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Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP

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In order to examine the effect of HTLV-I proviral load on the pathogenesis of HAM/TSP, we measured the HTLV-I proviral load in peripheral blood mononuclear cells (PBMC) from a large number of HAM/TSP patients and asymptomatic HTLV-I carriers. To measure the proviral load, we used an accurate and reproducible quantitative PCR method using a dual-labeled fluorogenic probe (ABI PRISM 7700 Sequence Detection System). The mean ± standard error of mean (s.e.m.) HTLV-I proviral copy number per 1×10^4 PBMC was 798 ± 51 (median 544) in 202 HAM/TSP patients; 120 ± 17 (median 34) in 200 non HAM-related (general) asymptomatic HTLV-I carriers (RC); and 496 ± 82 (median 321) in 43 asymptomatic HTLV-I carriers genetically related to HAM/TSP patients (FA). The prevalence of HAM/TSP rises exponentially with log (proviral load) once the proviral load exceeds 1% PBMC. The HTLV-I proviral load of female patients with HAM/TSP was significantly higher than that of male patients, however there was no significant difference in proviral load between sexes in RC. There was a significant correlation between the proviral load and the concentration of neopterin in CSF of HAM/TSP patients. These results indicate that the HTLV-I proviral load in PBMC may be related to the inflammatory process in the spinal cord lesion. The increased proviral load in FA suggests the existence of genetic factors contributing to the replication of HTLV-I *in vivo*.

Keywords: human T-cell lymphotropic virus type I (HTLV-I); HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP); asymptomatic carrier; proviral load; quantitative PCR

Introduction

Human T-cell lymphotropic virus type I (HTLV-I) is an agent causing HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al*, 1985; Osame *et al*, 1986). Endemic areas of HTLV-I exist in southern Japan, the Caribbean, Central and South America, and Africa. Furthermore, high HTLV-I prevalence was found in intravenous drug users and patients with sexually transmitted diseases especially among African Americans and Hispanics in USA (Khabbaz *et al*, 1992). Since estimated prevalence of HAM/TSP is less than 1% of HTLV-I-infected individuals (Kaplan *et al*, 1990), most HTLV-I carriers remain asymptomatic lifelong. This shows HTLV-I infection alone is not sufficient to cause HAM/TSP, but occurrence of HAM/TSP may require additional factors such as the virus-host interaction (Hollsberg and Hafler, 1995) and difference in the HTLV-I proviral load.

It has previously been demonstrated that the replication of HTLV-I is increased in HAM/TSP patients as compared with asymptomatic HTLV-I carriers (Yoshida *et al*, 1989; Gessain *et al*, 1990; Kira *et al*, 1991; Kubota *et al*, 1993). However the reported sample number and accuracy of quantifi-

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cation using polymerase chain reaction (PCR) were not sufficient to allow firm conclusions to allow the risk of HAM/TSP to be correlated with the proviral load. Spontaneous proliferation of peripheral blood mononuclear cells (PBMC) from HAM/TSP patients, polyclonal lymphocyte proliferation *in vitro* (Usuku *et al*, 1998; Jacobson *et al*, 1988; Itoyama *et al*, 1988; Eiraku *et al*, 1992; Machigashira *et al*, 1997), an increased humoral and cellular immune responses against HTLV-I (Osame *et al*, 1987; Jacobson *et al*, 1990; Parker *et al*, 1992; Jacobson, 1996) suggested that increased replication of HTLV-I and immune response to HTLV-I associated with pathogenesis of HAM/TSP. It was also expected that analysis of HTLV-I proviral load might provide a useful tool to predict clinical outcome and assess the efficacy of treatment as observed in human immunodeficiency virus (HIV) infection. The plasma RNA level (viral load) has been found to be a valid predicator of the rate of clinical progression of HIV-related disease and reported to be useful to assess the efficacy of antiretroviral drugs (O'Brien *et al*, 1996; Saag *et al*, 1996; Mellors *et al*, 1997).

In this report, we adopted an accurate and reproducible quantitative PCR method (Heid *et al*, 1996) and measured the amount of HTLV-I proviral DNA in PBMC of a large number of HAM/TSP patients and asymptomatic HTLV-I carriers in order to clarify the influence of HTLV-I proviral load on the pathogenesis of HAM/TSP. We obtained evidence that the median proviral load in PBMC of HAM/TSP patients was about 16-fold higher than that of general asymptomatic HTLV-I carriers. In addition, we found that the proviral load of asymptomatic HTLV-I carriers in family members of HAM/TSP patients is higher than that of non HAM/related (general) asymptomatic HTLV-I carriers. The proviral load was independent of age at blood sampling, age at onset and duration of illness. However, HTLV-I proviral load was correlated with concentration of neopterin, a biochemical marker of inflammation, in CSF.

Results

Accuracy of quantitative PCR

Several DNA samples were prepared from the various mixtures of MT-2 cells and PBMC from a HTLV-I-seronegative healthy donor, and the HTLV-I proviral load was measured five times in each case. For estimating the accuracy of this quantitative PCR system, we calculated the coefficient of variance (CV%) in each copy number (Figure 1). The mean CV% was 25.8%. The lower limit of detection was one copy per 10^4 PBMC.

Quantification of HTLV-I proviral of HAM, FA, and RC and risk of HAM

As shown in Table 1, we estimated the absolute copy number of HTLV-I proviral DNA per 10^4 PBMC. The copy numbers in HAM varied from 1 to 2942, those of RC varied from ND (not detectable by this assay) to 2485, and those of FA varied from ND to 2079. The mean ± s.e.m. and median of the copy number was 798 ± 51 and 544 in HAM, and all HAM subjects had a proviral load above the lower limit of detection. As regards RC and FA, the values were 120 ± 17 (median 34), 496 ± 82 (median 321), respectively. The median copy number of HAM patients was about 16-fold higher than that of RC. It was noteworthy that the copy number in FA was significantly higher than that of RC. The differences were statistically significant between RC and HAM, and between RC and FA, respectively. ($P<0.01$, Kruskal-Wallis test and Scheffe's F test) (Figure 2). No RC donor was aged over 65 years. However there was a statistically significant difference among respective age matched groups (each decade) in HAM and RC (data not shown).

There was no statistically significant correlation between copy number of HTLV-I proviral DNA and

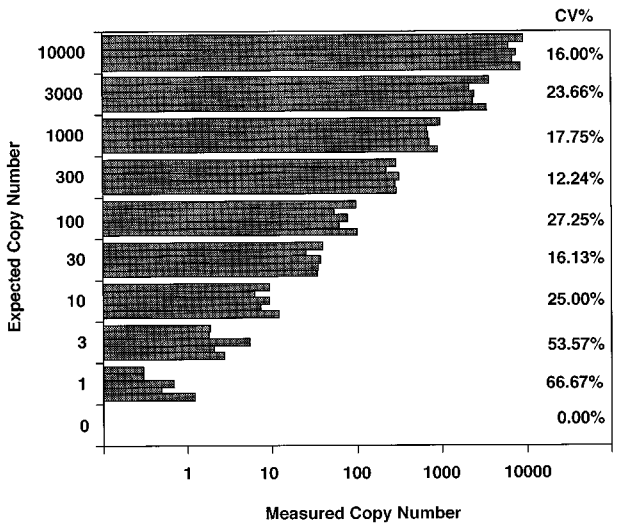

Figure 1 The expected copy number of HTLV-I proviral DNA was estimated by various mixture of MT-2 cells and PBMC from HTLV-I seronegative healthy donor. Each sample was assayed five times. The copy number indicates HTLV-I proviral load per 10^4 cells. CV% means (s.d. of the copy number in five assays / mean of the copy number in five assays) ×100.

Table 1 Quantification of HTLV-I proviral DNA.

Subjects	*Mean ± s.e.m.*	*Median*	*Range*
HAM (*n*=202)	798 ± 51	544	1 ~2942
FA (*n*=43)	496 ± 82	321	ND ~2079
RC (*n*=200)	120 ± 17	34	ND ~2485

HTLV-I copy number per 10^4 PMBC was represented. *n*=number of subjects. HAM=patients with HAM. FA=asymptomatic HTLV-I carriers in genetic relatives of patients with HAM. RC=non HAM-related (general) asymptomatic HTLV-I carriers. ND=not detectable by this assay.

age at blood sampling in HAM ($r=-0.096$, $P=0.22$) and RC ($r=0.081$, $P=0.25$, Spearman's rank correlation) (Figure 3).

The amount of HTLV-I proviral DNA of female patients with HAM (mean $\pm$ s.e.m. 849 ± 60, median 615) was significantly higher than that of male patients (678 ± 91, 421) ($P<0.05$, Mann-Whitney *U*-test), however there was no significant difference in proviral load between the sexes in RC (data not shown).

The distribution of proviral load (Figure 2) in HAM patients and asymptomatic HTLV-I carriers (RC) can be used to estimate, by Bayes' theorem, the risk (i.e. the prevalence) of HAM at a given proviral load. Proviral loads under 1% (100 copies per 1×10^4 PBMC) appear to have little influence on the risk of HAM (Figure 4A); above this apparent threshold, the risk of HAM rises exponentially (Figure 4B).

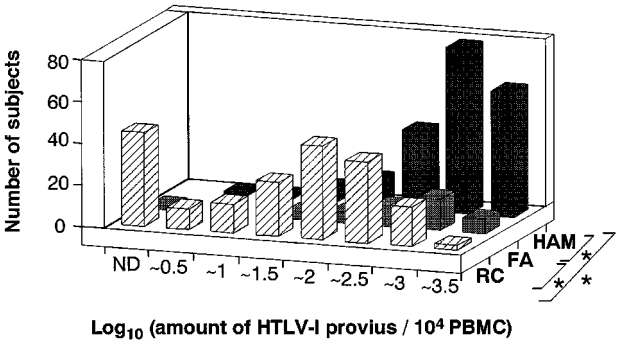

Figure 2 Distribution pattern of HTLV-I proviral DNA in PBMC. HTLV-I copy number per 10^4 PBMC was represented on a logarithmic scale. ND represents not detectable by this assay. * Means *P* value <0.01 by Scheffe's F.

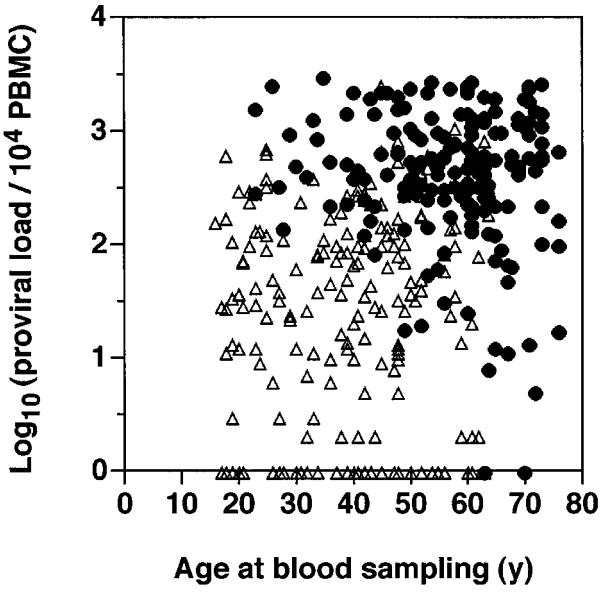

Figure 3 Correlation of HTLV-I proviral load with age at blood sampling in HAM and RC. Black circles indicate HTLV-I proviral load of HAM patients and white triangles indicate HTLV-I proviral load of RC donors. HTLV-I copy number per 10^4 PBMC was represented on a logarithmic scale.

Relation of HTLV-I proviral DNA load to clinical status in HAM

There was no statistically significant correlation between the copy number of HTLV-I proviral DNA and either age at onset or the duration of illness in HAM (Figure 5). However some patients with ages at onset after 60 years old showed low amount of HTLV-I proviral DNA. The amounts of HTLV-I proviral DNA were not significantly different between patients with a past history of blood transfusion and patients without it (data not shown).

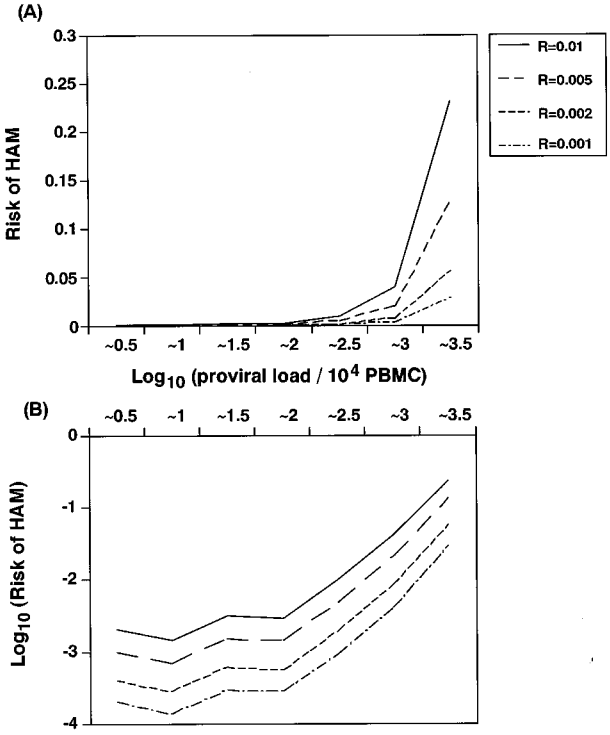

Figure 4 Estimation of risk of HAM at different proviral loads. By Bayes' theorem, using the conventional notation for conditional probability:

$$p(HAM|L) = p(HAM) \times p(L|HAM)/(p(HAM) \times p(L|HAM) + p(RC) \times p(L|RC))$$

Where p(HAM)=R=rate of HAM in the HTLV-In positive population as a whole, and p(L|HAM)=proportion of HAM patients with a given load (L). R is unknown but estimated to lie between 0.001 and 0.01 (Osame *et al*, 1990b); four curves are therefore shown, at different values of R. Whatever the prevalence (R) of HAM in the HTLV-I population, the risk of HAM rises sharply at high proviral loads (**A**). The risk rises exponentially above an apparent threshold load of 1% PBMC (**B**).

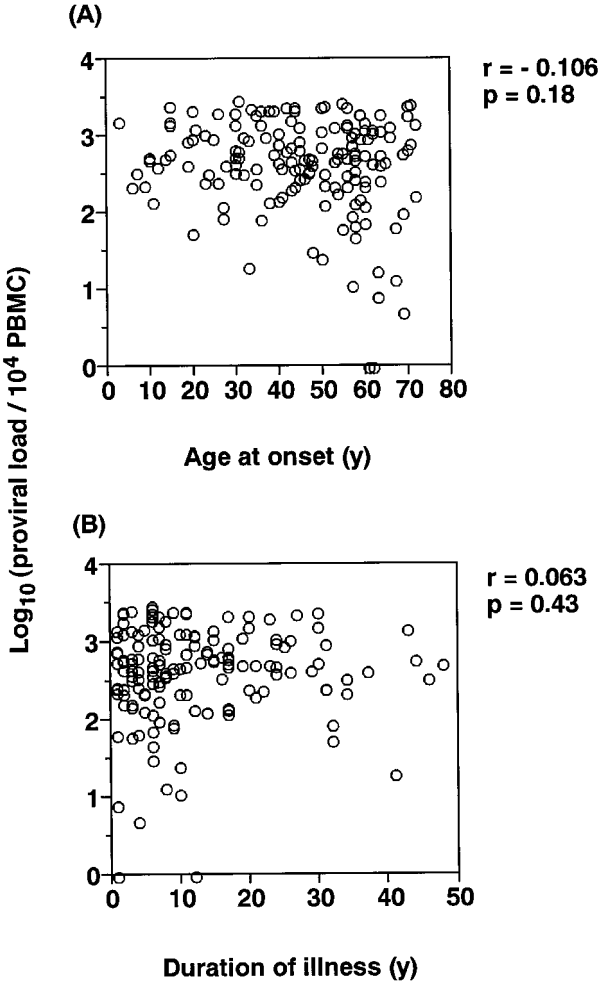

Figure 5 Correlation of HTLV-I proviral load to clinical status in HAM: (**A**) age at onset and (**B**) duration of illness. HTLV-I copy number per 10 ^{4} PBMC was represented on a logarithmic scale. There was no statistically significant correlation in either case.

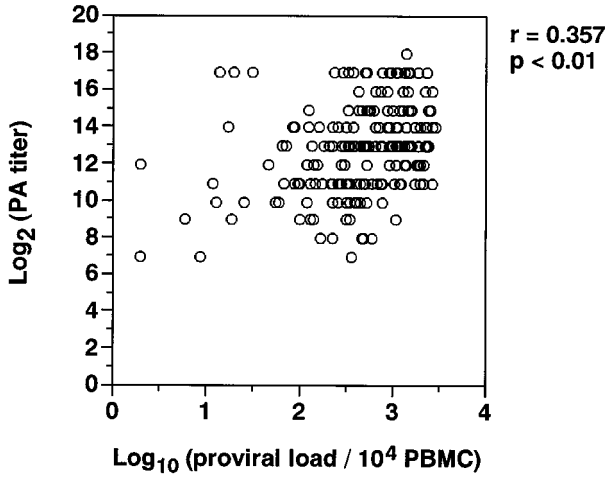

Figure 6 Correlation between HTLV-I proviral load and titer of anti-HTLV-I antibody in sera measured by PA method. HTLV-I copy number per 10^4 PBMC and titer of antibody were represented on a logarithmic scale.

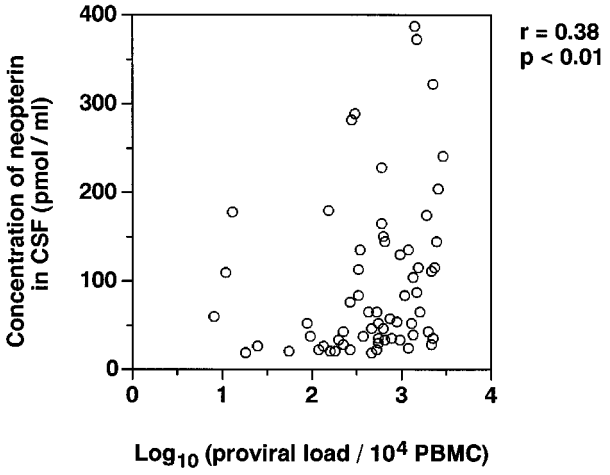

Figure 7 Correlation between HTLV-I proviral load in PBMC and neopterin concentration in CSF. HTLV-I copy number per 10^4 PBMC was represented on a logarithmic scale. Normal value of neopterin in CSF was below 30 pmol/ml.

Correlation between HTLV-I proviral DNA load and laboratory findings

There was a significant correlation between the amount of HTLV-I proviral DNA and antibody titer in sera from HAM patients (r=0.357, $P<0.01$, Spearman's rank correlation) (Figure 6). This significant correlation was also observed in RC (data not shown). The amount of HTLV-I proviral DNA significantly correlated with the concentration of neopterin in CSF of HAM (r=0.38, $P<0.01$, Spearman's rank correlation) (Figure 7).

Discussion

It has previously been reported that the replication of HTLV-I was increased in HAM patients as compared with asymptomatic HTLV-I carriers using Southern blot analysis and quantitative PCR method (Yoshida *et al*, 1989; Gessain *et al*, 1990; Kira *et al*, 1991; Kubota *et al*, 1993). These observations suggested that increased replication of HTLV-I was associated with pathogenesis of HAM. In HIV-1 infection, plasma HIV-1 RNA amount (viral load) is one of highly predictive markers of both progression to AIDS and death. HIV viral load decreases rapidly after initiation of effective antiretroviral therapy, so it provides a valuable tool for the management of individual patients (O'Brien *et al*, 1996; Saag *et al*, 1996; Mellors *et al*, 1997).

In this study, we measured HTLV-I proviral load in a large number of subjects using a precise quantitative PCR method (Figure 1) and confirmed increased load of HTLV-I provirus in HAM patients. The estimated median HTLV-I proviral copy number of HAM patients was about 16-fold higher than those of asymptomatic carriers (RC) (Table 1 and Figure 2). Kubota *et al*, (1993) previously reported that HTLV-I proviral load was 2–20 copies per 100 PBMC of HAM patients (n=6) using quantitative PCR, and then median was 14-fold higher than those of asymptomatic HTLV-I carriers (n=8). Hashimoto *et al*, (1998) recently reported that HAM patients (n=10) had median 5.6% HTLV-I positive cells, and asymptomatic HTLV-I carriers (n=3) had median 1.1% HTLV-I positive cells in PBMC detecting by *in situ* PCR method. These results are consistent with our estimation. We also examined HTLV-I proviral load in asymptomatic HTLV-I carriers in families of HAM/TSP patients (Table 1 and Figure 2). The HTLV-I median proviral load of the genetic relatives of HAM patients (FA) was about nine-fold higher than those of none-HAM related asymptomatic carriers (RC). This observation is the first report as far as we know. Furukawa *et al* (1992) previously examined the frequency of detection of the integrated HTLV-I proviral DNA in PBMC of HAM families using Southern blot analysis. Although absolute copy numbers of HTLV-I proviral DNA were not measured, the frequency of detection in HAM families was significantly higher than that among general asymptomatic carriers. Our results are therefore consistent with this finding. An increased proviral load observed in FA suggests that genetic factors, such as HLA (Usuku *et al*, 1988) may be related to the high proviral load of genetic relatives.

The HTLV-I proviral load of female patients with HAM was significantly higher than that of male patients. Although the reason for this difference remains unknown, the difference corresponds with the epidemiological observation that the ratio of male to female HAM patients is about 1 : 2. Interestingly, it has been reported that danazol which is an attenuated androgen (male hormone) was useful agent in the management of HAM (Harrington *et al*, 1991; Melo *et al*, 1992).

Lymphocytic infiltration in the spinal cord lesions in HAM has been demonstrated by immunohistological study (Izumo *et al*, 1992; Umehara *et al*, 1993). High values of neopterin, which is a valuable biochemical marker of inflammation and the degree of T-cell activation in the central nervous system (CNS), were reported in HAM patients previously (Nomoto *et al*, 1991; Ali *et al*, 1992). These results suggested that HAM was a chronic inflammatory disorder which could be mediated by continuous activation of inflammatory cells in the CNS (Ijichi *et al*, 1993). Our present findings that significant correlation between HTLV-I proviral load of PBMC and CSF neopterin concentrations (Figure 7) supported the idea that proviral load influences the degree of inflammation in the spinal cord lesion. It is postulated that increased replication of HTLV-I may be a basic factor which enhances the incidence of subsequent unknown factors associated with pathogenesis; e.g., augmentation of adhesion molecules to spinal cord (Ichinose *et al*, 1992; 1994; Dhawan *et al*, 1993, propagation of activated T cells or autoreactive T cell (Sonoda *et al*, 1987; Nagai *et al*, 1996), and increased production of cytokines (Umehara *et al*, 1994). The strong, chronically activated anti-HTLV-I immune response, which appears to be in equilibrium with the high proviral load, may cause bystander damage to resident cells in the CNS, which are not themselves infected (Ijichi *et al*, 1993; Moritoyo *et al*, 1996; Matsuoka *et al*, 1998).

Although the increased replication of HTLV-I seems to associate with pathogenesis of HAM (Figure 4), the high proviral load itself is not necessary to cause HAM in some patients, since small number of patients (less than 10%), particularly those with ages at onset after 60 years old, had low proviral load (Figure 5). There are two possible explanations of these findings. First, disturbance of the immune system associated with aging may predispose to HAM regardless of low proviral load. Second, vulnerability of tissue associated with aging may decrease the threshold level against inflammation. It is also controversial whether HTLV-I proviral load transiently increases at early phase of onset. To resolve this point, it is necessary to follow up asymptomatic HTLV-I carriers until they develop HAM (i.e., prospective study).

In summary, we have determined the load of HTLV-I proviral DNA in a large number of HAM patients and asymptomatic HTLV-I carriers. The proviral load of HAM was substantially higher than that of asymptomatic HTLV-I carriers. It appears that proviral load is profoundly related to the pathogenesis of HAM, and influences the development and progression of HAM. Based on the knowledge about proviral load, further detailed analysis should enable us to predict the risk of progression to HAM from asymptomatic HTLV-I carriers and prognosis of HAM. It is also expected that the analysis of proviral load will be useful to evaluated the effect of treatment for HAM.

Materials and methods

Subject

Study populations consisted of 202 patients with HAM/TSP (HAM), 43 asymptomatic HTLV-I carriers in families of the HAM/TSP patients (FA) and 200 HTLV-I asymptomatic healthy carriers (RC) selected randomly from blood donors of Kagoshima Red Cross Blood Center, residing in Kagoshima,

Japan. FA consisted of 43 genetic relatives of HAM/TSP patients. The diagnosis of HAM/TSP was done according to WHO diagnosis guidelines (Osame, 1990a). Sex and ages of subjects were as follows: HAM group, 61 males and 141 females, 23–74 (mean 57) years old; FA group, 14 males and 29 females, 25–82 (mean 50) years old; RC group, 93 males and 107 females, 16–64 (mean 40) years old. The titer of anti-HTLV-I antibody was measured using particle-agglutination (PA) test (Serodia auto HTLV-I; Fujirebio, Japan). All samples were taken under informed consent.

Isolation and cryopreservation of PBMC and DNA extraction

PBMC were isolated from either heparinized peripheral blood (HAM and FA) or peripheral blood withdrawn with the addition of acid-citrate-dextrose solution (RC) by Ficoll-Hypaque (Pharmacia Biotech, Sweden) density gradient centrifugation, previously reported (Nagai *et al*, 1996). The isolated PBMC were kept in liquid nitrogen until use.

Genomic DNA was extracted from the frozen PBMC by either a spin column procedure using QIAamp blood kit (QIAGEN, Germany) in HAM and FA or phenol-chloroform procedure in RC. The concentration of extracted DNA was adjusted to 10 ng/μl for working solution.

Generation of standard DNA

Standard curve material consisted of extracted DNA from HTLV-I negative PBMC for β-actin and TARL-2 for pX. As single PBMC has two copies of β-actin, we assumed that one nanogram of DNA contains to 333 copies of β-actin gene. TARL-2 is an HTLV-I-infected cell line, which has single copy of HTLV-I proviral DNA (Tateno, 1987). We assumed that one nanogram of DNA contains to 167 copies of pX gene. The DNAs extracted from HTLV-I negative PBMC and TARL-2 were serially diluted respectively. The dilution series represented 5×10^3, 1×10^4, 2×10^4, 4×10^4 copies per 10 μl for β-actin, and 10, 1×10^2, 1×10^3, 1×10^4,copies per 10 μl for pX.

Primers and probes

The primer set for HTLV-I pX region was 5′-CAAACCGTCAAGCACAGCTT-3′ positioned at 7140–7159 and 5′-TCTCCAAACACGTAGACTGGGT-3′ positioned at 7362–7341 (Nucleotide coordinates are numbered according to the HTLV-I reference sequence on the Genbank database). The primer set for β-actin was 5′-TCACCCACACTGTGCCCATCTACGA-3′ positioned at 2141–2165 and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′ postitioned at 2435–2411. The TaqMan probe consists of an oligonucleotide with a 5′-reporter dye and 3′-quencher dye. The fluorescent reporter dye, FAM (6-carboxy-fluorescein), is covalently linked to the 5′ end of the oligonucleotide. The reporter is quenched by TAMRA (6-carboxy-tetramethyl-rhodamine), at the 3′ end. The probe for HTLV-I pX region was 5′-TTCCCAGGGTTTGGACAGAGTCTTCT-3′ positioned at 7307–7332 and for β-actin was 5′-ATGCCCTCCCCCATGCCATCCTGCGT-3′ positioned at 2171–2196.

Quantitative polymerase chain reaction

10 μl of DNA solution were added to 40 μl of reaction mixture, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM EDTA, 60 nM ROX (passive reference dye to normalize reporter signal) 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 (UNG) and 1.25 U of Taq polymerase (AmpliTaq Gold; Perkin Elmer Applied Biosystems, USA). AmpliTaq Gold is activated by heating and used for 'hot start' PCR. The use of UNG and dUTP instead of dTTP in the PCR is designed to minimize carryover from previous PCR products. The amplification of standard DNA and sample DNA was carried put on a 96-well reaction plate (Perkin Elmer Applied Biosystems, USA), and all samples were performed in triplicate. The standard DNA was always placed on a same plate. The 96-well reaction plate was placed in an ABI PRISM 7700 Sequence Detector (Perkin Elmer Applied Biosystems, USA). The thermal cycler conditions were as follows: 50°C for 2 min (for activation of UNG), 95°C for 10 min (for inactivation of UNG and activation of Taq polymerase), and then 45 cycles of 95°C for 15 s (denaturation), 58°C for 1 min (annealing and extension) in case of pX; or 40 cycles at 95°C for 15 s, at 60°C for 1 min in case of β-actin.

The 5′ nuclease activity of Taq polymerase cleaves a nonextendible hybridization probe during the extension phase of PCR. And then a specific fluorescent signal is generated and measured at each cycle. At any given cycle within the exponential phase of PCR, the amount of product was proportional to the initial number of template copies. The threshold cycle (C) was the cycle where the sequence detention application began to detect the increase in signal associated with exponential growth of PCR product. The C was dependent on the starting template copy number. Based on the C of four known concentrations, a standard curve was produced. The concentration of the unknown sample was determined by plotting the C on the standard curve.

The amount of HTLV-I proviral DNA was calculated by the following formula: copy number of HTLV-I (pX) per 1×10^4 PBMC=[(copy number of pX)/(copy number of β-actin/2)] $\times10^4$.

Clinical evaluation and laboratory data of patients with HAM

Clinical status, i.e. age at blood sampling, age at onset, and duration of illness were evaluated in the cases of HAM.

Anti-HTLV-I antibody was measured using PA test and CSF neopterin levels were measured by high-pressure liquid chromatography (HPLC) with fluorimetric detection as previously reported (Nomoto *et al*, 1991).

Statistical analysis
Mann-Whitney's *U*-test, Kruskal-Wallis test, Scheffe's F, and Spearman's rank correlation were used for statistical analysis. All the statistical manipulation were made on log-transformed proviral load data.

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