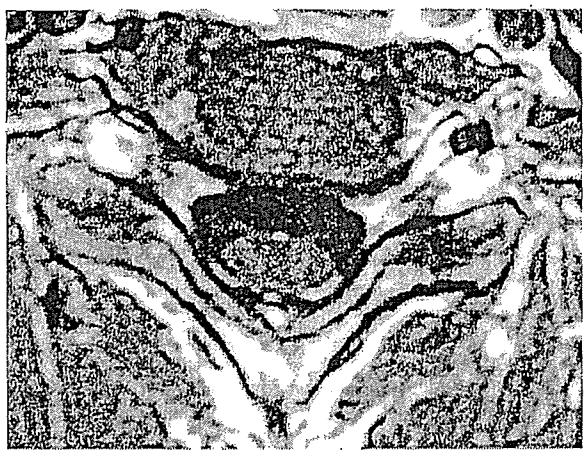

Fig. 1. *Gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) enhanced T2-weighted magnetic resonance imaging at C6–7 levels.* High intensity signals in C6 and C7 dorsal roots were observed on Gd-DTPA enhanced T2-weighted imaging (axial view), indicating on-going inflammation.

Eight months after the onset, she gradually developed varying degrees of numbness and sensory deficit in the extremities, face, and trunk in an asymmetrical distribution, as well as mild muscle weakness and difficulty in swallowing. Her parents were healthy and unrelated. Although there were no neurological disorders in her family, her younger brother underwent right leg amputation because of thromboangiitis obliterans (Buerger's disease). She has a medical history of atopic dermatitis. Personal history includes 11 years of cigarette smoking averaging 30 pieces per day, occasional alcoholic beverage drinking and no toxic substance use. On admission, she presented with dysphagia and finger swelling with Raynaud's phenomenon. Neurological examination revealed facial numbness and hypoesthesia in the right V1 and V2 distributions of the trigeminal nerve, and decreased temperature sensitivity in the left upper and right lower limbs. Decreased superficial sensation was also seen in the distal parts of both upper and left lower limbs. There was absence of deep tendon reflexes and severe impairment of vibratory and position sense in the distal limbs, but Romberg and Babinski signs were not demonstrated. A systemic survey did not detect any malignancy. Blood test disclosed only mild inflammatory signs (ESR: 22 mm/h, IgM: 305 mg/dl, CRP: <0.05). Her serum was positive for anti-centromere antibodies (21.4 U/ml, normal range <10.0) and antinuclear antibody (ANA) (1:640 with a speckled pattern), but negative for anti-SS-A, anti-SS-B and anti-Scl-70 antibodies. CSF analysis showed a protein level of 81.5 mg/dl (IgG: 9.9 mg/dl, IgG index: 0.94) and a white cell count of 2/μL with 100% lymphocytes. Upper gastrointestinal imaging revealed lower esophageal and gastric hypomotility. Biopsy of the minor salivary gland showed sialadenitis with infiltration of mononuclear cells. However, salivary and lacrimal secretion tests as well as salivary gland scintigraphy showed no apparent abnormality. Skin biopsy demonstrated thickening of the dermis. Based on these findings, we diagnosed her as having limited SSc.

2.2. Neurophysiological and neuroradiological examinations

Standard nerve conduction study of the upper and lower limbs (median, ulnar, tibial and sural nerves) was performed. Although motor conduction velocity (MCV), compound muscle action potential (CMAP) and latency studies were normal, sensory action potentials (SNAP) were markedly decreased. Somatosensory evoked potentials (SEPs) showed delayed cortical N20 and P40 potentials but normal inter-peak latency between N20 and P40 (N20–P40), suggesting a lesion of the dorsal root and/or peripheral nerves. In accordance with the elevated CSF protein level, cervical magnetic resonance imaging (MRI) demonstrated gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) enhancement of the C6 and C7 dorsal roots, indicating on-going inflammation (Fig. 1).

2.3. Sural nerve biopsy and anti-neural antibodies

Sural nerve biopsy showed marked decrease in myelinated fibers, mostly those of large diameter, as well as axonal degeneration and infiltration of macrophages, but no signs of vasculitis (Fig. 2). Teased fiber morphology classification according to Dyck et al. [5] was as follows: A+B 18%, C 0%, D 0%, E 77%, F 5% (A: normal; B: myelin irregularity; C: demyelination; D: demyelination and remyelination; E: axonal degeneration; F: remyelination; A and B, considered to be normal, are expressed as A+B). All the anti-neuronal antibodies and anti-ganglioside antibodies examined i.e. both IgG and IgM anti GM1, GQ1b, GD1b antibodies, IgM anti SGPG antibody and anti-Yo, Hu, Ri, CRMR-5, Tr, Ma-2, and amphiphysin antibodies were not detected in her serum (data not shown).

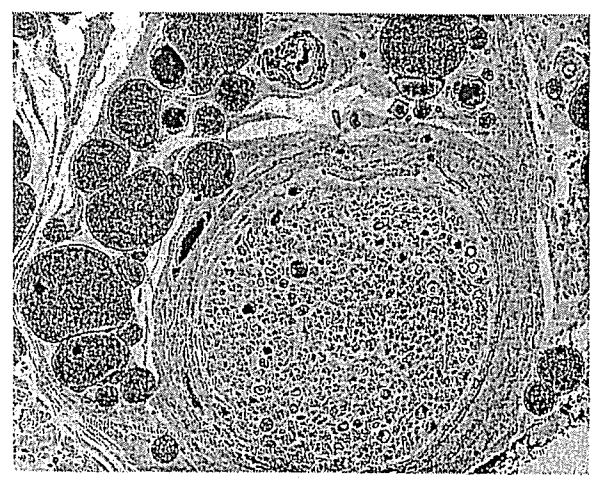

Fig. 2. *Sural nerve pathology of the patient.* Biopsy specimen of the left sural nerve showed marked decrease in myelinated fibers, mostly those of large diameter, as well as axonal degeneration and infiltration of macrophages. However, there were no signs of vasculitis. (Hematoxylin–eosin staining, magnification ×50).

2.4. Cytokine measurement by multiplex bead array assays

Cytokines in serum and CSF were analyzed in duplicate by using Multiplex bead kits (BioSource International, Inc. Camarillo, CA, USA) and Luminex® system (Luminex Corporation, Austin, TX, USA) according to the manufacturers' specifications. Analyzed cytokines were as follows: interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF and tumor necrosis factor-α (TNF-α).

2.5. Treatment and outcome

After admission, an initial treatment of high-dose methylprednisolone (1000 mg per day) was administered for 3 days, followed by the conventional dosage of oral prednisone (1 mg/kg/day). Although she felt mild improvement with regards to cold sensitivity in the distal limbs, she developed an apparent Romberg sign, ataxic gait and athetosis of the hands after this treatment. We, therefore administered high-dose (400 mg/kg/day for 5 days) intravenous gammaglobulin (IVIg) therapy. After starting IVIg, she showed noticeable improvement in neurological symptoms, especially the ataxic gait, as well as improvement in morning stiffness and reduction of CSF protein level.

3. Discussion

It is well known that connective tissue diseases are common causes of peripheral neuropathy in a number of patients, mainly due to ischemia secondary to systemic vasculitis [1]. In SSc, the most frequently observed clinical peripheral nervous system disease is trigeminal neuropathy (19%) [6]. Other peripheral neuropathies are also observed although as minor complications (5.6%) [3]. It has been suggested that SSc accounts for only about 1% of all vasculitic neuropathies due to connective tissue diseases [7]. Limited systemic sclerosis is typically preceded by Raynaud's phenomenon, and involves cutaneous sclerosis distal to the elbows with gastrointestinal and pulmonary fibrosis, and anti-centromere antibody positivity [8]. Our case showed all of these findings except for pulmonary fibrosis. The diagnosis of cutaneous limited fibrosis was also supported by skin biopsy. Although Sjögren's syndrome is known to occasionally present with sensory neuropathy with prominent ataxia reflecting kinesthetic sensory impairment [9,10], our case did not fulfill the diagnostic criteria of Sjögren's syndrome [11].

The unique clinical characteristic of our case was severe and progressive sensory impairment, with ataxia as the dominant clinical feature. Neurophysiological findings supported the sensory involvement with preservation of motor nerves. Since electrodiagnostic findings in patients with vasculitic neuropathy such as polyarteritis nodosa (PN) show sensorimotor axonal neuropathy, mainly due to ischemic mononeuropathy or to multiple mononeuropathies, rather than a distal symmetric neuropathy, our case was clearly different from such conditions. Indeed, sural nerve biopsy of this patient showed no vasculitis, although loss of myelinated fibers mostly those of large diameter, axonal degeneration and infiltration of macrophages were observed. Blood test disclosed only mild inflammatory signs, and neither systemic inflammation nor autoantibodies associated with vasculitis, such as antineutrophil cytoplasmic antibodies (ANCA), were observed. However, an elevated protein level without pleocytosis, and high IL-1β and GM-CSF in the CSF of this patient (Table 1) indicated on-going inflammation consistent with cervical MRI findings that demonstrated Gd-DTPA enhancement of the C6 and C7 dorsal roots. Clinical symptoms such as ataxia also suggested that the site of pathology was presumably the dorsal root ganglion (DRG) and dorsal roots. Interestingly, cytokine analysis using Multiplex bead kits and Luminex® system revealed increased IL-4 levels, which is probably associated with her atopic dermatitis, only in her serum but not in CSF. This indicates that IL-4 did not play a dominant role in her CSAN (Table 1). Although she did not respond to corticosteroid therapy, IVIg caused marked improvement in neurological symptoms, especially the ataxic gait, as well as improvement in morning stiffness, and reduction of CSF protein level.

Although its precise mechanisms of action are not entirely known, IVIg has been reported to be effective in many autoimmune neuromuscular diseases including complications of connective diseases [12]. IVIg may have immunomodulatory effects via autoantibodies, complements, Fc receptors, cytokines, adhesion molecules and T-cells in addition to antigen-specific effects in this case [13]. Indeed, we detected increased amounts of IL-1β, IL-2, IL-4, and TNF-α in the serum, and IL-1β and GM-CSF in the CSF of this patient. Since IVIg treatment modulates the production of IL-2, TNF- α and GM-CSF by peripheral blood mononuclear cells and attenuates the intensity of inflammation [14], IVIg could be effective in improving reversible neuronal dysfunction mediated by these cytokines.

Table 1
Cytokine levels in CSF and serum of this case measured by a multiplexed fluorescent bead-based immunoassay

Cytokine (pg/ml)	Serum	CSF
IL-1β	**694.49***	**265.62**
IL-2	**978.57**	33.47
IL-4	**902.13**	53.85
IL-6	N.D.	39.10
IL-8	23.55	18.12
IL-10	13.25	26.40
GM-CSF	88.41	**212.14**
TNF-α	**389.5**	N.D.
IFN-γ	N.D.	39.57

*Values higher than mean+2SD of 32 patients with non-inflammatory neurological diseases (10 motor neuron disease and 22 spinocerebellar ataxia) are shown in bold type. N.D.: not detected.

In conclusion, our case suggested that CSAN could be associated with limited SSc. Both the neurophysiological examination and spinal cord MRI were useful in supporting the clinical diagnosis. In addition, our data suggested the possibility of IVIg as a potentially efficacious therapy for CSAN patients complicated by collagen disease without vasculitis, who did not respond to corticosteroid therapy.

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BRIEF COMMUNICATION

Victoria Ward · Branwen J. Hennig · Kouzo Hirai · Hideki Tahara · Akihiro Tamori · Ritu Dawes · Mineki Saito · Charles Bangham · Henry Stephens · Anne E. Goldfeld · Warunee Kunachiwa · Nipapan Leetrakool · Julian Hopkin · Sarah Dunstan · Adrian Hill · Walter Bodmer · Peter C. L. Beverley · Elma Z. Tchilian

Geographical distribution and disease associations of the CD45 exon 6 138G variant

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Abstract CD45 is crucial for normal lymphocyte signalling, and altered CD45 expression has major effects on immune function. Both mice and humans lacking CD45 expression are severely immunodeficient, and single-nucleotide polymorphisms in the CD45 gene that cause altered splicing have been associated with autoimmune and infectious diseases. Recently, we identified an exon 6 A138G polymorphism resulting in an increased proportion of activated CD45RO T cells and altered immune function. Here we report a significantly reduced frequency of the 138G allele in hepatitis C Japanese patients and a possibly reduced frequency in type I diabetes. The allele is widely distributed in the Far East and India, indicating that it may have a significant effect on disease burden in a large part of the human population.

Keywords CD45 · A138G · Alternative splicing · Type I diabetes · Hepatitis C

CD45 (leucocyte common) antigen is a haemopoietic cell-specific tyrosine phosphatase which undergoes complex alternative splicing. In humans, naive T cells express CD45RA-containing isoforms, while most memory/effector T cells express the low molecular weight CD45RO isoform. The function of the isoforms remains obscure, although it is clear that CD45 is an important immunomodulator which regulates many signalling pathways in the immune response, from establishing a threshold for T cell antigen receptor (TCR) signalling to modulating

V. Ward · W. Bodmer
Cancer and Immunogenetics Laboratory, Weatherall Institute of Molecular Medicine, Cancer Research UK, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

B. J. Hennig · A. Hill
Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, Oxford OX3 7BN, UK

K. Hirai · H. Tahara · A. Tamori
Osaka City University Graduate School of Medicine, 1-4-3, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

R. Dawes · P. C. L. Beverley · E. Z. Tchilian (✉)
The Edward Jenner Institute for Vaccine Research, Compton, Berks RG20 7NN, UK
e-mail: elma.tchilian@jenner.ac.uk
Tel.: +44-1635-577929
Fax: +44-1635-577901

M. Saito
Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

C. Bangham
Department of Immunology, Faculty of Medicine, Imperial College, Norfolk Place, London W2 1PG, UK

H. Stephens
Institute of Urology and Nephrology, University College London Medical School, Mortimer Street, London W1N 8AA, UK

A. E. Goldfeld
Center for Blood Research, Institute for Biomedical Research, Boston, MA 02115, USA

W. Kunachiwa · N. Leetrakool
Department of Clinical Immunology, Chiang Mai University, Chiang Mai, Thailand

J. Hopkin
Swansea Clinical School, University of Wales, Swansea, UK

S. Dunstan
Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam

cytokine production and responses, as well as regulating cell survival (Hermiston et al. 2003; Irie-Sasaki et al. 2003).

The importance of CD45 expression for normal lymphocyte development was first demonstrated in CD45-deficient mice (Kishihara et al. 1993; Byth et al. 1996), and animals with genetic polymorphisms influencing the balance of isoforms expressed show altered disease susceptibility and production of cytokines (Subra et al. 2001). It is likely therefore that genetic variants altering CD45 expression or the balance of different isoforms would affect disease susceptibility or progression in humans. This has been found to be the case. Individuals lacking CD45 expression exhibit severe combined immunodeficiency (Kung et al. 2000; Tchilian et al. 2001b), while the exon 4 C77G variant, which profoundly affects alternative splicing (Thude et al. 1995; Zilch et al. 1998), has been associated with altered susceptibility to various autoimmune and infectious diseases (Jacobsen et al. 2000; Tchilian et al. 2001a; Schwinzer et al. 2003; Vogel et al. 2003; Vyshkina et al. 2004). C77G carriers are relatively rare (allele frequency from 0 to 3.5% in Europe and North America), which has hampered more sophisticated studies of the influence of this variant on disease progression or immune function.

Table 1 Frequency of the CD45 exon 6 A138G polymorphism in control and disease groups from different regions

Disease group	Total number	AA (%)	AG (%)	GG (%)	% G allele frequency
Japanese Osaka controls	314	184	117	13	22.8
Hepatitis C cases	338	237	91	10	16.4**
Diabetes type I cases	190	132	52	6	16.8*
Cervical cancer cases	69	38	27	4	25.4
Japanese Kagoshima controls	79	54	25	0	15.8
HTLV HC	80	57	19	4	16.9
HAM/TSP	79	57	17	5	17.1
Japanese Wakayama	594	374	198	22	20.4
Vietnam malaria controls	225	164	55	6	14.9
Malaria cases	332	237	86	9	15.7
Vietnam dengue controls	226	158	66	2	15.5
Dengue cases	303	197	101	5	18.3
Dengue fever	108	74	32	2	16.7
Dengue haemorrhagic fever	60	43	17	0	14.2
India controls (lepromatous)	42	33	9	0	10.7
Leprosy cases (lepromatous)	62	51	11	0	8.9
Leprosy controls (tuberculoid)	59	52	7	0	5.9
Leprosy cases (tuberculoid)	62	49	13	0	10.5
India TB controls	180	155	22	3	7.8
India TB cases	263	231	32	0	6.1
Thai controls	146	95	43	6	18.8
Cambodian controls	20	12	8	0	20.0
Chinese controls	560	349	187	24	21
Peruvian controls	19	12	6	1	21.0

The overall genotype distribution and allele frequencies are shown. Statistically significant differences (assessed by continuity corrected chi-square) in the frequency of G allele carriers between cases and respective controls are indicated with asterisks. There were no truly significant departures from Hardy–Weinberg equilibrium in any of the populations tested. Ethical approval was obtained for all cohorts genotyped, from the relevant authorities. The presence of the A138G allele was detected using alkaline-mediated differential interaction (AMDI), which is based on alkaline-mediated detection of ARMS-PCR products using double-stranded DNA specific dyes (Bartlett et al. 2001; Stanton et al. 2003) in all Japanese, Thai, Cambodian and Peruvian samples. Two separate reaction mixes containing the forward (GCAGAAGTGCTTGAAGATT) and one of the two reverse primers GCATAGTCAGACCTGAGCT (for the A allele) and GCATAGTCAGACCTGAGCC (for the G allele) were used. Annealing temperatures were 52°C and 55°C for the A and G alleles, respectively. All of the Indian and Vietnamese samples were genotyped using the Sequenom Mass-Array MALDI-TOF primer extension assay (Jurinke et al. 2002) (http://www.sequenom.de/) under standard conditions. Primers for A138G were P1: ACGTTGGATGACCTCCAACACCACCATCAC, P2: ACGTTGGATGGAAG ACACTACTAGAGCAGC and extension P: ACACCACCATCACAGCGAAC. Where different samples from the same population had been typed by the Sequenom and AMDI techniques, there was no more than a 1% difference in frequencies between the two samples typed using the different techniques

HTLV HC Human T lymphotropic virus in healthy controls, *HAM/TSP* HTLV-1-associated myelopathy/tropical spastic paraparesis patients, *TB* tuberculosis

*p=0.019; **p=0.0028

We recently described another CD45 polymorphism, exon 6 A138G, which is common at least in Japan (Stanton et al. 2003). This variant showed a relatively strong protective effect in Graves' disease and hepatitis B, and a suggestive, but not significant, reduction in the frequency of the variant in hepatitis C patients (Boxall et al. 2004). Preliminary data indicate that 138G individuals have more activated CD45RO T cells and synthesize more interferon gamma (IFN-γ) (Boxall et al. 2004). The high frequency of 138G-carrying individuals in Japan suggests that the G allele might also affect susceptibility and pathogenesis in other autoimmune or infectious diseases and might have a wider geographical distribution.

We therefore extended our earlier studies by analysing an additional 138 Japanese controls and 165 further hepatitis C patients all from the Osaka region. We also examined the frequency of the A138G single-nucleotide polymorphism (SNP) in patients with type I diabetes and cervical cancer, a malignant disease with viral aetiology. As shown in Table 1, we have now analysed a total of 314 controls and 338 hepatitis C cases.

Our previous data have been combined with those added in this study, as there was no significant heterogeneity between the two sets of samples. The overall frequency of the presence of the G vs A allele (namely, AA vs AG + GG) is significantly different in the hepatitis C patients compared to the controls, p=0.0028 (odds ratio=0.6, 95% confidence interval=0.43–0.84), using Yates continuity corrected chi-square. Even when using the Bonferroni correction to allow for multiple comparisons (12 in this case), the p value remains significant at p=0.0336. In this case, however, the correction is conservative because there is an a priori suggestion of an association between hepatitis C and another CD45 variant C77G (our unpublished results), and our previous data suggested an effect of the A138G variant, and also because of the effect of the A138G variant on immune phenotype and function (Boxall et al. 2004).

There is a suggestion of a decreased frequency of the G allele in type I diabetes p=0.019, which, however, would not remain significant when the number of comparisons is taken into account. Nevertheless, it is intriguing that, given our previous data on Graves' disease, the A138G may have effects on two autoimmune disease. Interestingly, both Graves' disease and diabetes are influenced by another polymorphism, namely, in the CTLA-4 gene, suggesting that their pathogenesis may share immunological mechanisms and that A138G should be added to the list of common alleles with low penetrance, in addition to the major histocompatibility genes, which have already been shown to be factors in the development of autoimmune diseases (Rioux and Abbas 2005). There was no significant difference between the cervical cancer patients and controls.

We also analysed the frequency of this polymorphism in another Japanese population from the Kagoshima region of southern Japan, where human T lymphotropic virus type I (HTLV-1) infection is endemic (Jeffery et al. 2000). No significant difference in the frequency was found between 79 controls, 80 asymptomatic healthy carriers (HTLV HC) and 79 HTLV-1-associated myelopathy/tropical spastic paraparesis patients (HAM/TSP). The relatively large difference in the frequency of the A138G allele between the Kagoshima and Osaka controls is puzzling and needs more investigation, especially as 594 samples from the southern county of Wakayama prefecture (Shirakawa et al. 1997) showed a frequency of 20.4% for the A138G polymorphism which is very similar to that found in Osaka. It may be interesting in the future to see if this difference between the Kagoshima and other Japanese regions can be confirmed and, if so, whether it is specific only to the A138G CD45 variant or is reflected in the frequency of other polymorphisms.

Table 2 CD45 A138G gene frequency in different geographical locations

Location	Total number	% G allele frequency
Osaka (Japan)	314	22.8
Kagoshima (Japan)	238	16.6
Wakayama (Japan)	594	20.4
Vietnam	1254	16.2
Thailand	146	18.8
China	560	21
Cambodia	20	20
India	668	7.5
Peru	19	21

Data from disease and control groups were combined when no significant difference between them was found

We further investigated the frequency of the A138G SNP in other Far Eastern populations, examining cohorts of Vietnamese malaria (Tran et al. 1996) and dengue cases and controls (Loke et al. 2001; Cao et al. 2002; Phuong et al. 2004). The variant allele is present in Vietnam at a frequency of 15%, but there was no significant difference between the 332 malaria cases and 225 controls. Nor was there any significant difference between 303 dengue cases and 226 respective controls nor between 108 Dengue fever and 60 Dengue haemoraghic fever cases in a second well characterised cohort (Cao et al. 2002; Phuong et al. 2004). The G allele was detected in populations of 146 Thais and 20 Cambodians with frequencies of 18.8% and 20% respectively. In a large cohort of Chinese samples (Peisong et al. 2004) we also found a variant frequency of 21%. These data indicate that many Far Eastern Oriental populations have a relatively high frequency of the 138G allele.

We also tested samples from India and found an overall frequency for the A138G SNP of about 7.5%. No significant differences in the genotype distributions between lepromatous or tuberculoid leprosy patients and their respective matched controls from Calcutta were found (Roy et al. 1999). Similarly no significant difference in the overall frequency amongst the 263 Indian tuberculosis (TB) patients and 180 controls from Madurai was found. Interestingly, no G138G homozygotes were detected amongst the TB patients. However, this is not statistically

significant and so more samples clearly need to be analysed to establish whether there is a possible recessive effect in TB.

Since the A138G polymorphism was not found in cohorts of Africans and at a frequency of less than 1% in Western Europeans (Stanton et al. 2003), it was of interest, given the opportunity, to test samples from South American Peruvian Quechua Indians where we found the G allele at a high frequency in a small cohort of 21 individuals (21%) (Delgado et al. 2004). A summary of our overall data on the geographical distribution of the A138G allele is shown in Table 2. In cases where there are no differences in frequency between disease and control groups (Kagoshima, Vietnam and India), we have combined the data to calculate an overall frequency.

In summary, our data show a clearly significant protective effect of the 138G allele in hepatitis C. There may also be a similar effect in diabetes in Japanese from Osaka. The effects of the CD45 variants on disease are most likely subtle and not easy to establish in multifactorial diseases because of the likely complex interactions with other genetic and environmental factors. In both autoimmune and infectious diseases, large numbers of genes controlling immune responses have been implicated in contributing to the control of incidence and severity. These include genes that affect the threshold for triggering immune responses and genes that affect the quality of responses through cytokine production (Rioux and Abbas 2005). Both the threshold of response and cytokine signalling can be influenced by CD45 variants (Hermiston et al. 2003; Boxall et al. 2004; Do et al. 2005).

In the present study, we show that the A138G CD45 polymorphism is present at a high frequency in the Far East and at a lower frequency in India. Given its wide geographical distribution and the evidence of relevant functional effects, the variant 138G allele may well have a considerable impact on human disease.

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Flow cytometry evaluation of the T-cell receptor Vβ repertoire among human T-cell lymphotropic virus type-1 (HTLV-1) infected individuals: Effect of interferon alpha therapy in HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Mineki Saito [a,*], Hirohisa Nose [a], Koichiro Usuku [b], Amir H. Sabouri [a], Toshio Matsuzaki [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, Kagoshima, Japan*

[c] *Department of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan*

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Abstract

Human T-cell lymphotropic virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is chronic inflammatory disease of the spinal cord characterized by perivascular lymphocytic cuffing and parenchymal lymphocytic infiltration. In this study using flow cytometry, we have investigated the T-cell receptor (TCR) Vβ repertoire of peripheral blood T lymphocytes in 8 HAM/TSP patients, 10 HTLV-1 infected healthy carriers, and 11 uninfected healthy controls to determine if there is a biased usage of TCR Vβ. We found that TCR Vβ7.2 was under-utilized and Vβ12 was over-utilized in $CD4^+$ T cells of HTLV-1 infected individuals compared with healthy uninfected controls, whereas there were no such differences in $CD8^+$ T-cells. Comparison of Vβ repertoire changes before and after interferon-alpha (IFN-α) treatment for HAM/TSP revealed that one out of five patients showed dramatic decrease of specific Vβ in $CD8^+$ T cells. Our results suggest that dominant Vβ subpopulations in $CD4^+$ T cells evolved associated with chronic HTLV-1 infection, and IFN-α treatment for HAM/TSP does not induce a specific pattern of TCR Vβ changes.

Keywords: Interferon-α; T cell receptor Vβ repertoire; HTLV-1; HAM/TSP

1. Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) [1,2] is a replication-competent human retrovirus associated with adult T-cell leukemia (ATL) [3,4] and with a slowly progressive neurological disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5,6]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [7] observed only in a minority of HTLV-1 infected individuals [8]. Because the main pathological features of HAM/TSP are chronic inflammation in the spinal cord characterized by perivascular lymphocytic cuffing and parenchymal lymphocytic infiltration [9], it is widely assumed that the immune response causes the inflammatory spinal cord damage seen in HAM/TSP patients [10]. Analysis of the T-cell receptor (TCR)-variable (V) repertoire has been used for studying selective T-cell responses in autoimmune disease, alloreactivity in transplantation, and protective immunity against microbial and tumor antigens [11]. In HIV infection, prospective studies demonstrated that HIV infected subjects

* 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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with mobilization of a restricted mono-oligoclonal TCR repertoire during acute infection generally experience a rapid progression to AIDS, whereas a slower disease progression was associated with a broader TCR repertoire [12,13]. Alterations in certain TCR Vβ members are also detected in long-term non-progressors [14]. However, in HTLV-1 infection, there has been no study with regard to the TCR-V usage between infected individuals and normal healthy uninfected controls (NCs). In this study, we investigated the TCR Vβ usage in both $CD4^+$ and $CD8^+$ T cells of peripheral blood mononuclear cells (PBMCs) in HTLV-1 infected individuals (both HAM/TSP patients and healthy HTLV-1 carriers: HCs) and NCs by flow cytometry, to determine if there is a biased usage of the TCR Vβ repertoire. We also tested if the Vβ repertoire changed at all after interferon-alpha (IFN-α) treatment for HAM/TSP.

2. Patients and methods

2.1. Patients and cells

Eight patients with clinical diagnoses of HAM/TSP in accordance with World Health Organization criteria [15], 10 HCs and 11 NCs were enrolled in this study. All patients gave informed consent. Five out of the eight HAM/TSP patients received IFN-α treatment. All the individuals were from Kagoshima, which is an endemic area of HTLV-I infection in southern Japan. The duration of illness ranged from 2 to 25 years. In these patients, motor disability scores (rated from 0 to 13 as described in Section 2.3) ranged from 2 to 6. Fresh peripheral blood mononuclear cells (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.

2.2. Interferon-α treatment protocol and concomitant therapy

Three million international units (IU) of interferon-α (IFN-α: human lymphoblastoid interferon-HLBI, Sumiferon® by Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan) were administrated per one intramuscular injection. Drugs other than IFN-α such as muscle relaxants or vitamins were continued on the condition that the dosage regimen was kept constant before, during, and after therapy. Two patients received low-dose (5 and 10 mg daily, respectively) oral prednisolone throughout the study period. None of the other patients had received any immunomodulators.

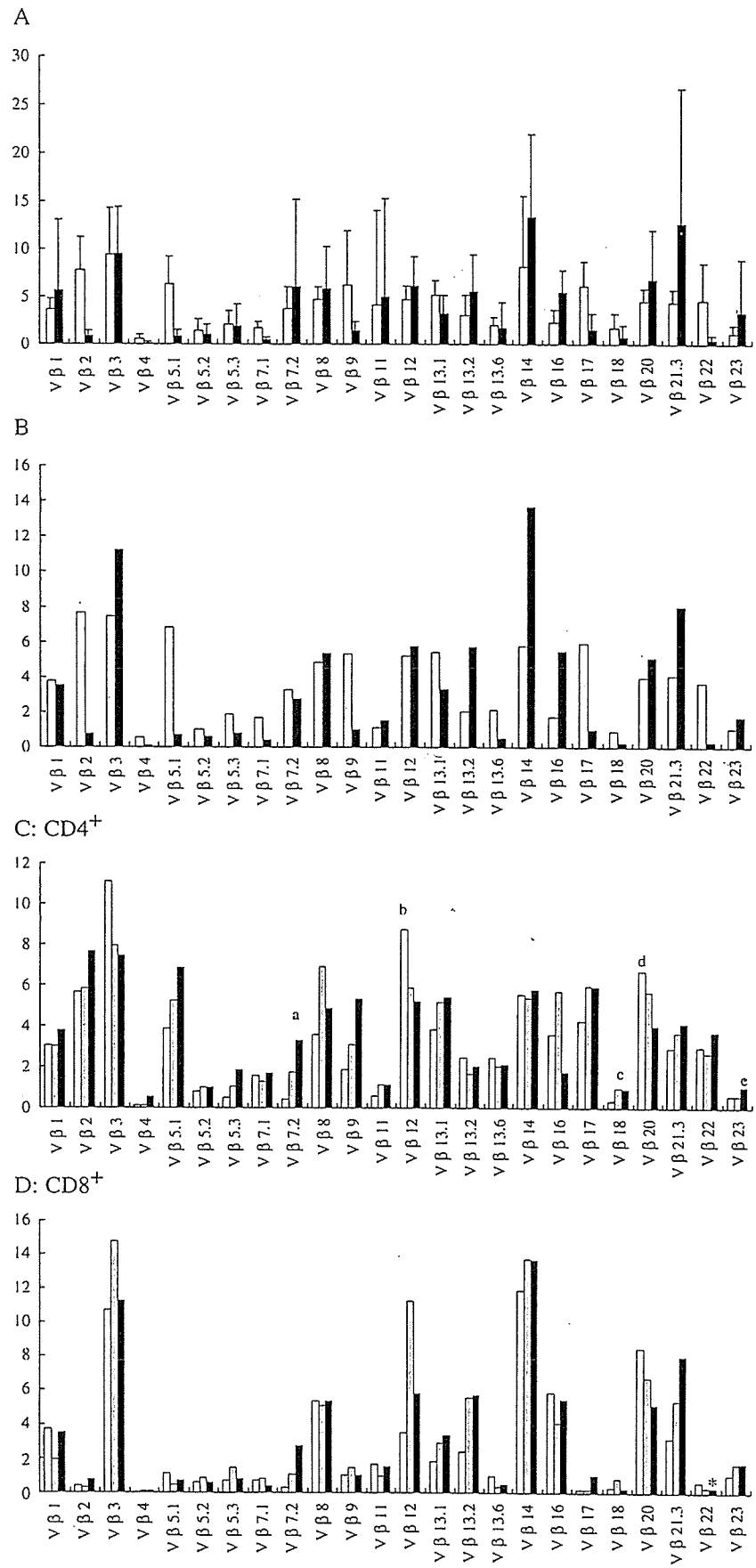
2.3. Clinical evaluation

The treating neurologists assessed the patients before therapy and 4 weeks after starting therapy. Motor dysfunction was evaluated based on the Osame Motor Disability Score (OMDS) that graded the motor dysfunction from 0 (normal walking and running) to 13 (completely bedridden) [16]. 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 scale: 0 = normal; 1 = slight; 2 = moderate; 3 = severe. The urinary disturbance score (UDS) represents the sum of all three symptoms [16]. An overall evaluation of efficacy (improvement of dysfunction) mainly on the basis of improvement of motor dysfunction as well as changes in urinary disturbances and neurological signs was also evaluated according to the scale previously used in the multicenter, randomized, double blind, and controlled study [16]. In this study, the entire HTLV-1 provirus load data were measured after the first 4 weeks of IFN-α administration was completed, by an investigator who was not involved in the patients' clinical care. Therefore, clinical neurologists did not have access to provirus load data.

2.4. Lymphocyte phenotyping and TCR Vβ repertoire analysis by flow cytometry

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 energy-coupled dye (ECD)-labeled anti-CD8 (SFCI21-ThyD3; Beckman Coulter) and phycoerythrin-Cy5 (PC5)-labeled anti-CD4 (13B8.2; Beckman Coulter). After washing the cells, the TCR Vβ repertoire staining was conducted using different anti-TCR Vβ antibodies from a Vβ monoclonal antibodies kit (IOTest Beta Mark, Beckman Coulter), which allows analysis of 24 TCR Vβ chains on previously fractionated $CD4^+$ and $CD8^+$ T cell subpopula-

Fig. 1. Comparison of TCR Vβ usage by $CD4^+$ and $CD8^+$ peripheral T-cell subsets among 8 HAM/TSP patients, 10 HTLV-1 infected healthy carriers and 10 normal uninfected controls. TCR Vβ usage of HAM/TSP patients, HTLV-1 infected healthy carriers (HCs) and normal HTLV-1 uninfected controls (NCs) by peripheral T-cell subsets were analyzed by flow cytometry using 24 anti-TCR Vβ monoclonal antibodies. *X* axis: Vβ chains, *Y* axis: frequency of expression (%). (A and B) TCR Vβ usage by $CD4^+$ (open bars) and $CD8^+$ (closed bars) peripheral T-cell subsets from 10 NCs. (A) The columns represent mean values (± 1 S.D.) of %Vβ expression frequency in each subset. (B) The columns represent median values of %Vβ expression frequency in each subset. (C and D) TCR Vβ usage of HAM/TSP patients (white bars), HCs (gray bars) and NCs (black bars) by peripheral T-cell subsets. The columns represent median values of %Vβ expression frequency in each group. (C) TCR Vβ usage by $CD4^+$ subset. (D) TCR Vβ usage by $CD8^+$ subset. (a) $p<0.05$ between HAM-NCs ($p=0.0021$) and HC-NCs ($p=0.024$). (b) $p<0.05$ between HAM-HCs ($p=0.022$) and HAM-NCs ($p=0.00070$). (c) $p<0.05$ between HAM-NCs ($p=0.047$). (d) $p<0.05$ between HAM-HCs ($p=0.037$) and HAM-NCs ($p=0.0028$). (e) $p<0.05$ between HAM-NCs ($p=0.043$). *$p<0.05$ between HAM-HCs ($p=0.024$) and HAM-NCs ($p=0.029$).


A
B
C: CD4+
D: CD8+
Vβ1
Vβ2
Vβ3
Vβ4
Vβ5.1
Vβ5.2
Vβ5.3
Vβ7.1
Vβ7.2
Vβ8
Vβ9
Vβ11
Vβ12
Vβ13.1
Vβ13.2
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tions using a four-color stain system. Isotype-matched mouse immunoglobulins were used as a control. Percentages of each Vβ member were determined by the reading on an EPICS® XL flow cytometer (Beckman Coulter) in the lymphocyte gate, based on forward versus side scatter.

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

To examine the HTLV-I provirus load, we carried out quantitative PCR using an ABI Prism 7700™ (PE-Applied Biosystems) with 100ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMCs as reported previously [17]. Using β-actin as an internal control, the amount of HTLV-I proviral DNA was calculated through the following formula: copy number of HTLV-I (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-I were determined by the particle agglutination method (Serodia-HTLV-I®, Fujirebio). The antibody titers were achieved by performing a serial dilution of the patient serum and noting the highest dilution at which agglutination was still present.

2.6. Statistical analysis

We made paired comparison of changes in T cell phenotypes and HTLV-I provirus load from before treatment to after treatment (week 4) by using the Wilcoxon signed rank test. For multiple comparisons, we used Sheffe's F to analyze statistical differences. The results represent the mean±standard deviation (S.D.) where applicable. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. TCR Vβ expression on T-cell subsets of controls and HTLV-I infected individuals

To compare the TCR repertoires in $CD4^+$ and $CD8^+$ T cells between HTLV-I infected and uninfected individuals, we first evaluated the 10 NCs. As reported by others [18], there is a marked usage of individual Vβ families, with several members making up the majority of the repertoire with marked individual differences. As shown in Fig. 1A and B, Vβ chains such as 2, 3, 5.1, and 14 in $CD4^+$ and Vβ chains 3 and 14 in $CD8^+$ were frequently used, whereas Vβ chains 4 and 18 in both $CD4^+$ and $CD8^+$ were under-utilized in our control group. All 24 TCR Vβ chains were represented in the T repertoires, but over utilization of specific Vβ (over 10%) was more frequently observed in $CD8^+$ subpopulations compared with $CD4^+$, suggesting that the T lymphocytes of healthy donors use TCR Vβ genes nonrandomly, but particular mobilizations may more easily occur in $CD8^+$ subpopulations.

TCR Vβ repertoire distribution of $CD4^+$ and $CD8^+$ subpopulations in HTLV-I infected individuals and NCs is shown in Fig. 1C and D. Basically, similar patterns of TCR Vβ mobilization were detected between HCs and NCs. As observed in NCs, Vβ chains 2, 3, 5.1, and 14 in $CD4^+$ and Vβ chains 3 and 14 in $CD8^+$ subpopulations were also the most used Vβ chains among HTLV-I infected individuals. However, we also observed some significant differences in the frequency of utilization ($p<0.05$) between HTLV-I infected individuals and NCs. In $CD4^+$ circulating cells, Vβ7.2 was under-utilized in HTLV-I infected individuals compared with NCs (HAM-NCs: $p=0.0021$, HCs-NCs: $p=0.024$) whereas Vβ chains 18 and 23 were under-utilized in HAM/TSP patients compared with NCs (Vβ18: $p=0.047$, Vβ23: $p=0.043$). In contrast, Vβ12 was more frequently utilized in HAM/TSP patients than in both HCs and NCs (HAM-HCs: $p=0.022$, HAM-NCs: $p=0.00070$) whereas Vβ20 was over-utilized in HAM/TSP patients compared with both HCs and NCs (HAM-HCs: $p=0.037$, HAM-NCs: $p=0.0028$). In $CD8^+$ circulating cells, significant differences in the frequency of utilization ($p<0.05$) remained for only Vβ22 , which were much more expressed in HAM/TSP patients than in both HCs and NCs (HAM-HCs: $p=0.024$, HAM-NCs: $p=0.029$).

3.2. Clonal-like dominance of specific Vβ observed in HTLV-I infected individuals

We identified skewed repertoires of TCR Vβ when each patient was evaluated separately. Significant expansions or reductions were observed for all 10 patients in both $CD4^+$ and $CD8^+$ subpopulations. Fig. 2 exemplifies the frequencies of Vβ usage of both $CD4^+$ and $CD8^+$ cells of four HTLV-I infected individuals. As shown in Fig. 2, a "clonal-like" dominance of specific Vβ, i.e. more than 25% of circulating cells expressed specific Vβ, was observed in both $CD4^+$ (HAM5 and HC5) and $CD8^+$ (HAM1 and HC1) subpopulations of HTLV-I infected individuals. In HC5 and HAM5, more than 25% of circulating $CD4^+$ cells expressed specific Vβ (HC5: Vβ8 37.2%, HAM5: Vβ2 34.6%, Vβ12 31.5%). However, in NCs, such a dominance of specific Vβ was observed only in $CD8^+$ subpopulations (data not shown). We expected that biased $CD4^+$ cell repertoires would occur associated with chronic HTLV-I infection.

3.3. Comparison of Vβ repertoires from HAM/TSP patients, before and after IFN-α

To investigate the effect of IFN-α therapy on the TCR Vβ peripherally circulating repertoire, the five HAM/TSP patients were evaluated after 4weeks of treatment. The changes in TCR mobilization observed for five patients in the $CD4^+$ and $CD8^+$ subpopulations. Although all patients showed fluctuations of Vβ chains, some declined and others expanded, only one patient with clinical improvement after therapy presented dramatic (more than 50%) alterations on

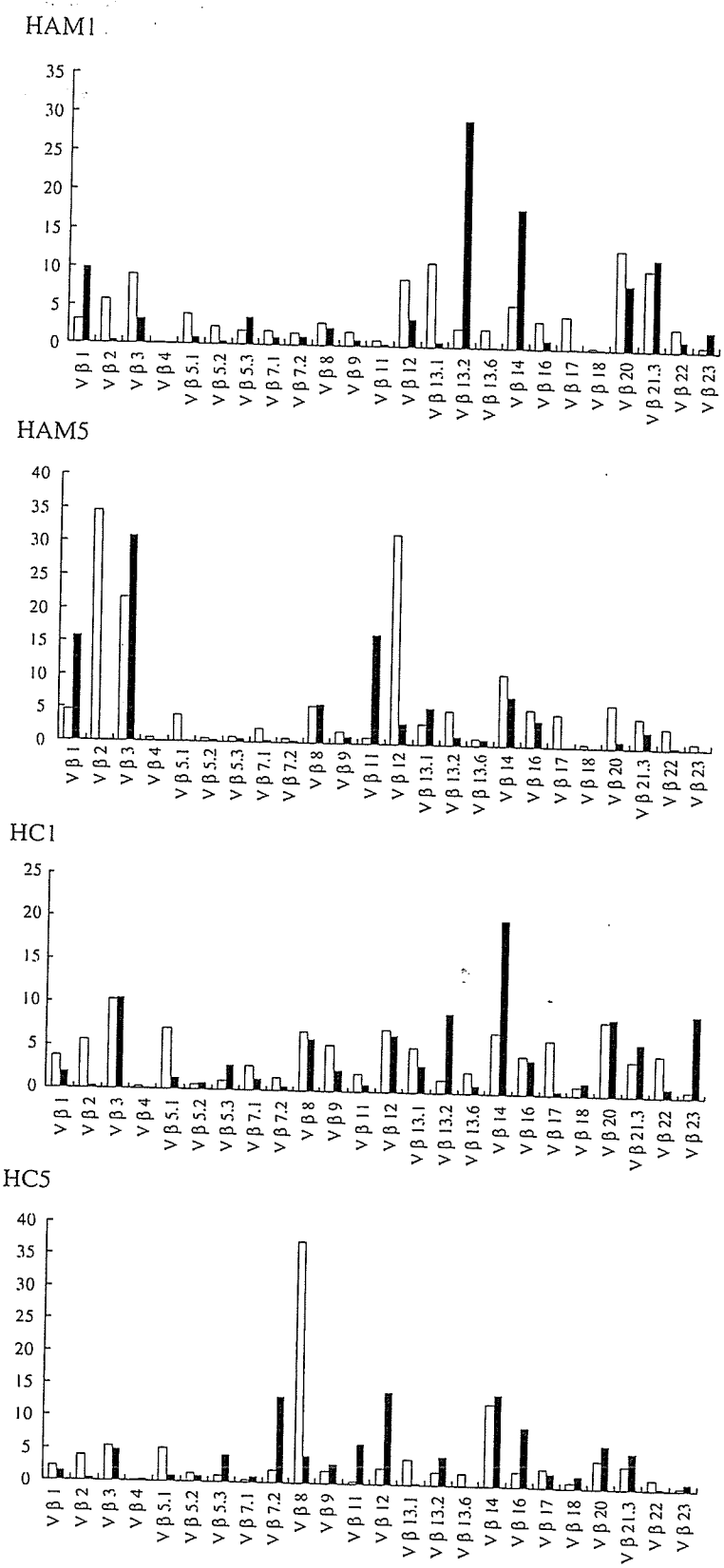

Fig. 2. Examples of "clonal-like" dominance of specific Vβ observed in HTLV-1 infected individuals. A "clonal-like" dominance of specific Vβ was observed both in $CD4^+$ (open bars) (HAM5 and HC5) and $CD8^+$ (closed bars) (HAM1 and HC1) subpopulations. However, "clonal-like" dominance in $CD4^+$ was observed only in HTLV-1 infected individuals. In HC5 and HAM5, over 30% of circulating $CD4^+$ cells expressed specific Vβ (HC5: Vβ8 37.2%, HAM5: Vβ2 34.6%, Vβ12 31.5%). *X* axis: Vβ chains, *Y* axis: frequency of expression (%).

Vβ7.2 frequencies in a $CD8^+$ population, when compared with values observed before IFN-α (data not shown).

4. Discussion

Because HAM/TSP is T cell-mediated inflammatory disease in the central nervous system (CNS) induced by chronic HTLV-1 infection and similar to organ-specific autoimmune diseases, it is possible that T cells recognizing viral antigen–or some other antigens such as neuronal antigen–should use a limited number of TCR chains. We have previously reported the evidence of an amino acid motif in the complementarity-determining region 3 (CDR3) of the TCR Vβ chain of freshly isolated $CD8^+$ T cells and muscle infiltrating cells that recognize the Tax11-19 peptide from HAM/TSP patients and HTLV-1 infected polymyositis patients [19,20], suggesting that chronic stimulation by HTLV-1 Tax antigen in vivo exerts strong selection for certain TCR structures. Therefore, it is interesting to identify TCR involved in the process of HAM/TSP development. However, there has so far been no study about TCR repertoire of PBMCs directly isolated from HTLV-1 infected individuals without any in vitro cultivation. In this study, we evaluated the TCR Vβ repertoire status of HTLV-1 infected individuals and NCs by four-color flow cytometry, which allows analysis of 24 TCR Vβ chains on both $CD4^+$ and $CD8^+$ subpopulations in a very simple way. At first, we investigated 10 NCs to define the normal frequency and distribution of TCR Vβ chains in PBMCs in the studied population. In accordance with previous observation [21], all known TCR Vβ gene members are expressed in our control subjects. We observed that Vβ chains 2, 3, 5.1, and 14 in $CD4^+$ and Vβ chains 3 and 14 in $CD8^+$ were dominant whereas Vβ chains 4 and 18 in both $CD4^+$ and $CD8^+$ were under-utilized in our NCs. However, in NCs, "clonal-like" dominance of specific Vβ, i.e. more than 25% of circulating $CD4^+$ cells expressed specific Vβ, was observed only in $CD8^+$ cells not in $CD4^+$ cells. Previous reports indicated that unlike $CD4^+$ T cells, $CD8^+$ T cells often expand clonally in the peripheral blood, and these expanded $CD8^+$ T cells are already detectable in young adults and become very frequent in elderly donors [22–24]. We found that TCR Vβ7.2 was under-utilized and Vβ12 was over-utilized in $CD4^+$ T cells of HTLV-1 infected individuals compared with NCs, whereas there were no such differences in $CD8^+$ T cells. In addition, "clonal-like" dominance of specific Vβ was observed in both the $CD4^+$ and $CD8^+$ T cell populations in HTLV-1 infected individuals, whereas such "clonal-like" dominance of specific Vβ was observed only in $CD8^+$ T cells in NCs. Because it has been suggested that HTLV-1 preferentially infects $CD4^+$ T lymphocytes [25] and in many cases this may lead to a benign clonal expansion of infected cell [26,27], our results suggest that $CD4^+$ T cell expansion would occur associated with chronic HTLV-1 infection, although the isolation of individually expanded $CD4^+$ T lymphocytes with subsequent molecular analysis is required to definitely confirm the presence of integrated HTLV-1 provirus in these cells.

We have also tested whether specific Vβ population(s) changes before and after 4 weeks administration of IFN-α in five HAM/TSP patients. In HIV infection, significant Vβ changes after highly active antiretroviral therapy (HAART) has already been reported by several groups [28–30]. In contrast, four out of five HAM/TSP patients tested showed no significant changes of specific Vβ after 4 weeks of IFN-α administration, neither on $CD4^+$ nor on $CD8^+$ cells, although some chains were slightly mobilized. Also, no pattern of specific Vβ changes was observed in either $CD4^+$ or $CD8^+$ circulating T cells in these patients. Only one patient presented more than 50% alterations in Vβ7.2 frequencies in $CD8^+$ population after IFN-α. Therefore, the effect of IFN-α was not restricted to specific T-cell populations.

In conclusion, we found that TCR Vβ7.2 was under-utilized and Vβ12 was over-utilized in the $CD4^+$ T cells of HTLV-1 infected individuals compared with healthy uninfected controls, and IFN-α treatment for HAM/TSP did not induce a specific pattern of TCR Vβ changes. These findings may provide useful information about the nature of HTLV-1 infection and assist in designing novel TCR-based immunotherapy for HAM/TSP in the future.

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Clinical symptoms and the odds of human T-cell lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) in healthy virus carriers: Application of best-fit logistic regression equation based on host genotype, age, and provirus load

Hirohisa Nose,[1] Mineki Saito,[1] Koichiro Usuku,[2] Amir H Sabouri,[1] Toshio Matsuzaki,[1] Ryuji Kubota,[3] Nobutaka Eiraku,[4] Yoshitaka Furukawa,[5] Shuji Izumo,[3] Kimiyoshi Arimura,[1] and Mitsuhiro Osame[1]

[1]Department of Neurology and Geriatrics, [2]Department of Medical Information Science, and [3]Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [4]Kagoshima University Health Service Center, Kagoshima, Japan; [5]Division of Blood Transfusion Medicine and Cell Therapy, Kagoshima University Hospital, Kagoshima, Japan

The authors have previously developed a logistic regression equation to predict the odds that a human T-cell lymphotropic virus type 1 (HTLV-1)–infected individual of specified genotype, age, and provirus load has HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) in southern Japan. This study evaluated whether this equation is useful predictor for monitoring asymptomatic HTLV-1–seropositive carriers (HCs) in the same population. The authors genotyped 181 HCs for each HAM/TSP-associated gene (tumor necrosis factor [TNF]-α −863A/C, stromal cell-derived factor 1 (SDF-1) +801G/A, human leukocyte antigen [HLA]-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup) and measured HTLV-1 provirus load in peripheral blood mononuclear cells using real-time polymerase chain reaction (PCR). Finally, the odds of HAM/TSP for each subject were calculated by using the equation and compared the results with clinical symptoms and laboratory findings. Although no clear difference was seen between the odds of HAM/TSP and either sex, family history of HAM/TSP or adult T-cell lenkemia (ATL), history of blood transfusion, it was found that brisk patellar deep tendon reflexes, which suggest latent central nervous system compromise, and flower cell-like abnormal lymphocytes, which is the morphological characteristic of ATL cells, were associated with a higher odds of HAM/TSP. The best-fit logistic regression equation may be useful for detecting subclinical abnormalities in HCs in southern Japan. *Journal of NeuroVirology* (2006) 12, 171–177.

Keywords: best-fit logistic regression equation; clinical symptoms; HAM/TSP; HTLV-1; HTLV-1 carriers

Address correspondence to Mineki Saito, MD, PhD, at present address, Department of Microbiology, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Ishikawa 920-0293, Japan. E-mail: mineki@kanazawa-med.ac.jp

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Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) (Poiesz *et al*, 1980; Yoshida *et al*, 1982) infection is of particular interest to the field of immunology as well as neurology because HTLV-1 is never eliminated from the host in spite of a vigorous cellular and humoral immune response against the virus, but causes no disease in a majority of infected subjects (asymptomatic HTLV-1–seropositive

carriers; HCs). Only approximately 2% to 3% develop adult T-cell leukemia (ATL) and another 2% to 3% develop chronic inflammatory diseases involving the central nervous system (HTLV-1–associated myelopathy/tropical spastic paraparesis; HAM/TSP) (Bangham, 2000), the eyes (Mochizuki *et al*, 1992; Nakao and Ohba, 1993), the lungs (Sugimoto *et al*, 1987; Matsuyama *et al*, 2003), the joints (Nishioka *et al*, 1989), or the skeletal muscles (Higuchi *et al*, 1993; Uchiyama, 1997; Saito *et al*, 2002). Therefore, evaluation of the individual risk for developing HTLV-1–associated diseases in each HC would certainly be of considerable importance in HTLV-1 endemic area. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa *et al*, 1996). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, our previous population association study in Kagoshima, HTLV-1 endemic southern Japan, revealed that high provirus load (Nagai *et al*, 1998; Yoshida *et al*, 1989), certain human leukocyte antigen (HLA) (Jeffery *et al*, 1999, 2000) and non-HLA (Sabouri *et al*, 2004; Vine *et al*, 2002) genes are closely associated with HAM/TSP development. Namely, HLA-A*02 and -Cw*08 genes were associated with a lower HTLV-1 provirus load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and B*5401 were associated with susceptibility to HAM/TSP (Jeffery *et al*, 1999, 2000). Because the function of class 1 HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTLs), these results imply that the efficient lysis of HTLV-1–expressing infected cells by HLA-A*02- or Cw*08-restricted CTLs reduce the risk of HAM/TSP, mainly through a reduction in provirus load. In the same cohort, we also determined the host genotype at over 100 single nucleotide polymorphisms (SNPs) in over 70 loci outside HLA class 1, and polymorphisms in at least 4 loci (tumor necrosis factor [TNF]-α, interleukin [IL]-15, SDF-1, and IL-10) were found to have statistically significant independent effects on the provirus load or the risk of HAM/TSP, or both (Sabouri *et al*, 2004; Vine *et al*, 2002). The TNF-α promoter −863 A allele predisposed to HAM/TSP (Vine *et al*, 2002), whereas SDF-1 +801A, IL-15 +191C (Vine *et al*, 2002), and IL-10 −592A alleles (Sabouri *et al*, 2004) conferred protection against HAM/TSP. In another study we reported the association between HTLV-1 *tax* gene sequence variation and the risk of HAM/TSP (Furukawa *et al*, 2000). The *tax* subgroup A was more frequently observed in HAM/TSP patients and this effect was independent of HLA-A*02. These results indicate that both host and viral genetic factors play a role in determining the risk of developing HAM/TSP. Based on these observations, we developed a best-fit logistic regression equation that can be used to predict the odds that an HTLV-1–infected individual of specified genotype (TNF-α −863A/C, SDF-1 +801G/A, HLA-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup), age, and provirus load in Kagoshima has HAM/TSP (Vine *et al*, 2002). In this study, to validate whether this multivariate logistic equation can be useful to identify HAM/TSP-related symptom in HCs, we calculated the odds in 181 consecutive HCs and the individual odds of these HCs were compared with their clinical parameters and laboratory findings.

Table 1 Characteristics of 181 asymptomatic HTLV-1 carriers participated in the study

	HCs (n = 181)
Age	46.5 ± 12.7
Sex	
Male	95
Female	86
Serum anti-HTLV-I antibody titer*	
(Mean ± SD)	×2932.6 ± 6447.4
(Median)	×1024
HTLV-I provirus load in PBMCs**	
(Mean ± SD)	240.8 ± 361.4
(Median)	82

*Anti-HTLV-1 antibodies were titrated by the particle agglutination method.
**HTLV-1 Tax copy number per 1×10^4 PBMCs.

Results

Demographic and clinical characteristics of healthy HTLV-1 carriers

A total of 181 consecutive HCs (95 men and 86 women) were completed the evaluation. Demographic and clinical characteristics of these HCs are given in Table 1. The age of the subjects enrolled ranged from 10 to 79 years with a mean age of 46.5 ± 12.7 years (men, 45.6 ± 13.2 years; women, 47.6 ± 12.2 years; mean ± SD). There were no abnormalities in complete blood cell count, electrolytes, glucose, renal and liver function tests, and the percentages of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in peripheral blood mononuclear cells (PBMCs). The HTLV-1 provirus load of HCs was 240.8 ± 361.4 copies/10^4 PBMCs (mean ± SD). There was no significant difference between the sexes in HTLV-1 provirus load (men, 235.2 ± 336.0; women, 247.0 ± 389.6; mean ± SD) and anti-HTLV-1 antibody titer (men, ×2254.3 ± 3644.6; women, ×3682.6 ± 8502.8; mean ± SD).

DNA analyses and odds of developing HAM/TSP

The numbers of subjects with each genotype of HAM/TSP associated genes are shown in Table 2. Frequencies of HLA-A*02 was slightly lower and TNF-α −863A allele was slightly higher than HCs of our previous analysis (Vine *et al*, 2002). Of 181 HCs, 17 (9.4%) had tax subgroup A and 164 (90.6%) had *tax* subgroup B. The frequency of *tax* subgroup A in these HCs was similar to our previous findings (14 out of 200 HC; 7.0%) (Furukawa *et al*, 2000). Based on these data, we calculated the odds for developing HAM/TSP by using the best-fit logistic regression equation for the risk of HAM/TSP in the

Table 2 Frequencies of genotypes and alleles for the different polymorphisms of HAM/TSP associated genes in 181 asymptomatic HTLV-1 carriers participated in the study

Genes	*Allele*	*Number of HCs*	*Genotype*	*Number of HCs*
TNF-α −863	A	80 (22.1)*	AA	18 (9.9)
	C	282 (77.9)	AC	43 (23.8)
			CC	120 (66.3)
	Total	362	Total	181
SDF-1 +801	G	241 (66.6)	GG	80 (44.2)
	A	121 (33.4)	GA	81 (44.8)
			AA	20 (11.0)
	Total	362	Total	181
HLA-Cw*08	Positive	27 (14.9)		
	Negative	154 (85.1)		
	Total	181		
HLA-A*02	Positive	64 (35.4)		
	Negative	117 (64.6)		
	Total	181		
Tax subgroup	Subgroup A	17 (9.4)		
	Subgroup B	164 (90.6)		
	Total	181		

*Numbers in parentheses are percentage.

Kagoshima HTLV-1–infected cohort as previously described (Table 3) (Vine *et al*, 2002). The median odds in HCs was 0.36, which was significantly lower than that of HAM/TSP patients (median: 21.0) in our previous analysis ($P < .0001$, by Mann-Whitney U test). (Vine *et al*, 2002).

Receiver operator characteristic (ROC) curve analysis

The receiver operating characteristic (ROC) curve was used to compare the diagnostic accuracy among anti-HTLV-1 antibody titer, HTLV-1 provirus load, and the odds for HAM/TSP calculated by the best-fit logistic regression equation. The area under the curve (AUC) of the ROC was used to estimate the predictive value of each parameter. Judged by their areas, the accuracy of odds for HAM/TSP (0.95) is much higher than that of HTLV-1 provirus load (0.88) and anti-HTLV-1 antibody titer (0.86). The cut-off value to differentiate HAM/TSP and HCs was also determined from the ROC curve. We have chosen HAM/TSP odds "0.78" as a cut-off value (Figure 1), which maximizes sensitivity to diagnose HAM/TSP and minimizes false-positive rate to misdiagnose HCs as HAM/TSP. Using this value, sensitivity and specificity of the HAM/TSP odds to diagnose HAM/TSP are 92.1% and 86.5%, respectively. After dividing our present 181 HCs by this cut-off value, then clinical parameters and laboratory findings were compared.

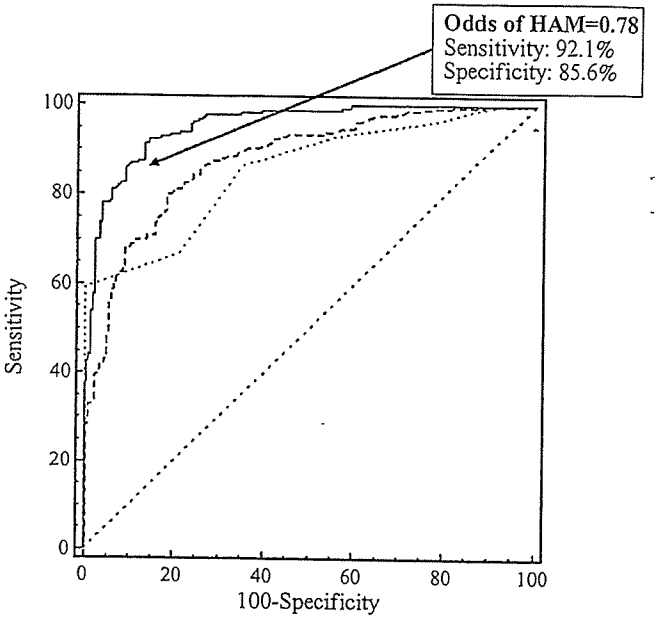

Figure 1 Receiver operating characteristic (ROC) curve of anti-HTLV-1 antibody titer, HTLV-1 provirus load, and the odds for HAM/TSP calculated by the best-fit logistic regression equation. ROC curve was constructed by plotting sensitivity against the false-positive rate (1-specificity) over a range of odds for HAM values or HTLV-1 provirus load or anti-HTLV-1 antibody titers by using our previously reported Kagoshima cohort data that consisted of 222 patients with HAM/TSP and 184 HCs (Vine *et al*, 2002). The cut off value to differentiate HAM/TSP and HCs was determined from the ROC curve as 0.78. Odds for HAM/TSP = 0.78 maximizes the sensitivity to diagnose HAM/TSP and minimizes the false-positive rate to misdiagnose HCs as HAM/TSP. Using this value, the sensitivity and specificity of the HAM/TSP odds required to diagnose HAM/TSP are 92.1% and 86.5%, respectively. ————: Odds for HAM/TSP; – – – – – –: $\log_{10}$ (HTLV-1 Tax copy number per 1×10^4 PBMCs); - - - - - - - - - -: serum anti-HTLV-I antibody titer.

Table 3 Best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-1–infected cohort ($n = 402$) (Vine *et al*, 2002)

Factor, condition	*ln (odds of HAM/TSP)*	*Odds ratio (P)*
Constant	−1.716	
Age	−(0.145 × age) + (0.003 × age²)	
Provirus load	+(0.460 × load) + (0.487 × load²)	
TNF-α −863A⁺	+3.057 − (4.616 × load) + (1.476 × load²)	
SDF-1 +801GA	−0.808	0.45 (0.042)
SDF-1 +801AA	−1.689	0.18 (0.003)
HLA-A*02⁺	−0.638	0.53 (0.043)
HLA-Cw*08⁺	−0.894	0.41 (0.046)
HTLV-1 subgroup B	−1.587	0.20 (0.017)

Example: An HTLV-1–infected individual in Kagoshima, 60 years old, with a $\log_{10}$ (provirus load) of 2.5 with the genotype TNF-α −863A⁺, SDF-1 +801AA, HLA-A*02⁻, HLA-Cw*08⁺, HTLV-1 subgroup B has a predicted ln odds of HAM/TSP of $-1.716 - (0.145 \times 60) + (0.003 \times 60^2) + (0.46 \times 2.5) + (0.487 \times 2.5^2) + 3.057 - (4.616 \times 2.5) + (1.476 \times 2.5^2) - 1.689 - 0.894 - 1.587 = 1.14975$. That is, this HTLV-1–infected individual's odds of developing HAM/TSP = exp(1.14975) = 3.157403.

Physical and neurological findings

First, the odds in HCs were compared with their demographic data and clinical variables to assess whether there was any association between the odds and each clinical parameter. As shown in Table 4,

Table 4 Comparison between the odds of HAM and clinical findings

	High odds (≥0.78)	*Low odds (<0.78)*	*P value*	*OR*	*95% CI*
$n = 181$	$n = 69$	$n = 112$			
Age (years)	53.4 ± 10.8	42.3 ± 12.1	<.001	N/A	N/A
Sex (male/female)	33/36	62/50	.41	N/A	N/A
Anti-HTLV-1 antibodies*	5123.7 ± 9495.7	1558.1 ± 2585.7	<.001	N/A	N/A
HTLV-1 provirus load**	505.1 ± 463.0	78.0 ± 101.6	<.001	N/A	N/A
Brisk patellar tendon reflexes	24/45	22/90	.036	2.18	1.11–4.31
Absent superficial abdominal reflexes	29/40	32/80	.089	1.81	0.97–3.4
Increased urinary frequency (≥10 times/day)	17/51	17/95	.15	1.86	0.88–3.96
Increased nocturia (≥2 times/night)	10/58	12/100	.58	1.44	0.58–3.53
Skin lesion	6/63	9/103	.90	1.09	0.37–3.21
Superficial lymph nodes swelling	6/63	8/104	.93	1.24	0.41–3.73
History of blood transfusion	2/67	5/107	.60	0.64	0.12–3.39
Family history of HAM/TSP or ATL	14/51	25/82	.93	0.90	0.43–1.89

The values are shown as the mean ± SD. N/A: not applicable.
*Anti-HTLV-1 antibodies were titrated by the particle agglutination method.
**HTLV-1 tax copy number per 1×10^4 PBMCs.

when we select odds for HAM/TSP = 0.78 as a cut-off value, there was no clear difference between the sex, family history of HAM/TSP or ATL, history of blood transfusion, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion, absent superficial abdominal reflexes (SARs), and the odds for HAM/TSP. However, brisk patellar deep tendon reflexes (PTRs), which may suggest latent central nervous system compromise, were more frequently observed in the HCs with higher odds (≥0.78) than the HCs with lower odds (<0.78) ($P = .036$, by χ^2-test with Yates correction). Absent superficial abdominal reflexes also tend to be more frequent in healthy carriers with higher odds, but P value did not reach statistical significance ($P = .089$, by χ^2-test with Yates correction).

Laboratory findings

Although no clear association was seen between the odds of HAM/TSP and either the complete blood cell count, electrolytes, glucose, renal or liver function tests, or the percentages of $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in PBMCs, both the absolute number and the percentage of flower cell-like abnormal lymphocytes (Ably), which is a morphological characteristic of ATL cells, were more frequently observed in healthy carriers with higher odds (≥0.78) than the healthy carriers with lower odds (<0.78) ($P = .011$ and .010, respectively by Mann Whitney U test) (Table 5).

Discussion

We have previously developed a logistic regression equation based on age, HTLV-1 provirus load, and genotypes of HAM/TSP–associated genes (TNF-α −863A/C, SDF-1 +801G/A, HLA-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup) to predict the odds that an HTLV-1–infected individual in Kagoshima has HAM/TSP (odds of HAM/TSP) (Vine *et al*, 2002). To compare the diagnostic value of this equation, HTLV-1 provirus load and anti-HTLV-1 antibody titer for predicting the risk that an HTLV-1–infected individual will develop HAM/TSP, we employed receiver operating characteristic (ROC) curve analysis.

Table 5 Comparison between the odds of HAM and laboratory findings

	Odds of HAM/TSP		*P value*
	≥0.78	*<0.78*	
	$n = 68$	$n = 110$	
WBC counts	5430.9 ± 1491.4	5560.9 ± 1333.7	0.36
Lymphocyte counts	1640.1 ± 708.1	1721.5 ± 537.0	0.39
Lymphocyte %	30.4 ± 9.8	31.8 ± 8.6	0.27
Abnormal lymphocyte* counts	51.7 ± 79.0	19.0 ± 39.4	0.011
Abnormal lymphocyte %	0.88 ± 1.23	0.36 ± 0.77	0.010
Atypical lymphocyte** counts	32.6 ± 53.2	36.8 ± 63.2	0.66
Atypical lymphocyte %	0.65 ± 1.02	0.65 ± 1.23	1.00
	Cluster of differentiation		
	$n = 65$	$n = 104$	
$CD4^+$ counts	820.0 ± 410.1	820.1 ± 266.7	0.34
$CD4^+$ %	49.1 ± 8.0	47.3 ± 6.9	0.28
$CD8^+$ counts	497.3 ± 233.7	532.0 ± 191.2	0.12
$CD8^+$ %	30.5 ± 7.1	30.8 ± 7.3	0.71
CD4/8 ratio	1.73 ± 0.62	1.65 ± 0.56	0.71
	$n = 59$	$n = 101$	
$CD4^+CD3^+$ counts	797.7 ± 428.8	753.0 ± 249.7	0.59
$CD4^+CD3^+$ %	45.4 ± 7.9	43.3 ± 7.7	0.21
$CD8^+CD3^+$ counts	411.1 ± 241.4	427.1 ± 168.2	0.95
$CD8^+CD3^+$ %	23.8 ± 6.5	24.5 ± 6.5	0.82
$CD4^+CD3^+/CD8^+$ $CD3^+$ ratio	2.13 ± 0.89	1.92 ± 0.70	0.20
$CD16^+CD56^+CD3^-$ counts	228.7 ± 117.7	257.6 ± 166.5	0.64
$CD16^+CD56^+CD3^-$ %	15.1 ± 7.7	14.7 ± 6.9	0.64
$CD16^+CD56^+CD3^+$ counts	35.8 ± 44.7	47.3 ± 57.8	0.56
$CD16^+CD56^+CD3^+$ %	2.25 ± 2.83	2.80 ± 3.39	0.42
	Blood chemistry		
	$n = 66$	$n = 109$	
LDH	363.2 ± 114.9	349.8 ± 92.4	0.25

Cell counts are per/mm^3.
*Abnormal lymphocyte: flower cell (ATL cell)-like lymphocytes (see Materials and Methods).
**Atypical lymphocyte: a reactive lymphocyte due to antigenic stimulation with increased size and presence of active DNA synthesis, i.e., lobulated or indented nucleus with slightly finer chromatin, and the cytoplasm vary in color being basophilic, dark blue, plasmacytic to pale gray.

The ROC curve analysis was also used to identify a threshold at which sensitivity is highest at the lowest possible false-positive rate for each valuable. Our results clearly suggested that "odds for HAM/TSP" is better parameter for predicting disease than both HTLV-1 provirus load and anti-HTLV-1 antibody titer. The ROC curve of the odds of HAM/TSP showed an area under the curve (AUC) of 0.95, and best cut-off value being 0.78. After dividing HCs into the higher odds group and the lower odds group by this cut-off value (0.78), we have compared different clinical and laboratory parameters between two groups.

The comparison between the odds for HAM/TSP and clinical parameters revealed that brisk PTR were more frequently observed in the HCs with higher odds ($\geq$0.78) than the HCs with lower odds (<0.78) ($P = .036$, by χ^2-test with Yates correction), although their neurological signs were subtle and none had any motor signs. In contrast, there was no statistically significant association between the odds of HAM/TSP and either sex, family history of HAM or ATL, history of blood transfusion, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion. Because deep tendon reflexes (DTRs) test the integrity of the neurological system such as neuromuscular junction, peripheral nerve, nerve root, spinal cord and certain supraspinal centers, these reflexes are routinely used by clinicians to evaluate the nervous system for anatomical diagnosis. Hyperactive DTRs suggest central nervous system compromise. Therefore, increased frequencies of brisk PTRs in HCs with higher odds suggest that the calculated odds of HAM/TSP could be used as an indicator of HAM/TSP-related symptom. However, limitations of DTR are its qualitative nature of the assessments based upon subjective grading, and limited inter-rater reliability. Therefore, we next compared the objective laboratory data between HCs with higher odds ($\geq$0.78) and the HCs with lower odds (<0.78).

In all the laboratory parameters tested, only flower cell–like abnormal lymphocytes (Ably), both in their absolute number and frequency, were more frequently observed in HCs with higher odds ($\geq$0.78) than the HCs with lower odds (<0.78), with statistically significant level ($P = .011$ and .010, respectively by Mann Whitney U test). Because the odds for HAM/TSP is strongly correlated with provirus load, our result is consistent with previous studies, which demonstrated the presence of circulating Ably in HCs and a correlation between Ably frequency and HTLV-1 provirus load (Hisada *et al*, 1998; Tachibana *et al*, 1992). It may therefore be possible that the higher odds of HAM/TSP is associated with "genetically determined" efficient proliferation of HTLV-1–infected cells *in vivo*. However, neither the absolute number nor the frequency of $CD4^+CD3^+$ T cells, which is the main reservoir of HTLV-1 provirus, was significantly greater in HCs with higher odds ($\geq$0.78) than in the HCs with lower odds (<0.78) ($P = .59$ and .21, respectively, by Mann Whitney U test). This result suggests the presence of clonal outgrowth of HTLV-1–infected cells and skewed T cell repertoire, which is probably due to a long history of constant antigenic exposure, in $CD4^+CD3^+$ T cells of HCs with higher odds. Indeed, a previous report indicated that HTLV-1 infection is characterized by perturbation in T cell receptor (TCR) Vβ usage and CDR3 size distributions in both $CD8^+$ and $CD4^+$ T cells with clonal expansions (Eiraku *et al*, 1998). Therefore, HCs with higher odds of HAM/TSP may have more sustained clonal expansions and immune activation than HCs with lower odds, and these condition may also induce higher Ably level. If this is the case, Ably will be a good marker for the efficient clonal expansion of HTLV-1–infected T-cells and increase the risk of HAM/TSP. It would be informative, in a further study, to test whether clonal proliferation of infected $CD4^+$ T cells as well as TCR repertoire is related to the odds of HAM/TSP.

In conclusion, our study shows the possibility that our best-fit logistic regression equation could be useful for detecting HAM/TSP-related symptoms within HCs in Kagoshima cohort. This provides important indications for the management of HCs in an endemic area. It is possible that selective antiretroviral therapy as well as the therapeutic agents designed to reduce the effects of proinflammatory cytokines will reduce the risk for developing HAM/TSP in individuals with a higher odds for HAM/TSP. Further follow-up study is warranted to confirm the present findings.

Materials and methods

Study population

This study includes 190 consecutive HTLV-1–infected asymptomatic individuals who attended the Kagoshima University Hospital HTLV-1 Carrier Consultation Clinic between February 1999 and November 2004. Participation was voluntary and written informed consent was obtained from each subject upon entry into the study. This study was approved by the ethics committee of the Kagoshima University Graduate School of Medical and Dental Sciences. Nine cases were diagnosed as ATL by examination and blood tests and were therefore excluded from study. All cases were Japanese and resided in Kagoshima Prefecture, an HTLV-1 endemic region in southern Japan. On the first visit, all study participants interviewed by one of the three consultant neurologists who were certified by the board of Japanese Society of Neurology, then received a physical and neurological examination as well as blood tests. The following demographic data and clinical variables were assessed: sex, past history of blood transfusion, family history of hematological malignancies, family history of HAM/TSP, deep tendon reflexes, superficial abdominal reflexes, pathological reflexes, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion, HTLV-1 provirus load, anti-HTLV-1