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Usefulness of proviral load measurement for monitoring of disease activity in individual patients with human T-lymphotropic virus type I–associated myelopathy/tropical spastic paraparesis

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High human T-lymphotropic virus type I (HTLV-I) proviral load in peripheral blood mononuclear cells (PBMCs) has been reported in patients with HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and the proviral load has been reported to fluctuate in individual patients during the course of the disease. Clinical symptoms usually became stable after a prolonged period of symptom progression. However, the authors have experienced having some patients whose clinical manifestations suddenly became worse during the course of the disease. To clarify the role of high proviral load and its fluctuation in the pathogenesis of HAM/TSP, the authors measured the proviral load of serially taken PBMCs as well as of cerebrospinal fluid (CSF) cells from patients with HAM/TSP on long-term follow-up and compared these with their clinical manifestations. There was a wide distribution of proviral load, from 0.3 to 37.8 copies/100 PBMCs; however, the proviral load in individual patients was relatively stable during the course of the disease. Eighty-three percent of the patients with clinical worsening showed an increase in proviral load at the time point when clinical worsening was recorded, or at the preceding time point. The proviral loads in CSF cells were higher than those in PBMCs in individual patients. The ratio of proviral loads in CSF cells/in PBMCs, but not the absolute load, in either compartment, was significantly associated with clinically progressive disease and with recent onset of HAM/TSP. These findings indicate that clinical progression of HAM/TSP is associated with increased proliferation or immigration of HTLV-I–infected lymphocytes in the central nervous system. *Journal of NeuroVirology* (2003) **9,** 29–35.

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Introduction

Human T-lymphotropic virus type I (HTLV-I) is known as a viral cause of HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al*, 1985; Osame *et al*, 1986), as well as adult T-cell leukemia/lymphoma (ATLL) (Yoshida *et al*, 1982). However, a precise pathogenic role of the virus in HAM/TSP has not been clarified yet. It has been reported that the HTLV-I proviral load in peripheral blood mononuclear cells (PBMCs) was higher in patients with HAM/TSP than in asymptomatic

HTLV-I carriers (Nagai *et al*, 1998; Jeffery *et al*, 1999), and this high proviral load or high expression of viral antigens has been suspected to be associated with the development of the disease (Hashimoto *et al*, 1998; Moritoyo *et al*, 1999; Nakamura, 2000; Jeffery *et al*, 2000).

HAM/TSP is a chronic disease and clinical manifestations of the patients are almost stable after a long period of symptom progression. However, in some patients, the clinical manifestations suddenly become worse during the course of the disease (Nakagawa *et al*, 1995). Although fluctuation of HTLV-I proviral load in PBMCs has been reported in the patients (Kubota *et al*, 1993), little is known about whether or not this fluctuation correlates with change of their disease progression. In order to clarify the meaning of this fluctuation in HAM/TSP, we measured proviral loads of serially taken PBMCs from patients with HAM/TSP on long-term follow-up and compared the loads with their clinical manifestations.

The site of the lesion in HAM/TSP is the central nervous system (CNS) compartment. The presence of HTLV-I–infected cells in the spinal cord lesions has been reported (Kubota *et al*, 1994; Hara *et al*, 1994; Moritoyo *et al*, 1996; Matsuoka *et al*, 1998; Aye *et al*, 2000), and this has been thought to have an important role in the pathogenesis of the disease (Ijichi *et al*, 1993; Izumo *et al*, 1997). On the other hand, it has been reported that neopterin concentration or HTLV-I antibody titer in cerebrospinal fluid (CSF) are associated with disease progression (Nakagawa *et al*, 1995). HTLV-I–infected cells have also been detected in CSF, and the ratio of HTLV-I-tax–positive cells/total mononuclear cells were higher in CSF than in peripheral blood (Moritoyo *et al*, 1999). These facts suggest that HTLV-I proviral load in CSF cells correlates more with the clinical manifestation than that in PBMCs. In order to clarify this point, we also measured proviral loads in CSF cells and analyzed the relationship among the proviral loads in CSF cells, those in PBMCs, and their clinical manifestations.

Results

Long-term follow-up of proviral load in PBMCs

Although there was a wide distribution of the proviral load, from 0.3 to 37.8 copies/100 PBMCs, the proviral load in individual patients was relatively stable during the course of the disease. However, 22 (71%) out of 31 patients showed a temporary increase in the proviral load of more than twofolds (Figure 1**A**). Concerning the change of motor disability, 12 (39%) out of 31 patients showed worsening of more than 1 grade on Osame's motor disability grading during follow up. Ten (83%) of them showed an increase of proviral load accompanied by worsening of their motor disability. The increase of the proviral load occurred at the time of, or preceding, clinical worsening, and the increase either continued for several years ($n = 6$) (Figure 1**B**) or only temporarily ($n = 4$) (Figure 1**C**). In two patients, the proviral load decreased and the motor disability improved with steroid therapy (Figure 1**D**). There was no significant correlation between motor disability and proviral load at the time of first sampling (Spearman $r = \approx.159$, $P = .383$) as well as the time of last sampling (Spearman $r = \approx.230$, $P = .207$).

Proviral load in CSF cells and PBMCs

We detected HTLV-I provirus in all samples of CSF cells. The proviral loads in CSF cells were clearly higher than those in PBMCs in all patients. In 11 out of 14 patients, the proviral load in CSF cells was more than two times that in PBMCs (Figure 2**A**). These 11 patients had less than 15 years' duration of illness. On the other hand, the duration of illness was more than 25 years in the three other patients with less than two times the proviral load in CSF cells. There was a significant correlation between the duration of illness and the ratio of proviral load in CSF cells/in PBMCs by exponential regression analysis ($P = .011$) (Figure 2**D**). However, the duration of illness did not significantly correlate with the proviral load in CSF cells (regression analysis, not significant; Spearman $r = .090$, $P = .745$) (Figure 2**C**), nor the proviral load in PBMCs (regression analysis, not significant; Spearman $r = .281$, $P = .310$) (Figure 2**B**). The ratio of proviral load in CSF cells to that in PBMCs increased in cases in the progressive stage of the disease at the time of sampling (Mann-Whitney $U = 3.00$, $P = .006$) (Figure 3**C**). However, the disease progression of HAM/TSP did not significantly correlate with the proviral load in CSF cells (Mann-Whitney $U = 22.0$, $P = .749$) (Figure 3**B**) or in PBMCs (Mann-Whitney $U = 10.0$, $P = .064$) (Figure 3**A**). There was no significant correlation between the motor disability and either the proviral load in CSF cells (Kruskal-Wallis test, $P = .898$), the proviral load in PBMCs (Kruskal-Wallis test, $P = .898$), or the ratio of proviral load in CSF cells to that in PBMCs (Kruskal-Wallis test, $P = .095$) (Figure 3**D**).

Discussion

High HTLV-I proviral load in PBMCs has been reported in patients with HAM/TSP (Nagai *et al*, 1998; Jeffery *et al*, 1999) and the proviral load has been reported to fluctuate in individual patients during the course of the disease (Kubota *et al*, 1993). Little is known, however, about the role of high proviral loads and its fluctuation in the pathogenesis of HAM/TSP. To clarify this point, we measured the proviral load of serially taken PBMCs from patients with HAM/TSP on long-term follow-up and compared the loads with their clinical manifestations. In the present study, we demonstrated that the proviral load in individual

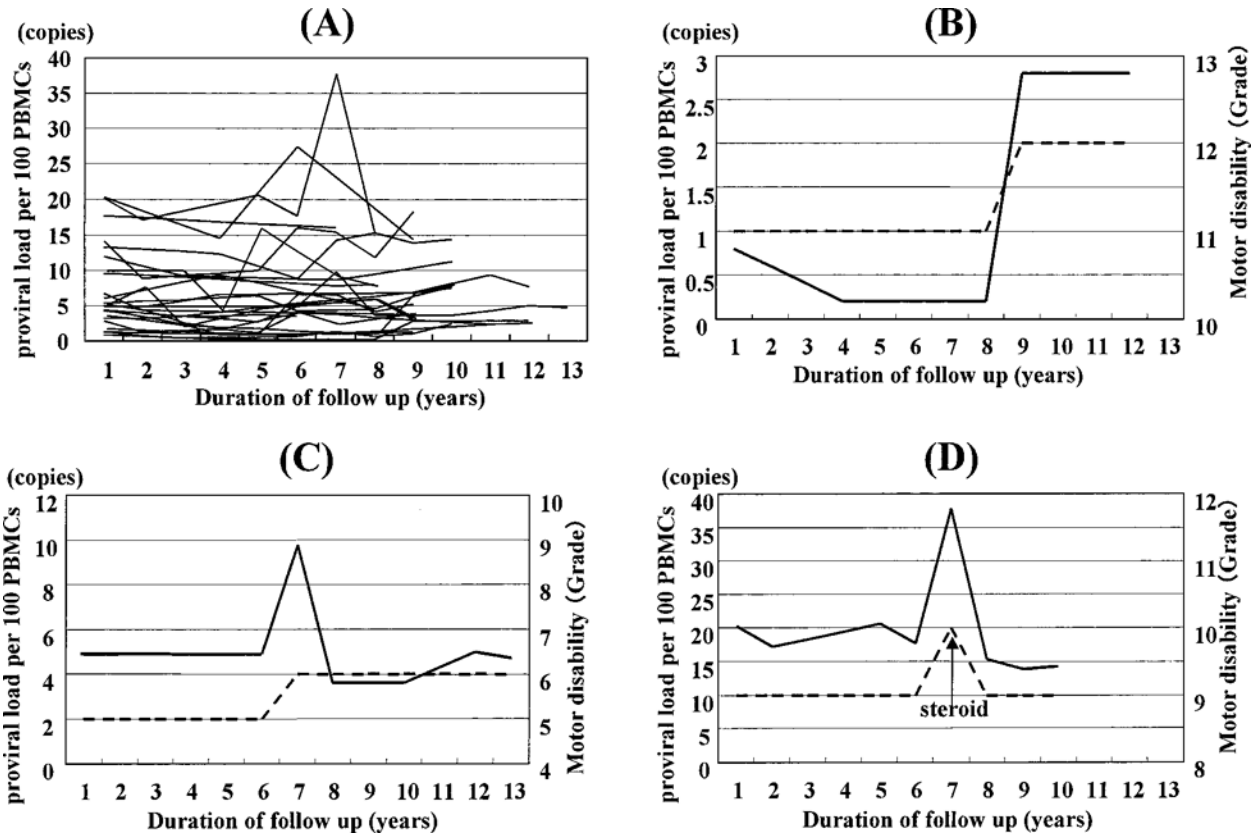

Figure 1 (**A**) Time course of HTLV-I proviral load per 100 PBMCs in all patients with HAM/TSP ($n = 31$). (**B**, **C**, **D**) Time course of proviral loads per 100 PBMCs and grades of motor disability in representative patients with HAM/TSP. (**B**) Increase of the proviral load occurred at the time point of, or preceding, clinical worsening, and the increase continued for several years ($n = 6$). (**C**) Increase of the proviral load occurred at the time point of, or preceding, clinical worsening, and the increase was temporal ($n = 4$). (**D**) Time course of proviral loads per 100 PBMCs and grades of motor disability in patients with steroid therapy ($n = 2$). The proviral load decreased and the motor disability improved after steroid therapy. (Solid line—: proviral load in 100 PBMCs; dashed line- - -: Grades of motor disability.)

patients was relatively stable during the course of the disease. The patient with a high proviral load in the early stage of the disease also showed a high proviral load in the late stage, and the patient with a low proviral load initially had a low proviral load during the course of the disease. This means that there may be a set-point level of proviral load in each patient with HAM/TSP. Both viral factors (Renjifo *et al*, 1996; Nakane *et al*, 2000; Furukawa *et al*, 2000) and host immune responsiveness (Jeffery *et al*, 1999, 2000; Nakamura, 2000) have been suspected to influence the proviral load. There is, however, no clear answer to the question of why the level of proviral load differs among individual patients. The level of the proviral load must therefore be determined by the relationship between the viral expression and the immune response against the virus. It is probable that an increase of HTLV-I–infected cells and their elimination by the immune response might reach dynamic equilibrium as is known in the case of human immunodeficiency virus (HIV)-1 infection (Perelson *et al*, 1996). Whether or not this kind of set-point level is also present in the asymptomatic HTLV-I carrier should also be examined.

Another important finding demonstrated in this study was a relationship between clinical worsening and temporary increase of the proviral load in some patients with HAM/TSP. The proviral load decreased with clinical improvement after steroid therapy. However, we could not get a statistically significant result among the whole patients, because there were some patients who showed apparent changes in the proviral load without any change in their motor disability grades. One possible reason is that there might indeed be some changes in the motor disability; however, our retrospective survey of clinical changes was not sensitive enough to detect such slight changes. Therefore, it is necessary to apply a more detailed grading of motor disability in order to study the precise relationship between changes in the proviral load and that of clinical manifestation. It is reasonable to suspect that a relationship exists between the increase in the proviral load and worsening of the disease, because an increase of HTLV-I–infected cells in the blood stream would mean an increase in the probability of these cells to invade into the spinal cord and to trigger inflammation. We have no clear idea why the proviral

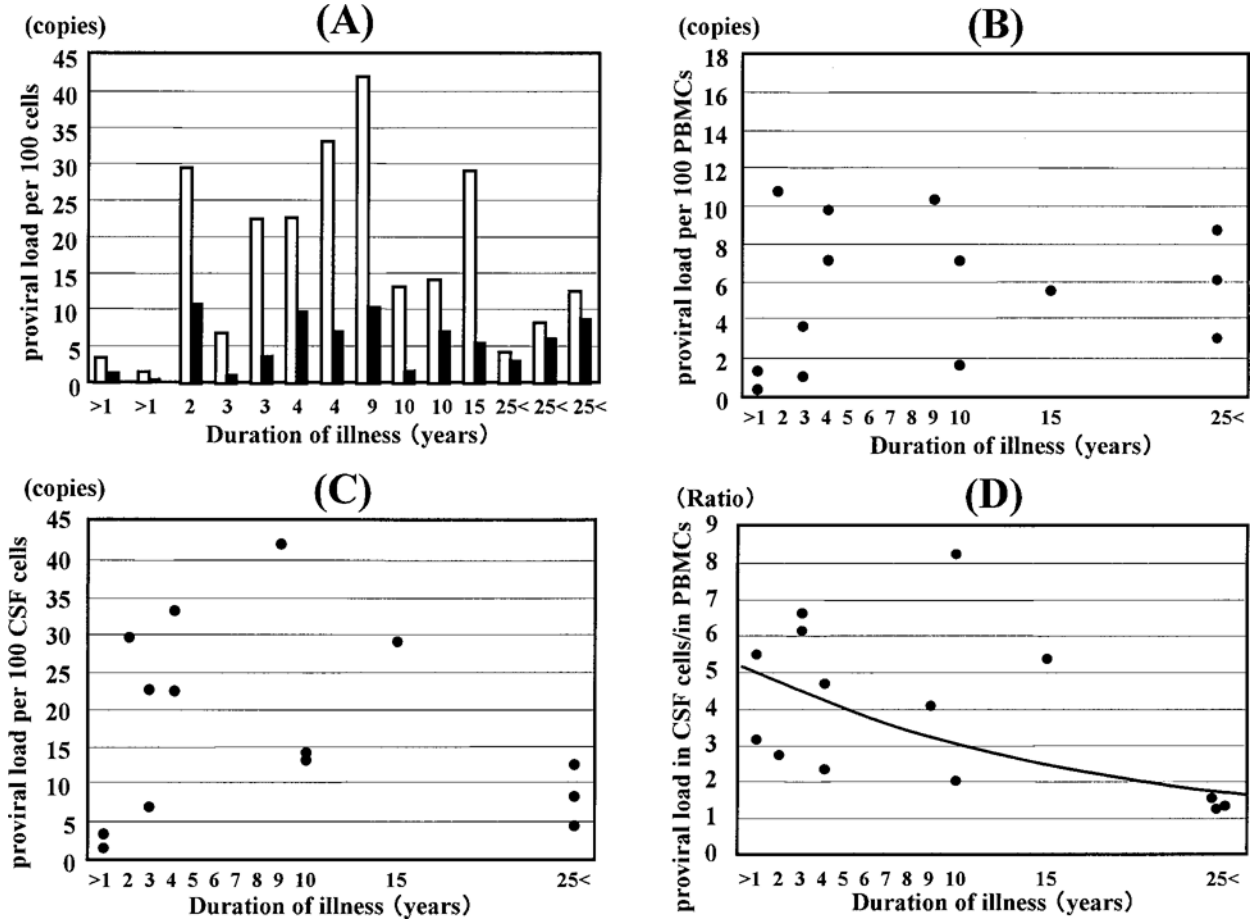

Figure 2 (**A**) Relationship between HTLV-I proviral load in CSF cells, those in PBMCs, and duration of illness. (Open bar ■: proviral load in CSF cells; solid bar ■: Proviral load in PBMCs.) (**B**) Relationship between HTLV-I proviral load in PBMCs and duration of illness. (**C**) Relationship between HTLV-I proviral load in CSF cells and duration of illness. (**D**) Relationship between the ratio of proviral load in CSF cells/in PBMCs and duration of illness. Statistical significance was obtained only in **D** by exponential regression ($P = .011$).

load increases temporarily during the course of the disease.

In HAM/TSP, HTLV-I–infected cells in the CNS compartment have been thought to have an important role in the pathogenesis of the disease. This suggests that HTLV-I proviral load in the CSF correlates more with clinical manifestation than that in PBMCs. Our present study clearly demonstrated that HTLV-I proviral load increases in the CNS compartment, the site of lesion in HAM/TSP. The higher proviral load in CSF cells than in PBMCs suggests that HTLV-I–infected cells have a high ability of invading and/or proliferating in the CNS compartment. It has been reported that HTLV-I–infected cells have activated adhesion molecules (Umehara *et al*, 1996; Al-Fahim *et al*, 1999; Kambara *et al*, 1999; Matsuoka *et al*, 2000), and endothelial cells in the spinal cord of the patients also have activated adhesion molecules (Umehara *et al*, 1996; Romero *et al*, 2000). These previous reports support the hypothesis that HTLV-I–infected cells have a high ability of invasion into the CNS compartment. We need to further study whether or not HTLV-I–infected cells have a high ability of proliferation in the CNS compartment. Though this high proviral load might be explained by the change of lymphocyte subsets, the CD4+/CD8+ ratio in CSF did not differ significantly compared to that of PBMCs as previously reported (Mori *et al*, 1988; Ijichi *et al*, 1989; Nagai *et al*, 2001). The ratio of proviral loads in CSF cells/in PBMCs was more elevated in patients in the progressive stage compared to those in the stable stage, and also in patients with shorter duration of illness. These suggest that the proviral load in CSF cells increases at the onset of the disease or in accordance with clinical worsening, and decreases after a long-standing stable state.

It has been reported that there was no significant correlation between motor disability and the proviral load in PBMCs (Nagai *et al*, 1998). In this study, we also could not show the correlation between the motor disability and the proviral load in CSF cells as well as in PBMCs.

Some patient with HAM/TSP has been reported to respond to treatments such as steroids, plasmapheresis, and interferon-μ (Matsuo *et al*, 1989; Osame *et al*, 1990; Nakagawa *et al*, 1996; Izumo *et al*, 1996);

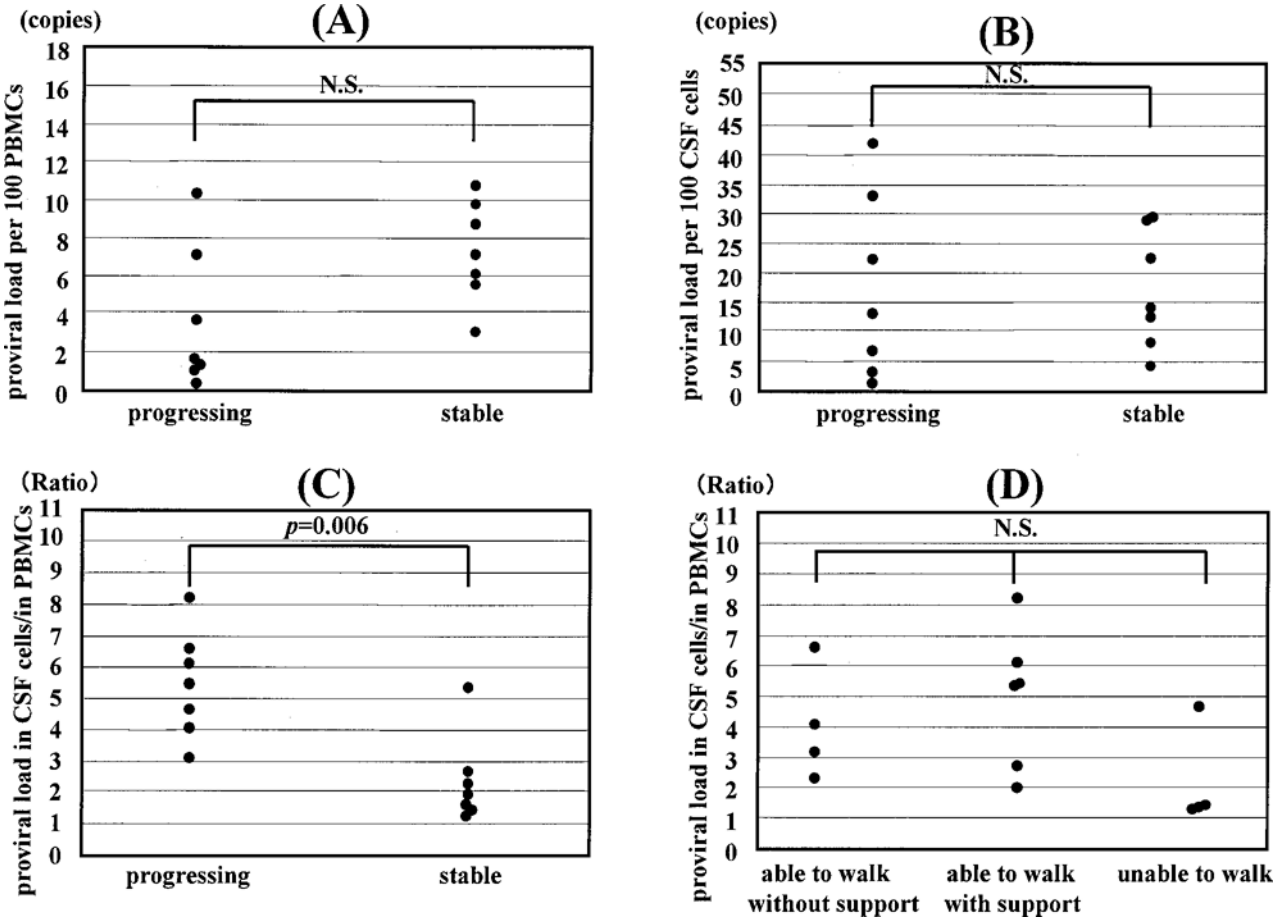

Figure 3 (**A**) Relationship between HTLV-I proviral loads in PBMCs and progression of the disease (Mann-Whitney $U = 10.0$, $P = .064$). (**B**) Relationship between HTLV-I proviral loads in CSF cells and progression of the disease (Mann-Whitney $U = 22.0$, $P = .749$). (**C**) Relationship between the ratio of proviral load in CSF cells/in PBMCs and progression of the disease (Mann-Whitney $U = 3.00$, $P = .006$). (**D**) Relationship between the ratio of proviral load in CSF cells/in PBMCs and motor disability (Kruskal-Wallis test, $P = .095$).

however, it is difficult to get a good response to treatment when the disease activity has diminished. Therefore, it is necessary to determine disease activity for the proper timing of treatments. In this study, we demonstrated a correlation between disease progression and increase of the proviral load in PBMCs and/or CSF cells in patients with HAM/TSP. This may indicate that long-term monitoring of the proviral load both in PBMCs and in CSF cells is useful for estimating disease progression and for further planning of appropriate treatment of individual patients with HAM/TSP.

Materials and methods

Sampling for long-term follow-up of proviral loads in PBMCs

PBMCs were serially collected and preserved from 378 patients, 113 males (age, $58.9 \approx 11.3SD$) and 265 females (age, $58.6 \approx 11.7SD$), who were diagnosed as HAM/TSP and followed-up in Kagoshima University Hospital from November 1985 to July 2001. Among these patients, there were 130 patients who have been followed-up for more than 4 years. The patients whose PBMCs were preserved more than four times were 31 of these 130 patients. We investigated all 31 patients, 11 males (age, $60.2 \approx 10.6SD$) and 20 females (age, $53.3 \approx 10.8SD$), in this study.

Sampling for proviral load analysis of CSF cells

CSF cells and PBMCs were taken at the same time from 14 patients with HAM/TSP, 2 males (age, $59.5 \approx 6.4SD$) and 12 females (age, $52.5 \approx 11.3SD$), who were admitted in Kagoshima University Hospital from May 2000 to July 2001.

Clinical evaluations of the patients

We retrospectively evaluated their duration of illness and motor disability at the time of sampling. The grades of motor disability were evaluated by using Osame's motor disability grading (Osame *et al*, 1990). Disease activity of HAM/TSP was also evaluated in 14 patients who were registered for proviral load analysis of CSF cells. These patients were divided into two groups according to the following: "progressing," patients who showed clinical worsening

of more than 1 grade on Osame's motor disability grading at the time of sampling since the last clinic visit ($n = 7$), and "stable," patients who did not show clinical worsening at the time of sampling, or who showed clinical worsening apparently caused by factors other than HAM/TSP, such as compression fracture of the spine ($n = 7$). These 14 patients were also divided into three groups based on the motor disability as follows: able to walk without any support ($n = 4$), able to walk with support ($n = 6$), and unable to walk ($n = 4$).

DNA extraction and quantitative PCR

We extracted DNA from these samples by using column extraction (QIAamp DNA Blood Mini Kit; QIAGEN, Germany). The concentration of extracted DNA was adjusted to 10 ng/μl for the working solution. Standard curve material for HTLV-I pX consisted of extracted DNA from HTLV-I infected rat T-cell line, TARL-2, which has a single copy of HTLV-I proviral DNA (Tateno, 1987). We assumed that 1 ng of DNA contains 167 copies of pX gene. The extracted DNAs were serially diluted to 10, $1 \approx 10^2$, $1 \approx 10^3$, $1 \approx 10^4$ copies per 10 μl for pX. We measured proviral load by using TaqMan PCR method targeting the pX region of HTLV-I (ABI PRISM 7700 Sequence Detection System; Perkin Elmer Applied Biosystems, USA). Polymerase chain reaction (PCR) for HTLV-I provirus was performed using the protocol of our previous report (Takenouchi *et al*, 1999), with some modification. The primer set for HTLV-I pX region was 5′-ACAAAGTTAACCATGCTTATTATCAGC-3′ and 5′-ACACGTAGACTGGGTTATCCGAA-3′. PCR for μ-actin gene was also performed simultaneously to calculate the proviral load per 100 PBMCs or per 100 CSF cells. The primer set for μ-actin was 5′-TCACCCACACACTGTGCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′. The TaqMan probe consists of an oligonucleotide with a 5′-reporter dye and 3′-quencher dye. The fluorescent reporter dye, FAM (6-carboxyfluorescein), is covalently linked to the 5′ end of the nucleotide. The reporter is quenched by TAMRA (6-carboxytetramethylrhodamine), at the 3′ end. The probe for HTLV-I pX region was 5′-TTCCCAGGGTTTGGACAGAGTCTTCT-3′ and for μ-actin was 5′-ATGCCCTCCCCCATGCCATCCTGCGT-3′. Ten microliters of DNA solution were added to 40 μl of reaction mixture, containing 5 μl of PCR buffer (10 X TaqMan PCR buffer; Perkin Elmer Applied Biosystems, USA), 3.5 mM $MgCl_2$, 0.3 μM each primer, 0.2 μM of TaqMan probe, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 400 μM dUTP, 0.5 U of uracil-*N*-glycosylase, and 1.25 U of Taq polymerase (AmpliTaq Gold; Perkin Elmer Applied Biosystems, USA). The thermal cycler conditions were as follows: 50℃ for 2 min, 95℃ for 10 min, and then 45 cycles of 95℃ for 15 s, 58℃ for 1 min in both case of pX and μ-actin. The HTLV-I proviral load was calculated by the following formula: copy number of HTLV-I provirus per 100 cells = [(copy number of pX)/(copy number of μ-actin/2)] $\approx$ 100.

Statistical analysis

We performed statistical analysis for comparisons among the clinical manifestations, the proviral loads in PBMCs, those in CSF cells, and the ratio of proviral loads in CSF cells/in PBMCs. We used the ratio to reduce effects of a passive increase of proviral load in CSF according to increase of proviral load in PBMC. The significance of differences was determined by Spearman *r* test, regression analysis, Mann-Whitney *U* test, or Kruskal-Wallis test. We set the level of statistical significance for *P* value at less than .05.

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