV-1 carriers gave strong bands corresponding to the specific band in Fig. 1A. However, no detectable signal was observed in any lane of MS DNA even after longer exposure. Thus, the HTLV-1 sequence was not detected in DNA of PBMC from nine patients with typical MS in Japan. Additional bands were also detected in lanes 12 and 13. These would represent small amounts of by-products such as single-stranded form, because these were also detected on the standard HTLV-1 DNA when excess template was used. Thus, these bands in Fig. 1B do not affect the conclusion.

The sensitivity of our PCR assay was estimated by dilution of the cloned HTLV-1 DNA against non-infected cellular DNA (Fig. 2). The results directly demonstrated that our assay could detect a few infected cells in 10^5 PBMC. This supports the view that our assay was rather sensitive and sufficient to derive a conclusion, although only one site for PCR was used.

Based on these considerations, our results on MS DNA imply that these MS patients either had no HTLV-1-infected cells or had, if any, less than 2–3 HTLV-1-infected cells among 10^5 mononuclear cells. If all MS patients were infected with HTLV-1, it would be very unlikely that they would all have HTLV-1 infection that is restricted to such a small fraction of cells, because all the patients had a long history of the disease. Reddy *et al.*[18] reported more frequent infection of HTLV-1 into adherent cells than into lymphocytes. Even if this were the case, most adherent cells in peripheral blood were collected in our samples, and therefore, our assay should

detect the HTLV-1 sequence if it is present in any type of cell.

We thus conclude that the nine MS patients tested here were not infected with HTLV-1. This conclusion is consistent with the absence of antibodies to HTLV-1 in these MS patients. Thus, no general correlation of MS with HTLV-1 infection was observed in Japanese non-endemic areas. The reason for the discrepancy between the observations of our group and other groups is not clearly understood. One may argue that a point mutation at a primer site may not pick up the HTLV-1 DNA in PCR, thus giving a negative result. But, such possibility seems unlikely because PCR can be operated with imperfectly matched primers.[23] As other possibilities, the discrepancy may reflect possible differences between MS patients in Japan and those in Western countries, or artifacts due to the conditions used for PCR assay. To clarify these differences, careful survey of large numbers of MS patients would be essential.

Our results can exclude a general association of MS with infection by HTLV-1 itself, but do not exclude possible association with a retrovirus related to HTLV-1, because a non-identical, but similar sequence at the primer sites might have given a negative result in PCR assay.

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