cause IL-10 mRNA expression was induced by HTLV-I Tax in both transiently and stably transfected Jurkat cells [28], it is likely that Tax directly transactivates the IL-10 promoter. The resulting overexpression of Tax in vivo may cause a Sjögren-like syndrome via an IL-10–mediated mechanism.

The implication of a heritable genetic basis for IL-10 production is supported by the concordance of IL-10 production in monozygotic twins, which suggests that genetic polymorphism could account for up to 75% of the observed variation in IL-10 production [29]. As was already mentioned, several studies have shown an association between particular polymorphisms in the human IL-10 promoter region and the outcome of certain viral infections, such as EBV [15], HBV [16], HCV [17], and HIV-1 [18]. In view of the immunomodulatory and anti-inflammatory effects of IL-10, we initially hypothesized that genetically determined lower production of IL-10 (associated with the allele −592 A) might influence disease susceptibility to HAM/TSP. This is the case for HIV-1 infection, because individuals with the IL-10 −592 AA genotype have been reported to be at higher risk of HIV-1 infection and rapid progression to AIDS [18]. In contrast, the present data show that, in HTLV-I infection, possession of the IL-10 −592 A allele prevented ~44.7% (SD, ±13.1%) of potential cases of HAM/TSP and was also a significant predictor for a lower provirus load in the entire cohort.

The −592 A/C SNP is located between the Sp1 and Ets binding site within the region between −652 and −571 nt that is necessary for IL-10 transcription [21]. It is of interest that previous reports have indicated that Tax transactivates the parathyroid hormone–related protein promoter by forming a ternary complex between Tax, Ets, and Sp-1, which acts on the promoter Sp-1 and Ets binding sites [30]. Another report showed that the HTLV-I LTR also contains a motif related to the Ets-binding sequence, named TRE-2S [31]. More important, 1 copy of the cyclic AMP response element (CRE)–like 21-bp sequence and TRE-2S in the HTLV-I LTR, contributes to the transactivation of viral gene via a ternary complex formed between Tax, Gli2 (TRE-S binding Gli oncogene family protein), and CRE-binding protein [32]. These findings suggest that a common mechanism of the HTLV-I Tax–mediated transactivation of the promoter of target genes ternary complexes formed with 2 different transcription factors. Furthermore, the results also suggest that the IL-10 promoter −592 A/C SNP, which lies between the Sp-1 and Ets binding sites, affects Tax-mediated transcription. Indeed, our cotransfection study using a Tax-expressing vector and Jurkat cells demonstrated that a IL-10 −592 luciferase vector carrying the high producer allele (C) showed higher Tax-mediated transcription than that of low producer allele (A), whereas a promoter fragment (fragment −571 to +120) that does not contain −592 SNP, as well as the neighboring Sp-1 and Ets binding site, was not transactivated by Tax. These findings suggested that HTLV-I Tax directly transactivates the IL-10 promoter and that the −592 A/C SNP affects Tax-induced transcription—that is, that the C allele is more effective than the A allele in mediating the Tax-induced transcription of IL-10. In future studies, it may be interesting to test whether Tax, Ets, and Sp-1 form a ternary complex on the IL-10 promoter and whether the −592 SNP affects this complex formation.

Among >90 non-HLA candidate gene loci that we have so far examined, the IL-10 −592 A/C SNP is the only non-HLA candidate gene locus associated with a significant reduction in both the provirus load and the risk of HAM/TSP. This observation is exactly analogous to the argument that we previously reported for HLA-A*02 and -Cw*08, where, in each case, possession of the allele was associated with both a significant reduction in provirus load in the HCs and a significant reduction in the risk of HAM/TSP [11, 12]. Thus, one possible mechanism for the observed IL-10 promoter effect is that increased the production of IL-10 reduces the efficiency of immune surveillance of HTLV-I infection—for example, by reducing the number or the activity of HTLV-I–specific cytotoxic T lymphocytes. However, the IL-10 promoter genotype remained a significant predictor of the risk of HAM/TSP even after taking the provirus load into account. This observation suggests that IL-10 increases the risk of HAM/TSP by another mechanism in addition to an apparent effect on provirus load.

In conclusion, we report that the IL-10 −592 A allele, which is associated with lower HTLV-I Tax–mediated transcriptional activity, influences both the provirus load in HTLV-I–infected individuals and the susceptibility to HAM/TSP in the Kagoshima cohort. This effect remains significant even after taking into account the other 2 known major predictors of HAM/TSP risk in this cohort—provirus load and HLA-A*02 genotype—which suggests a powerful argument in favor of a real physiological effect of this polymorphism. Further functional studies to clarify the role of IL-10 in HTLV-I infection may reveal immunotherapeutic strategies that would retard the development of HAM/TSP.

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Serum concentration and genetic polymorphism in the 5′-untraslated region of VEGF is not associated with susceptibility to HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) in HTLV-I infected individuals

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Abstract

HTLV-I- associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type I (HTLV-I) infection. It remains unknown why the majority of infected people remain healthy whereas only approximately 2–3% of infected individuals develop the disease. Recently, it has been reported that increased plasma concentrations of VEGF were significantly related to high ATL cell infiltration, and the viral transactivator Tax activates the VEGF promoter, linking the induction of angiogenesis to viral gene expression. To investigate whether VEGF promoter −634C/G single nucleotide polymorphism (SNP) and serum concentration of VEGF are associated with the development of HAM/TSP, we studied a group of 202 HAM/TSP patients, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs) in Kagoshima, Japan by using PCR-RFLP analysis. The serum concentration of VEGF was also compared among patients with HAM/TSP, ATL, HCs as well as with NCs. Our results indicate that both VEGF gene polymorphism and serum VEGF levels are not specifically associated with the risk of HAM/TSP in our cohort.

Keywords: VEGF; Single nucleotide polymorphism; HAM/TSP; HTLV-I; Disease susceptibility; Proviral load

1. Introduction

Human T-cell lymphotropic virus type I (HTLV-I) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-I is estimated to affect 10 million to 20 million people worldwide [5]. However, only a minority of infected individuals develops HAM/TSP, by mechanisms incompletely understood [6]. Since it has been reported that the subtype of the viral transactivator Tax is associated with the risk of developing HAM/TSP [7], many other reported findings suggest that host factors are most important to determine the risk of HAM/TSP.

Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis. Dysregulated VEGF expression has been implicated as a major contributor to the development of a number of common disease pathologies [8]. A recent report indicated that among seven common polymorphisms in the promoter region, genotype distribution of the −634C/G single nucleotide polymorphism (SNP) differed significantly ($P=0.011$) between patients with and without diabetic retinopathy, and that C allele was significantly increased in patients with retinopathy compared with those without retinopathy ($P=0.0037$) [9]. On the other hand, it has recently been reported that HTLV-I-transformed cells secrete VEGF and basic fibroblast growth factor (bFGF) proteins and induce

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angiogenesis in vitro, via HTLV-I Tax-induced transcriptional activation of the VEGF promoter [10]. Therefore, it may be possible that altered vessel permeability and activated endothelial cells are involved in the pathogenesis of HAM/TSP.

To test this possibility, we examined the serum concentration of VEGF as well as promoter gene polymorphism to assess its possible role in HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 202 patients with HAM/TSP, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs), all residing in HTLV-I endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [11]. Clinical characteristics of the patients are shown in Table 1. All samples were taken with the consent of the patients.

2.2. Isolation and cryopreservation of PBMC and DNA extraction

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using a Histopaque-1077 instrument (Sigma, Tokyo, Japan) and washed three times with phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PMBCs using a QIAamp blood kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

2.3. Genomic PCR analysis

In order to amplify a 469 base pair fragment containing the −634C/G SNP in VEGF promoter, 50 ng of genomic DNA was PCR-amplified with the primers (forward: 5′-TTG CTT GCC ATT CCC CAC TTG A-3′ and reverse: 5′-CCG AAG CGA GAA CAG CCC AGA A-3′) by 1 unit of Takara-Taq DNA polymerase® (Takara, Tokyo, Japan) in a final volume of 50 μl. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 60 s, and elongation at 72 °C for 60 s with a final extension at 72 °C for 10 min. The 15 μl of PCR product was then digested for 12 h using 5 units of *Bsm-FI* (New England Biolabs, MA) restriction enzyme, resulting in fragments of 338 and 131 bp in length if −634G is used or in fragments of 469 bp in length if −634C is used [9]. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of VEGF in serum

The serum VEGF concentration was measured in duplicate using a commercial ELISA kit (R&D Systems, Minneapolis, Minnesota). All samples were quickly frozen and stored at −80 °C until the time of the assay. The assay system used is sensitive to typically less than 9.0 pg/ml. Optical density at 450 nm was measured on the Immuno-Mini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and VEGF concentration was determined by linear regression from a standard curve using the VEGF supplied with the kit as standard. The intra-assay coefficient of variation (CV) of the VEGF ELISA was 6.7%, and the inter-assay CV was 8.8%.

2.5. Quantification of HTLV-I provirus load, CSF neopterin and anti-HTLV-I antibody titers

To examine the HTLV-I provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMC samples as reported previously [12]. In this method, the 5′ nuclease activity of Taq polymerase cleaves a nonextendible hybridization probe during the extension phase of PCR. This cleavage generates a specific fluorescent signal that is measured at each cycle. Based on the standard curve created by four known concentrations of template, the concentrations of unknown samples were determined. Using β-actin as an internal control, the amount of HTLV-1 proviral DNA was calculated by the following formula: copy number of HTLV-1 (pX) per 1×10^4 PBMC=[(copy number of pX)/(copy number of β-actin/2)] $\times 10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by HPLC with fluorometric detection methods [13]. Serum and CSF antibody titers to

Table 1
Clinical characteristics of HAM/TSP patients and asymptomatic HTLV-I carriers (HCs)

	Age	Male/Female	Anti-HTLV-I antibodies[a]	HTLV-I proviral load[b]	Neopterin in CSF[c]
HAM/TSP (n=202)	57.3 ± 11.9[d]	60/142	26,364 ± 41,347	725.2 ± 656.5	111.9 ± 112.4
HCs (n=202)	39.5 ± 12.9	96/106	1514 ± 1467	191.2 ± 312.9	N/A

N/A: not applicable.
[a] Anti-HTLV-1 antibodies were titrated by the particle agglutination method.
[b] Tax copy number per 1×10^4 PBMCs.
[c] Neopterin levels were evaluated by HPLC with fluorometric detection methods.
[d] The values are shown as the mean ± SD.

Table 2
Summary of VEGF -634C/G SNP data

Allele	HAM/TSP	HCs	NCs	p value[a]	Genotype	HAM/TSP	HCs	NCs	p value[b]
C	181 (44.8)[c]	183 (45.3)	93 (43.1)	0.89 (HAM-HCs)	CC	41 (20.3)	40 (19.8)	20 (18.5)	0.92 (HAM-HCs)
G	223 (55.2)	221 (54.7)	123 (56.9)	0.68 (HAM-Normal)	CG	99 (49.0)	103 (51.0)	53 (49.1)	0.91 (HAM-Normal)
				0.59 (HCs-Normal)	GG	62 (30.7)	59 (29.2)	35 (32.4)	0.84 (HCs-Normal)
Total	404	404	216			202	202	108	

HCs: asymptomatic HTLV-I carriers.
NCs: seronegative healthy controls.
[a] p values are calculated by χ^2-test with 2×2 contingency table.
[b] p values are calculated by χ^2-test with 2×3 contingency table.
[c] Numbers in parentheses are percentage.

HTLV-I were determined by a particle agglutination method (Serodia-HTLV-I®, Fujirebio).

2.6. Statistical analysis

Comparisons of genotype frequency among HAM/TSP patients, HCs and NCs were calculated by the chi-squared test. For multiple comparisons, we used Sheffe's F to analyze statistical difference. Mann–Whitney U-test was used to compare serum VEGF levels between the various clinical groups. Significance was considered at $p < 0.05$.

3. Results

3.1. VEGF promoter gene polymorphism in HAM/TSP patients, asymptomatic HTLV-I carriers and seronegative healthy controls

The functional promoter polymorphism at position −634C/G SNP in the VEGF promoter had been previously reported from Saitama, Japan to be associated with diabetic retinopathy with a significantly increased frequency of the CC genotype [9]. However, in the present study, no significant differences were observed among HAM/TSP patients, HCs and NCs genotype or gene frequencies (Table 2). In all groups (HAM/TSP patients, HCs and NCs) the genotype frequencies were distributed according to Hardy–Weinberg equilibrium. Interestingly, the allele and genotype frequencies of VEGF −634C/G SNP in Kagoshima population was very similar to previously reported type 2 diabetic patients with retinopathy, but not without retinopathy [9]. Recently reported allele and genotype frequencies of VEGF −634C/G SNP from Italian control population also showed similar results with our present study [14]. Thus −634C/G SNP in the VEGF promoter was not associated with the risk for HAM/TSP in Kagoshima population.

3.2. Serum concentration of VEGF among HAM/TSP, ATL patients, asymptomatic HTLV-I carriers and seronegative controls

There was no significant difference in serum VEGF levels among 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (228.22 ± 156.99) and 12 NCs (209.89 ± 159.02) (Fig. 1). Two ATL patients with organ infiltration of ATL cell showed relatively high serum VEGF levels (ATL1: 652.0 pg/ml; ATL 2: 857.5 pg/ml) than other patients, consisting with previous reports [15,16].

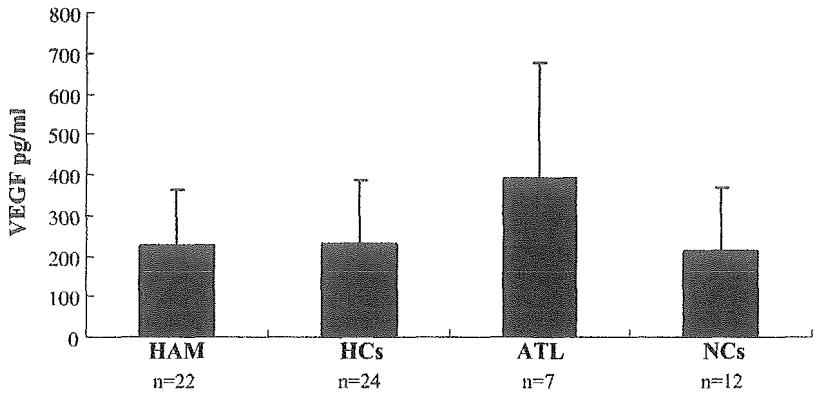

Fig. 1. Serum concentration of VEGF among HAM/TSP patients, ATL patients, asymptomatic HTLV-I carriers and seronegative controls. Serum VEGF levels from 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (HCs) (228.22 ± 156.99) and 12 seronegative healthy controls (NCs) (209.89 ± 159.02) were determined using ELISA. Bars show the mean ± standard deviation in each group.

Table 3
VEGF −634C/G SNP genotype and HTLV-I provirus load

	CC	CG	GG
HAM (n=202)	743.6 ± 110.9	704.0 ± 76.9	750.4 ± 93.4
HCs (n=202)	224.8 ± 59.0	200.0 ± 33.6	155.0 ± 27.6
All patients combined	441.6 ± 65.3	423.1 ± 42.9	411.0 ± 51.7

The values are shown as the mean tax value (tax copies/10^4 PBMCs) ± SE.

3.3. The VEGF −634 SNP is not a significant predictor of the HTLV-I proviral load in HAM/TSP patients and asymptomatic HTLV-I carriers

To test whether VEGF −634C/G SNP genotype is a significant predictor of the HTLV-I proviral load, we measured the proviral load of HTLV-I and compared it with VEGF −634C/G genotype in HAM/TSP patients and HCs. Our data indicated that there was no association between VEGF −634C/G genotype and HTLV-I proviral load (Table 3), CSF neopterin levels as well as serum HTLV-I antibody titers (data not shown) in our population. Also, the clinical course and disability of HAM/TSP were not specifically associated with the polymorphism and serum VEGF levels in HAM/TSP patients (data not shown).

4. Discussion

HTLV-I infection is of particular interest to the field of immunology as well as neurology because it persists at a remarkably high level despite a vigorous cellular and humoral immune response, and causes inflammatory demyelinating disease only in a minority of infected people. Although certain Tax subtypes were recently reported to carry different risks of HAM/TSP [7], viral factors alone are not sufficient to predict disease. Our recent observations as well as many reported findings strongly suggest that the outcome of HTLV-I infection mainly depends upon a host of genetic factors [17]. Especially, our recent case/control study in Kagoshima strongly supports this idea. In the Kagoshima population, possession of the HLA-class I genes, HLA-A*02 and Cw*08, each independently halve the odds of developing HAM/TSP, whereas possession of the HLA-class I gene, HLA-B*5401 and the HLA-class II gene, HLA-DRB1*0101, predispose a person to HAM/TSP [18,19]. Since HLA itself does not explain the entire disease onset of HAM/TSP, and a non-HLA candidate gene approach has already been shown to be successful in identifying markers in other infectious diseases [20,21], we are now focusing on non-HLA gene polymorphisms as candidate genes that are associated with development of HAM/TSP.

VEGF is a specific mitogen and survival factor for endothelial cells and a key promoter of angiogenesis in physiological and pathophysiological conditions, and promotes inflammatory processes by causing vascular leakage and mobilizing leukocytes [8]. Increased concentrations of free VEGF have been measured in a variety of autoimmune and infectious inflammatory diseases, including rheumatoid arthritis [22], POEMS syndrome [23,24], and Kawasaki disease [25,26]. More interestingly, VEGF expression was consistently upregulated in both acute and chronic multiple sclerosis plaques [27], suggesting that VEGF exacerbate the inflammatory response in autoimmune diseases of the central nervous system and migration of inflammatory cells into the lesions. Since HTLV-I-transformed cells secrete VEGF and bFGF proteins and induce angiogenesis in vitro via HTLV-I Tax-mediated transcriptional activation of VEGF promoter [10] and HAM/TSP is also associated with inflammatory cell infiltrations into central nervous system (CNS), we investigated the influence of VEGF gene polymorphism as well as serum concentration of VEGF in HTLV-I infection.

In the present study, there were no significant differences in any VEGF −634C/G genotypes between HAM/TSP patients and HCs. Also, there were no correlations between serum VEGF levels and CSF neopterin levels as well as serum anti-HTLV-I antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with the VEGF −634C/G polymorphism and serum VEGF levels in HAM/TSP patients, although two ATL patients with organ infiltration showed relatively higher concentration of VEGF in the serum. Taken together, our present results suggest that VEGF −634C/G genotype as well as serum concentrations of VEGF are not susceptibility factors for the development of HAM/TSP. It is still possible that VEGF might have an important role in the affected spinal cord lesion of HAM/TSP, as reported in both acute and chronic MS plaques [27], although further studies are needed to clarify this point.

In conclusion, our results indicate that VEGF in serum is not the suitable factor to evaluate the risk and disease activity of HAM/TSP.

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Degenerate specificity of HTLV-1–specific CD8+ T cells during viral replication in patients with HTLV-1–associated myelopathy (HAM/TSP)

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Human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is an inflammatory neurologic disease caused by HTLV-1 infection, in which HTLV-1–infected CD4+ T cells and HTLV-1–specific CD8+ T cells may play a role in the disease pathogenesis. Patients with HAM/TSP have high proviral loads despite vigorous virus-specific CD8+ T-cell responses; however, it is unknown whether the T cells are efficient in eliminating the virus in vivo. To define the dynamics of HTLV-1–specific CD8+ T-cell responses, we investigated longitudinal alterations in HTLV-1 proviral load, amino acid changes in an immunodominant viral epitope, frequency of HTLV-1–specific T cells, and degeneracy of T-cell recognition in patients with HAM/TSP. We showed that the frequency and the degeneracy of the HTLV-1–specific CD8+ T cells correlated well with proviral load in the longitudinal study. The proviral load was much higher in a patient with low degeneracy of HTLV-1–specific T cells compared to that in a patient with comparable frequency but higher degeneracy of the T cells. Furthermore, in a larger number of patients divided into 2 groups by the proviral load, those with high proviral load had lower degeneracy of T-cell recognition than those with low proviral load. Sequencing analysis revealed that epitope mutations were remarkably increased in a patient when the frequency and the degeneracy were at the lowest. These data suggest that HTLV-1–specific CD8+ T cells with degenerate specificity are increased during viral replication and control the viral infection. (Blood. 2003;101:3074-3081)

Introduction

Virus-specific CD8+ T cells recognize short peptide fragments bound to the cleft of major histocompatibility complex (MHC) class I molecules, which are endogenously processed within virus-infected cells.[1,2] The CD8+ T cells play a pivotal role in controlling viral infection in both the acute and chronic phase. Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that preferentially and persistently infects CD4+ lymphocytes in vivo.[3] Even though HTLV-1 infection is lifelong, fewer than 1% of the individuals infected with HTLV-1 develop a neurologic disease termed HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or a hematologic disease termed adult T-cell leukemia (ATL); the vast majority of individuals remain in the asymptomatic carrier state.[4-6]

HAM/TSP is an inflammatory disease in the spinal cord where inflammatory cells, predominantly CD8+ T cells, infiltrate the perivascular area.[7] Patients with HAM/TSP show spastic paraparesis and sphincter dysfunction with mild sensory dysfunction, which are consistent with the pathologic changes in the spinal cord.[8] It has been demonstrated that the HTLV-1 proviral load and the frequency of HTLV-1–specific CD8+ T cells are increased in the peripheral blood in patients with HAM/TSP compared with HTLV-1 carriers.[9-11] In the cerebrospinal fluid in patients with HAM/TSP, HTLV-1–specific CD8+ T cells accumulate and HTLV-1–expressing CD4+ T cells are increased.[11,12] Furthermore, HTLV-1–specific CD8+ T cells and the expression of HTLV-1 gene products have been demonstrated in the central nervous system.[13,14] Therefore, it has been suggested that these cells may play an important role in the pathogenesis of HAM/TSP.[15]

HTLV-1–specific T cells show strong killing activity toward HTLV-1–infected cells in vitro and the frequency is extraordinarily high in the circulation of patients with HAM/TSP,[10] reaching over 10% of the CD8+ cell population.[16] The CD8+ T-cell response is preferentially directed against the HTLV-1 Tax protein. In HLA-A2+ patients especially, it has been shown that HTLV-1 Tax 11-19 is an immunodominant epitope and is one of the strongest binding peptides to the HLA-A2 molecule.[17] Despite the vigorous T-cell response to the virus, patients with HAM/TSP show increased proviral load. Therefore, the question of whether the increased HTLV-1–specific CD8+ T cells are effective in killing virus-infected cells in vivo arises. To answer this question, a longitudinal study was conducted to examine the viral state and virus-specific CD8+ T-cell responses in patients with HAM/TSP, in which alterations in proviral load, amino acid changes in the epitope, HTLV-1–specific T-cell frequency, and degeneracy of antigen recognition of the T cells were investigated. The high frequency of the circulating HTLV-1–specific CD8+ T cells to the immunodominant Tax 11-19 and increased proviral load afford a unique opportunity to examine detailed virus-host immunity interactions

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focusing on a single epitope in bulk peripheral blood mononuclear cells (PBMCs) in patients with HAM/TSP.

Patients, materials, and methods

Patients

Fifty-three patients with HAM/TSP who had HLA-A*02 haplotype and were residing in Kagoshima, an endemic area in Japan, were included in this study. The diagnosis was based on the neurologic symptoms of the patients and seroreactivity to HTLV-1 according to the World Health Organization guidelines. These patients had not been treated with any antiretroviral drugs. PBMCs were separated by Ficoll gradient centrifugation from the heparinized blood of the patients, which was taken under informed consent and stored in liquid nitrogen until use. HLA-A*02 typing was carried out by polymerase chain reaction (PCR) sequence-specific primer reaction as previously described.[18] In a longitudinal study, 3 patients were chosen for the precise detection of peptide-specific $CD8^+$ T cells by flow cytometry. They had over 2% increase in HTLV-1–specific $CD8^+$ T cells and had HLA-A*0201 haplotype after having their blood drawn several times. Approval was obtained from the Kagoshima University Ethics Committee for this study. Informed consent was provided according to the Declaration of Helsinki.

Peptides

The amino acid sequence of HTLV-1 Tax 11-19 is LLFGYPVYV.[19] L-Alanine–substituted peptides for Tax 11-19 at positions 4, 5, 6, and 8 (altered peptide ligand; APL) were synthesized using Fmoc solid-phase methodology (Kurabo, Osaka, Japan). Amino acid residues in these positions are expected to bind the T-cell receptor (TCR).[20] The synthetic peptides were designated as G4A, Y5A, P6A, and Y8A, respectively. Influenza virus M1 peptide (GILGFVFTL) was used as a control peptide that binds to HLA-A*02.[19] HLA-A*0201-binding affinity of the peptides can be predicted via the Internet (http://bimas.dcrt.nih.gov/molbio/hla_bind/).[21] The estimated dissociation half-time is 2406 minutes by Tax 11-19, G4A, Y5A, and P6A and 437 minutes by Y8A and 550 minutes by M1 peptide. Purity of the peptides was over 90% by high-performance liquid chromatography (HPLC) analysis. The synthetic peptides were resolved in 50% dimethyl sulfoxide (DMSO) in phosphate-buffered saline at 1 mM.

Intracellular cytokine detection by flow cytometry

The assay was conducted by a modified protocol as previously described.[16] Briefly, Hmy2.C1R cells transfected with HLA-A*0201 (Hmy-A2) were prepulsed with 1 μM Tax 11-19 or APL for 1 hour and washed. Cryopreserved PBMCs were quickly thawed and washed. Then 5×10^5 PBMCs were cocultivated with the same number of peptide-prepulsed Hmy-A2 cells for 6 hours. Brefeldin A (Sigma, Tokyo, Japan) was added to the cells at a final concentration of 10 μg/mL at the beginning of the culture to minimize the expression of HTLV-1 protein in the infected cells, which may lead to activation of HTLV-1–specific T cells.[22] After culture, cells were harvested, washed, and stained with antihuman CD8 antibody conjugated with PC5 (Beckman-Coulter, Tokyo, Japan) at 4°C for 20 minutes. Cells were washed and fixed with 4% paraformaldehyde for 5 minutes, then washed again. The cells, resuspended in 50 μL permeabilization buffer containing 0.1% saponin (Sigma), were stained with antihuman interferon γ (IFN-γ) antibody conjugated with fluorescein isothiocyanate (FITC; Pharmingen, San Diego, CA) for 20 minutes. Epics-XL flow cytometer and SYSTEM II software were used for fluorescent signal detection and data analysis (Beckman-Coulter). Lymphocytes were readily distinguished from Hmy-A2 cells by size and were gated on forward and side scatter image. Ten thousand $CD8^+$ cells were further gated and the proportion of IFN-γ^+ cells in the $CD8^+$ cell population was analyzed. The frequency of peptide-specific $CD8^+$ T cells was obtained by subtracting the percentage of IFN-γ^+ cells without peptide from that with a peptide. The degeneracy of T-cell recognition is evaluated according to the degree by which the T cell recognizes altered peptides from a cognate peptide at the T-cell clone level. Therefore, the total degeneracy of virus-specific T-cell population in the PBMCs could be evaluated by relative T-cell recognition of an APL against the cognate peptide. The relative T-cell recognition of an APL against Tax 11-19 was given by the following formula: (frequency of APL-specific T cells)/(frequency of Tax 11-19–specific T cells) × 100. The functional T-cell avidity to the antigen was estimated as the peptide concentration required for half-maximum IFN-γ production.

Quantitative PCR for HTLV-1 proviral load

The method used has been previously described.[9] Briefly, gDNA was extracted from PBMCs by using Qiagen DNA extraction kit (Qiagen, Tokyo, Japan). Approximately 100 μg DNA was subjected to a real-time PCR using a *Taq*Man probe. The fluorescent signal was detected by an ABI PRISM 7700 sequence detector (Applied Biosystems, Chiba, Japan). The copy number of the target gene in the sample was estimated by the standard curves. HTLV-1 copy number per 10^4 PBMCs was calculated according to the following formula: (copy number of HTLV-I *tax* gene in the sample)/((copy number of β-*globin* gene in the sample)/2) × 10.[4] The assay was conducted in triplicate.

Sequencing analysis of HTLV-1 *tax* gene

The method was previously described.[18] Briefly, 100 ng DNA was amplified by 35 cycles of PCR using primers PX01+: 5′-TCGAAACAGCCCTGCA-GATA-3′ at position 7257-7276 and PX22−: 5′-TGGTGGGCAAA-CAGTCTTCG-3′ at position 7928-7947. One microliter of the first PCR products was further used for 20 cycles of nested PCR using internal primers PXI1+: 5′-ATACAAAGTTAACCATGCTT-3′ at position 7274-7293 and PXI1−: 5′-GGGTTCCATGTATCCATTTC-3′ at position 7644-7663. Amplified DNA products were purified using QIA quick purification kit (Qiagen). The purified *tax* gene was subcloned into pCR-Blunt II-TOPO cloning vector (Invitrogen, Burlingame, CA). The vector was linearized by *Eco*RI digestion, which does not cut the *tax* gene, and purified by the QIA quick purification kit. The *tax* gene was sequenced with PXI1+ or PXI1− primer using dye terminator DNA sequencing kit (Applied Biosystems) in an automatic sequencer (377 DNA Sequencer, Applied Biosystems). Approximately 50 clones were sequenced in each sample and mutations in the *tax* gene encoding Tax 11-19 peptide were confirmed by sequencing in the reverse direction.

Results

Frequency of HTLV-1–specific $CD8^+$ T cells correlated with the proviral load

To optimize a concentration of peptide, which induced cytokine production in antigen-specific T cells, we conducted a peptide titration assay. IFN-γ production in bulk PBMCs in response to Tax 11-19 peptide appeared at 0.001 nM and reached the maximum over 1 nM, whereas weak responses to some analog peptides were observed at 1000 nM (data not shown). Therefore, we used peptides at 1 μM in the subsequent study. Figure 1 shows a representative IFN-γ production from $CD8^+$ cells in PBMCs from patient no. 31.

A high frequency of Tax 11-19–specific $CD8^+$ T cells, with a lesser extent to APLs, and a low frequency to antigen-presenting cells (APCs) without peptide (NP, background) were detected. The background was 0.42% ± 0.42% (mean ± SD) in 53 HLA-A2 HAM/TSP patients. Peptide-specific $CD8^+$ T-cell frequencies were estimated by subtraction of percentage of IFN-γ^+ cells without peptide from that with a peptide. The Tax 11-19–specific $CD8^+$ T-cell frequency, measured in 53 HAM/TSP patients, was 2.01% ± 3.97% (mean ± SD). We chose 3 patients who had high frequencies of over 2% and had their blood drawn at least 4 separate times for the longitudinal analysis.

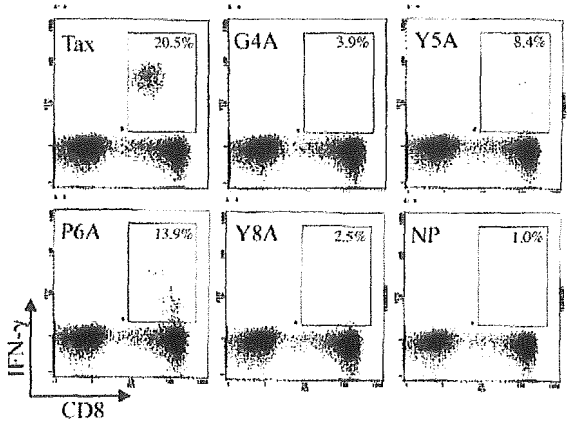

Figure 1. Representative analysis of Tax 11-19– and APL-specific CD8⁺ T cells in PBMCs from patient no. 31 on 3/5/97 by flow cytometry. PBMCs were cocultured with APCs prepulsed with either Tax 11-19, APL, or no peptide (NP) for 6 hours in the presence of brefeldin A, a reagent to accumulate synthesized proteins in the Golgi apparatus. The culture cells were stained with anti-CD8 and anti–IFN-γ antibodies. The lymphocytes were segregated from the APCs by size on the forward and side scatter and IFN-γ⁺ cells were gated as shown. The number in the gate indicates the frequency of IFN-γ⁺ cells in CD8⁺ cell population. Tax is the cognate Tax 11-19 peptide and APL was designated as G4A (glycine at position 4 of Tax 11-19 was substituted by alanine).

As shown in Figure 2A-B, in patient no. 31, the alterations in proviral load and frequency of HTLV-1 Tax 11-19–specific CD8⁺ T cells during 8 years ranged from 506 to 674 copies/10^4 PBMCs and from 6.7% to 19.5%, respectively. The CD8⁺ T cells were effectively increased in parallel with the slight increase in the proviral load. The alterations in patient no. 38 were from 1595 to 2225 copies/10^4 PBMCs and from 2.2% to 3.1%, respectively; the increase in virus-specific T-cell frequency was small. In patient no. 48, the alterations were from 93 to 605 copies/10^4 PBMCs and from 0.8% to 5.5%, respectively. The degree of the increase in virus-specific T-cell frequency to the increase of proviral load was different in each patient; however, HTLV-1 Tax 11-19–specific CD8⁺ T-cell frequency correlated well with the proviral load in each patient, suggesting that the HTLV-1 Tax 11-19–specific CD8⁺ T cells proliferate to control viral replication.

Degeneracy of T-cell recognition in HTLV-1–specific CD8⁺ T cells correlated with the proviral load

To test whether T-cell recognition of the antigen may be altered during the clinical course, especially when the proviral load was increased, a combination of intracellular cytokine detection and APL was used. With this method, the degeneracy of T-cell recognition in the HTLV-1–specific CD8⁺ T-cell population can be evaluated. For example, in Figure 1, the Tax 11-19–specific CD8⁺ T cells were relatively tolerated with the alanine substitution at position 6, and, to a lesser extent at position 5, whereas rarely at positions 4 and 8. The relative T-cell responses to the APL against those to the Tax 11-19 peptide were calculated according to the formula mentioned in "Patients, materials, and methods." For example, the calculated value was 14.9% for G4A, 37.9% for Y5A, 66.2% for P6A, and 7.7% for Y8A from the data in Figure 1. The

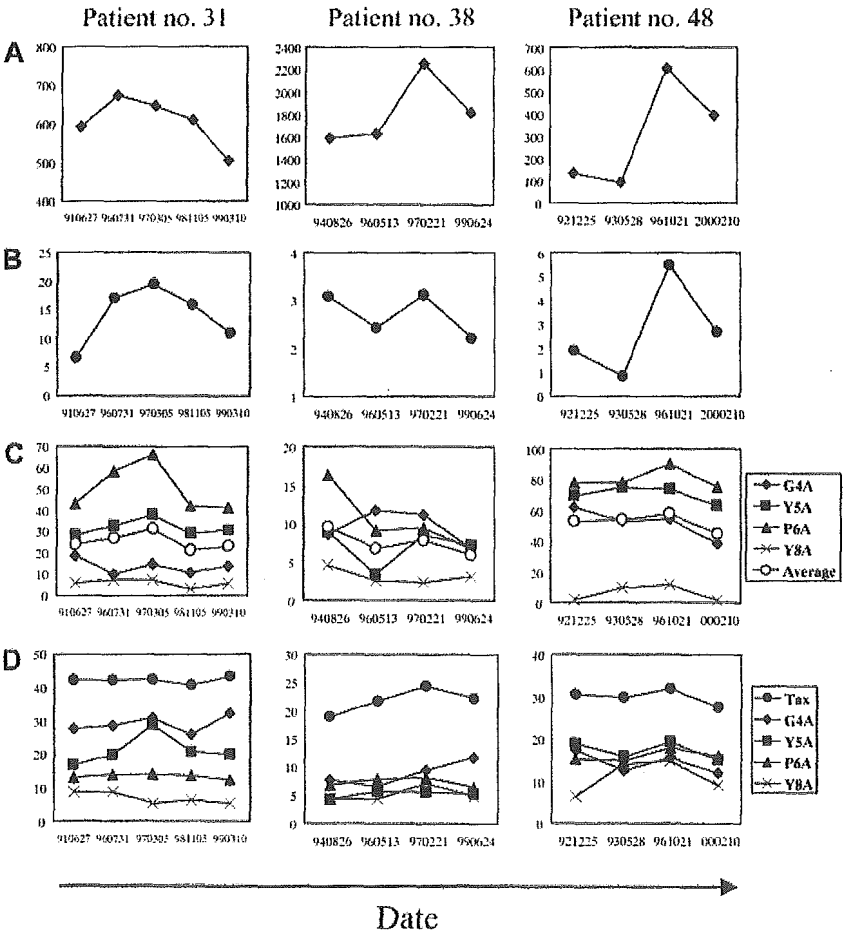

Figure 2. Longitudinal analysis in 3 HLA-A*0201⁺ patients with HAM/TSP. The x-axis indicates the date when the PBMCs were obtained. (A) HTLV-1 proviral load (♦) measured by a quantitative real-time PCR (copy/10^4 PBMCs). (B) Frequency of HTLV-1 Tax 11-19–specific CD8⁺ T cells in CD8⁺ cell population (●), which were measured as shown in Figure 1 (%). (C) Degeneracy of T-cell recognition in HTLV-1 Tax 11-19–specific CD8⁺ T cells at each time point. Frequencies of T cells that recognized either cognate Tax 11-19 peptide or APL were measured as shown in Figure 1. The relative CD8⁺ T-cell frequency to APL against that to cognate Tax 11-19 was calculated. The y-axis indicates the relative percentage (%). (D) MFI of IFN-γ–FITC produced by T cells responding to cognate Tax 11-19 or APL (unit).

Table 1. Comparison of proviral load, frequency and degeneracy of HTLV-1 Tax 11-19-specific T cells, and epitope variant among 3 patients with HAM/TSP

Patient no.	Proviral load, copy/10^4 PBMCs	Frequency, % in $CD8^+$ cells	Degeneracy,* %	Epitope variant,† %
31	606.0 ± 64.0‡	14.0 ± 5.1§	25.4 ± 18.6‖	3.4 ± 2.5
38	1823.0 ± 303.1¶	2.7 ± 0.5	7.5 ± 3.8#	8.8 ± 10.5
48	306.5 ± 240.5	2.7 ± 2.0	52.3 ± 30.1	4.8 ± 2.3

The numbers indicate mean ± SD.

*Degeneracy of HTLV-1 Tax 11-19-specific T cells is accessed by the average of relative T-cell responses to APLs during the time course.

†Percentage of epitope variant is given by the division of the number of variants by the number of clones sequenced in each patient.

‡*P*, as estimated by the Mann-Whitney *U* test, is significantly different between patients no. 31 and no. 38 (‡$P < .0001$), no. 31 and no. 38 (§$P < .0006$), no. 31 and no. 48 (§$P < .0006$), no. 31 and no. 38 (‖$P < 0.0005$), no. 31 and no. 48 (‖$P < .0001$), no. 38 and no. 48 (¶$P < .0001$), and no. 38 and no. 48 (#$P < .0001$).

relative T-cell response to the APL was plotted according to the time course in the 3 patients (Figure 2C) and mean fluorescence intensity (MFI) of IFN-γ–FITC in the peptide-specific $CD8^+$ T cells was graphed (Figure 2D). Although the relative T-cell response in patient no. 31 varied according to each APL, P6A was highly tolerated over 40%, and Y5A recognition was approximately 30%, but the recognitions for G4A and Y8A were low. In patient no. 38, the relative APL recognition was low compared to that in patient no. 31, in whom the responses to all the APLs were less than 17%. In patient no. 48, the T-cell response was relatively high to all the APLs except for Y8A, which reached up to 90% for P6A. This indicated that T-cell recognition of HTLV-1 Tax 11-19 peptide is strict in patient no. 38, but not in patient no. 48. These data suggest that not only the virus-specific T-cell frequency but also the extent of degeneracy in T-cell responses differ among the patients.

In the longitudinal study (Figure 2C), it is striking that the recognition for some APLs correlated with the frequency of the virus-specific T cells and the proviral load. This was particularly dramatic in patient no. 31, who showed a high frequency of HTLV-1 Tax 11-19–specific $CD8^+$ T cells. For example, the T-cell recognitions of P6A and Y5A were well associated with the proviral load and the virus-specific T cell frequency (Figure 2A-C), whereas the recognition of G4A and Y8A was not changed. In patient no. 38, the relative T-cell responses to P6A and Y5A correlated with the proviral load; however, the G4A recognition correlated with neither the proviral load nor the T-cell frequency (Figure 2A-C). In patient no. 48, the curves of $CD8^+$ T-cell recognition of P6A and G4A correlated with the curves of the proviral load and the frequency, but were not clear in the response to Y5A and Y8A (Figure 2A-C). Although alterations in MFI of IFN-γ–FITC were not apparent in the response to Tax, P6A and Y8A peptide in patient no. 31, the MFI to Y5A was increased when the virus-specific T-cell frequency peaked (Figure 2B,D). In patient no. 38, it was hard to find any associations between the MFI and the proviral load or the T-cell frequency. In patient no. 48, all the MFI curves except for that to Y8A showed the same tendency as the curves for the proviral load and the virus-specific T-cell frequency (Figure 2A-B,D). These results suggest that, in some patients, when the proviral load was increased, the virus-specific T cells secrete cytokines more efficiently to some analog peptides compared to when the proviral load was decreased.

To compare the effect of HTLV-1–specific T cells on the proviral load among these 3 patients, we calculated the average of each factor during the time course (Table 1). Patient no. 38 had the highest proviral load with the lowest frequency and degeneracy of the virus-specific T cells. Patient no. 48 had the same frequency of the HTLV-1–specific T cells as patient no. 38, but increased degeneracy of 52.3% for the APLs and the lowest proviral load. Patient no. 31 had extremely high virus-specific T-cell frequency of 14.0%, but the proviral load was higher than that in patient no. 48. This patient showed a moderate extent of degeneracy in T-cell recognition among all the patients included.

To investigate whether or not the degeneracy of T-cell recognition is associated with the proviral load as observed in patients no. 38 and no. 48, we measured degeneracy and proviral load in a series of 22 patients with HAM/TSP who had HLA-A*02 haplotype. The proviral load of these patients ranged from 2230 to 130 copies/10^4 PBMCs, with the median of 481 copies/10^4 PBMCs. We divided the patients into 2 groups according to the following: patients with proviral load more than the median were included in high proviral load group (n = 11), whereas patients with proviral load less than the median were included in low proviral load group (n = 11). As shown in Figure 3, the patients with low proviral load showed an increased degeneracy of HTLV-1 Tax 11-19–specific T cells as compared to the patients with high proviral load. This was observed when Y5A peptide was used, but not when the other peptides were used. These data suggest that virus-specific T cells with increased degeneracy have an advantage to control viral infection.

To test whether the increased degeneracy of T-cell recognition resulted from increased avidity of Tax 11-19–specific $CD8^+$ T cells to the APCs, we conducted antigen titration assays using wild-type Tax 11-19 peptide at various concentrations ranging from 0.001 to 1000 nM.

As shown in Figure 4A, the antigen concentration required to reach the half-maximum number of IFN-γ^+ cells were almost the

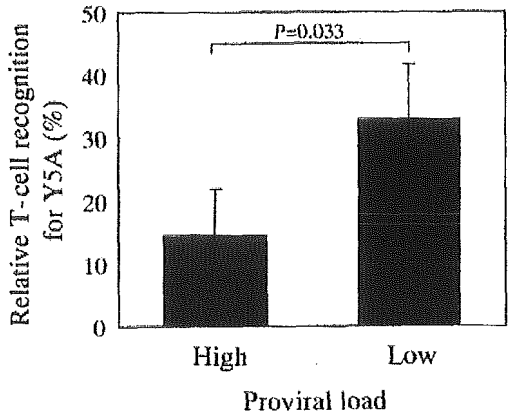

Figure 3. HAM/TSP patients with high proviral load have HTLV-1 Tax 11-19–specific T cells with low degeneracy of T-cell recognition. Twenty-two patients with HAM/TSP who had HLA-A*02 were divided into 2 groups according to whether their proviral load is higher or lower than the median proviral load (481 copies/10^4 PBMCs). The high proviral load group (mean, 1190.0 copies/10^4 PBMCs; n = 11) had significantly higher proviral load than the low proviral load group (mean, 250.2 copies/10^4 PBMCs; n = 11; $P < .0001$, Mann-Whitney *U* test). The relative recognition of HTLV-1 Tax 11-19–specific T cells for Y5A was higher in the patient group with low proviral load than in the patient group with high proviral load ($P = .033$; Mann-Whitney *U* test). There were no significant differences between the 2 groups for other APLs including G4A, P6A, and Y8A (data not shown). The column and vertical bar indicate the mean and SE, respectively.

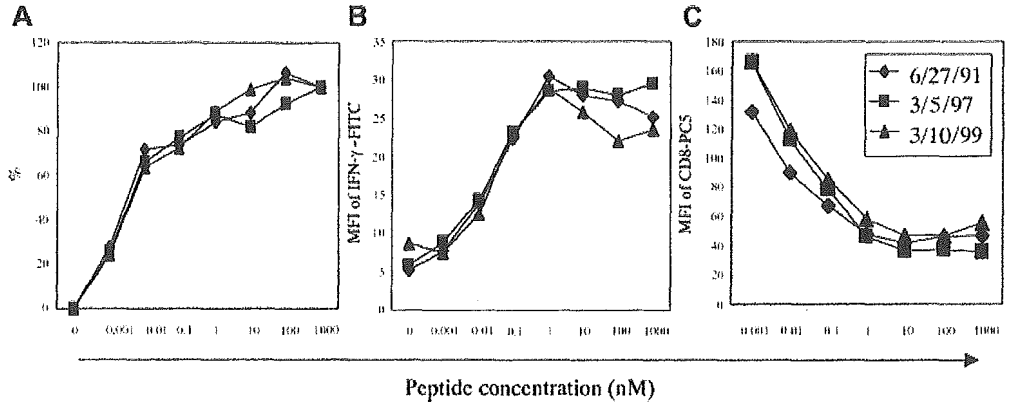

Figure 4. Functional antigen avidity test in Tax 11-19–specific CD8⁺ T cells in PBMCs from patient no. 31. Samples were chosen at 3 time points, when the HTLV-1 proviral load was either high or low as shown in Figure 2A. The x-axis indicates HTLV-1 Tax 11-19 peptide concentration. (A) Frequencies of the HTLV-1 Tax 11-19–specific CD8⁺ T cells in CD8⁺ cell population, which were measured as in Figure 1. The y-axis indicates relative percentage as standardized by the frequency at 1000 nM in each sample. The frequencies at 1000 nM were 6.3% on 6/27/91, 18.5% on 3/5/97, and 10.6% on 3/10/99. (B) The MFI of IFN-γ–FITC produced by the HTLV-1 Tax 11-19–specific CD8⁺ T cells. (C) The MFI of CD8-PC5 in the Tax 11-19–specific CD8⁺ T cells.

same as in patient no. 31 (3.2 pM on June 27, 1991, 4.1 pM on March 5, 1997, and 4.5 pM on March 10, 1999). During the time course the curves of MFI for IFN-γ–FITC were almost the same (Figure 4B), suggesting that the amounts of IFN-γ production in the Tax 11-19–specific CD8⁺ T cells are the same among the samples. Additionally, the curves of MFI for CD8-PC5 were almost the same at different times (Figure 4C), suggesting that down-regulation of CD8 molecules after engaging with peptide/MHC may be almost the same. Similar titration curves were observed in patients no. 38 and no. 48 (data not shown). Collectively, it seems likely that the changes of degeneracy of T-cell recognition did not result from alterations in T-cell avidity.

HTLV-1–specific T-cell frequency was higher in patients with HAM/TSP than in asymptomatic HTLV-1 carriers

It would be of interest to know if degeneracy of T-cell recognition in HTLV-1–specific T cells differed between patients with HAM/TSP and asymptomatic HTLV-1 carriers. Therefore, we measured frequencies of HTLV-1 Tax 11-19–specific CD8⁺ T cells in CD8⁺ T-cell population both in 47 patients with HAM/TSP and 32 asymptomatic HTLV-1 carriers, who had HLA-A*02 haplotype. The frequency was 1.90% ± 2.68% (mean ± SD) and 0.25% ± 0.21%, respectively. The frequency of HTLV-1 Tax 11-19–specific CD8⁺ T cells was significantly higher in HAM/TSP patients than in the carriers ($P < .0001$; Figure 5). Unfortunately, it was difficult to investigate degeneracy of HTLV-1 Tax 11-19–specific T cells in the carriers because the frequency of the T cells in the carriers was too small to evaluate T-cell degeneracy.

Variant epitopes were increased in a patient with low frequency and limited degeneracy in the virus-specific T cells

To determine if degeneracy or frequency of the HTLV-1–specific CD8⁺ T cells may have an effect on the viral epitope variations, the HTLV-1 *tax* gene containing the sequence coding Tax 11-19 peptide was sequenced at 3 time points in the 3 patients (Table 2).

For this experiment 2 DNA samples with low virus-specific T-cell frequencies and one sample with the highest frequency during the time course were chosen. In patient no. 31, the amino acid mutation rate in the Tax 11-19 ranged from 2.0% to 6.3%. In patient no. 48, the mutation rate was also not widely changed from 2.1% to 6.3%. However, in patient no. 38 who had relatively high proviral load and low frequency of the HTLV-1–specific T cells (Figure 2; Table 1), the variation rate reached 20.4% in the sample drawn on June 24, 1999. In this time point, the frequency and the degeneracy of the virus-specific T cells were the lowest in the time course (Figure 2B-C). The numbers of amino acid substitutions of Tax11-19 in the overall 445 clones sequenced were 2, 1, 3, 9, 4, 1, 3, 2, and 1 in the order of the amino acid position, respectively (Table 2). This indicates that the substitutions were frequently observed at positions 4 and 5. The substitution of glycine by arginine at position 4 was most frequently observed with 9 of 26 variants (34.6%). When we predicted the HLA-A2–binding affinity of the variants through the Internet, only 6 of 26 variants would make a lower HLA-A2–binding affinity than Tax 11-19 (Table 2). There was no accumulation of variant epitope during the time course as seen in HIV infection.[23-25]

Discussion

In this longitudinal study, we have shown that: (1) the HTLV-I Tax 11-19–specific CD8⁺ T-cell frequency correlated with the proviral load during the time course of the disease; (2) the T cells showed an

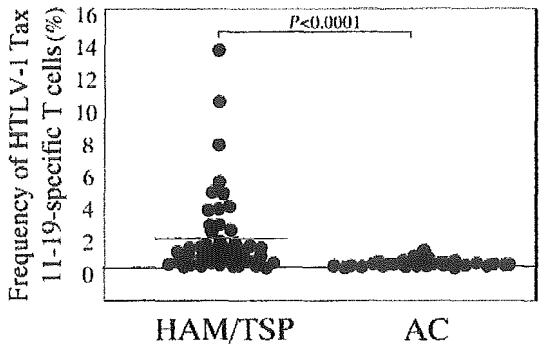

Figure 5. HTLV-1 Tax 11-19–specific T-cell frequency is higher in patients with HAM/TSP than in asymptomatic HTLV-1 carriers. Frequency of HTLV-1 Tax 11-19–specific CD8⁺ T cells in CD8⁺ cell population was measured both in 47 patients with HAM/TSP and 32 asymptomatic HTLV-1 carriers (AC). The mean ± SD was 1.90% ± 2.68% and 0.25% ± 0.21%, respectively. The HAM/TSP patients had significantly higher frequency of HTLV-1 Tax 11-19–specific T cells than the carriers ($P < .0001$, Mann-Whitney *U* test).

Table 2. Longitudinal analysis of variants in an HTLV-1 immunodominant epitope, Tax 11-19, in 3 patients with HAM/TSP

Patient	Date	Proviral load*	Frequency†	Peptide sequence‡	Peptide frequency	Percentage of variant epitope§	Predicted HLA-A2 binding affinity‖
31	6/27/91	594	6.67	LLFGYPVYV	48/49	2.0	2406.2
				LLF**R**YPVYV	1/49		2406.2
	3/5/97	646	19.46	LLFGYPVYV	45/48	6.3	2406.2
				PLFGYPVYV	1/48		31.1
				LL**Y**GYPVYV	1/48		2081.0
				LLF**R**YPVYV	1/48		2406.2
	3/10/99	506	11.00	LLFGYPVYV	48/49	2.0	2406.2
				LLFG**H**PVYV	1/49		2406.2
38	5/13/96	1631	2.43	LLFGYPVYV	48/51	5.9	2406.2
				LL**L**GYPVYV	1/51		2406.2
				LLFGYP**I**YV	2/51		2406.2
	2/21/97	2255	3.12	LLFGYPVYV	52/52	0.0	2406.2
	6/24/99	1810	2.20	LLFGYPVYV	39/49	20.4	2406.2
				L**P**FGYPVYV	1/49		15.7
				LL**L**GYPVYV	1/49		2406.2
				LLF**R**YPVYV	5/49		2406.2
				LLFG**N**PVYV	1/49		2406.2
				LLFG**H**PVYV	1/49		2406.2
				LLFGYPV**H**V	1/49		437.5
48	5/28/93	93	0.83	LLFGYPVYV	45/48	6.3	2406.2
				LLF**R**YP**A**YV	1/48		2406.2
				LLFG**F**PVYV	1/48		9143.4
				LLFGYPV**C**V	1/48		437.5
	10/21/96	606	5.47	LLFGYPVYV	48/51	5.9	2406.2
				LLF**R**YPVYV	1/51		2406.2
				LLFGY**L**VYV	1/51		5534.1
				LLFGYPVY**E**	1/51		0.5
	2/10/00	395	2.70	LLFGYPVYV	47/48	2.1	2406.2
				PLFGYPVYV	1/48		31.1

Total numbers of variants of Tax 11-19 are 2, 1, 3, 9, 4, 1, 3, 2, and 1, in the order of amino acid position, respectively.
*HTLV-1 proviral load is shown as copies/10^4 PBMCs.
†HTLV-1 Tax 11-19-specific T-cell frequency in $CD8^+$ cell population is shown.
‡Amino acid sequence is presented by the single letter. Underlined amino acids indicate mutations.
§Percentage of variant epitope in the sequenced clones is shown at each time point.
‖HLA-A2-binding affinity by dissociation half-time (minutes) of each peptide is predicted through the Internet (http://bimas.dcrt.nih.gov/moibio/hla_bind/).

increased degeneracy of T-cell recognition when the T-cell frequency and the proviral load were increased; (3) in 2 patients with comparable virus-specific T-cell frequency, the proviral load was much higher in the patient who had low degeneracy of the T cells as compared to the other patient who had high degeneracy and the patients with high proviral load had lower degeneracy than those with low proviral load; (4) the degeneracy of T-cell recognition was independent of the T-cell avidity; (5) the frequency of HTLV-1 Tax 11-19–specific T cells is significantly higher in HAM/TSP patients than in the asymptomatic HTLV-I carriers; (6) the epitope variants were remarkably increased in a patient when the frequency and the degeneracy of the T cells were the lowest in the time course; (7) the amino acid changes in the epitope predominantly occurred in the central positions of the epitope; and (8) there is no accumulation of any variant epitope during the time course of the disease.

When the proviral load was increased, HTLV-1–specific $CD8^+$ T cells were increased and the T-cell population that recognized APLs substituted at positions 5 and 6 was increased in patients no. 31 and no. 38 (Figure 2A-C). Then, according to the decrease in proviral load, both the frequency and the degeneracy of virus-specific T cells were decreased. This suggests that the fine specificity of HTLV-1–specific $CD8^+$ T cells is altered according to the viral load in vivo and that the degenerate specificity may give virus-specific T cells an advantage to eliminate the virus. When we compared several factors including viral load, frequency and degeneracy of virus-specific T cells among the patients, patients no. 38 and no. 48 had comparable virus-specific T-cell frequencies (Table 1), and MFI curves of IFN-γ–FITC in the T cells were similar using an antigen titration assay (data not shown). These results suggest that the degree of virus-specific T-cell responses in the 2 patients were almost the same in the given experimental conditions. However, patient no. 38 with limited degeneracy showed 6 times higher proviral load than patient no. 48 who had high degeneracy. Furthermore, in the experiment in which a greater number of patients with HAM/TSP were examined, the patients with high proviral load had lower degeneracy of T-cell recognition than those with low proviral load. Again, it is possible that the increase in degeneracy of the HTLV-1–specific T-cell responses tend to reduce the proviral load in vivo. In chronic viral infections, the virus-specific T-cell repertoire is diversified in the acute phase, whereas in the persistent phase, the T cells show a narrow TCR repertoire, suggesting that memory T cells may have a strict antigen recognition.[26,27] Other experiments demonstrate that naïve cytotoxic T lymphocytes (CTLs) show heterogeneous diversity, whereas virus-specific memory CTLs show limited diversity.[28-30] In the study presented, IFN-γ was detected as a marker of antigen-specific T cells in the culture condition, in which PBMCs were cocultivated with APCs for 6 hours; therefore, the IFN-γ^+ cells may be memory or effector T cells.[31] Furthermore, the functional antigen avidities were comparable in the HTLV-1 Tax11-19–specific $CD8^+$ T cells during the time course (Figure 4A). Therefore, memory or effector T cells specific for HTLV-1 Tax

11-19 could show degenerate specificity of T-cell recognition during viral replication in chronic viral infections.

The increased frequency and degeneracy of HTLV-1–specific $CD8^+$ T cells in response to the increased proviral load may have another biologic role. If virus-specific $CD8^+$ T cells have limited degeneracy, they could eliminate wild-type viruses. However, some viruses with a mutation at the epitope would escape from the T cells, thereby increasing the proportion of the mutant viruses. The rate of epitope variants in patient no. 38 on June 24, 1999 was as high as 20.4%, whereas the rates were 0% or 5.9% at other time points (Table 2). However, the degree of IFN-γ production from the virus-specific T cells was similar throughout the time course (as shown in Figure 4B for patient no. 31). At the time point, the degeneracy of the T-cell population was the lowest, around an average of 6% during the course investigated (Figure 2C, June 24, 1999). The presented data support the hypothesis that the heterogeneous responses of virus-specific $CD8^+$ T cells have an advantage to eliminate mutant viruses over homologous T-cell responses.[26,32] Therefore, it seems likely that the degenerate specificity of virus-specific T-cell responses are defensive against mutant viruses in chronic viral infections.

The HTLV-1–specific T-cell frequency correlated well with the proviral load in each individual. Recently, it has clearly been shown that HTLV-1 mRNA load correlates with proviral DNA load and virus-specific $CD8^+$ T-cell frequency by a quantitative method.[33] If the increased proviral load during the clinical course in our patients suggests an elevated level of HTLV-1 mRNA load, then increased Tax protein expression would effectively stimulate the Tax 11-19–specific T cells in vivo (Figure 2A-B). In HTLV-1 infection, it has been suggested that HTLV-1 predominantly replicates as a provirus via mitotic proliferation of the infected cells in vivo.[3] High virus-specific T-cell response observed in patients with HAM/TSP suggests that the immune system is continuously exposed to the viral antigens in vivo.[10] Recently, it has been shown that circulating $CD4^+$ T cells infected with HTLV-1 rarely express viral antigens; however, they readily express the viral proteins in a 6-hour ex vivo culture and are subsequently killed by the $CD8^+$ CTLs in vitro.[34] Patient no. 31 showed a marked increase of HTLV-1–specific T-cell frequency (2.9 times elevation from 6.7% to 19.5%) corresponding to the increase of proviral load (1.3 times elevation from 506 to 674 copies/10^4 PBMCs) during the course (Figure 2A-B), suggesting that the T cells proliferated by a stimulation from HTLV-1. The proviral load was then reduced, implying that the T cells killed the virus-infected cells in vivo. However, the proviral load finally reached a final level of 506 copies/10^4 PBMCs despite continuous high virus-specific T-cell frequency on March 10, 1999. This suggests that silent HTLV-1–infected cells, which do not express the viral antigens, may survive in the body of the infected individual.

A marked increase in proviral load is a virologic hallmark in patients with HAM/TSP as compared to asymptomatic HTLV-1 carriers.[9] However, it is still controversial as to whether HTLV-1–specific T cells are increased in patients with HAM/TSP as compared to the carriers. It has been reported that circulating HTLV-1–specific T cells are significantly increased in patients with HAM/TSP as compared to asymptomatic HTLV-1 carriers with the use of CTL assay, limiting dilution assay, and intracellular cytokine assay.[10,11,16] However, it has also been reported that HTLV-1–specific T cells are increased both in HAM/TSP patients and asymptomatic HTLV-1 carriers.[35] It was not clear what causes this discrepancy. One possibility may be that the numbers of subjects included were small in both studies. In this study with a larger number of subjects, HTLV-1 Tax 11-19–specific T-cell frequency was significantly increased in the patients with HAM/TSP as compared to the carriers. It has been shown that HTLV-1 Tax 11-19–specific T cells accumulate and are activated in cerebrospinal fluid in patients with HAM/TSP as compared to the periphery and that $CD8^+$ T cells accumulate in the spinal cords of patients with HAM/TSP.[7,11,13] Collectively, these data suggest that HTLV-1–specific $CD8^+$ T cells may play an important roll in the pathogenesis of HAM/TSP.[15]

Crystal structure analysis of the HLA-A2/Tax 11-19/ TCR complex in a Tax 11-19–specific T-cell clone clearly shows that the amino acid at position 5 fits into the pocket formed by the TCR Vα and Vβ and makes numerous contact sites with the TCR,[20] suggesting that the amino acid is a primary TCR contact residue. The sequence analysis of an immunodominant epitope Tax 11-19 revealed that amino acid mutations accumulated in positions 4 and 5 and were observed in 13 of 26 variant epitopes (50%; Table 2). However, the mutated variants at the HLA-A2 anchor positions 2 and 9 were observed in only 2 of 26 variants (7.7%), which resulted in reduction in the binding affinity to HLA-A2.[21] In the patients with HAM/TSP, amino acid changes in the epitope preferentially occurred at TCR-binding positions rather than at HLA anchor positions. This is a marked contrast to HIV infection, where viral variants with a change at the HLA anchor positions become dominant during the time course because the majority of the variants readily escape from host immunity.[23-25] The reasons why amino acid substitutions preferentially occurred at the TCR-binding sites rather than at the HLA-binding sites in HTLV-1 infection are not clear. On the other hand, alterations in amino acids around position 5 of the epitope may provide a chance for the virus to escape from the HTLV-1–specific T cells. In the 3 patients, the T-cell recognition of APL substituted at positions 5 and 6 were increased when the proviral load was increased, although the increase was slight in patient no. 48 who had higher degeneracy of T-cell recognition than the others (Figure 2A-C). Therefore, the increased degeneracy of T-cell recognition preferentially directing amino acids at positions 5 and 6 may have an advantage to eliminate mutant viruses at these positions.

The naturally occurring variant epitope altered from glycine to arginine at position 4 of Tax 11-19 epitope, designated as G4R (LLFRYPVYV), is of interest, because the variant was predominantly investigated among the 3 patients (9 of 26 variants; Table 2). This mutation has previously been demonstrated in HLA-A*02^+ HAM/TSP patients and asymptomatic HTLV-1 carriers with different ethnic origins and in HLA-A*02^+ ATL patients.[18,36] In the present study, the variant did not become dominant among the patients during the time course of the disease. The predicted HLA-A2–binding affinity of G4R is comparable to that of cognate Tax 11-19 peptide (Table 2). If the variant peptide is endogenously processed and expressed efficiently with HLA on the infected cells, it is expected that G4R-specific $CD8^+$ T cells might emerge and kill the cells infected with the mutant virus. Therefore, we conducted an experiment to investigate whether G4R-specific $CD8^+$ T cells may emerge during the time course. However, $CD8^+$ T cells in PBMCs did not recognize the G4R peptide during the course in the 3 patients (data not shown), suggesting that the nonpredominance of G4R did not result from an appearance of G4R-specific $CD8^+$ T cells. It has been demonstrated that Tax protein acts as a *trans*-activator on its long terminal repeat and activates a number of cellular genes such as interleukin 2 through nuclear transcription factor NF-κB.[37] Thus, a mutation of Tax protein may give an advantage or a disadvantage to the infected cells for proliferation.

A *trans*-activation assay using the mutant gene that causes G4R mutation reveals that the *trans*-activation activity disappeared.[36] Therefore, a possible reason for nonpredominance of G4R despite lack of G4R-specific $CD8^+$ T cells is that the loss of function of Tax protein may lead to a decreased expression of the viral proteins and low replication rate of the mutant virus.

In conclusion, HTLV-1 persistently infects humans despite a vigorous virus-specific T-cell response, in part because the majority of the virus-infected cells rarely express viral antigens in vivo. During viral replication, HTLV-1–specific $CD8^+$ T cells show increases in both frequency and degeneracy. Thereafter, the interaction between the T cells and the virus may transiently reach an equilibrium state. This high immune response to the virus may play a crucial role in the pathogenesis of HAM/TSP. The elucidation of the underlying mechanisms by which HTLV-1–infected cells rarely express the viral antigens in vivo will be beneficial in designing patient therapies.

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Human T-Cell Lymphotropic Virus Type I (HTLV-I)–Related Clinical and Laboratory Findings for HTLV-I–Infected Blood Donors

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Summary: Clinical and laboratory findings were examined for 111 human T-cell lymphotropic virus type I (HTLV-I)–infected blood donors. HTLV-I provirus loads in subjects with a family history of adult T-cell leukemia (ATL) or HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) tended to be higher than those in subjects without a family history of these conditions. There were 3 asymptomatic patients with ATL, 4 with a history of uveitis, 7 with hyperreflexia in the lower limbs, and 3 with urinary frequency in the night. The mean CD4 cell/CD8 cell ratio ± SD was significantly lower ($p < .0001$) in subjects with hyperreflexia in the lower limbs (1.3 ± 0.2) than in subjects without any clinical abnormalities (1.7 ± 0.6), suggesting that subjects with hyperreflexia in the lower limbs already have some immunologic abnormalities. The concordance of HTLV-I infection between husband and wife was lower in this study than in a previous study. HTLV-I–related inflammatory symptoms were more frequent ($p = .021$, Fisher exact test; OR = 9.5; 95% CI, 1.7–53.5) in HTLV-I *tax* A–infected donors (3 [50%] of 6 donors) than in HTLV-I *tax* B–infected donors (10 [9.5%] of 105 donors), suggesting different risks of HTLV-I–related symptoms according to the virus genotype. **Key Words:** HTLV-I—Blood donors—Preclinical symptoms—Sexual transmission—HTLV-I subgroup.

Human T-cell lymphotropic virus type I (HTLV-I) was first isolated from lymphocytes of patients with cutaneous T lymphoma (1) in 1980 and was identified as the causative agent of adult T-cell leukemia (ATL) in 1982 (2). A retrospective study (3) and a follow-up study (4) of blood recipients revealed HTLV-I infection by blood transfusion from HTLV-I–infected people. In 1985, the association of tropical spastic paraparesis (TSP) with HTLV-I was demonstrated (5), and independent work in Japan also found an association between HTLV-I and spastic paraparesis called HTLV-I–associated myelopathy (HAM) (6); the condition is now called HAM/TSP. Although ATL rarely if ever develops after HTLV-I infection by blood transfusion, HAM/TSP was found to develop after HTLV-I infection by blood transfusion (7). After the nationwide screening of blood donors in Japan that started in 1986, a significant reduction in the number of patients with transfusion-associated HAM/TSP was observed (7). Most (≈97%) HTLV-I–infected individuals develop no associated disease. However, in Japan, ≈2%–3% of infected individuals develop ATL, and a further 0.25% develop HAM/TSP.

We have been studying the mechanism why most HTLV-I–infected individuals remain healthy and others develop ATL or HAM/TSP. Regarding the host factors, the HLA subtype was shown to influence the outcome of HTLV-I infection (8). We also showed that viral factor is

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associated with different risk for HAM/TSP (9). There are two HTLV-I *tax* subgroups (*tax* A and *tax* B) in Kagoshima, and HTLV-I *tax* A is more frequently observed in individuals with HAM/TSP than in healthy carriers (9). From April 1999, the Japan Red Cross Transfusion Service started to inform blood donors of HTLV-I infection status when they expressed their willingness to know serology results at the time of blood donation. Many HTLV-I carriers notified by the Red Cross visited our clinic between May 1999 and June 2001. We wanted to know if the frequency of preclinical symptoms of HAM/TSP differed between HTLV-I subgroups in these blood carriers. The Miyazaki cohort study analyzed the natural history of HTLV-I infection (10), and the risks of ATL (11) and heterosexual transmission of HTLV-I (12) were reported. However, preclinical symptoms of HAM/TSP were not reported to be associated with HTLV-I infection based on self-reported symptoms (13).

The aim of this study was originally to detect early-stage HAM/TSP to identify the risks and the pathogenetic mechanism of HAM/TSP. Therefore, all cases in this study were examined for their neurologic findings by board-certified neurologists and were analyzed for HTLV-I provirus load and viral subgroups. We also examined viral load according to the presence or absence of a family history of HTLV-I–related diseases and tested for evidence of heterosexual transmission of HTLV-I.

MATERIALS AND METHODS

HTLV-I–Infected Red Cross Blood Donors

One hundred twenty-eight HTLV-I–infected blood donors, who were notified of their HTLV-I–positive status by the Japan Red Cross Transfusion Service, visited our clinic between May 1999 and June 2001. Antibody to HTLV-I was detected by particle agglutination testing and confirmed by enzyme immunoassay and indirect immunofluorescence assay at the Red Cross. Of these 128 HTLV-I–infected individuals, 111 gave written informed consent for this study before the collection of blood samples. To identify the risks and the pathogenetic mechanisms of HAM/TSP, we tried to detect cases of early-stage HAM/TSP in these blood donors. All cases were checked for neurologic findings by three board-certified neurologists. We also checked family history, past history, physical examination findings, and laboratory findings. Approval was obtained from the institutional review board for these studies, and informed consent was provided according to the Declaration of Helsinki.

Antibody to HTLV-I was reexamined in our laboratory by particle agglutination testing (Fujirebio, Tokyo, Japan).

Cell Surface Staining

Surface markers were stained for phycoerythrin-labeled antibody to CD4 or CD8 (Becton Dickinson, Tokyo, Japan) and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

Provirus Load Measurement

The HTLV-I provirus load in peripheral blood mononuclear cells (PBMCs) of HTLV-I–infected subjects was measured by quantitative polymerase chain reaction (PCR) analysis using the ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Tokyo, Japan) as previously described (14). The amount of HTLV-I provirus DNA was calculated as follows: copy number of HTLV-I *tax*/10,000 PBMCs = (copy number of *tax*)/(copy number of β-actin/2) × 10,000.

Viral Subgroup by Restriction Fragment Length Polymorphism Analysis of the HTLV-I *tax* Gene

The viral subgroup of the HTLV-I *tax* gene was determined as we previously described (9). Nested PCR analysis of the extracted DNA was performed. The first PCR analysis was done using primers PXO1+ and PXO2–, and the second PCR analysis was done using primers PXI3+ and PXI3–. Since substitution at the nucleotide position of 8344 in the *tax* A gene created an *Acc*II site, we typed the given sample as *tax* A when the nested PCR product was digested using *Acc*II. When undigested, the sample was typed as *tax* B (9).

Statistical Analysis

The Student *t* test, Welch *t* test, Mann–Whitney *U* test, and Fisher exact test were used for statistical analysis.

RESULTS

Background of HTLV-I–Infected Blood Donors

All HTLV-I–infected blood donors were unaware that they were infected with HTLV-I until notification by the Red Cross. There were 67 male and 44 female blood donors, and the mean age of the blood donors was 45 years (44 years, male donors; 46 years, female donors). Of the 111 HTLV-I–positive subjects who gave written informed consent for our analysis, 10 had a family history of ATL and two had a family history of HAM/TSP in their related family members. One of them had a family history of both ATL and HAM/TSP. Seven other subjects had a family history of hematologic malignancies such as leukemia or lymphoma, but it was not clear if they had ATL (Table 1). WBC count, lymphocyte percentage, CD4 cell percentage, CD8 cell percentage, and CD4 cell/CD8 cell ratio did not differ significantly between HTLV-I–infected blood donors with and without a family history of ATL, HAM/TSP, or hematologic malignancies by the Student *t* test.

HTLV-I–Related Diseases or Symptoms in HTLV-I–Positive Subjects

Of the 111 HTLV-I–positive subjects, four had a history of uveitis (Table 2). As regards the diagnosis of ATL, there were three subjects (case 1, 37-year-old

TABLE 1. *Family history and laboratory findings*

Family history	No. of cases	WBC[a] (/μL)	Lymphocyte[a] (%)	CD4[a] (%)	CD8[a] (%)	CD4/8 ratio[a]	Anti-HTLV-I antibody[b]	Median HTLV-I provirus load[c]
ATL	10[d]	6130 ± 1844	29 ± 6	54 ± 11	28 ± 8	2.1 ± 1.0	2048	307
HAM/TSP	2[d]	5650 ± 636	29 ± 8	47 ± 8	38 ± 6	1.2 ± 0.4	1280	857
Hematologic malignancy	7	5057 ± 1196	29 ± 12	49 ± 5	32 ± 6	1.6 ± 0.5	512	87
No family history	93	5506 ± 1710	30 ± 9	49 ± 8	30 ± 8	1.8 ± 0.8	1024	79
Total	111	5539 ± 1689	30 ± 9	49 ± 8	30 ± 8	1.8 ± 0.8	1024	88

[a] Mean ± SD is shown for white blood cell (WBC) count, lymphocyte %, CD4 %, CD8% and CD4/8 ratio.
[b] Anti-HTLV-I antibodies were measured by particle agglutination test and the median HTLV-I antibody titer is shown.
[c] HTLV-I provirus load is shown as median provirus load (copy number of tax in 10,000 PBMC).
[d] One case had both family history of adult T-cell leukemia and HTLV-1–associated myelopathy/tropical spastic paraparesis.
No statistical difference was observed in laboratory findings between subjects with family histories and without family history.

male; case 2, 59-year-old female; and case 3, 45-year-old male) who had >5% abnormal lymphocytes in the circulation; two of these three subjects had monoclonal proliferation of HTLV-I–infected lymphocytes by Southern blot analysis. These three subjects had an increased CD4 cell count and CD4 cell/CD8 cell ratio in the peripheral blood but no clinical symptoms such as lymphoadenopathy or hepatosplenomegaly. According to Shimoyama's criteria (15), two of them were diagnosed with smoldering ATL, and the other was diagnosed with chronic ATL but not treated because of indolent disease activity. One of them had a family history of ATL. As regards the neurologic findings, seven (6.3%) of the 111 HTLV-I–positive subjects had hyperreflexia in the lower limbs, and three had urinary frequency during the night, both of which are neurologic features of HAM/TSP. However, pathologic reflexes such as Babinski reflex or Chaddock reflex were not observed in any subject. The WBC count, lymphocyte percentage, CD4 cell percentage, and CD4 cell/CD8 cell ratio were all significantly higher and the CD8 cell percentage was significantly lower in the three subjects with ATL than in HTLV-I–positive subjects without any HTLV-I–related findings, such as history of uveitis, hyperreflexia in the lower limbs, or urinary frequency in the night (Table 2). In HTLV-I–positive subjects with hyperreflexia in the lower limbs, the lymphocyte percentage and CD4 cell/CD8 cell ratio were significantly lower and the CD8 cell percentage was significantly higher than in subjects without any HTLV-I–related findings. In HTLV-I–positive subjects with urinary frequency in the night, the lymphocyte percentage was significantly lower than in subjects without any HTLV-I–related findings.

HTLV-I Provirus Load

The distribution of HTLV-I provirus load is shown in Figure 1. The median HTLV-I provirus load was 88 *tax* copies/10,000 PBMCs (≈0.88% of PBMCs, assuming one provirus copy per cell), and the mean provirus load was 313 *tax* copies/10,000 PBMCs. In males, the median HTLV-I provirus load was 88 *tax* copies/10,000 PBMCs, and the mean provirus load was 329 *tax* copies/10,000 PBMCs. In females, the median HTLV-I provirus load was 98 *tax* copies/10,000 PBMCs, and the average provirus load was 290 *tax* copies/10,000 PBMCs. There was no significant difference in HTLV-I provirus load between males and females. The HTLV-I provirus load in association with family history is shown in Table 1. The median HTLV-I provirus load in HTLV-I–positive sub-

TABLE 2. *Clinical symptoms and laboratory findings*

Diagnosis or symptom	No. of cases	WBC[a] (/μL)	Lymphocyte[a] (%)	CD4[a] (%)	CD8[a] (%)	CD4/8 ratio[a]	Anti-HTLV-I antibody[b]	HTLV-I provirus load[c]
ATL	3	10133 ± 1206**	43 ± 11*	72 ± 6**	15 ± 2**	4.7 ± 0.9**	1024	1871
Uveitis in past history	4	4205 ± 1678	32 ± 7	51 ± 7	33 ± 9	1.6 ± 0.5	1536	206
Hyper-reflexia in the lower limbs	7[d]	5528 ± 1716	22 ± 6*	44 ± 7	36 ± 6*	1.3 ± 0.2*	1024	467
Urinary frequency during the night	3[d]	4966 ± 1553	19 ± 5*	45 ± 3	38 ± 2	1.2 ± 0.1	1024	686
Other carriers	95	5473 ± 1536	31 ± 9	49 ± 7	30 ± 7	1.7 ± 0.6	512	79

[a] Mean ± SD is shown for white blood cell (WBC) count, lymphocyte %, CD4 %, CD8% and CD4/8 ratio.
[b] Anti-HTLV-I antibody were measured by particle agglutination test and the median HTLV-I antibody titer is shown.
[c] HTLV-I provirus load is shown as median provirus load (copy number of tax in 10,000 PBMC).
[d] One case had both hyper-reflexia in the lower limbs and urinary frequency and the HTLV-I virus load was 686.
** $p < .01$, and * $p < .05$ by Student t test compared with carriers without physical findings.

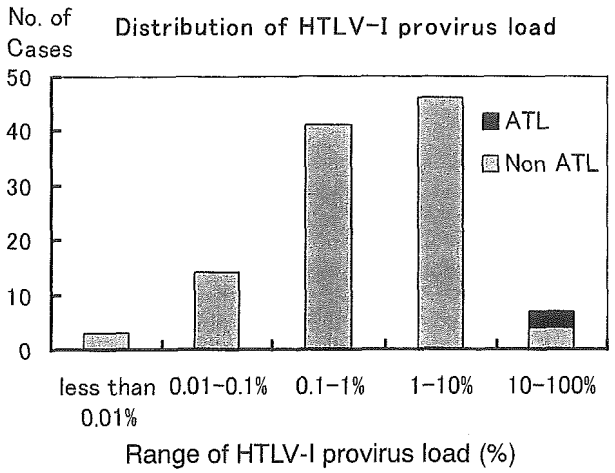

FIG. 1. Distribution of human T-cell lymphotropic virus type I (HTLV-I) provirus load. HTLV-I provirus load is shown as the percentage of cells infected with HTLV-I, assuming that each infected cell contains one copy of HTLV-I provirus. The number of cases with the range of each HTLV-I provirus load is shown. Hatched bar = non–adult T-cell leukemia (ATL) cases; solid bar = ATL cases.

jects with a family history of ATL (n = 10; 307 *tax* copies/10,000 PBMCs) tended to be higher than that in HTLV-I–positive subjects without a family history of ATL, HAM/TSP, or hematologic malignancies (n = 93; 79 *tax* copies/10,000 PBMCs), but this finding was of borderline significance by the Mann–Whitney U test (p = .058). The median HTLV-I provirus load in HTLV-I–positive subjects with a family history of HAM/TSP (n = 2; 857 *tax* copies/10,000 PBMCs) was also higher than that in HTLV-I–positive subjects without a family history of ATL, HAM/TSP, or hematologic malignancies. However, the number of subjects with a family history of HAM/TSP was too small (n = 2) for a meaningful statistical analysis. The median HTLV-I provirus loads in HTLV-I–infected subjects with HTLV-I–related findings are shown in Table 2. The median load in subjects with ATLV or HTLV-I–infected subjects with hyperreflexia in the lower limbs or urinary frequency tended to be higher than that in carriers without these findings. However, the number of subjects with ATL and urinary frequency was too small for Mann–Whitney U testing. In addition, the provirus load was not significantly different between HTLV-I–infected subjects with hyperreflexia in the lower limbs and carriers without any HTLV-I–related findings, perhaps because of the small number.

Concordance of HTLV-I Infection Between Husband and Wife

There were 17 male HTLV-I–infected subjects whose wives were tested for HTLV-I infection: only two wives were found to be infected with HTLV-I (Fig. 2). The mean age of the 17 husbands was 43 years, and the two husbands whose wives were infected with HTLV-I were 30 and 54 years old. The median viral load in the 17 husbands was 151 provirus copies/10,000 PBMCs, and the viral load in two husbands with infected wives were 328 and 302 provirus copies/10,000 PBMCs. On the other hand, there were only two HTLV-I–infected women whose husbands' HTLV-I seropositivity status could be ascertained; both husbands were seropositive for HTLV-I.

HTLV-I *tax* Gene Subgroup

Of the 111 HTLV-I–infected subjects, six (5.5%) were infected with HTLV-I *tax* A, and three (50%) of them had some HTLV-I–related symptoms (Table 3). Two of the HTLV-I *tax* A–infected subjects had hyperreflexia in the lower limbs, and one of these two HTLV-I–infected subjects had urinary frequency without cystitis. One other HTLV-I–infected subject had a history of uveitis, which has been reported to be associated with HTLV-I infection (16). Of the 105 HTLV-I *tax* B–infected subjects, only 10 (9.5%) had HTLV-I–related symptoms. Hyperreflexia in the lower limbs was observed in five (4.8%) of the 105 HTLV-I–infected subjects, and two other HTLV-I–infected subjects had urinary frequency in the night. The three other HTLV-I *tax* B–infected subjects (2.9%) had a history of uveitis. The frequency of

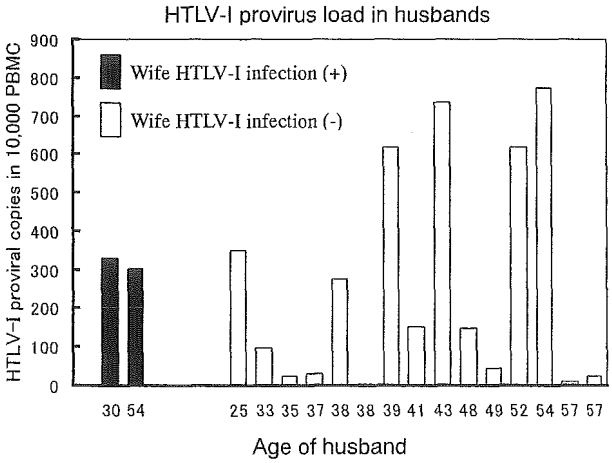

FIG. 2. Human T-cell lymphotropic virus type I (HTLV-I) provirus load in husbands with infected wives or uninfected wives is shown. HTLV-I provirus load is shown as the number of HTLV-I–infected cells in 10,000 peripheral blood mononuclear cells (PBMCs), assuming that each infected cell contains one copy of HTLV-I provirus. Solid bar = HTLV-I provirus load in a husband whose wife is infected with HTLV-I; grey bar = HTLV-I provirus load in a husband whose wife is not infected with HTLV-I. Numbers on the x axis represent the age of each husband.

TABLE 3. *HTLV-I tax subgroups and HTLV-I related inflammatory symptoms*

Symptoms	tax A	tax B	p value	Odds ratio	95% CI
Cases with hyper-reflexia in the lower limbs, *n*	2[a]	5			
Cases with urinary frequency in the night, *n*	1	2			
Cases with uveitis in past history, *n*	1	3			
Total cases with above symptoms, *n*	3	10			
Cases without above symptoms, *n*	3	95	.021	9.5	1.7–53.5

[a] One case had both exaggerated tendon reflex and urinary frequency.
p value, 2-tailed Fisher exact test.
CI, confidence interval.

HTLV-I–related inflammatory symptoms was significantly different between *tax* A and *tax* B HTLV-I–infected subjects ($p = .021$ by Fisher exact test; OR, 9.5; 95% CI, 1.7–53.5). The median HTLV-I provirus load in HTLV-I *tax* A–infected subjects was 68 copies/10,000 PBMCs (0.68%), and that in *tax* B HTLV-I–infected subjects was 88 copies/10,000 PBMCs (0.88%).

DISCUSSION

There have been several studies on the natural history of HTLV-I infection. The Miyazaki Cohort Study identified risk factors for ATL (11), heterosexual transmission of HTLV-I (12), and so on. In our study, to identify the risks and pathogenetic mechanisms of HAM/TSP, we tried to detect cases of early-stage HAM/TSP in blood donors, and neurologic findings were examined in all cases by three board-certified neurologists. We also examined laboratory findings including HTLV-I provirus load and HTLV-I *tax* subgroups and ascertained family history. It is possible that blood donors who have a family history of leukemia or lymphoma worry about themselves and tend to visit the Red Cross Transfusion Service to check on the condition of their health. Because we checked the family history of ATL, HAM/TSP, and other hematologic malignancies, we were able to control for a family history of ATL and HAM/TSP in this study.

As regards the HTLV-I provirus load, the mean HTLV-I provirus load in this study was 313 copies/10,00 PBMCs (≈3.13% of PBMCs, assuming one provirus copy per cell), and the median provirus load was 0.88%. The mean provirus load in the male population was 3.29%, and that in the female population was 2.9%. These results are consistent with those of the Miyazaki Cohort Study, in which the mean provirus load was 3.5% in total, 3.3% in the male population, and 3.7% in the female population, with no significant differences between males and females (17).

As regards HTLV-I–related diseases, first, there were four HTLV-I–infected subjects with a history of uveitis. Uveitis was reported to be associated with HTLV-I infection (16). The prevalence of HTLV-I–associated uveitis was reported to be 1.12 cases per 1,000 people in the north part of Kyushu (18) and 0.5–1 case per 1,000 people in Kagoshima (19). Our observation that four of 111 HTLV-I–infected subjects had a history of uveitis suggests that HTLV-I–associated uveitis is usually a transient disease, not lifelong as in ATL or HAM/TSP, and that HTLV-I–infected subjects not infrequently have a history of HTLV-I–associated uveitis.

Second, as regards ATL, there were three subjects with >5% abnormal lymphocytes in the circulation. Two of them had smoldering ATL and one had chronic ATL according to Shimoyama's criteria (15). Although there may be debate whether we should have included these subjects without any clinical symptoms as having ATL, we must follow these subjects carefully. We did not treat these subjects with ATL, because it has not been established that patients with smoldering ATL or chronic ATL who do not have elevated serum LDH and creatinine levels and decreased albumin levels are benefited by chemotherapy. It is of interest that HTLV-I–infected subjects with a family history of ATL tended to have a higher HTLV-I provirus load than HTLV-I–infected subjects without a family history, although this difference was only of borderline statistical significance. In other studies, increased HTLV-I provirus load also was observed in the family members of subjects with HAM/TSP (14,20). These findings suggest that there is also a genetic predisposition to a higher provirus load in family members of patients with ATL, and we should carefully follow these subjects.

Third, there were seven HTLV-I–infected blood donors with hyperreflexia in the lower limbs and three with urinary frequency in the night (one of them had both hyperreflexia and urinary frequency). In a report by the Retrovirus Epidemiology Donor Study Group (21), hyperreflexia was more frequently observed in HTLV-I–positive donors than in HTLV-I–negative donors. Because we did not test neurologic findings in subjects