almost certain a priori that there will be significant differences between populations in the genetic contribution to susceptibility to HAM/TSP, since HLA-B*5401 is prevalent in Japan and elsewhere in East Asian populations, but is virtually absent from many other populations. Since HLA-B*5401 has an important influence on the risk of disease in Kagoshima (Jeffery *et al.*, 2000), its presence in the population is certain to influence the risk associated with other HLA alleles, and the absence of HLA-B*5401 in other populations with endemic HTLV-1 infection will alter the relative importance of other genes to the risk of developing HAM/TSP.

We first examined the HTLV-1 provirus load in Iranian HAM/TSP patients and HCs, since one of the major risk factors for developing HAM/TSP is the provirus load (Nagai *et al.*, 1998). The median HTLV-1 provirus load of Iranian HAM/TSP patients was twofold greater in HAM/TSP patients than in HCs, whereas that of Japanese HAM/TSP patients was 13-fold greater than in HCs. Interestingly, despite differences in the methods of DNA extraction between the two study groups (whole blood-derived DNA for Iranian samples vs PBMC-derived DNA for Japanese samples), the HTLV-1 provirus load in Iranian HCs was still significantly higher than Japanese HCs ($P=0{\cdot}004$, Mann–Whitney U test). This may be the main cause of the smaller observed ratio of median provirus load between HAM/TSP patients and HCs in the Iranian study group. To investigate the reason for this difference between the two populations, we further analysed the frequencies of certain HLA alleles and the HTLV-1 *tax* subgroup in the Iranian population.

In the Kagoshima population, possession of either of the HLA class I genes HLA-A*02 or HLA-Cw*08 was associated with a statistically significant reduction in both HTLV-1 provirus load and the risk of HAM/TSP (Jeffery *et al.*, 1999, 2000). However, in Mashhadi Iranian subjects, both HLA-A*02 and HLA-Cw*08 had no effect on either the risk of HAM/TSP or provirus load. In contrast, HLA-DRB1*0101 was associated with increased susceptibility to HAM/TSP both in Kagoshima ($P=0{\cdot}049$) and Iran ($P=0{\cdot}035$). In HAM/TSP, $CD4^+$ cells are the predominant cells present early in the active lesions (Umehara *et al.*, 1993) and are also the HTLV-1-infected cells in the inflammatory spinal cord lesions (Moritoyo *et al.*, 1996). Moreover, HLA-DRB1*0101 restricts $CD4^+$ T-cell immunodominant epitopes of HTLV-1 *env* gp21 (Yamano *et al.*, 1997; Kitze *et al.*, 1998). Therefore, it is possible that HLA-DRB1*0101 is associated with susceptibility to HAM/TSP via an effect on $CD4^+$ T-cell activation and subsequent bystander damage in the central nervous system (Ijichi *et al.*, 1993; Bangham, 2000). However, since possession of HLA-DRB1*0101 was associated with a significantly lower provirus load in the Japanese HAM/TSP patients but not in the Iranian HAM/TSP patients, the underlying mechanism involving HLA-DRB1*0101 may not be the same between Iranian and Japanese HTLV-1-infected individuals. Differences in other genetic factors, including non-HLA genes, may also be important for explaining the observed differences between the populations.

Another possible explanation of the observed differences in the present study is that certain HLA genotypes are associated with different effects on different subtypes of the virus. In human papilloma virus (HPV) infection, the association of the DRB1*1501–DQB1*0602 haplotype with HPV-related cervical carcinoma was reported to be specific for the viral type HPV-16, suggesting that specific HLA haplotypes may influence the immune response to specific virus-encoded epitopes and affect the risk of viral disease (Apple *et al.*, 1994). To test this possibility, we sequenced almost the entire region of the *tax* gene in 20 Mashhad Iranian HTLV-1-infected individuals (10 HAM/TSP and 10 HCs) and compared the sequence with that of two Japanese strains, *tax* subgroups A and B. Although we could not identify any amino acid differences in the Tax11–19 immunodominant epitope between the Iranian and Japanese *tax* subgroups A and B, we found that Iranian HTLV-1 possessed 10 different nucleotides in the *tax* region compared with Japanese *tax* subgroup B. Among these, nt 7897, 7959, 8208 and 8344 were identical to *tax* subgroup A. Therefore, Iranian *tax* sequences have four additional different amino acids compared with Japanese *tax* subgroup A and six additional different amino acids compared with Japanese *tax* subgroup B. These findings suggest that both the lack of consistency of host genetic influences and the smaller difference in median provirus load between HAM/TSP patients and HCs in Iran may be due in part to different strains of HTLV-1. Our present observation that HLA-A*02 was associated with a lower provirus load only in the *tax* subgroup B-infected subjects in Kagoshima, but not in *tax* subgroup A-infected subjects, is consistent with this hypothesis. Further studies to examine functional differences between Iranian and Japanese HTLV-1 Tax proteins will provide important information to clarify this point.

The interaction between different genes and/or environmental factors is also likely to contribute to the observed differences between the two populations. For example, co-infection with *Strongyloides stercoralis* (Gabet *et al.*, 2000) can affect the HTLV-1 provirus load. In Japan, *S. stercoralis* infection is endemic in the southwestern islands Amami and Ryukyu, but is rarely reported on the mainland including Kagoshima (Arakaki *et al.*, 1992). However, there are no data on the prevalence of *S. stercoralis* infection in Mashhad, Iran, and therefore future epidemiological studies are necessary to clarify this possibility.

It seems likely that the same evolutionary selection pressures that induce polymorphisms in 'infection-resisting genes' have contributed to marked allele-frequency differences at the same loci. When geographical variation in pathogen polymorphism is superimposed on this host genetic heterogeneity, considerable variation in detectable allelic associations is likely to result in the different

populations. In other words, genetic resistance to infectious diseases that is formed by complex host genetic effects is complicated further by pathogen diversity and environmental factors. Considering this background of complexity, the most practical approach to finding reliable results may be first to identify disease-associated genes in a single large population, and secondly to analyse subsequently whether a similar effect is found in other ethnic populations, as we have shown in this study.

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ApaI polymorphism of vitamin D receptor gene is associated with susceptibility to HTLV-1-associated myelopathy/tropical spastic paraparesis in HTLV-1 infected individuals

Mineki Saito[a,*], Nobutaka Eiraku[b], Koichiro Usuku[c], Yasuyuki Nobuhara[a], Wataru Matsumoto[a], Daisuke Kodama[a], Amir H. Sabouri[a], Shuji Izumo[d], Kimiyoshi Arimura[a], Mitsuhiro Osame[a]

[a]*Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[b]*Kagoshima University Health Service Center, 1-21-24 Korimoto, Kagoshima 890-8580, Japan*

[c]*Department of Medical Information Science, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[d]*Department of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

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Abstract

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type-1 (HTLV-1) infection. It remains unknown why the majority of infected people remain healthy, whereas only approximately 2–3% of infected individuals develop the disease. The active form of vitamin D has immunomodulatory effects, and allelic variants of the vitamin D receptor (VDR) appear to be associated with differential susceptibility to several infectious diseases. To investigate whether VDR single nucleotide polymorphisms (SNPs) are associated with the development of HAM/TSP, we studied four VDR SNPs in a group of 207 HAM/TSP patients and 224 asymptomatic HTLV-1 seropositive carriers (HCs) in Kagoshima, Japan, by using PCR-RFLP analysis. We found that *ApaI* polymorphism of VDR is associated with the risk of HAM/TSP, although this polymorphism did not affect the provirus load of HTLV-1 in either HAM/TSP patients or HCs.

Keywords: Vitamin D receptor; Single nucleotide polymorphism; HAM/TSP; HTLV-1; Provirus load

1. Introduction

Human T-cell lymphotropic virus type-1 (HTLV-1) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-1 is estimated to affect 10–20 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. Our case/control studies in Kagoshima strongly support this hypothesis. In the Kagoshima population, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 provirus load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP [8,9]. Further analysis to look at non-HLA host genetic factors revealed that the TNF-α promoter-863 A allele predisposed to HAM/TSP, whereas SDF-1 +801A 3′ UTR, and IL-15 191 C alleles conferred protection against this disease

* Corresponding author. Tel.: +81 99 275 5332; fax: +81 99 265 7164.
E-mail address: mineki@m3.kufm.kagoshima-u.ac.jp (M. Saito).

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[10], suggesting that non-HLA gene polymorphism also affects the risk for developing HAM/TSP.

It is well known that the active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$), is involved in the maintenance of mineral homeostasis [11]. The effect of 1,25-$[OH]_2D_3$ is mediated by its receptor, which is the ligand-dependent transcription factor, and the vitamin D receptor (VDR) gene consists of nine exons with a number of polymorphisms [12]. To date, over 30 studies to test an association between polymorphisms of VDR and osteoporosis were reported, with about half confirming the association and the remainder failing to confirm [13]. Not only for mineral homeostasis, 1,25-$[OH]_2D_3$ is also implicated in the regulation of the immune system [11]. The immunomodulatory actions of the 1,25-$[OH]_2D_3$ are mediated by interaction with VDR, which is expressed in resting and activated lymphocytes [14]. Therefore, VDR may also interact to determine the risk of infectious diseases. Some studies have revealed a close association between VDR polymorphisms and the outcome of certain infectious diseases such as tuberculosis [15], hepatitis B virus (HBV) [15] and leprosy [16]. Most importantly, reports by Barber et al. and Nieto et al. showed that individuals with the VDR *BsmI* BB and *FokI* heterozygotes, respectively, were associated with rapid progression to AIDS among HIV-1 seropositive intravenous drug users [17,18], suggesting that particular polymorphisms in the VDR contribute to the host immune reaction against viral infection. Since the most commonly studied VDR polymorphisms include a *Fok*I restriction fragment-length polymorphism (RFLP) in exon 2 (alleles F/f or nucleotides C/T), *Bsm*I (B/b or nucleotides T/C) and *Apa*I (A/a or nucleotides T/G) variants in intron 8, and a *Taq* I (T/t or nucleotides T/C) variant in exon 9, with lowercase alleles indicating the presence of restriction sites, we genotyped these four SNPs and analyzed the effect of each SNP on the risk of HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 207 patients with HAM/TSP and 224 asymptomatic HTLV-1 seropositive carriers (HCs), all residing in HTLV-1 endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [19]. 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 PBMCs 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. PCR-RFLP analysis

Fifty nanograms of genomic DNA was amplified by PCR in a total volume of 25 μl using the primer and PCR conditions described by Harris et al. [20] for the *FokI*, by Wilkinson et al. [21] for the *TaqI* and *BsmI*, and Niino et al. [22] for the *ApaI* polymorphism of VDR. The primer sequences were as follows: 5′ -AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′ and 5′ -ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ for *FokI*; 5′ -GGG ACG ATG AGG GAT GGA CAG AGC-3′ and 5′ -GGA AAG GGG TTA GGT TGG ACA GGA-3′ for *TaqI*; 5′ -AAC TTG CAT GAG GAG GAG CAT GTC-3′ and 5′ -GGA GAG GAG CCT CTG TCC CAT TTG-3′ for *BsmI*; 5′ -GTC GCT GAG GGA TGG-3′ and 5′ -GTC GGC TAG CTT CTG GAT-3′ for *ApaI*. After PCR amplification, the 15 μl of PCR product was digested for 12 h with an excess of restriction enzyme. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of HTLV-1 provirus load, CSF neopterin and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-

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

	Age	Male/female	Anti-HTLV-1 antibodies[a]	HTLV-1 provirus load[b]	Neopterin in CSF[c]
HAM/TSP (n=207)	57.0±12.1[d]	62/145	×26,458±41,433	719.3±709.2	112.7±112.6
HCs (n=224)	39.8±13.1	109/115	×1478±1453	131.9±243.0	N/A

N/A: not applicable.
HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
[a] Anti-HTLV-1 antibodies were titrated by the particle agglutination method. The antibody titers were achieved by performing a serial dilution of the patient serum and noting the highest dilution at which agglutination is still present.
[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±S.D.

Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMC samples as reported previously [23]. Using β-actin as an internal control, the amount of HTLV-1 provirus 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)]$\times10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by high-performance liquid chromatography (HPLC) with fluorimetric detection methods [24]. Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio). The antibody titers were achieved by performing a serial dilution of the patient serum and noting the highest dilution at which agglutination is still present.

2.5. Statistical analysis

Comparisons of genotype frequency between HAM/TSP patients and HCs were calculated by the chi-squared test. For multiple comparisons of the HTLV-1 provirus load measured in HAM/TSP and HCs individuals, subdivided according to their *ApaI* genotype, we used one-factor ANOVA when variance of each group was equal by Bartlett test. If variance of each group was different, Kruskal–Wallis test was employed. Linkage disequilibrium (LD) was calculated between SNPs as previously described [25] by using SNPAlyze software ver. 3.2 (Dynacom, Mobara, Japan), which provides a D prime (D') value between 0 and 1. A D' value of 0 indicates no LD between the two markers and a D' value of 1 indicates complete LD between two markers. Significance was considered at $p<0.05$.

3. Results

3.1. Vitamin D receptor gene polymorphism in HAM/TSP patients and asymptomatic HTLV-1 carriers

Initially, we genotyped 118 patients with HAM/TSP and 129 HCs for each SNP. There were no significant differences in the distribution of the *FokI, TaqI* and *BsmI* genotypes and allele frequencies between 118 HAM/TSP patients and 129 HCs (Table 2). In contrast, the *ApaI* genotypes (AA, Aa, aa) showed a significant difference in frequency ($\chi^2=8.04$ on 2 *df*, $p=0.018$). We therefore further analyzed a total of 207 cases of HAM/TSP and 224 HCs for *ApaI* polymorphism and identified a significant association between AA genotype and reduced risk of HAM/TSP ($\chi^2=10.48$ on 2 *df*, $p=0.0012$, Odds ratio=0.28, 95%CI=0.13–0.63). In both HAM/TSP patients and HCs, the genotype frequencies were distributed according to the Hardy–Weinberg equilibrium. Previously reported allele and genotype frequencies of *ApaI* polymorphism from a Japanese normal control population showed similar results with the HCs group of our present study [22].

3.2. AA genotype is associated with a lower risk for HAM/TSP only in female gender and FokI heterozygotes

Interestingly, the protective effect of *ApaI* AA genotype was observed only in the female subjects (72 HAM/TSP patients and 59 HCs, $\chi^2=7.11$ on 2 *df*, $p=0.029$) but not in the male subjects (38 HAM/TSP patients and 50 HCs, $\chi^2=4.31$ on 2 *df*, $p=0.116$). Because a series of the three polymorphisms (*BsmI*, *ApaI* and *TaqI* SNPs) in the 3′

Table 2
Frequencies of genotypes and alleles for the different polymorphisms in the vitamin D receptor gene

SNP	Allele	HAM/TSP	HCs	*P* value[a]	Genotype	HAM/TSP	HCs	*P* value[b]
ApaI	A	105 (25.4)[c]	143 (31.9)	0.034*	AA	8 (3.9)	28 (12.5)	0.0053*
	a	309 (74.6)	305 (68.1)		Aa	89 (43.0)	87 (38.8)	
					aa	110 (53.1)	109 (48.7)	
	total	414	448		total	207	224	
FokI	F	141 (59.7)	157 (60.9)	0.80	FF	44 (37.3)	50 (38.8)	0.97
	f	95 (40.3)	101 (39.1)		Ff	53 (44.9)	57 (44.2)	
					ff	21 (17.8)	22 (17.1)	
	total	236	258		total	118	129	
BsmI	B	28 (11.9)	32 (12.4)	0.85	BB	2 (1.7)	1 (0.8)	0.70
	b	208 (88.1)	226 (87.6)		Bb	24 (20.3)	30 (23.3)	
					bb	92 (78.0)	98 (76.0)	
	total	236	258		total	118	129	
TaqI	T	208 (88.1)	228 (88.4)	0.93	TT	92 (78.0)	100 (77.5)	0.78
	t	28 (11.9)	30 (11.6)		Tt	24 (20.3)	28 (21.7)	
					tt	2 (1.7)	1 (0.8)	
	total	236	258		total	118	129	

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
[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.
* $P<0.05$.

Table 3
Linkage disequilibrium (LD) between four vitamin D receptor polymorphisms in HTLV-1 infected subjects

VDR SNP	*FokI*-HAM	*BsmI*-HAM	*TaqI*-HAM
ApaI-HAM	0.23444	**0.93886**	**1.0000**
	FokI-HAM	0.58781	0.44849
		BsmI-HAM	**0.91828**
			TaqI-HAM
ApaI-HCs	0.05065	**0.87419**	**0.93298**
	FokI-HCs	0.59933	0.41685
		BsmI-HCs	**0.88339**
			TaqI-HCs
ApaI-All	0.03603	**0.90706**	**0.96805**
	FokI-All	0.59305	0.43561
		BsmI-All	**0.90042**
			TaqI-All

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
LD was calculated between SNPs, which provides a *D* prime (*D'*) value between 0 and 1.
D' values between two SNPs in each group were shown.
No LD=0, complete LD=1, strong LD=0.7–1.0 (values in bold).

UTR of the VDR gene have been shown to be in strong LD with one another in Western countries [26], we analyzed the presence of the LD in our subjects. As a result, the *BsmI*, *ApaI* and *TaqI* polymorphisms are in strong LD whereas the *FokI* polymorphism at the translation initiation site in exon2 of the VDR gene was not in significant linkage with the other polymorphisms (Table 3). Since previous report by Nieto et al. showed clear association between combined genotypes for *FokI* and *BsmI* polymorphisms and a faster progression to AIDS among HIV-1 seropositive intravenous drug users, despite no significant linkage between two polymorphisms in their population [18], we also tested whether the *FokI* genotype affect the observed protective effect of *ApaI* genotype against HAM/TSP development. When we analyzed the distribution of the *ApaI* polymorphism in the 274 HTLV-1 infected individuals with the non-Ff genotype (homozygous FF and ff), we observed that there were no significant differences in the distribution of the *ApaI* genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs. In contrast, in individuals with the Ff genotype (heterozygous, 53 HAM/TSP patients and 57 HCs), the risk ratios for progression to HAM/TSP in HTLV-1 infected individuals without AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals with AA genotype (Table 4). Therefore, the protective effect of *ApaI* AA genotype was observed only in the *FokI* heterozygotes (Ff) but not in homozygotes (FF and ff).

3.3. *Vitamin D receptor gene ApaI polymorphism is not a significant predictor of the HTLV-1 provirus load in HAM/TSP patients and asymptomatic HTLV-1 carriers*

To test whether VDR gene *ApaI* polymorphism is a significant predictor of the HTLV-1 provirus load, we measured the provirus load of HTLV-1 and compared it with *ApaI* genotypes (AA, Aa, aa) in HAM/TSP patients and HCs. Our data indicated that there was no association between *ApaI* genotypes and HTLV-1 provirus load (Table 5), CSF neopterin levels, as well as serum HTLV-1 antibody

Table 4
Vitamin D receptor *ApaI* allele/genotype frequencies in relation to *FokI* genotypes in HTLV-1 infected individuals

FokI genotype	*ApaI* allele	HAM/TSP	HCs	OR (95% CI)	*P* value
Homozygous (FF and ff)	A	41 (31.5)	49 (34.0)	0.89 (0.54–1.48)	0.66
	a	89 (68.5)	95 (66.0)	1.12 (0.68–1.86)	0.66
	total	130	144		
Heterozygous (Ff)	A	25 (23.6)	37 (32.5)	0.64 (0.35–1.17)	0.14
	a	81 (76.4)	77 (67.5)	1.56 (0.86–2.83)	0.14
	total	106	114		
FokI genotype	*ApaI* genotype	HAM/TSP	HCs	OR (95% CI)	*P* value
Homozygous (FF and ff)	AA	3 (4.6)	7 (9.7)	1 (reference)	
	Aa	35 (53.8)	35 (48.6)	2.33 (0.56–9.76)	0.24
	aa	27 (41.5)	30 (41.7)	2.10 (0.49–8.94)	0.31
	Aa+aa	62 (95.4)	65 (90.3)	2.23 (0.55–8.99)	0.25
	total	65	72		
Heterozygous (Ff)	AA	1 (1.9)	10 (17.5)	1 (reference)	
	Aa	23 (43.4)	17 (29.8)	13.53 (1.58–116.0)	0.0044*
	aa	29 (54.7)	30 (52.6)	9.67 (1.16–80.4)	0.014*
	Aa+aa	52 (98.1)	47 (82.5)	11.06 (1.36–89.7)	0.0062*
	total	53	57		

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
Numbers in parentheses are percentage.
OR: odds ratio; 95% CI: 95% confidence interval.
* $P<0.05$.

Table 5
Vitamin D receptor *ApaI* polymorphism and HTLV-1 provirus load

	AA	Aa	aa	*P* value
HAM (207)	541.6±222.2 (8)	745.8±79.6 (89)	710.7±65.2 (110)	0.727[a]
HCs (224)	103.8±32.4 (28)	129.7±31.5 (87)	140.9±20.4 (109)	0.799[b]
All patients combined (431)	201.1±61.4 (36)	441.3±48.9 (176)	427.1±39.3 (219)	0.718[b]

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
The values are shown as the mean tax value (tax copies/10^4 PBMCs)±S.E.
[a] *P* value by one-factor ANOVA.
[b] *P* value by Kruskal–Wallis test.

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-1 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. The outcome of HTLV-1 infection depends on both host genetic and viral factors. However, although different virus strains (denoted HTLV-1 subgroups) can influence the risk of developing HAM/TSP [7], the impact of HTLV-1 viral sequence variation in determining the risk of developing HAM/TSP in Kagoshima is not sufficient to predict disease. Our recent observations, as well as many reported findings, strongly suggest that the outcome of HTLV-1 infection mainly depends upon a host of genetic factors [27].

In addition to its role in calcium and skeletal homeostasis, 1,25-$[OH]_2D_3$ plays an important role in immune system modulation [11]. The 1,25-$[OH]_2D_3$ promotes monocyte differentiation, stimulates cell-mediated immunity, and inhibits lymphocyte proliferation and secretion of cytokines such as interleukin (IL)-2, granulocyte-macrophage colony-stimulating factor and interferon-γ from T cells, and IL-12 from macrophages and B cells [28–31]. The inhibiting effect of vitamin D on the immune response appears to target Th1 cells by preventing their activation and cytokine production [28]. Interestingly, previous studies indicated that the Th1 cell response is predominant in HAM/TSP [32,33] and 1,25-$[OH]_2D_3$ has the potential to suppress cell proliferation through binding to the VDR overexpressed in HTLV-1 infected T cells [34,35]. Therefore, it is interesting to test whether VDR gene polymorphism is associated with the risk for developing HAM/TSP.

In the present study, the *ApaI* polymorphism of VDR showed a significant difference in allele frequency, and AA genotype was associated with the reduced risk of HAM/TSP in HTLV-1 infected individuals, whereas there were no significant differences in the distribution of the *FokI*, *TaqI* and *BsmI* genotypes and allele frequencies. Interestingly, the protective effect of *ApaI* AA genotype was observed only in females, which is one of the risk factor for developing HAM/TSP. The HTLV-1 provirus load of female patients with HAM/TSP was significantly higher than that of male patients [23] and the ratio of male to female HAM/TSP patients is about 1:2, as shown in the present study. However, there was no significant difference between any VDR genotypes and HTLV-1 provirus load in either HAM/TSP patients or HCs. Also, there were no correlations between CSF neopterin levels or serum anti-HTLV-1 antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with any VDR polymorphisms in HAM/TSP patients. This was strikingly different from the HLA-A*02 [8] and Cw*08 [9], which were associated with both the risk of HAM/TSP and lower provirus load in HCs. Since *ApaI* polymorphism is located in intron 8 and is not affecting any splicing site and/or transcription factor binding site [36], it is unlikely that this polymorphism is directly associated with the functional difference of VDR itself. Linkage disequilibrium with truly functional polymorphism elsewhere in the VDR gene or other gene(s) may be associated with the susceptibility to HAM/TSP via gender-specific mechanism other than an apparent effect on provirus load. In our population, significant LD was found among the *BsmI*, *ApaI* and *TaqI* polymorphisms located in the 3′ UTR of the VDR gene, but *FokI* polymorphism was not in LD with these three polymorphisms. However, the *FokI* genotype affects the observed protective effect of *ApaI* genotype against HAM/TSP, as observed in HIV-1 infected intravenous drug users for progression to AIDS [18]. In HTLV-1 infected individuals with the *FokI* Ff genotype, the risk ratios for progression to HAM/TSP without *ApaI* AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals with AA genotype, whereas there were no significant differences in the distribution of the *ApaI* genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs which were non-Ff genotype (homozygous FF and ff). These findings provide strong evidence to suggest that genetic variations at the VDR locus may affect the outcome of HTLV-1 infection. Recent transmission-disequilibrium test on family-based study also showed a significant association of tuberculosis with SNP combinations *FokI–ApaI* by the increased transmission to affected offspring [37]. It is possible that the presence of unidentified, associated functional alleles that lies on this haplotype background influence the susceptibility to HAM/TSP, although further studies are needed to clarify this point.

In conclusion, our results indicate that VDR *ApaI* polymorphism is a novel non-HLA host genetic factor to evaluate the risk of HAM/TSP. The functional significance

of this observation may reveal immunotherapeutic strategies that would retard the development of HAM/TSP in the future.

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A prospective uncontrolled trial of fermented milk drink containing viable *Lactobacillus casei* strain Shirota in the treatment of HTLV-1 associated myelopathy/tropical spastic paraparesis

Toshio Matsuzaki [a], Mineki Saito [a,*], Koichiro Usuku [b], Hirohisa Nose [a], Shuji Izumo [c], Kimiyoshi Arimura [a], Mitsuhiro Osame [a]

[a] *Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[b] *Department of Medical Information Science, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[c] *Department of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

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Abstract

Ten patients with human T-cell lymphotropic virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) were treated in an uncontrolled preliminary trial by oral administration of viable *Lactobacillus casei* strain Shirota (LcS) containing fermented milk. HTLV-1 provirus load, motor function, neurological findings, and immunological parameters were evaluated after 4 weeks. Although LcS did not change the frequencies or absolute numbers of all the examined cell surface phenotypes of peripheral blood mononuclear cells, NK cell activity was significantly increased after 4 weeks of oral administration of LcS preparation. Improvements in spasticity (modified Ashworth Scale scores) and urinary symptoms were also seen after LcS treatment. No adverse effect was observed in all the 10 patients throughout the study period. Our results indicated that LcS may be a safe and beneficial agent for the treatment of HAM/TSP; therefore randomized controlled studies are warranted.

Keywords: Immunomodulation; *Lactobacillus casei* strain Shirota; NK cell activity; HAM/TSP; HTLV-1

1. Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [1–3]. Although the precise mechanism causing HAM/TSP is still obscure, virus–host immunological interactions are considered to be the most important cause of this disease, because (1) the median HTLV-1 provirus load is more than 10 times higher in HAM/TSP patients than in healthy HTLV-1 carriers (HCs), and high provirus load is also associated with an increased risk of progression to disease [4]; (2) the titer of anti-HTLV-1 antibody often reaches a very high level in HAM/TSP patients, and correlates well with a HTLV-1 provirus load [4]; (3) in HAM/TSP patients, large populations of activated T cells both in peripheral blood mononuclear cells (PBMCs) [5] and cerebrospinal fluid (CSF) [6], and spontaneous proliferation of PBMCs in vitro [7–9] have been reported; (4) HTLV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) are abundant and activated in PBMCs in HAM/TSP patients [10], and these CTLs are preferentially accumulated in CSF cells [11,12]; and (5) it

* Corresponding author. Tel.: + 81 99 275 5332; fax: +81 99 265 7164.
E-mail address: mineki@m3.kufm.kagoshima-u.ac.jp (M. Saito).

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has been shown that HTLV-1 Tax_{11-19}-specific $CD8^+$ T cells have the potential to produce proinflammatory cytokines [13], whereas possession of the HLA-A*02 allele was associated with protection against HAM/TSP as well as a lower provirus load [14]. To regulate such immunologic processes, some therapeutic trials with agents such as prednisolone [15,16], plasma exchange [17], and interferon (IFN)-α [18–22], have been conducted and reported to be effective.

Lactobacillus casei strain Shirota (LcS), a type of lactic acid bacteria, was originally isolated from the human intestine and has been used commercially for a long time to produce fermented milk [23]. It was previously reported that LcS activates macrophages [24], NK cells [25], and cytotoxic T cells [26], and also exhibits a marked inhibitory effect against tumors [23,27], type II collagen-induced arthritis in DBA/1 mice [28], and infectious pathogens such as *Listeria monocytogenes* [29], herpes simplex virus [30], and influenza virus [31]. Adverse effects and toxic reactions to LcS are rarely reported. On the other hand, previous reports indicated that the NK cell activity and the percentages of NK cell subsets was significantly decreased in HAM/TSP patient compared with that in uninfected normal controls [32,33]. In our previous study, we also reported that one of the inhibitory NK cell receptor, NKG2A, was significantly decreased in frequency in HAM/TSP patients but not in HCs [34]. Since inhibitory NK cell receptors such as NKG2A can down-regulate antigen-mediated T-cell effector functions including cytotoxic activity and cytokine release, decreased NK cell function and NKG2A+ T cells may be associated with impaired regulation of T cell-mediated antiviral immune responses in HAM/TSP patients. These observations therefore raise the possibility that LcS could improve the symptoms of HAM/TSP via activating NK cell subsets.

To conduct a preliminary investigation of the efficacy of LcS for HAM/TSP–and to see if controlled trials are warranted–we evaluated in an uncontrolled trial with 10 HAM/TSP patients. After daily oral administration of LcS preparation that contains approximately 8×10^{10} viable LcS for 4 weeks, the effects of spasticity, motor disabilities, urinary symptoms and immunological parameters were evaluated.

2. Patients and methods

2.1. Study population

Our study constituted a prospective open evaluation of LcS over a 4-week period. Ten patients with clinical diagnoses of HAM/TSP in accordance with World Health Organization criteria [35] were enrolled in this study. All patients gave informed consent.

2.2. Patients and cells

Clinical characteristics of the patients are shown in Table 1. The duration of illness ranged from 7 to 24 years.

Table 1
Summary of demographic and clinical characteristics for 10 patients with human T lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Patient	Age, years	Sex	Disease duration, years	HTLV-1 antibody titer	Provirus load before Tx	Provirus load after Tx[a]	Concomitant Tx[b]	MAS before Tx	MAS after Tx[c]	OMDS before Tx	OMDS after Tx[d]	UDS before Tx	UDS after Tx[e]	Overall evaluation of efficacy[f]
HAM1	34	F	19	×131072	1757	1397	VC 375mg/day	2	1	4	3	2	0	good
HAM2	62	M	14	×32768	634	777	PSL 5mg/day, VC 375mg/day	+1	0	6	6	3	0	good
HAM3	50	F	17	×2048	907	779	None	2	1	5	4	3	0	good
HAM4	45	F	15	×16384	2942	471	PSL 5mg/day, VC 375mg/day	+1	0	5	3	6	3	excellent
HAM5	60	F	7	×8192	204	194	PSL 5mg/day, VC 375mg/day	3	4	4	4	1	0	fair
HAM6	47	M	18	×2048	716	849	PSL 10mg/day, VC 375mg/day	2	1	6	6	5	4	fair
HAM7	46	M	24	×16384	278	361	None	3	1	4	3	2	0	good
HAM8	55	F	10	×65536	2882	524	None	2	1	4	3	6	3	good
HAM9	41	F	17	×65536	1073	1263	None	3	0	2	2	3	1	good
HAM10	57	F	13	×32768	245	387	None	2	1	4	4	2	1	Fair

LcS, *Lactobacillus casei* strain Shirota; OMDS, Osame Motor Disability Score; UDS, urinary disturbance score; MAS, modified Ashworth scale.

[a] At 4 weeks after the first administration of LcS; for the changes in HTLV-1 provirus load, $p=0.401$, by the Wilcoxon signed rank test.

[b] VC, vitamin C; PSL, predonisolone.

[c] At 4 weeks after the first administration of LcS; for the change in MAS, $p=0.015$, by the Wilcoxon signed rank test.

[d] At 4 weeks after the first administration of LcS; for the change in OMDS, $p=0.157$, by the Wilcoxon signed rank test.

[e] At 4 weeks after the first administration of LcS; for the change in UDS, $p=0.0085$, by the Wilcoxon signed rank test.

[f] The evaluation was mainly based on improvement in motor dysfunction, urinary disturbances, and neurologic signs, as judged by the attending neurologists (see Patients and methods).

In these patients, motor disability scores rated from 0 to 13 (as described in Table 2), and ranged from 2 to 6 (Table 1). Fresh PBMCs were isolated on a Histopaque-1077 (Sigma) density gradient centrifugation, washed twice in RPMI 1640 with 5% heat-inactivated fetal calf serum (FCS), then stored in liquid nitrogen until use.

2.3. Treatment protocol

All patients were given LcS preparation, which contains approximately 4×10^{10} viable LcS, orally twice a day for 4 weeks. The drugs other than LcS such as muscle relaxants or vitamins were continued on the condition that the dosage regimen was kept constant before, during, and after treatment. Four patients (HAM2, HAM4, HAM5 and HAM6) received low-dose (5–10 mg daily) oral prednisolone throughout the study period. None of the patients–with the exception of these four–had received any immunomodulators.

2.4. Concomitant therapy

The drugs other than LcS such as vitamin C or low dose (5–10 mg daily) oral *prednisolone* were continued on condition that the dosage regimen was kept constant before, during, and after therapy. Four patients (HAM2, HAM4, HAM5 and HAM6) out of 10 had received low dose oral prednisolone throughout study period. None of the patients except for these four had received any immunomodulators (Table 1).

2.5. Clinical evaluation

Assessments were performed on subjective symptoms, degree of spasticity, disability grading, sphincter disturbance and adverse effects before and 1 month after administration of LcS. The clinical findings of each patient were assessed by the blinded consultant neurologists, i.e. the examining neurologist for post-clinical scores was blinded to the pre-clinical scores. Spasticity of the lower limbs was graded with the modified Ashworth scale (MAS) [36]. Motor dysfunction was evaluated based on the Osame Motor Disability Score (OMDS), which grades motor dysfunction from 0 (normal walking and running) to 13 (complete bedridden) (see Table 2). The severity of the three main symptoms of urinary disturbance, i.e. increased frequency of urination, feeling of residual urine, and urinary incontinence were scored using the following scale: 0=normal; 1=slight; 2=moderate; 3=severe. The urinary disturbance score (UDS) represents the sum of all three symptoms. An overall evaluation of efficacy (improvement of dysfunction) based mainly on the improvement of motor dysfunction, as well as changes in urinary disturbances and neurological signs, was also evaluated according to the following scale, which had been previously used in the multicenter, randomized, double blind, and controlled study for IFN-α [21]: (1) excellent: patients showing two or more grades of improvement on the OMDS; (2) good: patients with one grade of improvement on the OMDS, or patients with an improvement of motor dysfunction with no change on the OMDS, but apparent improvement on more than two items other than motor dysfunction; (3) fair: patients exhibiting an improvement of motor dysfunction with no change on the OMDS, or patients with no improvement in motor dysfunction but with an improvement on one or more of the other items examined; (4) poor: patients with no improvement; and (5) none: patients with continuing clinical deterioration. In this study, the entire HTLV-1 provirus load data was measured after 4 weeks of LcS administration were completed, by an investigator who was not involved in the patients' clinical care. Clinical neurologists did not have access to provirus load data.

Table 2
Osame Motor Disability Score (OMDS) for HAM/TSP

Grade	Disability
0	Normal gait and running
1	Normal gait but runs slow
2	Abnormal gait (staggering or spastic)
3	Abnormal gait and unable to run
4	Needs support (handrails) while using stairs but limited to 10 m
5	Needs one-hand support in walking
6	Needs two-hand support in walking
7	Needs two-hand support in walking but is limited to 10 m
8	Needs two-hand support in walking but limited to 5 m
9	Unable to walk even with support but can crawl in hands and knees
10	Crawls with hands
11	Unable to crawl but can turn sideways in bed
12	Unable to crawl but can move the toes
13	Completely bedridden

2.6. Lymphocytes phenotyping by flow cytometric analysis

After thawing, cells were washed three times with phosphate-buffered saline (PBS) and fixed in a PBS containing 2% paraformaldehyde (Sigma) for 20 min, and then resuspended in PBS at 4 °C. Fixed cells were washed with PBS containing 7% of normal goat serum (Sigma). Cells were then incubated for 15 min at room temperature with various combinations of fluorescence-conjugated monoclonal antibodies (mAb) as follows: fluorescein isothiocyanate (FITC)-labeled anti-CD27 (M-T271; Pharmingen, CA), FITC-labeled anti-CD3 (UCHT1; Beckman Coulter, CA), FITC-labeled anti-pan γδ T cell (Immu 510; Beckman Coulter, CA), Cy-Chrome-labeled anti-CD45RA (HI100; Pharmingen), phycoerythrin (PE)-labeled anti-CXCR3 (1C6/CXCR3; Pharmingen), PE-labeled anti-CD16 (3G8; Beckman Coulter), PE-labeled anti-NKG2A (Z199; Beckman Coulter), phycoerythrin-Cy5

(PC5)-labeled anti-CD4 (13B8.2; Beckman Coulter), PC5-labeled anti-CD56 (N901; Beckman Coulter), and energy-coupled dye (ECD)-labeled anti-CD8 (SFCI21ThyD3; Beckman Coulter). Isotype-matched mouse immunoglobulins were used as a control. The phenotype was determined by flow cytometry (EPICS® XL; Beckman Coulter, Tokyo, Japan) in the lymphocyte gate, based on forward versus side scatter.

2.7. NK cell-mediated cytotoxic assays

The erythroleukemia cell line K562 was maintained in RPMI 1640 medium, and supplemented with 10% FBS. Target cells (1×10^6 viable K562 cells) were labeled with $Na_2^{51}CrO_4$ (100 μCi) for 1 h at 37 °C, washed, and resuspended at a concentration of 5×10^4 cells/ml in a culture medium. Effectors and labeled targets were incubated in triplicates in 0.2 ml volume at E/T ratio of 50:1 in round-bottom 96-well plates, and incubated for 4 h. Percentage of lysis was determined by the following formula: % specific lysis = $100 \times (\text{exp} - \text{spont})/(\text{max} - \text{spont})$, where experimental (exp) release represents the radioactivity from the experimental wells, maximum (max) release represents counts from detergent-lysed targets, and spontaneous (spont) release represents background release from wells with targets alone.

2.8. Quantification of HTLV-1 provirus load and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out a quantitative PCR using ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMCs as reported previously [12]. We chose the pX gene of HTLV-1, which has four open reading frames and encodes the unique regulatory and accessory proteins, for quantitative PCR because pX gene is retained by defective proviruses that are sometimes present in the infected individuals. Using β-actin as an internal control, the amount of HTLV-1 proviral DNA was calculated through 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. Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio).

2.9. Statistical analysis

We made a paired comparison of changes in T cell phenotypes, HTLV-1 provirus load, NK cell activity, OMDS, and UDS scores between LcS treatments (before and 4 weeks after LcS treatment) by using a Wilcoxon signed rank test. The results represent the mean ± standard deviation (S.D.) where applicable. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clinical improvement of HAM/TSP with LcS treatment

We studied 10 HAM/TSP patients who received a LcS preparation, which contains approximately 4×10^{10} viable LcS, orally once a day for 4 weeks. All 10 patients showed the rate of excellent to fair response by overall evaluation of efficacy (see Patients and methods). Motor dysfunction (OMDS) was improved after treatment in 5 out of 10 patients ($p = 0.157$, Wilcoxon signed rank test). One patient (HAM 4) who had needed a walking cane before treatment became able to walk without assistance after treatment. Urinary symptoms (UDS) were improved in all 10 patients ($p = 0.0085$, Wilcoxon signed rank test; Table 1). Nine out of 9 patients showed decreased spasticity on neurological examination ($p = 0.015$, Wilcoxon signed rank test; Table 1). No adverse effect and laboratory findings were observed. The concomitant administration of prednisolone was not associated with the specific clinical outcome against LcS treatment (Table 1).

3.2. Laboratory findings

Simultaneous staining with CD45RA and CD27 mAbs has been demonstrated to identify subpopulations of human $CD8^{high+}$ T cells that were distinct for both phenotypic and functional properties [37]. From this staining, unprimed naïve T cells were defined as $CD45RA^+CD27^+$, antigen-experienced effector T cells that parallel with CTLs were defined as $CD45RA^+CD27^-$, and memory T cells were defined as $CD45RA^-CD27^+$. The $CD45RA^-CD27^-$ population was found to contain both effector and memory type T cells. In accordance with this phenotype, $CD45RA^+CD27^-$–but not $CD45RA^-CD27^+$–T cells have cytolytic activity without previous in vitro stimulation [37]. Our data have shown that the frequency as well as the absolute number of all the T cell phenotypes examined were not significantly changed in patients who received 4 weeks of daily oral LcS administration (Table 3). Interestingly, although the frequency as well as the absolute number of $CD16^+CD56^+/CD3^-$ NK cells and inhibitory NK receptor $NKG2A^+$ cells, which was a selectively decreased on T cells in HAM/TSP patients [34], showed no significant change after 4 weeks of LcS treatment, NK cell activity, which was reported to be decreased in HAM/TSP patients [32,33], was significantly increased after LcS treatment (Table 3). However, the concomitant administration of prednisolone was not associated with specific changes of NK cell activity against LcS administration (Fig. 1).

3.3. Provirus load of HTLV-1

The change in the HTLV-1 provirus load was measured by competitive PCR before and 4 weeks after LcS administration. The copy number of HTLV-1 per 1×10^4 PBMCs was not significantly changed after 4 weeks of LcS

Table 3
Absolute number, frequency of cell subsets and HTLV-1 provirus copy number in peripheral blood mononuclear cells (PBMCs) from patients with human T lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) who received *Lactobacillus casei* strain Shirota daily for 4 weeks

Cell type	Before treatment		After treatment		P^a	
	Absolute count, cells × 10^2/mm^3	Frequency, %	Absolute count, cells × 10^2/mm^3	Frequency, %	Absolute count	Frequency
$CD4^+$	5.79±4.54	26.38±12.71	5.96±3.16	27.13±11.09	0.674	0.575
$CD8^{high+}$	3.89±1.61	20.47±6.93	4.72±2.02	22.60±9.01	0.208	0.327
Naive in $CD8^{high+}$	1.13±1.71	5.24±7.11	0.93±1.06	4.25±4.08	1.000	0.674
Memory in $CD8^{high+}$	4.84±2.60	24.05±6.44	5.25±2.13	24.47±7.37	0.401	0.889
Effector in $CD8^{high+}$	7.56±4.46	37.35±10.81	7.57±2.56	36.10±9.93	1.000	0.674
Effector/memory in $CD8^{high+}$	5.99±2.36	33.36±14.54	7.21±3.47	34.07±13.78	0.263	0.889
$CXCR3^+$	4.38±2.38	21.07±4.28	3.93±1.16	18.78±6.57	0.735	0.398
$CXCR3^+$ in $CD4^+$	2.03±2.13	8.26±5.06	1.60±0.75	7.46±3.28	0.866	0.612
$\delta\gamma T^+$	0.40±0.25	2.28±1.46	0.47±0.34	2.35±1.83	0.208	0.674
$NKG2A^+$	0.72±0.43	3.68±1.84	0.74±0.37	3.84±2.39	0.674	0.889
$CD16^+CD56^+/CD3^-$	3.14±1.32	18.63±11.33	3.38±1.73	16.13±8.81	0.575	0.635
NK cell activity (%)[b]	26.54±16.13		39.43±15.48		**0.015**	
HTLV-1 provirus load[c]	867.38±874.62		641.75±343.12		0.401	

Data are mean±S.D. of 10 HAM/TSP patients (except for NK cell activity, $n=9$). Significant P value is in bold.
[a] Calculated using the Wilcoxon signed rank test.
[b] NK cell activity measured against K562 target cells at E/T ratio of 50/1 by the Cr release assay ($n=9$).
[c] HTLV-1 (pX) copy number per 1×10^4 PBMC by quantitative PCR.

treatment (Table 3). HTLV-1 antibody titers in sera and CSF also showed no significant change after 4 weeks of LcS treatment (data not shown).

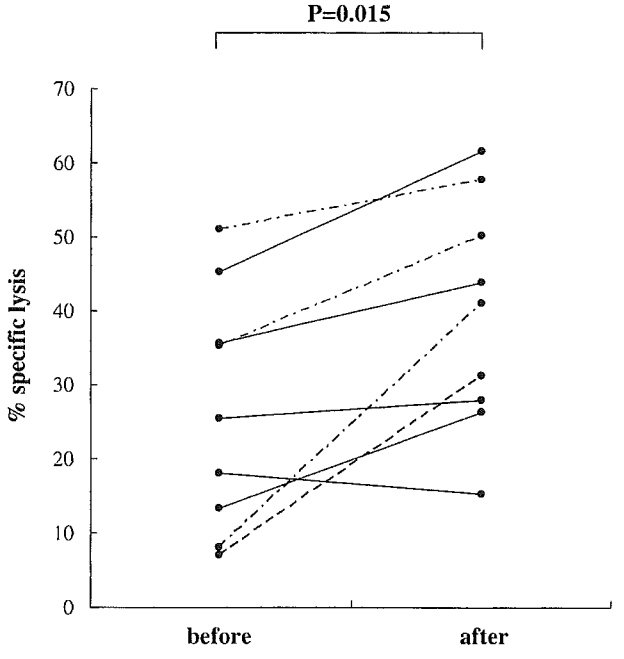

Fig. 1. Increased NK cell activity in PBMCs of HAM/TSP patients after oral administration of fermented milk drink containing *Lactobacillus casei* strain Shirota (LcS). The NK cell activity was significantly increased after LcS treatment ($n=9$, $p=0.015$, Wilcoxon's signed rank test). Each patient was given LcS preparation, which contains approximately 4×10^{10} viable LcS, orally twice a day for 4 weeks. NK cell activity was measured against K562 target cells at E/T ratio of 50/1 by the Cr release assay. Four patients out of ten received low dose oral prednisolone throughout the study period. None of the patients except for these four had received any immunomodulators. ------ Patients who received 5 mg/day oral prednisolone. ----: Patient who received 10 mg/day oral prednisolone.

4. Discussion

In this study, we demonstrated that clinical improvement was observed in all the HAM/TSP patients following 4 weeks of daily oral administration of LcS. Especially, improvement of urinary symptoms (UDS) was observed in all the 10 patients examined ($p=0.0085$, Wilcoxon signed rank test). LcS was also effective for motor dysfunction (OMDS) and spasticity (MAS) on lower extremity. Nine out of 10 patients showed decreased spasticity on neurological examination whereas OMDS score was improved only in 5 out of 10 patients. Thus, differences in OMDS score did not reach statistical significance ($p=0.157$, Wilcoxon signed rank test) whereas MAS scores did ($p=0.015$, Wilcoxon signed rank test). Although transient and mild diarrhea was previously reported in minor population (3 out of 65, 4.6%) after LcS administration [38], no adverse effect was observed in our patients.

Laboratory findings revealed that the NK cell activity in HAM/TSP patients was significantly increased after LcS treatment, but that HTLV-1 provirus load as well as the frequencies and absolute numbers of all the cell phenotypes examined were not significantly changed after treatment. In contrast, we have previously reported that the frequency of memory cells ($CD45RA^-CD27^+$) within $CD8^{high+}$, $CXCR3^+$ cells and HTLV-1 provirus load were significantly decreased after IFN-α treatment [22]. These findings suggest the possibility that NK cell activity is involved in the mechanism of clinical efficacy in LcS treatment, whereas Th1 type T cells and decreased HTLV-1 proviral load is associated with IFN-α effectiveness. Our present observation is consistent with the previous observation by Nagao et al. [39], who reported that in a healthy human subject, the frequency and number of NK cells, $CD4^+$ T

cells or $CD8^+$ T cells and T cell responsiveness to mitogens (Con A and PHA) were not significantly changed after 3 weeks of oral intake of LcS preparation containing approximately 4×10^{10} viable LcS. They also showed that the NK cell activity was significantly increased, and the enhancement of NK cell activity was particularly prominent in the low-NK cell activity individuals. Interestingly, our HAM/TSP case with most obvious clinical improvement (HAM 4) showed the lowest NK activity of all 10 patients before LcS treatment. However, another HAM/TSP case (HAM6) with low levels of NK activity before LcS treatment also showed the marked enhancement of NK cell activity after LcS but this patient did not show the obvious clinical improvement as seen in HAM4. Therefore, the level of enhanced NK cell activity after treatment did not always correlate with the level of clinical improvement. Since both of these patients (HAM 4 and 6) received low dose oral prednisolone throughout study period, this may also suggest that the concomitant administration of prednisolone *is* not specifically associated with the clinical outcome against LcS administration.

In general, probiotic bacteria such as LcS favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, and may boost immune function, and possibly increase resistance to infection. Although the mechanism of increased NK cell activity after oral administration of LcS is not clear, several studies have reported an effect of LcS on the stimulation of phagocytic activity of circulating immune cell populations which participate in innate immunity. Stimulated phagocytic cells may also play a role in potentiate acquired immunity via presentation of the antigen or synthesis of cytokines. Previous histological examination in an animal study showed that LcS was taken up by M cells in Peyer's patches [40], suggesting that LcS may be degraded in gut-associated lymphoid tissue and their signal from immuno-competent cells leads to a systemic effect such as stimulation of phagocytosis, synthesis of cytokines, IgA secretion, enhancement of NK cell activity, etc. Our data indicates that NK cell numbers were not significantly changed after LcS treatment. Also, we could not detect any significant changes of frequencies and absolute numbers of all the cell phenotypes examined, including the NK inhibitory receptor NKG2A positive cells and γδT cells. It is therefore likely that increased NK cell activity might be regulated by increased expression levels of cytotoxic molecules–rather than the changes of activating receptors and/or inhibitory receptors on NK cells–although further studies are necessary to clarify this point.

Previous studies have indicated that therapies effective for HAM/TSP such as prednisolone [15,16], plasma exchange [17], and interferon-α [18–22] have problems associated with a high frequency of adverse effects; furthermore, IFN-α and plasmapheresis usually require hospitalization, are very expensive and the clinical effects usually last only a few months. Since HAM/TSP is a chronic and progressive disorder, establishment of a chronic and safe treatment is essential. Also, innate immunity is thought to be important in protecting the host against many viral infections [41], and a combination treatment of acquired immunity oriented agents like IFN-α and innate immunity oriented agents like LcS might be a more desirable approach for HAM/TSP treatment. Since previous reports have indicated that the NK cell activity was significantly decreased in HAM/TSP patients by an unknown mechanism [32,33], the augmentation of a decreased host innate immune system by LcS might be a suitable approach for treating HAM/TSP.

In conclusion, our present results suggest that oral LcS may be comparatively effective, easy and safe, and is therefore a good candidate for maintenance treatment for HAM/TSP. However, in our present study, the number of patients is small and the study was conducted in an open, uncontrolled manner. Randomized controlled studies are warranted for the evaluation of LcS in HAM/TSP treatment.

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Human T Cell Leukemia Virus Type I-Infected Patients with Hashimoto's Thyroiditis and Graves' Disease

Takehiro Matsuda, Mariko Tomita, Jun-Nosuke Uchihara, Taeko Okudaira, Kazuiku Ohshiro, Takeaki Tomoyose, Tomoki Ikema, Masato Masuda, Mineki Saito, Mitsuhiro Osame, Nobuyuki Takasu, Takao Ohta, and Naoki Mori

Division of Molecular Virology and Oncology (T.M., M.T., J.-N.U., T.Ok., N.M.), Graduate School of Medicine, Division of Child Health and Welfare (T.Oh.) and Division of Endocrinology and Metabolism (T.T., T.I., M.M., N.T.), Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan; Department of Internal Medicine (K.O.), Naha Prefectural Hospital, Naha 902-8531, Japan; and Department of Neurology and Geriatrics (M.S., M.O.), Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8520, Japan

Context: Autoimmune thyroid diseases have been reported to be associated with human T cell leukemia virus type I (HTLV-I) infection. HTLV-I proviral load is related to the development of HTLV-I-associated myelopathy/tropical spastic paraparesis and has also been shown to be elevated in the peripheral blood of HTLV-I-infected patients with uveitis, arthritis, and connective tissue disease.

Objective: The objective of the study was to evaluate the proviral load in HTLV-I-infected patients with Hashimoto's thyroiditis (HT) or Graves' disease (GD) and ascertain the ability of HTLV-I to infect thyroid cells.

Patients and Methods: A quantitative real-time PCR assay was developed to measure the proviral load of HTLV-I in peripheral blood mononuclear cells from 26 HTLV-I-infected patients with HT, eight HTLV-I-infected patients with GD, or 38 asymptomatic HTLV-I carriers. Rat FRTL-5 thyroid cells were cocultured with HTLV-I-infected T cell line MT-2 or uninfected T cell line CCRF-CEM. After coculture with T cell lines, changes in Tax and cytokine mRNA expression were studied by RT-PCR.

Results: HTLV-I proviral load was significantly higher in the peripheral blood of patients with HT and GD than asymptomatic HTLV-I carriers. In the peripheral blood from HTLV-I-infected patients with HT, HTLV-I proviral load did not correlate with the thyroid peroxidase antibody or thyroglobulin antibody titer. After coculture with MT-2 cells, FRTL-5 cells expressed HTLV-I-specific Tax mRNA. These cocultured FRTL-5 cells with MT-2 cells expressed IL-6 mRNA and proliferated more actively than those cocultured with CCRF-CEM cells.

Conclusion: Our findings suggest the role of the retrovirus in the development of autoimmune thyroid diseases in HTLV-I-infected patients. (***J Clin Endocrinol Metab* 90: 5704–5710, 2005**)

HUMAN T CELL LEUKEMIA virus type I (HTLV-I) is a human retrovirus highly endemic in southern Japan, intertropical Africa, Melanesia, Latin America, and the Caribbean basin (1). HTLV-I is the etiological agent of adult T cell leukemia (ATL) (2) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), an inflammatory disease of the central nervous system (3, 4), and has also been implicated in several other inflammatory disorders, such as uveitis (5), chronic arthropathy (6), pulmonary alveolitis (7), and Sjögren's syndrome (8). Furthermore, transgenic mice expressing Tax protein, a transactivator encoded by HTLV-I, develop proliferative synovitis (9) and exocrinopathy affecting lacrimal and salivary glands, features similar to those of Sjögren's syndrome in humans (10).

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Abbreviations: ATL, Adult T cell leukemia; CTLA-4, cytotoxic T lymphocyte antigen-4; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, Graves' disease; HAM/TSP, human T cell leukemia virus type I-associated myelopathy/tropical spastic paraparesis; HT, Hashimoto's thyroiditis; HTLV-I, human T cell leukemia virus type I; MMC, mitomycin C; PBMC, peripheral blood mononuclear cell; Tg, thyroglobulin; TPO, thyroid peroxidase; TRAb, TSH receptor antibody; WST, water-soluble tetrazolium salt.

The possibility that HTLV-I may cause thyroid diseases was initially raised by reports of Hashimoto's thyroiditis (HT) in HTLV-I carriers and patients with HAM/TSP (11, 12). Graves' disease (GD) has also been observed in HTLV-I carriers (13, 14). Epidemiological studies have demonstrated that HTLV-I seropositivity is a risk factor for thyroid disorders in Japan. Kawai *et al.* (12) reported that the prevalence of HTLV-I antibody in HT patients resident of Tokushima and Kochi prefectures, Japan, was 6.3%, which was significantly higher than the expected frequency of 2.2%. Mizokami *et al.* (15) also reported that the prevalence of HTLV-I antibody was significantly higher in patients with either antithyroid antibody-positive chronic thyroiditis or GD than the expected frequency in Fukuoka prefecture, Japan. Mine *et al.* (16) found that the frequency of antithyroid antibodies in blood donors with HTLV-I antibody was significantly higher than that in control donors without the antibody. Akamine *et al.* (17) also found a high prevalence of positivity for thyroid autoantibodies in ATL patients and HTLV-I carriers.

Several findings support the hypothesis of the etiopathogenic role of HTLV-I in thyroid diseases: HTLV-I envelope protein and Tax mRNA have been detected in follicular epithelial cells of the thyroid tissues of a patient with HT (18); Tax mRNA was also found in infiltrating lymphocytes in the interfollicular space (18); and HTLV-I proviral DNA and

HTLV-I have been detected in thyroid tissues of patients with HT and GD (18, 19).

T lymphocytes, especially CD4+ T cells, are the main target of HTLV-I *in vivo* and carry the majority of the HTLV-I proviral load (20). The HTLV-I proviral load in peripheral blood mononuclear cells (PBMCs) is higher in patients with HAM/TSP than asymptomatic HTLV-I carriers (21), and the equilibrium set point of the proviral load is suspected to determine the development of the disease (22). We postulated that HTLV-I proviral load also influences the initiation and course of autoimmune thyroid diseases. To test our hypothesis, we measured this marker in PBMCs from HTLV-I-infected patients with HT and GD. To better understand the pathogenic mechanisms of HTLV-I-associated thyroid disorders, we determined whether HTLV-I could infect thyroid cells, and we characterized cell proliferation and cytokine gene expression in these cells after HTLV-I infection, using FRTL-5 rat thyroid cells.

Patients and Methods

Clinical samples

Blood samples were collected from 116 HTLV-I-infected patients, 38 asymptomatic carriers (33 females and five males, 21–79 yr old), 26 patients with HT (19 females and seven males, 37–80 yr old), eight patients with GD (seven females and one male, 40–59 yr old), 21 patients with HAM/TSP (17 females and four males, 31–74 yr old), and 23 patients with ATL (18 females and five males, 44–87 yr old). The diagnosis of HT was based on the presence of positive thyroid autoantibodies [thyroid peroxidase (TPO) and/or thyroglobulin (Tg)] and at least one of two additional criteria (hypothyroidism and/or goiter). Antibodies to TPO and Tg were determined by RIAs using commercially available kits (Cosmic, Tokyo, Japan). The patients with HT were treated with L-thyroxine. GD was diagnosed on the basis of history and signs of hyperthyroidism with diffuse goiter and the laboratory findings, including elevated serum T_4 and T_3 concentrations, undetectable serum TSH, and positive TSH receptor antibody (TRAb). TRAb was measured as TSH binding inhibitory Ig. One patient had ophthalmopathy. The patients with GD were treated with methimazole or propylthiouracil. Diagnosis and classification of the clinical subtypes of ATL were made based on the criteria of the Lymphoma Study Group (23) and were then confirmed in all cases by Southern blot hybridization analysis with detection of monoclonal integration of HTLV-I provirus into the genome. Diagnosis of HAM/TSP was based on the World Health Organization diagnosis guidelines (24). PBMCs donated by HTLV-I-seronegative healthy individuals (one female and two males, 25–29 yr old) served as normal controls. These control subjects did not have a history of thyroid or autoimmune diseases. PBMCs were isolated from heparinized blood by density gradient centrifugation. Seropositivity for HTLV-I was obtained by ELISA and particle agglutination assays. The screening of serum HTLV-I antibody was studied in all patients who visited our clinic at the University of the Ryukyus. All patients, HTLV-I asymptomatic carriers, and HTLV-I-seronegative healthy controls were Japanese, and they were living in Okinawa and Kagoshima prefectures (HTLV-I endemic areas), Japan. All individuals gave written informed consent for their participation.

Measurement of HTLV-I proviral load

DNA was prepared from each sample using a blood and tissue genomic DNA minikit, according to the protocol recommended by the manufacturer (Viogene-Biotek Corp., Hsichih, Taiwan) and stored at −80 C until use. The concentration of extracted DNA was adjusted to 10 ng/μl of the working solution. A quantitative real-time PCR assay was developed to measure the proviral load of HTLV-I in PBMCs. The HTLV-I copy number was referenced to the actual amount of cellular DNA by quantification of β-actin gene. The forward and reverse primers used for HTLV-I pX region were 5′-CAAACCGTCAAGCACAGCTT-3′ positioned at 7140–7159 and 5′-TCTCCAAACACGTAGACTGGGT-3′ positioned at 7362–7341. The internal HTLV-I pX TaqMan probe (5′-TTCCCAGGGTTTGGACAGAGTCTTCT-3′) was located between positions 7307 and 7332 of the genome, and carried a 5′ reporter dye FAM (6-carboxy fluorescein) and a 3′ quencher dye TAMRA (6-carboxy tetramethyl rhodamine). To quantify the human β-actin gene, the forward and reverse primers 5′-TCACCCACACTGTGCCCATCTACGA-3′ positioned at 2141–2165 and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′ positioned at 2435–2411, and the β-actin TaqMan prove (5′-ATGCCCTC-CCCCATGCCATCCTGCGT-3′ positioned at 2171–2196) were used. PCR was performed with 5 μl DNA template with the use of the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and target gene assay mix containing each respective forward and reverse primer and TaqMan probe. The PCR conditions were as follows: 1 cycle at 50 C for 2 min and 95 C for 10 min and 45 cycles of denaturation at 95 C for 15 sec and annealing/extension at 58 C for 1 min. PCR was carried out in triplicate for each sample. HTLV-I provirus DNA cloned into the plasmid served as the control template and the β-actin gene as the internal control. Data were quantified as mean values from the relative standard curve according to the instructions provided by the manufacturer (Applied Biosystems). Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. The amount of HTLV-I proviral DNA was calculated by the following formula: copy number of HTLV-I (pX) per 1×10^4 PBMCs = [(copy number of pX)/(copy number of β-actin/2)] $\times 10^4$.

Cell culture and HTLV-I infection in vitro

FRTL-5 cells are a continuous line of rat thyroid cells and were grown in the Coon's modified Ham's F-12 medium containing 5% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) with the addition of a mixture of six hormones: bovine thyroid-stimulating hormone (10 mU/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), hydrocortisone (10 nM), and insulin (10 μg/ml). All hormones were purchased from Sigma-Aldrich (St. Louis, MO). MT-2 cells, obtained by coculturing peripheral leukemic cells from an ATL patient with normal umbilical cord leukocytes (25), were used as an HTLV-I-infected T cell line. MT-2 cells contain proviral HTLV-I DNA and produce viral particles. CCRF-CEM cells were used as the uninfected T cell line. These T cells were treated with 100 μg/ml mitomycin C (MMC) for 1 h at 37 C. After washing three times with PBS, they were cultured with an equal number of FRTL-5 cells in Coon's modified Ham's F-12 medium containing 5% FBS. The culture medium was changed on the third day after coculture. FRTL-5 cells were harvested at 3 and 7 d, followed by RNA extraction as described below.

RT-PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer, and the amount of total RNA was determined by measuring absorbance at 260 nm. First-strand cDNA was synthesized from 5 μg total cellular RNA in a 20-μl reaction volume using an RNA PCR kit (Takara Shuzo, Kyoto, Japan) with random primers. Thereafter cDNA was amplified using a multiplex PCR kit for rat inflammatory cytokine gene (Maxim Biotech, Inc., San Francisco, CA) according to the instructions provided by the manufacturer. Product sizes were 351 bp for TNFα, 294 bp for IL-1β, 453 bp for IL-6, 250 bp for TGFβ, and 532 bp for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels of Tax and β-actin mRNAs were analyzed as described previously (26). Product sizes were 203 bp for Tax and 548 bp for β-actin. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Cell proliferation assay

FRTL-5 cells (1×10^4 cells/well) were cultured with or without MMC-treated MT-2 or CCRF-CEM (1×10^4 cells/well) cell line in 96-well culture plates in Coon's modified Ham's F-12 medium containing 5% FBS for 1, 3, or 5 d. The data were obtained by triplicate experiments. Four hours before terminating the culture, 10 μl of the cell proliferation reagent water-soluble tetrazolium salt (WST)-8, a tetrazolium salt (Wako Chemicals, Osaka, Japan) were added to each well. At the end of incubation, absorbance at 450 nm was measured using an automated microplate reader. Measurement of the mitochondrial dehydrogenase-

mediated cleavage of WST-8 to formazan dye indicates the level of proliferation.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was analyzed by Mann-Whitney *U* test. The Spearman's rank correlation coefficient was used to describe the association between different variables. The Student's *t* test was performed for comparisons of growth of uninfected FRTL-5 cells and that of HTLV-I-infected FRTL-5 cells.

Results

Quantification of HTLV-I proviral DNA in asymptomatic HTLV-I carriers, HTLV-I-infected patients with HT or GD, HAM/TSP, and ATL

As shown in Fig. 1, we estimated the absolute copy number of HTLV-I proviral DNA per 10^4 PBMCs. First, proviral load was quantified in three healthy volunteers (seronegative), 21 HAM/TSP patients, and 23 ATL patients. The provirus was undetectable in all healthy noncarriers (Fig. 1B), whereas HAM/TSP and ATL patients were positive for HTLV-I with a proviral load of 1986 ± 198 copies (range 879-4137 copies) and 2791 ± 320 copies (range 874-6175 copies), respectively (Fig. 1A). The provirus loads in smoldering-, chronic-, acute-, and lymphoma-type ATL patients were 1561 ± 268, 2683 ± 782, 3098 ± 468, and 3248 ± 893, respectively. The copy numbers in asymptomatic carriers varied from 0.4 to 347, those of HTLV-I-infected patients with HT varied from 2 to 1076, and those of HTLV-I-infected patients with GD varied from 29 to 1222 (Fig. 1B). The mean ± SD and median of the copy number was 60 ± 11 and 39 in asymptomatic carriers. With regard to HTLV-I-infected patients with HT and GD, the values were 276 ± 53 (median 199) and 303 ± 137 (median 200), respectively. The median copy number of HTLV-I-infected patients with HT and GD was about 5-fold higher than that of asymptomatic carriers. The differences were statistically significant between asymptomatic carriers and HTLV-I-infected patients with HT and between asymptomatic carriers and HTLV-I-infected patients with GD, respectively (Mann-Whitney *U* test) (Fig. 1B). There was no significant correlation between copy number of HTLV-I proviral DNA and antibody titer of either Tg ($P = 0.6535$) or TPO ($P = 0.4703$) in HTLV-I-infected patients with HT (Spearman's rank correlation) (Fig. 2). Among the HTLV-I-infected patients with GD, the correlation between copy number of HTLV-I proviral DNA and TRAb titer was not observed (data not shown).

Detection of HTLV-I Tax mRNA in FRTL-5 cells cocultured with HTLV-I infected T cells

FRTL-5 cells were cocultured with either MT-2 or CCRF-CEM cells. After cocultivation for 3 d, FRTL-5 cells were washed extensively and exchanged with fresh medium. After the cells were cultured for further 4 d, they were washed thoroughly. At 3 and 7 d after cocultivation, FRTL-5 cells were harvested for assessment by RT-PCR for expressing HTLV-I viral antigen. Because T cell lines were pretreated extensively with MMC, these MMC-treated T cells could not proliferate, as determined by WST-8 assay. These specimens of FRTL-5 cell at 3 and 7 d of culture contained no viable MT-2 cells. As shown in Fig. 3A, FRTL-5 cells cocultured with MT-2 cells showed strong expression of Tax mRNA. In contrast, FRTL-5 cells cocultured with CCRF-CEM cells did not express Tax mRNA. To determine whether the Tax cDNA sequence was amplified from residual MT-2 cells that had been added after MMC treatment, PCR amplification of a human PTHrP exon 3 sequence was done, using these DNA samples. The human PTHrP sequence was amplified from MT-2 DNA by PCR. However, the human PTHrP sequence was not detected in any of the cocultured rat FRTL-5 cells, which suggests that residual MT-2 cells in these samples were not amplified (data not shown). These results suggest that the HTLV-I can be transmitted into FRTL-5 cells from HTLV-I producing MT-2 cells.

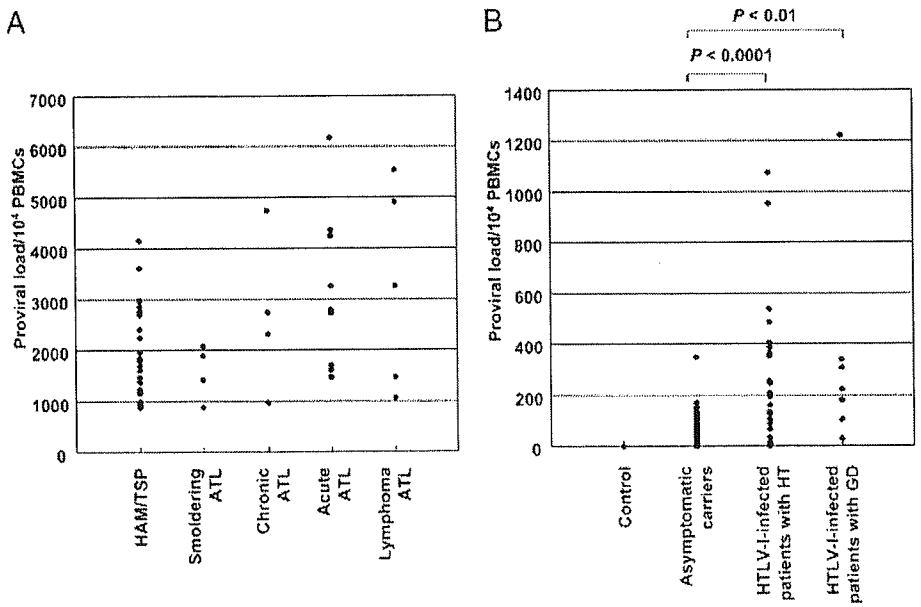

FIG. 1. HTLV-I proviral load in the peripheral blood of HAM/TSP and ATL (A) and healthy individuals without HTLV-I, asymptomatic carriers, and HTLV-I-infected patients with HT or GD (B). Data are HTLV-I copy number per 10^4 PBMCs.