Jpn. J. Cancer Res. 81, 238–246, March 1990

Amplification of HTLV-1-related Sequences among Patients with Neurological Disorders in Highly Endemic Nagasaki: Lack of Evidence for Association of HTLV-1 with Multiple Sclerosis

Daisuke Nakayama,[1] Shigeru Katamine,[1] Hajime Kanazawa,[2] Noritoshi Shibuya,[2] Ken'ichiro Kawase,[1] Ryozo Moriuchi,[1] Tsutomu Miyamoto[1] and Shigeo Hino[1,3]

[1]*Department of Bacteriology, Nagasaki University School of Medicine, 12-4 Sakamoto, Nagasaki 852 and* [2]*Kawatana National Hospital, 2005-1 Shimogumi, Kawatana, Higashi-sonogi, Nagasaki 859-36*

We studied DNA sequences homologous to HTLV-1 in peripheral blood mononuclear cells (PBMC) of 30 patients with neurological disorders in Kyushu, where HTLV-1 is highly endemic. The regions of HTLV-1 amplified by means of polymerase chain reaction (PCR) included U3, U5, *gag*, *pol*, *env* and *pX*. Our system specifically detected HTLV-1 sequences only from HTLV-1-positive cells and was sufficiently sensitive to detect one proviral copy per 10^3 PBMC. All PCR-positive cases were seropositive, including 4 cases of tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (4/4), and one case each of myasthenia gravis (1/7) and multiple sclerosis (1/8). The PCR-positive rate of patients, excluding 4 TSP/HAM cases, was 8% (2/26), which is similar to the seroprevalence of the adult population in the area. The data suggest that multiple sclerosis is not associated with prototype HTLV-1.

Key words: HTLV infections — Human T-cell leukemia virus — Multiple sclerosis — Polymerase chain reaction

Human T-lymphotropic virus type 1 (HTLV-1), a human retrovirus, is closely linked with adult T-cell leukemia (ATL)[1–5] and tropical spastic paraparesis or HTLV-1-associated myelopathy (TSP/HAM), a slowly progressive spastic paraparesis with elevated antibodies to HTLV-1 in cerebrospinal fluid (CSF) as well as serum.[6,7] These diseases cluster in areas endemic for HTLV-1, including southwestern Japan,[8–10] Africa[11] and the Caribbean basin,[12] and develop sporadically in the United States and other developed countries.[13]

Koprowski *et al.*[14] suggested the association of HTLV-1 or a related virus with another neurological disorder, multiple sclerosis (MS), based on the high prevalence of antibodies to HTLV-1, and the presence of HTLV-1-related RNA in T-cells of CSF of MS patients. However, others could not find anti-HTLV-1 antibody in sera or HTLV-1 RNA in CSF cells of patients with MS.[15,16] Using the newly developed polymerase chain reaction (PCR) as a technique to detect specific DNA sequences, at least 2 recent reports have suggested the presence of DNA sequences homologous to HTLV-1 in peripheral blood mononuclear cells (PBMC) of MS patients.[17,18] Neither of these PCR reports could demonstrate a positive association of the whole span of HTLV-1 genome with MS. Moreover, areas with high incidence of MS[19] are not associated with endemic areas of HTLV-1. Actually, the MS patients studied in these reports had originated from Sweden or the United States where HTLV-1 is not endemic. It seems worthwhile to study how and to what extent HTLV-1 or related sequences are involved in MS as well as other neurological disorders in HTLV-1 endemic areas.

This prompted us to investigate the presence or absence of HTLV-1 or related sequences in patients with neurological disorders in Nagasaki. The Nagasaki prefecture is located at the western side of Kyushu, a southwestern island of Japan, where HTLV-1 is most heavily endemic; the prevalence of HTLV-1 carriers in Nagasaki is above 6% in the population over 40 years of age.[20] We applied PCR to DNA of PBMC of 30 patients in Nagasaki using 6 primer pairs covering the whole span of the HTLV-1 genome. We detected positive signals in carriers, ATL and TSP/HAM patients, but could not demonstrate a significant correlation of HTLV-1 integration with any other neurological disorder, including MS.

MATERIALS AND METHODS

Cells We obtained peripheral blood samples of 30 in- or out-patients with various neurological disorders at Kawatana National Hospital, Nagasaki, Japan. Blood samples were coded at the hospital, and the identity and clinical data of each patient were disclosed to the group of people working in the laboratory only after all the work had been finished. Some patients, including most of the MS and TSP/HAM patients, were bled twice at a 1–3 week interval and both specimens were included in the coded samples. The control group consisted of blood

[3] To whom correspondence should be addressed.

samples of two ATL patients, 30 HTLV-1 carriers and 70 normal healthy individuals determined by antibody screening. We also used two CD4+ T-cell lines infected with HTLV-1, MT-2[21] and TL-Su,[22] and 2 CD4+ uninfected T-cell lines, Jurkat and CEM.[23]

Detection of anti-HTLV-1 antibodies We screened serum antibodies to HTLV-1 by the use of a commercial gelatin particle agglutination kit (Serodia-ATLA, Fujirebio, Tokyo), paired with indirect immunofluorescence targeting a 1:4 mixture of MT-2 and CEM cells. Indeterminate samples were confirmed by western-blot analysis using MT-2 cell lysates as antigens. The sera with positive signals at p19, p24 and p28 bands were judged as positive.[24]

Isolation of DNA PBMC were isolated by Ficoll-Conray density gradient centrifugation followed by two washings with phosphate-buffered saline.[25] High-molecular-weight DNA of PBMC was isolated by the method described previously[26] with minor modifications. Briefly, PBMC were lysed with buffer containing 0.5% SDS, 10 m*M* Tris-HCl (pH 7.5), 2 m*M* EDTA and 150 m*M* NaCl, and digested with 0.5 mg/ml of proteinase K for 2 h at 50°C and then overnight at 37°C. The lysates were extracted once each with phenol, phenol/chloroform and chloroform, and dialyzed against 1×TE (10 m*M* Tris-HCl, pH 8.0 and 1 m*M* EDTA). Then the samples were treated with 20 μg/ml of RNase A, followed by proteinase K digestion and phenol/chloroform extraction. The extracted DNA was precipitated with ethanol and finally dialyzed against 1×TE.

PCR Oligonucleotides used as primers were synthesized by the phosphoramidite method[27] on a synthesizer (Applied Biosystems 380B, Foster City, CA). Base sequences of the primers were derived from published sequences of the complete HTLV-1 genome, λATK-1[28] (all the nucleotide positions in this report are based on the λATK-1).

Sample DNA, 1 μg each, was added to a cocktail adjusted to final concentrations of 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* each of dATP, dGTP, TTP and dCTP, 100 pmol of each primer and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 100 μl, and subjected to 25 cycles of amplification.[29] To prevent evaporation, 100 μl of mineral oil was added. In each cycle of PCR, the mixture was denatured at 94°C for 1 min (3 min for the first cycle), annealed at 60°C for 2 min and then extended at 72°C for 2 min on a DNA thermal cycler (Perkin Elmer Cetus).

Analysis of PCR-amplified products A 10 μl aliquot of the reaction mixture was loaded on a composite gel containing 1.3% NuSieve/0.7% Seakem agaroses (FMC, Rockland, ME) and electrophoresed in Tris/borate/EDTA (TBE) buffer, pH 8.3. DNA on the gel was transferred onto a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA), and hybridized with a suitable probe labeled with ^{32}P by using random primers.[30] Hybridization was performed under high stringency in rapid hybridization buffer (Amersham, UK) at 65°C for 2 h. Membranes were then washed to a final stringency of 0.1×SSC (1× is 0.15 *M* NaCl/0.015 *M* sodium citrate, pH 7.0)/1% SDS at 65°C for 60 min followed by exposure to Konica X-ray film (Konica, Tokyo) with two Kodak X-Omatic regular intensifying screens (Kodak, Rochester, NY) at −80°C.

We used 4 restriction fragments, A–D, of a cloned HTLV-1 proviral DNA isolated from TL-Su cells (R. Moriuchi, unpublished) as probes, which correspond to the U3/U5, *gag, pol, env/pX* regions, respectively. The probe A was a 0.8-kilobase (kb) *Sma*I-*Sma*I fragment (nucleotides [nt]: 32–850); probe B, a 1.1-kb *Sma*I-*Sma*I fragment (nt: 851–1932); probe C, a 1.2-kb *Sma*I-*Bam*HI fragment (nt: 3932–5093); probe D, a 2.2-kb *Bam*HI-*Sma*I fragment (nt: 6099–8308).

RESULTS

Strategy to select regions for PCR amplification Since a hypothetical virus associated with neurological disorders, such as MS, may be partially homologous to HTLV-1, we picked various regions for amplification to cover the whole span of the prototype HTLV-1 provirus, namely U3, U5, *gag, pol, env,* and *pX* (Table I). Since sequence alterations can affect primer annealing, we selected only consensus regions of the viral genome based on previously reported sequence data.[31–35] Primer length, G+C content, and intrastrandal complementarity were also regarded for primer selection.

Specificity of PCR To ensure that specific signals were successfully generated from HTLV-1-positive cells, we tested the selected 6 sets of primer pairs on template DNA from various HTLV-1-positive and negative cells. Jurkat (Fig. 1a), PBMC from a normal individual (b) and CEM (c) were uniformly negative. In contrast, PBMC from an ATL patient (d), MT-2 (e) and TL-Su (f) gave dominant hybridization signals of predicted sizes for each region of HTLV-1. MT-2 cells generated only a faint signal for the *env* region relative to other regions (though not visible in Fig. 1e, lane 5, it was positive in an over-exposed film). In all probability, this is due to the integration of multiple defective copies of HTLV-1 genomes in MT-2 cells.[36] We further confirmed the specificity of PCR on DNA of 30 HTLV-1 carriers and 70 normal individuals. All samples of carriers were PCR-positive for the four regions, namely U5, *gag, pol* and *pX* regions. In contrast, all samples from normal individuals were negative (data not shown).

Table I. Nucleotide Sequences of Oligonucleotide Primers and Their Locations in the HTLV-1 Proviral Genome

Primer pair	Strand	Location	Sequence (5′→3′)	Predicted size of PCR products
U3	+	99–123	ACTAAGGCTCTGACGTCTCCCCCCG	250
	−	348–324	CTGAACTGTCTCCACGCTTTTATAG	
U5	+	601–625	GTTCTGCGCCGTTACAGATCGAAAG	155
	−	755–730	TGTGTACTAAATTTCTCTCCTGAGAG	
gag	+	802–820	ATGGGCCAAATCTTTTCCC	216
	−	1017–995	GAGTAGGCTGGCTAGGAGGGAGT	
pol	+	4319–4338	GTTTCACCCATTGCGGACAG	179
	−	4498–4479	AATGGGTAATGTCGCCTTGC	
env	+	6116–6139	GCGGTACCGGTGGCGGTCTGGCTT	150
	−	6264–6241	ACTATTGCTTGAGTTAACTGGGAA	
pX	+	7302–7326	CCCACTTCCCAGGGTTTGGACAGAG	203
	−	7504–7481	CTGTAGAGCTGAGCCGATAACGCG	

"+" primers are complementary to the minus strand, and "−" primers to the plus strand.
Base numbers are based on λATK-1.[29]

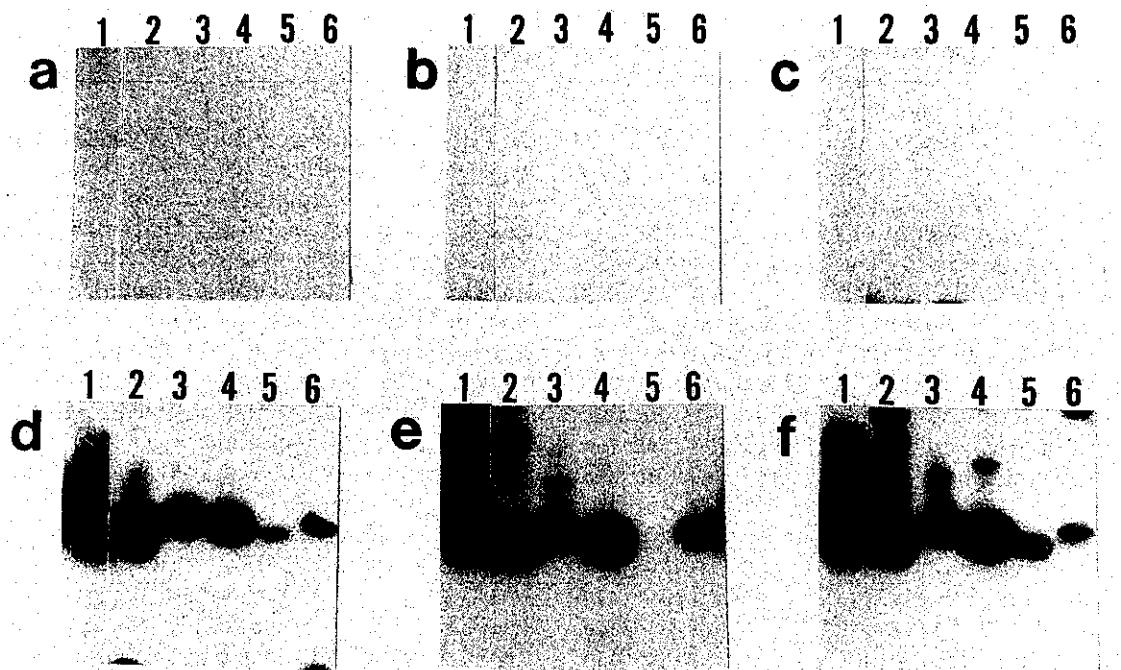

Fig. 1. Specificity of PCR analysis for HTLV-1 sequences. One μg each of genomic DNA from HTLV-1-negative cells (a, Jurkat; b, PBMC of a normal individual; c, CEM) and -positive cells (d, PBMC of an ATL patient; e, MT-2; f, TL-Su) was amplified through 25 cycles of PCR using primers corresponding to U3, U5, *gag*, *pol*, *env* and *pX* regions (see Table I). Amplified products were southern-transferred and hybridized with ^{32}P-labeled restriction fragments in the respective regions of the cloned HTLV-1 proviral DNA. Lanes: 1, U3; 2, U5; 3, *gag*; 4, *pol*; 5, *env*; 6, *pX*.

These results indicated that each set of primer pairs specifically detected HTLV-1 proviral DNA in the cells tested. We considered the endemic HTLV-1 is reasonably homogeneous and similar to the prototype λATK-1, since all positive signals obtained from the 30 healthy carriers corresponded with the predicted lengths (data

Table II. Summary of Thirty Patients with Neurological Disorders

Diagnosis	Abbrev.	No. cases		
		Female	Male	Total
Multiple sclerosis	MS	4	4	8
Spinal progressive muscular atrophy	SPMA	0	2	2
Tropical spastic paraparesis/ HTLV-1 associated myelopathy	TSP/HAM	4	0	4
Spinocerebellar degeneration	SCD	2	5	7
Myasthenia gravis	MG	5	2	7
Chronic inflammatory demyelinating polyneuropathy	CIDP	0	1	1
Systemic lupus erythematosus	SLE	0	1	1
Total		15	15	30

Table III. Anti-HTLV-1 Antibody of Sera and CSF, and HTLV-1 Proviral Sequences of PBMC in Patients with Neurological Disorders

Patient No., age, sex	Initial symptom	Diagnosis	HTLV-1 antibody		PCR					
			serum	CSF	U3	U5	*gag*	*pol*	*env*	*pX*
1, 31F	optic neuritis	MS	−	ND	−	−	−	−	−	−
2, 63M	muscle weakness	SPMA	−	ND	−	−	−	−	−	−
3, 64F	paraplegia	TSP/HAM	+	+	+	+	+	+	+	+
4, 58F	ataxia	SCD	−	ND	−	−	−	−	−	−
5, 38F	optic neuritis	MS	−	ND	−	−	−	−	−	−
6, 34M	optic neuritis	MS	−	ND	−	−	−	−	−	−
7, 62F	paraplegia	TSP/HAM	+	+	+	+	+	+	+	+
8, 47M	muscle weakness	SPMA	−	ND	−	−	−	−	−	−
9, 42M	ataxia	SCD	−	ND	−	−	−	−	−	−
10, 57F	ataxia	SCD	−	ND	−	−	−	−	−	−
11, 69F	diplopia	MG	+	ND	−	+	+	+	+	+
12, 38F	optic neuritis	MS	−	ND	−	−	−	−	−	−
13, 55F	diplopia	MG	−	ND	−	−	−	−	−	−
14, 37M	sensory disorder	CIDP	−	ND	−	−	−	−	−	−
15, 55M	ataxia	SCD	−	ND	−	−	−	−	−	−
16, 35M	diplopia	MG	−	ND	−	−	−	−	−	−
17, 35M	optic neuritis	MS	−	ND	−	−	−	−	−	−
18, 42M	optic neuritis	MS	−	ND	−	−	−	−	−	−
19, 48F	paraplegia	TSP/HAM	+	+	+	+	+	+	+	+
20, 71F	paraplegia	TSP/HAM	+	+	+	+	+	+	−	+
21, 39M	ptosis	MG	−	ND	−	−	−	−	−	−
22, 17M	fever	SLE	−	ND	−	−	−	−	−	−
23, 36F	paraplegia	MS	−	ND	+	−	−	−	−	−
24, 32F	ptosis	MG	−	ND	−	−	−	−	−	−
25, 39F	diplopia	MG	−	ND	−	−	−	−	−	−
26, 41F	diplopia	MG	−	ND	−	−	−	−	−	−
27, 27M	ataxia	SCD	−	ND	−	−	−	−	−	−
28, 28M	ataxia	SCD	−	ND	−	−	−	−	−	−
29, 55M	ataxia	SCD	−	ND	−	−	−	−	−	−
30, 35M	optic neuritis	MS	+	−	+	+	+	+	+	+

See Table II for abbreviations of diagnoses. ND: not done.

not shown). This assumption is consistent with previous reports demonstrating that HTLV-1 isolated from various parts of the world shows no more than 2% difference in homology.[37]

Sensitivity of PCR To estimate the sensitivity of PCR we tried to obtain a cell sample with one copy of HTLV-1 DNA per cell. Since the MT-2 and TL-Su cells contain multiple copies of the proviral DNA, we selected a PBMC sample of an ATL patient which contained more than 90% of ATL cells by morphological criteria. A southern blot analysis of the cellular DNA after digestion with *Eco*RI, which does not have a restriction site within the proviral genome, indicated a single monoclonal integration of HTLV-1 genome per cell.

Serial 10-fold dilutions of the template ATL-DNA, 0.3 μg to 0.3 pg/PCR reaction, were used to test the sensitivity of assay system. With 25 cycles of PCR, southern blot hybridizations probed with the respective restriction fragments of cloned HTLV-1 DNA were at least 100 times more sensitive than that by direct reading after enthidium bromide staining of the electrophoresed gels (data not shown). For southern blots, the endpoint dilutions of DNA preparations which gave positive hybridization signals were 3 ng for the *env* region, and 0.3 ng for the other 5 regions (data not shown). We estimated that the sensitivity of our PCR is sufficient to detect 10^3 DNA copies for *env* and 10^2 copies for the other regions of HTLV-1. Since we inoculated cellular DNA corresponding to 0.1 ml of blood per reaction, the sensitivity of our system is calculated to be 10^2 cells (10^3 cells for the *env* region) with a single copy of integrated HTLV-1 genome/ml blood, or approximately 0.1% of mononuclear cells in blood. The reproducibility of our technique was confirmed by the internal control, and the results of the double samplings from most of the TSP/HAM and MS patients. All blood samples obtained at second bleeding gave consistent results with the first in PCR, as in the case of antibodies.

Patients with neurological disorders Patients we tested consisted of 8 MS, 2 spinal progressive muscular atrophy (SPMA), 4 TSP/HAM, 7 spinocerebellar degeneration (SCD), 7 myasthenia gravis (MG), 1 chronic inflammatory demyelinating polyneuropathy (CIDP) and 1 systemic lupus erythematosus (SLE) patients (Table II). All patients had been brought up and were residing in Kyushu. They comprised 15 females and 15 males with an average age of 41 (range, 17–71 years). The age at onset of the diseases ranged from 13 to 65 years, and the duration, from 1 to 24 years. All patients had received clinical care and neurologic evaluation for at least 1 year and were diagnosed according to strict criteria[38,39] by a group of experienced neurologists.

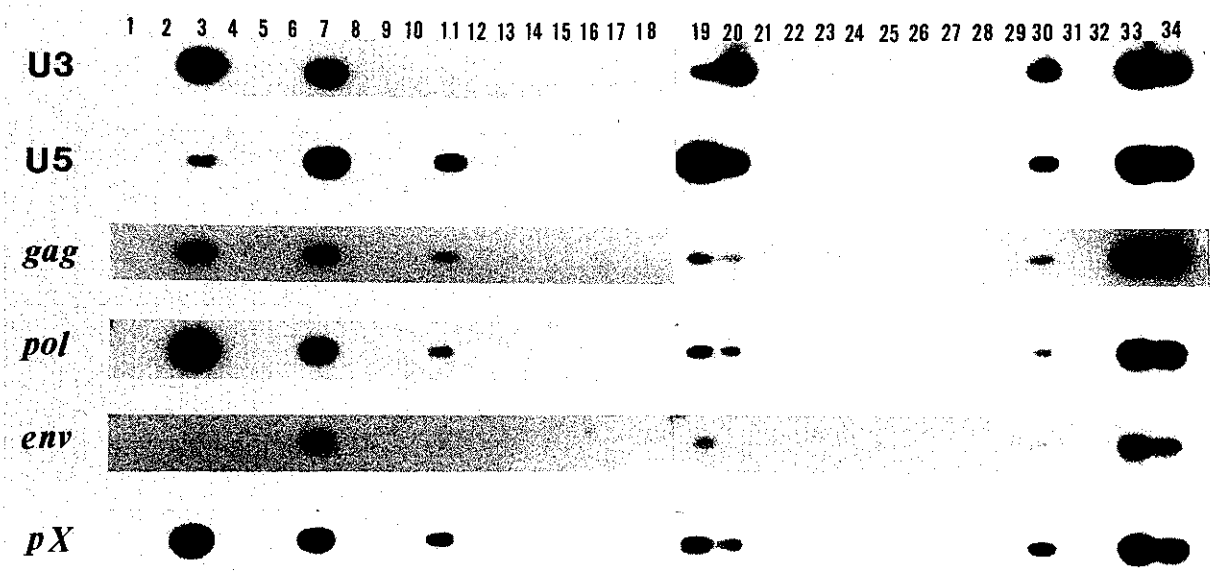

Fig. 2. Southern blot analysis of PCR products for HTLV-1-related sequences in patients with various neurological disorders. One μg each of genomic DNA from PBMC of the patients was subjected to PCR and analyzed by southern blottings probed with the ^{32}P-labeled restriction fragments. Each panel shows the fraction of the predicted size of PCR products (U3, 250; U5, 155; *gag*, 216; *pol*, 179; *env*, 150; *pX*, 203 bps). Final scoring was done in conjunction with the reading of over-exposed films. Lanes: 1–30, patients with neurological disorders; 31, no-DNA reagent control; 32, a normal individual; 33 and 34, ATL patients.

Antibody to HTLV-1 Among these 30 patients, 6 were seropositive for antibodies to HTLV-1. Each set of paired sera gave consistent results. Seropositive cases consisted of 4 TSP/HAM patients, an MS patient and an MG patient. In addition, samples of cerebrospinal fluid (CSF) of all 4 TSP/HAM patients were positive for antibodies to HTLV-1. CSF of the seropositive MS patient was anti-HTLV-1-negative (Table III). The two control ATL patients were seropositive and a normal individual was seronegative.

Clinical aspects of TSP/HAM and MS patients All 4 patients diagnosed as TSP/HAM were typical cases of TSP/HAM including anti-HTLV-1 antibody in CSF (Table III). Most (7/8) MS patients were typical MS cases with prominent signs of intracranial lesions and remissions. Although case #30 was seropositive and had spastic paraparesis below T6, he also had retrobulbar optic neuritis. But this was not diagnostic for MS or TSP/HAM, since neuroophthalmologic manifestations have been reported in TSP/HAM cases.[40] Thus case #30 was diagnosed as MS, since he was negative for anti-HTLV-1 antibody in CSF and his history contained a spontaneous remission.

Association of HTLV-1 with neurological disorders Three of 4 cases having been diagnosed as TSP/HAM (#3, #7, and #19) were PCR-positive in all 6 regions of HTLV-1 genome tested, and the remaining case (#20) was positive except for the *env* region (Fig. 2). Since our PCR system has 1/10 sensitivity in the *env* region compared to other regions, the negative result may be due to the lower sensitivity of the *env* assay.

Of 8 patients with MS, 6 seronegative cases were consistently negative in every region selected for PCR. The seropositive case (#30) was positive in all genomic regions we tested. The remaining one seronegative case (#23) was positive only for the U3 region. The only seropositive case (#11) of 7 MG pstients was positive in PCR except for the U3 region. The remaining 23 cases including 7 SCD, 2 SPMA, 1 CIDP and 1 SLE cases were uniformly PCR-negative in all regions we tested as well as seronegative. Since the U3 region gave discrepant results for unknown reasons in some of the control groups including healthy carriers and non-carriers screened by immunological methods, we have less confidence in the results with U3 region at this point. Thus, our PCR system consistently detected DNA sequences of U5, *gag, pol, env* and *pX* regions of HTLV-1 in all seropositive cases, and was negative in all seronegative patients (Table III).

DISCUSSION

In order to test the possible association of HTLV-1 with MS in Nagasaki where HTLV-1 is highly endemic, we tested 30 patients with various neurological disorders including 4 TSP/HAM and 8 MS patients using the PCR technique. Prepared primer pairs specifically amplified DNA fragments with predicted sizes from template DNAs of cells containing HTLV-1 proviruses, but not from those of HTLV-1-negative cells. The sensitivity of our PCR was 10^2 DNA copies per PCR reaction, except for *env* region where the sensitivity was 10^3 copies. The reason for the lower sensitivity of *env* with our primer pair is not known.

One of the major problems in the use of PCR for diagnostic purposes is laboratory contamination.[41] We used disposable materials as far as possible, and specifically designated reagents and pipetting devices for PCR use. We performed the DNA isolation and the PCR preparation in different rooms in the laboratory, both isolated from those used for recombinant research dealing with HTLV-1. Furthermore, the samples from second bleeding of the same patients were included in the coded samples as internal controls. Thus, we feel that our results should have been uninfluenced by laboratory contamination.

All of the TSP/ HAM patients (4/4) were PCR-positive. This is not surprising because the presence of antibodies in sera and CSF was a criterion for the diagnosis. Other positive cases included one each of MS and MG patients, who were both seropositive. The positive rate of patients, excluding 4 TSP/HAM cases, was 8% (2/26), which is equivalent to the seroprevalence of the adult population in the area. The remaining MS cases, 7 in all, were PCR-negative as well as seronegative. Although the sero- and PCR-positive MS case had distinct spastic paraparesis, the diagnosis was consistent with MS bacause of the presence of retrobulbar palsy and a remission, and the absence of antibody in CSF. Thus, the seropositive MS and MG patients might well be coincidental carriers. Thus, we could not find positive evidence suggesting the association of MS with HTLV-1.

Although a proviral HTLV-1 DNA was isolated from PBMC of a Caribbean patient with a chronic neurological disorder resembling MS,[42] the patient seems to have had TSP/HAM since he was seropositive and had spastic paraparesis; history of remission was not described in the report. MS is more prevalent in areas such as Iceland and Nordic countries than in Japan,[19] and in Japan it is not more common in the southwest area where HTLV-1 is highly endemic.[3, 8–10, 43]

Reddy *et al.*[17] found HTLV-1-related sequences by PCR followed by direct cloning. They selected *gag* (nucleotides [nt]: 841–1375) and *env* (nt: 5662–6129). The PCR conditions they used were denaturing at 94°C for 2 min, annealing at 55°C for 1 min, and extension at 70°C for 2 min, for 36–40 cycles. They found the HTLV-1-related sequences in 6/6 MS patients in 2 of 6 experi-

ments. Greenberg *et al.*[18] also reported the presence of HTLV-1-related sequences by PCR in MS patients. They amplified regions including *gag* (nt: 1388–1957), *pol* (nt: 2779–3015), *env* (nt: 5251–5782 [*env*1] and 5777–6103 [*env*2]). Their PCR condition was denaturing at 94°C for 15 s, annealing at 53°C for 30 s, and extension at 68°C for 30 s, for 30 cycles. Six of the 21 MS patients they tested were positive for *pol* region, but only 1 was positive for *env*1 region, 3 for *env*2 region, and none for *gag* and LTR regions. They suggested the possible association of an exogenous human retroviral agent partially homologous to but distinct from HTLV-1 with some MS patients.

We selected 6 regions in the HTLV-1 genome including *gag* (nt: 802–1017) and *env* (nt: 6116–6264), and used PCR conditions of denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min for 25 cycles. Our negative results on MS patients might have been due to the higher stringency or lower sensitivity of the assay. However, we think that the sensitivity of our PCR is adequate for two reasons. First, all 30 samples of HTLV-1 carriers were PCR-positive. Second, when we increased the number of cycles to 50 several normal samples became positive only with the U3 region, but none of the negative samples became positive with the U5, *gag*, *pol* and *pX* regions. These data suggested that the higher sensitivity may cause false-positive results. Although our PCR had lower sensitivity than those of other reports, it may be superior as regards specificity to HTLV-1. It seems unlikely that MS develops only in carriers with very few infected cells in PBMC, if MS is etiologically associated with HTLV-1.

Alternatively, the choice of amplified region may have caused the difference, if we assume that the putative MS virus has homologies only in some parts of HTLV-1 which we did not test. However, the *gag* region we selected overlapped with that of Reddy *et al.* Furthermore, the molecular clones Reddy *et al.* obtained contained only minor point mutations in the *gag* and *env* regions they selected, suggesting that the sequences are fairly well conserved. At the *env* region, the data obtained by Reddy *et al.* and Greenberg *et al.* were not consistent with each other, even though the region selected by the latter nearly covered that by the former. Thus, the possibility of a putative MS virus with limited local homology with HTLV-1 can not be supported, either. Our data do not support the idea that most MS is associated with HTLV-1 or a closely related virus.

ACKNOWLEDGMENTS

The authors thank Dr. S. Ikeda of the Department of Hematology, Nagasaki University School of Medicine, for supplying ATL cells. This study was supported in part by a Grant-in-Aid for Cancer Research by the Ministry of Education, Science and Culture.

(Received October 5, 1989/Accepted January 5, 1990)

REFERENCES

1) Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K. and Uchino, H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*, **50**, 481–492 (1977).
2) Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. and Gallo, R. C. Detection and isolation of type-C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA*, **77**, 7415–7419 (1980).
3) Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S. and Miyoshi, I. Adult T cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA*, **78**, 6476–6480 (1981).
4) Yoshida, M., Miyoshi, I. and Hinuma, Y. Isolation and characterization of retrovirus from cell lines of human adult T cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA*, **79**, 2031–2035 (1982).
5) Yoshida, M., Seiki, M., Yamaguchi, K. and Takatsuki, K. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. *Proc. Natl. Acad. Sci. USA*, **81**, 2534–2537 (1984).
6) Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L. and Calender, A. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet*, **ii**, 407–410 (1985).
7) Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., Matsumoto, M. and Tara, M. HTLV-I associated myelopathy, a new clinical entity. *Lancet*, **i**, 1031–1032 (1986).
8) Hinuma, Y., Komoda, H., Chosa, T., Kondo, T., Kohakura, M., Takenaka, T., Kikuchi, M., Ichimaru, M., Yunoki, K., Sato, I., Matsuo, R., Takeuchi, Y., Uchino, H. and Hanaoka, M. Antibodies to adult T-cell leukemia-virus-associated antigen (ATLA) in sera from patients with ATL and controls in Japan: a nation-wide seroepidemiologic study. *Int. J. Cancer*, **29**, 631–635 (1982).
9) Tajima, K., Tominaga, S., Suchi, T., Kawagoe, T., Komoda, H., Hinuma, Y., Oda, T. and Fujita, K. Epidemiological analysis of the distribution of antibody to adult T-cell leukemia-virus-associated antigen: possible horizontal transmission of adult T-cell leukemia virus. *Gann*, **73**, 893–901 (1982).
10) Hino, S., Yamaguchi, K., Katamine, S., Sugiyama, H.,

Amagasaki, T., Kinoshita, K., Yoshida, Y., Doi, H., Tsuji, Y. and Miyamoto, T. Mother-to-child transmission of human T-cell leukemia virus type-I. *Jpn. J. Cancer Res.*, **76,** 474–480 (1985).
11) Fleming, A. F., Yamamoto, N., Bhusnurmath, S. R., Maharajan, R., Schneider, J. and Hunsmann, G. Antibodies to ATLV (HTLV) in Nigerian blood donors and patients with chronic lymphatic leukaemia or lymphoma. *Lancet,* **ii,** 334–335 (1983).
12) Blattner, W. A., Kalyanaraman, V. S., Robert-Guroff, M., Lister, T. A., Galton, D. A. G., Sarin, P. S., Krawford, M. H., Catovsky, D., Greaves, M. and Gallo, R. C. The human type-C retrovirus, HTLV, in blacks from the Caribbean region, and relationship to adult T-cell leukemia/lymphoma. *Int. J. Cancer,* **30,** 257–264 (1982).
13) Blattner, W. A., Blayney, D. W., Robert-Guroff, M., Sarngadharan, M. G., Kalyanaraman, V. S., Sarin, P. S., Jaffe, E. S. and Gallo, R. C. Epidemiology of human T-cell leukemia/lymphoma virus. *J. Infect. Dis.*, **147,** 406–416 (1983).
14) Koprowski, H., DeFreitas, E. C., Harper, M. E., Sandberg-Wollheim, M., Sheremata, W. A., Robert-Guroff, M., Saxinger, C. W., Fainberg, M. B., Wong-Staal, F. and Gallo, R. C. Multiple sclerosis and human T-cell lymphotropic retroviruses. *Nature,* **318,** 154–160 (1985).
15) Hauser, S. L., Aubert, C., Burks, J. S., Kerr, C., Lyon-Caen, O., de The, G. and Brahic, M. Analysis of human T-lymphotropic virus sequences in multiple sclerosis tissue. *Nature,* **322,** 176–177 (1986).
16) Karpas, A., Kämpf, U., Sidèn, Å., Koch, M. and Poser, S. Lack of evidence for involvement of known human retroviruses in multiple sclerosis. *Nature,* **322,** 177–178 (1986).
17) Reddy, E. P., Sandberg-Wollheim, M., Mettus, R. V., Ray, P. E., DeFreitas, E. and Koprowski, H. Amplification and molecular cloning of HTLV-I sequences from DNA of multiple sclerosis patients. *Science,* **243,** 529–533 (1989).
18) Greenberg, S. J., Ehrlich, G. D., Abbott, M. A., Hurwitz, B. J., Waldmann, T. A. and Poiesz, B. J. Detection of sequences homologous to human retroviral DNA in multiple sclerosis by gene amplification. *Proc. Natl. Acad. Sci. USA,* **86,** 2878–2882 (1989).
19) Kurtzke, J. F. Epidemiologic contributions to multiple sclerosis: an overview. *Neurology,* **30,** part 2, 61–79 (1980).
20) Hino, S., Kawamichi, T., Funakoshi, M., Kanamura, M., Kitamura, T. and Miyamoto, T. Transfusion-mediated spread of the human T-cell leukemia virus in chronic hemodialysis patients in a heavily endemic area, Nagasaki. *Gann,* **75,** 1070–1075 (1984).
21) Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. and Hinuma, Y. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature,* **294,** 770–771 (1981).
22) Sugamura, K., Fujii, M., Kannagi, M., Sakitani, M., Takeuchi, M. and Hinuma, Y. Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. *Int. J. Cancer,* **34,** 221–228 (1984).
23) Barrè-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science,* **220,** 868–871 (1983).
24) Maeda, Y., Imai, J., Kiyokawa, H., Kanamura, M. and Hino, S. Performance certification of gelatin particle agglutination assay for anti-HTLV-1 antibody: inconclusive positive results. *Jpn. J. Cancer Res.*, **80,** 915–919 (1989).
25) Katsuki, T. and Hinuma, Y. Characteristics of cell lines derived from human leukocytes transformed by different strains of Epstein-Barr virus. *Int. J. Cancer,* **15,** 203–210 (1975).
26) Maniatis, T., Fritsch, E. F. and Sambrook, J. (ed.) "Molecular Cloning: A Laboratory Manual" (1982). Cold Spring Harbor Laboratory, New York.
27) Adams, S. P., Kavka, K. S., Wykes, E. J., Holder, S. B. and Galluppi, G. R. Hindered dialkylamino nucleoside phosphite reagents in the synthesis of two DNA 51-mers. *J. Am. Chem. Soc.*, **105,** 661–663 (1983).
28) Seiki, M., Hattori, S., Hirayama, Y. and Yoshida, M. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc. Natl. Acad. Sci. USA,* **80,** 3618–3622 (1983).
29) Saiki, R. K., Scharf, S., Faloona, F., Millis, K. B., Horn, G. T., Erlich H. A. and Arnheim, N. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* **230,** 1350–1354 (1985).
30) Freinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132,** 6–13 (1983).
31) Seiki, M., Hattori, S. and Yoshida, M. Human adult T-cell leukemia virus: molecular cloning of the provirus DNA and the unique terminal structure. *Proc. Natl. Acad. Sci. USA,* **79,** 6899–6902 (1982).
32) Josephs, S. F., Wong-Staal, F., Manzari, V., Gallo, R. C., Sodroski, J. G., Trus, M. D., Perkins, D., Patarca, R. and Haseltine, W. A. Long terminal repeat structure of an American isolate of type I human T-cell leukemia virus. *Virology,* **139,** 340–345 (1984).
33) Ratner, L., Josephs, S. F., Starcich, B., Hahn, B., Shaw, G. M., Gallo, R. C. and Wong-Staal, F. Nucleotide sequence analysis of a variant human T-cell leukemia virus (HTLV-Ib) provirus with a deletion in pX-I. *J. Virol.*, **54,** 781–790 (1985).
34) Hiramatsu, K., Nishida, J., Naito, A. and Yoshikura, H. Molecular cloning of the closed circular provirus of human T cell leukaemia virus type I: a new open reading frame in the *gag-pol* region. *J. Gen. Virol.*, **68,** 213–218 (1987).
35) Malik, K. T. A., Even, J. and Karpas, A. Molecular

cloning and complete nucleotide sequence of an adult T cell leukaemia virus/human T cell leukaemia virus type I (ATLV/HTLV-I) isolate of Caribbean origin: relationship to other members of the ATLV/HTLV-I subgroup. *J. Gen. Virol.*, **69**, 1695–1710 (1988).

36) Kobayashi, N., Konishi, H., Sabe, H., Shigesada, K., Noma, T., Honjo, T. and Hatanaka, M. Genomic structure of HTLV (human T-cell leukemia virus): detection of defective genome and its amplification in MT-2 cells. *EMBO J.*, **3**, 1339–1343 (1984).

37) Watanabe, T., Seiki, M. and Yoshida, M. HTLV type I (U.S. isolate) and ATLV (Japanese isolate) are the same species of human retrovirus. *Virology*, **133**, 238–241 (1984).

38) Rose, A. S., Ellison, G. W., Myers, L. W. and Tourtellotte, W. W. Criteria for the clinical diagnosis of multiple sclerosis. *Neurology*, **26**, part 2, 20–22 (1976).

39) Report: Scientific group on HTLV-I infections and associated diseases. Regional Office for the Western Pacific of the World Health Organization (1989).

40) Arimura, Y., Arimura, K., Osame, M. and Igata, A. Neuroophthalmological abnormalities in HTLV-I-associated myelopathy. *Neuroophthalmology*, **7**, 243 (1987).

41) Kwok, S. and Higuchi, R. Avoiding false positives with PCR. *Nature*, **339**, 237–238 (1989).

42) Reddy, E. P., Mettus, R. V., DeFreitas, E., Wroblewska, Z., Cisco, M. and Koprowski, H. Molecular cloning of human T-cell lymphotropic virus type I-like proviral genome from the peripheral lymphocyte DNA of a patient with chronic neurologic disorders. *Proc. Natl. Acad. Sci. USA*, **85**, 3599–3603 (1988).

43) Shibasaki, H. Epidemiology of multiple sclerosis. *Adv. Neurol. Sci.*, **33**, 778–789 (1989) (in Japanese).

Note added in proof: Since submission of this paper, other groups have reported that HTLV-1 sequences are not commonly found in Caucasian[1)] and Japanese[2)] MS patients.

1) Bangham, C. R. M., Nightingale, S., Cruickshank, J. K. and Daenke, S.; Richardson, J. H., Wucherpfennig, K. W., Endo, N., Rudge, P., Dalgleish, A. G. and Hafler, D. A. PCR analysis of DNA from multiple sclerosis patients for the presence of HTLV-1. *Science*, **246**, 821–824 (1989).

2) Watanabe, T., Iwasaki, Y., Tashiro, K. and Yoshida, M. No evidence of HTLV-1 infection in Japanese multiple sclerosis patients in polymerase chain reaction. *Jpn. J. Cancer Res.*, **80**, 1017–1020 (1989).