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

antibody titer, complete blood cell count, differential leukocyte count, electrolytes, glucose, renal and liver function tests, and percent of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in peripheral blood mononuclear cells (PBMCs).

Provirus load measurement and anti-HTLV-1 antibody titers

To assay the HTLV-1 provirus load, we carried out a quantitative polymerase chain reaction (PCR) method using ABI Prism 7700 (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) extracted from PBMCs using a QIAamp blood kit (Qiagen), according to the manufacturer's instructions (Nagai *et al*, 1998). 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 *tax* per 1×10^4 PBMCs = [(copy number of *tax*)/(copy number of β-actin/2)] $\times$ 10^4. All samples were tested in triplicate. The lower limit of detection was one copy of HTLV-1 *tax* per 10^4 PBMCs. Serum antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1; Fujirebio). Namely, 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.

Laboratory methods

Complete blood cell count, differential leukocyte count, electrolytes, glucose, renal and liver function tests, and the percentages of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in PBMCs were measured on all fresh samples at the Kagoshima University Hospital Clinical Laboratory. Peripheral blood smears were obtained by smearing one drop of fresh blood onto a glass slide. All the slides were fixed by methanol and stained with Giemsa, and read by observers who were blinded to clinical information. The identification of flower cell (ATL cell)–like abnormal lymphocytes (Ably) followed the criteria by Sacher *et al* (1999). Namely, we classified the cells as Ably when they fulfilled the following criteria: the absence of azurophil granules; the presence of nuclear folding or lobulation; and at least two of the following characteristics: nuclear chromatin condensation, nuclear to cytoplasmic ratio of >80%, and/or cell size >1.5 times that of small lymphocytes. The number of abnormal lymphocytes and atypical lymphocytes in Table 5 were calculated as follows: (1) a trained medical technologist blind to subject HTLV serostatus performed three 100–white cell differential counts on a total of 300 leukocytes, then percentage of Ably or atypical lymphocytes was obtained. (2) The findings obtained by a trained medical technologist were reviewed by a board-certified hematologist to confirm the findings. (3) Using percentage of Ably or atypical lymphocytes and absolute WBC counts, the number of abnormal lymphocytes and atypical lymphocytes were calculated.

Restriction fragment length polymorphism (RFLP) analysis of the HTLV-1 tax gene

To identify the HTLV-1 *tax* gene subgroup (*tax* subgroup A or B), we carried out a PCR-RFLP analysis as previously described. (Furukawa *et al*, 2000). For RFLP analysis, 4 μl of the PCR product was digested with 5 U of *AccII* (Takara, Tokyo, Japan) in a 10-μl volume at 37°C for 1 h followed by electrophoresis on 2% Nusieve agarose gel. Positive and negative controls of known samples of *tax* gene subgroups A and B, confirmed by direct sequencing analysis, were included in all the experiments.

HLA typing

PCR sequence-specific primer reactions were performed to detect HLA-A*02 and HLA-Cw*08 as previously described (Bunce *et al*, 1995; Olerup and Zetterquist, 1992).

Receiver operator characteristic (ROC) curve analysis

Receiver operator characteristic (ROC) curve was constructed by plotting sensitivity against the false-positive rate (1-specificity) over a range of values of either the odds of HAM/TSP or the HTLV-1 provirus load or the anti-HTLV-1 antibody titers. These curves were constructed with data from our previously reported Kagoshima cohort, which consisted of 222 patients with HAM/TSP and 184 HCs (Vine *et al*, 2002). The area under the curve (AUC) of the ROC was used to estimate the predictive value of each parameter. The AUC is classified as low if the area is between 0.5 and 0.7; as moderate, if between 0.7 and 0.9; and as high, if greater than 0.9. The cut-off value to differentiate HAM/TSP and HCs was also determined from the ROC curve.

Statistical analysis

The chi-squared test, the Mann-Whitney U test, and the odds ratio were used for statistical analysis. Significance was considered at $P < 0.05$.

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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)

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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

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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-1 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.

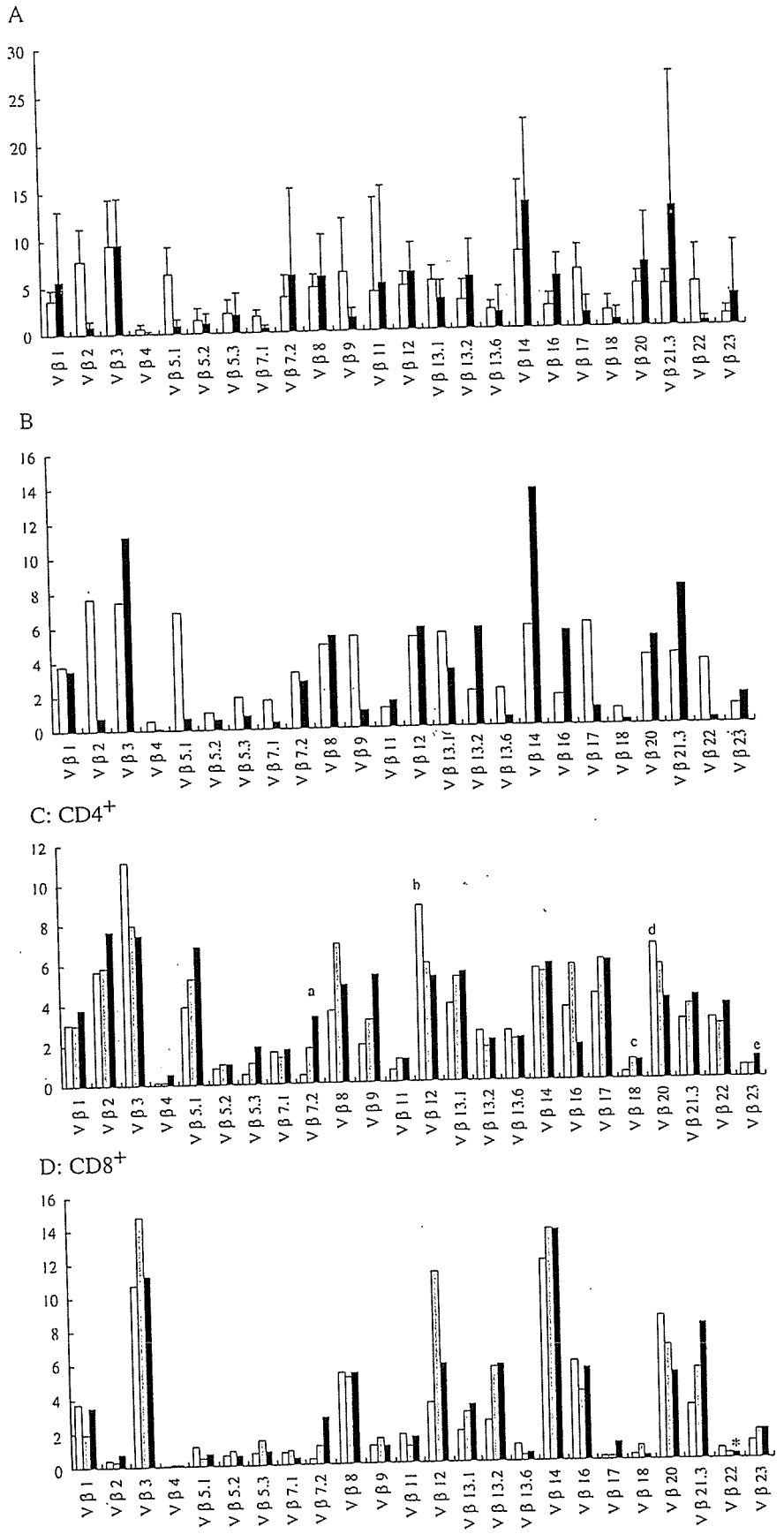
2.3. Clinical evaluation

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
Vβ13.6
Vβ14
Vβ16
Vβ17
Vβ18
Vβ20
Vβ21.3
Vβ22
Vβ23
a
b
c
d
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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-1 provirus load and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out quantitative PCR using an ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng 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-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 the 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 was still present.

2.6. Statistical analysis

We made paired comparison of changes in T cell phenotypes and HTLV-1 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-1 infected individuals

To compare the TCR repertoires in $CD4^+$ and $CD8^+$ T cells between HTLV-1 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-1 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-1 infected individuals. However, we also observed some significant differences in the frequency of utilization ($p<0.05$) between HTLV-1 infected individuals and NCs. In $CD4^+$ circulating cells, Vβ7.2 was under-utilized in HTLV-1 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-1 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-1 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-1 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-1 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 4 weeks 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

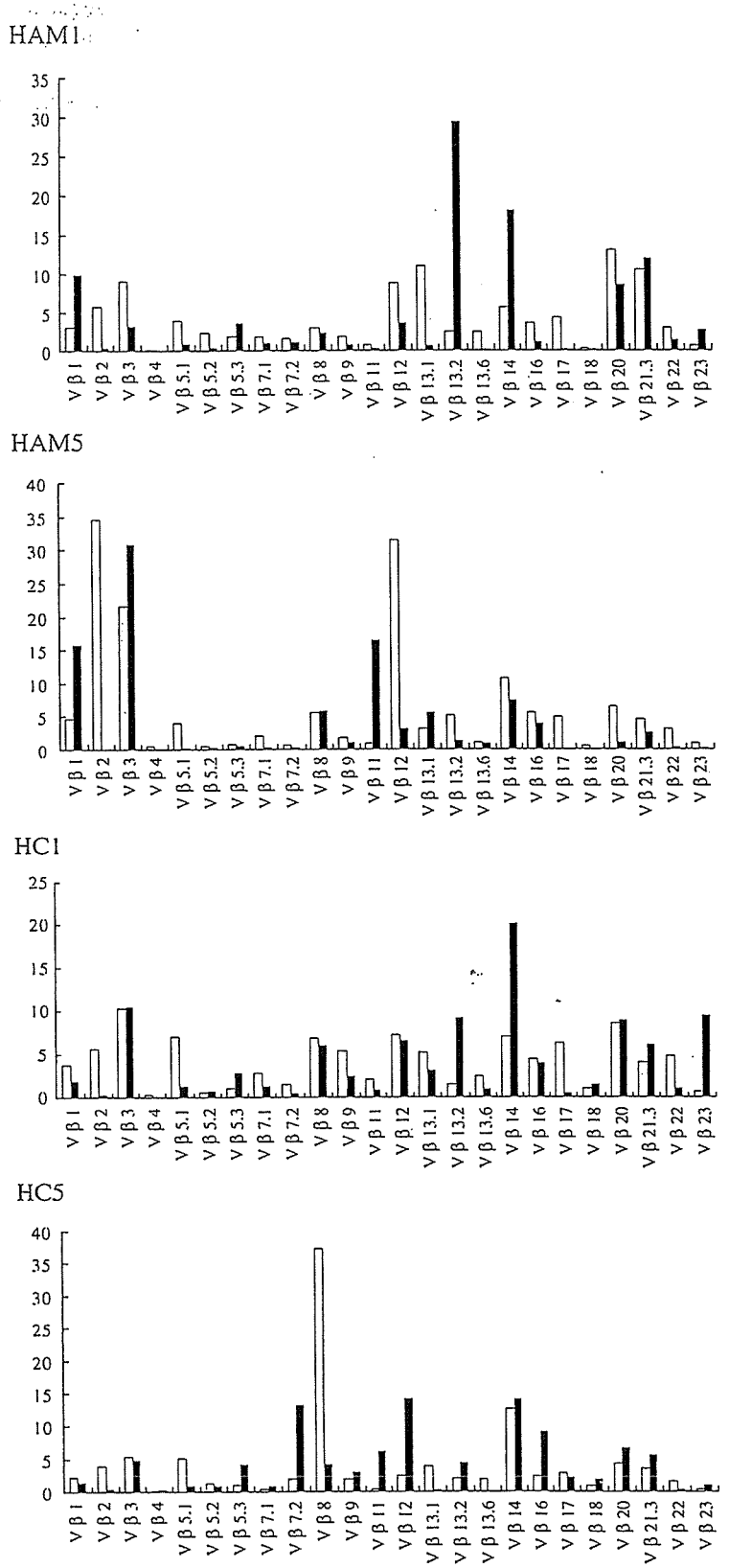

Fig. 2. Examples of "clonal-like" dominance of specific Vβ observed in HTLV-1 infected individuals. A "clonal-like" dominance of specific Vβ was observed both in $CD4^+$ (open bars) (HAM5 and HC5) and $CD8^+$ (closed bars) (HAM1 and HC1) subpopulations. However, "clonal-like" dominance in $CD4^+$ was 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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MRI studies of spinal visceral larva migrans syndrome

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Abstract

We report serial MR findings in four patients with myelitis caused by visceral larva migrans syndrome due to *Toxocara canis* or *Ascaris suum* infection. MR imaging revealed spinal cord swelling with or without gadolinium enhancement in three patients. T2-weighted images showed high signal intensities preferentially located in both lateral and posterior columns. Antihelmintic and corticosteroid treatment yielded improvement in neurologic deficits and spinal lesions. However, one patient with *T. canis* infection relapsed associated with reappearance of MRI abnormalities.

Keywords: Visceral larva migrans syndrome; *Toxocara canis*; *Ascaris suum*; Myelitis; MRI

1. Introduction

Visceral larva migrans (VLM) syndrome is a zoonotic disease caused by the migration or presence in human tissue of helminth larva from low-order animals. VLM syndrome most commonly affects the liver, skin, lungs and eyes [1], but involvement of the central nervous system is rare. The most common cause of VLM syndrome is the dog ascarid, *Toxocara canis* (*T. canis*) [2]. Prevalence of *T. canis* infection in dogs and the resulting ground contamination is relatively high in many countries. Recently, an outbreak of visceral larva migrans due to *Ascaris suum* (*A. suum*) infection has been reported in Kyushu, Japan [3].

Several reports of myelitis caused by VLM syndrome due to either *A. suum*, or *T. canis* infection have been reported [4–11]. However, long-term prognosis of the disease has not been well understood. In this paper, we report four cases of parasitic myelitis (2 patients with *T. canis* infection, and 2 patients with *A. suum* infection).

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2. Subjects and methods

For screening of parasite infection, multiple dot ELISA for 12 parasite antigens, *A. suum*, *T. canis*, *Dirofilaria immitis*, *Anisakis simplex*, *Gnathostoma doloresi*, *Strongyloides ratti*, *paragonimus westermanii*, *Paragonimus miyazakii*, *Fasciola hepatica*, *Clonorchis sinensis*, *Spirometra erinacei* and *Cysticercus cellulosae*, was performed. For evaluation of the treatment, semiquantitative analysis by microplate ELISA using antigens of *T. canis* and *A. suum* was done.

3. Case report

3.1. Patient 1

A 37-year-old woman who lived in Kagoshima prefecture in southern Kyusyu, Japan, noticed muscle convulsion of the right leg in December 2000. In January 2001, she had muscle weakness and dysesthesia of the left leg. Similar symptoms developed on the right leg from April 2001 resulting in paraplegia by May 2001. She was admitted to our clinic May 2001. On neurological

examination, she had spastic paraplegia associated with sensory impairment in all modalities below the T4 level. She also had urinary retention and bowel dysfunction. Deep tendon reflexes were exaggerated in both upper and lower limbs, and Babinski signs were positive bilaterally. Sagittal T1-weighted image (T1WI) revealed swelling of the cervical spinal cord (Fig. 1a). Sagittal T2-weighted image (T2WI) of the cervical and thoracic cord showed high signal intensity at the same levels (Figs. 1b, c). Brain MRI revealed a high intensity lesion in the left parietal white matter (Fig. 1d). Biochemical and hematological tests were normal except for eosinophilia (WBC: 6600/mm^3, eosinophil 11%, baso 1%, mono 4%, lympho 29%, neutro 55%). Cerebrospinal fluid examination disclosed the following results; mononuclear cells 38/mm^3, protein 149 mg/dl, glucose 43 mg/dl. Oligoclonal band was negative, and myelin basic protein was not elevated. At this point, she was diagnosed with an inflammatory myelitis of unknown etiology, and was started on intravenous methylprednisolone treatment (1000 mg/day for 3 days) followed by oral prednisolone treatment. Follow-up MRI revealed a decrease in the spinal cord swelling and high intensity signals. On multiple dot ELISA for 12 parasite antigens, both serum and CSF strongly bound to *T. canis* only. Parasite eggs were not found on repeated stool examinations. A diagnosis of myelitis caused by *T. canis* infection was then made. Albendazole (600 mg/day) was given for 2 courses of 4 weeks each with a 2-week interval between courses. Thereafter, high intensity signals in the spinal cord on T2WI remarkably decreased associated with improvement of muscle weakness in the lower limbs (Figs. 2a, b). Although she continued rehabilitation, she noticed worsening of paraparesis 6 months later. Spinal MRI revealed swelling of the spinal cord from the upper cervical to the lower thoracic cord levels with high intensity signals on T2WI (Figs. 2c, d). Cerebrospinal fluid examination disclosed the following; cells 53/mm^3 (eosinophils 1%, lymphocytes 94%, monocytes 5%), protein 150 mg/dl, glucose 43 mg/dl, and IgG index 0.82. Antibody titer to *T. canis* was not elevated. Again, she was treated with intravenous methylprednisolone (1000 mg/day for 3 days) followed by oral prednisolone. Although she became able to sit on a chair, severe paraparesis remained. Three weeks after, CSF examination revealed a decrease in cell count (21/mm^3), and protein (39 mg/dl). Repeat spinal MRI revealed marked reduction of spinal cord swelling with high intensity areas.

3.2. Patient 2

A 40-year-old woman who lived in Kagoshima prefecture, in southern Kyusyu, Japan, noticed acute hypoesthesia and dysesthesia in the lower limbs on June 9, 2003. One week later, muscle weakness in the right lower limb developed and then worsened. She visited us in July of the same year. On neurological examination, she had spastic paraplegia (predominantly in the right lower limb) associated with superficial sensory impairment below the T10 level, although deep sensation was preserved. She had urinary retention and bowel dysfunction. Deep tendon reflexes were exaggerated in the

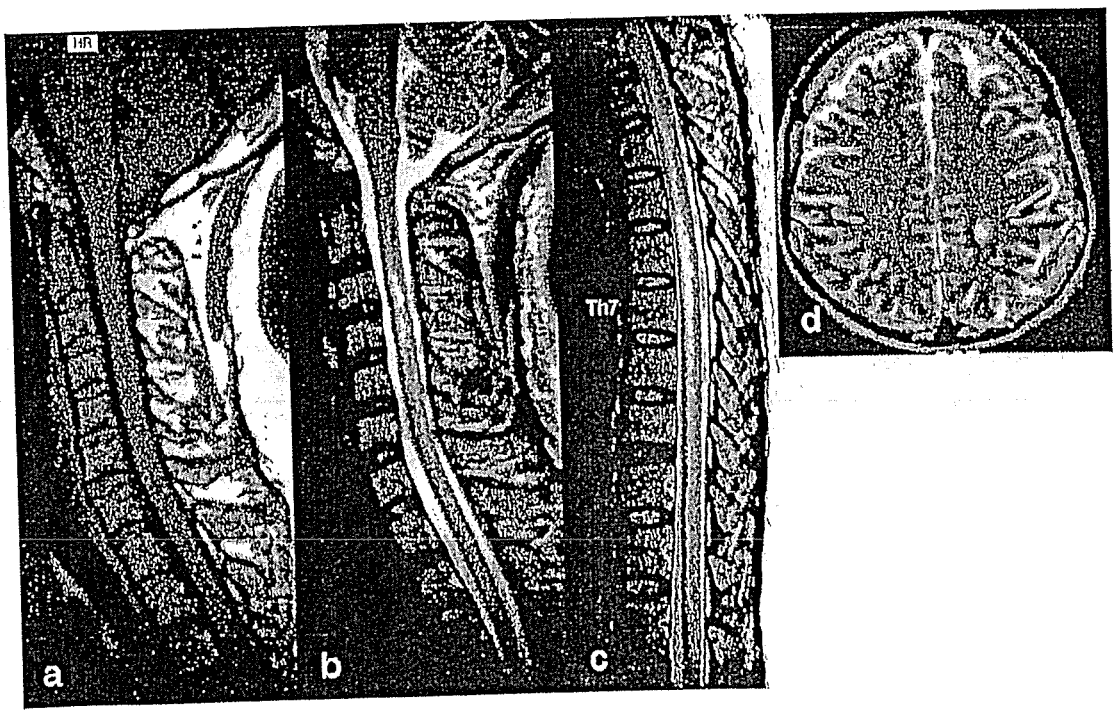

Fig. 1. MRI before treatment (patient 1). (a) Sagittal T1-weighted image (T1WI) demonstrating swelling of the cervical spinal cord. (b, c) Sagittal T2-weighted image (T2WI) showing high signal intensity in the entire spinal cord. (d) Brain MRI demonstrating a high signal intensity area in the left parietal white matter.

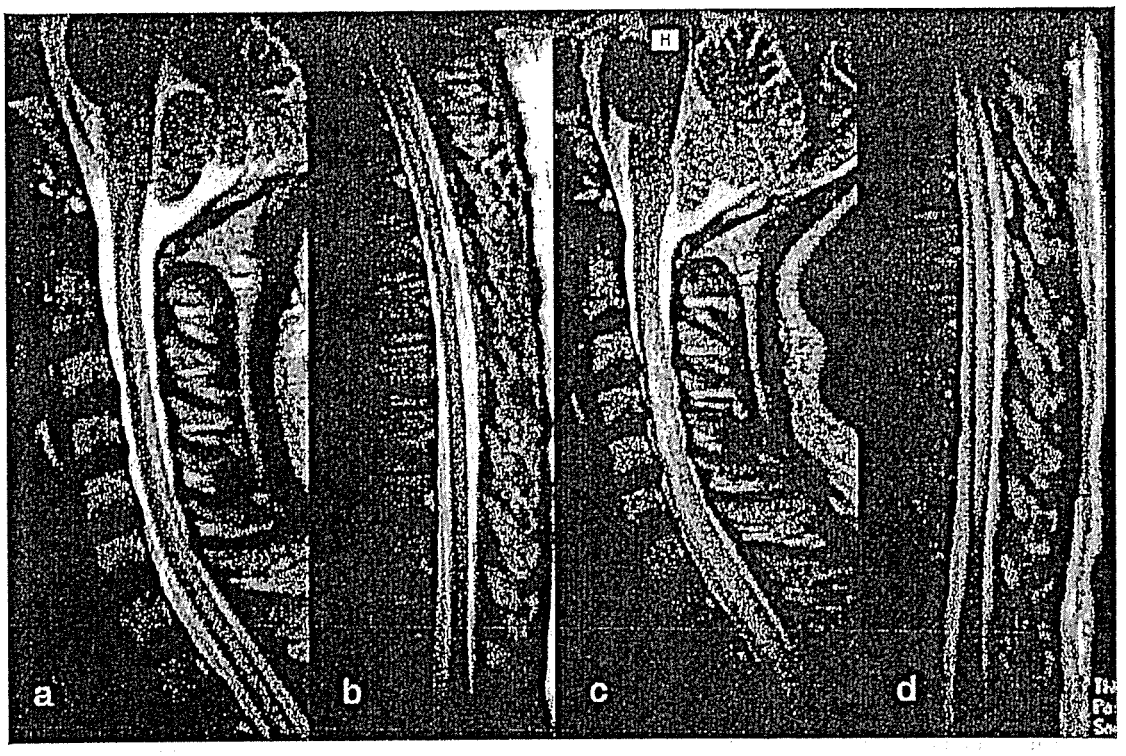

Fig. 2. Follow-up MRI after treatment (patient 1). (a) Sagittal T2WI shows improvement but still residual high-signal intensity in the posterior aspects of the cervical cord. (b) Sagittal T2WI showing disappearance of the high signal intensity area in the thoracic cord. (c, d) On relapse, sagittal T2WI showing swelling with high intensity signal throughout the spinal cord.

lower limbs and Babinski signs were positive bilaterally. Lhermitte's sign was positive. Her gait was unstable. Sagittal T1WI revealed swelling of the spinal cord from T1 to T7 (Fig. 3a). Sagittal T2WI revealed high signal intensity at the corresponding levels (Fig. 3b). After the administration of gadolinium, focal enhancement was noted (Figs. 3c, d). Brain

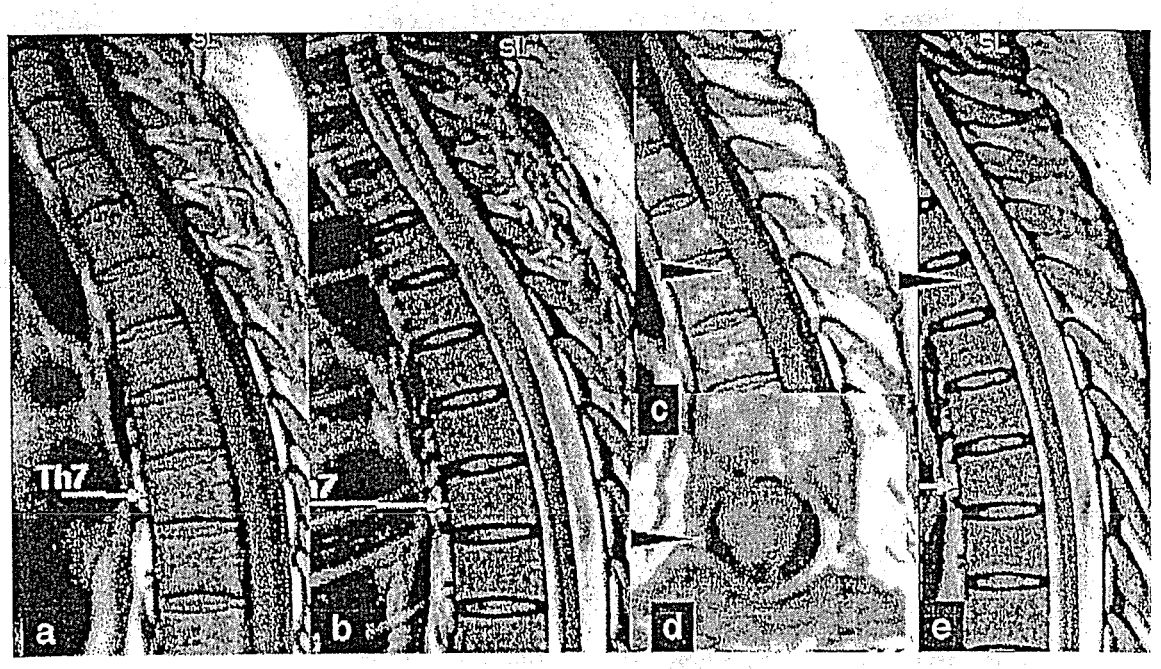

Fig. 3. MRI before (a–d) and after (e) treatment (patient 2). (a) Sagittal T1WI demonstrates swelling of the cord from T3 to T6. (b) Sagittal T2WI shows high signal intensity in the same levels. (c, d) Post-contrast sagittal (c) and axial (d) T1WI show focal enhancement at the T4 level (arrowhead). (e) After the second treatment, sagittal T2WI showing decreased swelling with residual high signal intensity of the spinal cord.

MRI was unremarkable. Biochemical and hematological tests were normal except for hyper IgEemia (802 IU/ml, normal 3–304 IU/ml). Cerebrospinal fluid examination disclosed the following: cells 9/mm^3 (eosinophils 10%, mononuclear cells 90%), protein 31 mg/dl, glucose 43 mg/dl, IgG index 0.73. Oligoclonal band was negative, and myelin basic protein was not elevated. At this point, she was diagnosed with an inflammatory myelitis of unknown etiology, and then had intravenous methylprednisolone treatment (1000 mg/day for 3 days) followed by oral prednisolone treatment. Follow-up MRI revealed decrease of spinal cord swelling and high intensity signals. On multiple dot ELISA, both serum and CSF strongly bound to *T. canis* and *A. suum*, and weakly to *Dirofilaria immitis*, *Anisakis simplex*, *Gnathostoma doloresi*, and *Strongyloides ratti*. Microplate ELISA was positive for positive for *T. canis* only. Parasite eggs were not found on repeated stool examinations. A diagnosis of myelitis caused by *T. canis* infection was then made. Albendazole (600 mg/day) with oral prednisolone (30 mg/day) was given daily for 2 courses of 4 weeks each with a 2-week interval between each course. Thereafter, high intensity signals in the spinal cord on T2WI remarkably decreased (Fig. 3e) associated with improvement of muscle weakness in the lower limbs.

3.3. Patient 3

A 54-year-old man who lived in Kagoshima prefecture, noticed muscle weakness in the right lower limb, and numbness in bilateral lower limbs on April 15, 2003. Three days later, he had facial numbness followed 5 days later by muscle weakness in the lower limbs. He consulted us April 28, 2003. On neurological examination, he had spastic paraplegia associated with numbness of the face and extremities. He also had urinary retention and bowel dysfunction. Deep tendon reflexes were exaggerated in both upper and lower limbs; however, Babinski signs were negative bilaterally. Sagittal T2WI showed high signal intensity at the spinal cord from C2 to T3 (Fig. 4a) without gadolinium enhancement. Brain MRI was unremarkable. Biochemical and hematological tests were normal except for eosinophilia (WBC: 4700/mm^3, eosinophil 8%, baso 2%, mono 6%, lympho 38%, neutro 46%) and hyper IgEemia (991 IU/ml). Cerebrospinal fluid examination disclosed a normal cell count 2/mm^3 (mononuclear cell), protein 38 mg/dl, glucose 43 mg/dl. Oligoclonal band was negative, and myelin basic protein was not elevated. EEG showed disorganization of α wave activity, and spikes after hyperventilation. On multiple dot ELISA, both serum and CSF strongly bound to *A. suum* only. Parasite eggs were not found on repeated stool examinations. At this point, she was diagnosed myelitis caused by *A. suum* infection. She was given daily for 2 courses of 4 weeks each with a 2-week interval between courses. Thereafter, there was improvement of muscle weakness in the lower limbs associated with decrease of high intensity signals in the cervical cord on MRI (Fig. 4b).

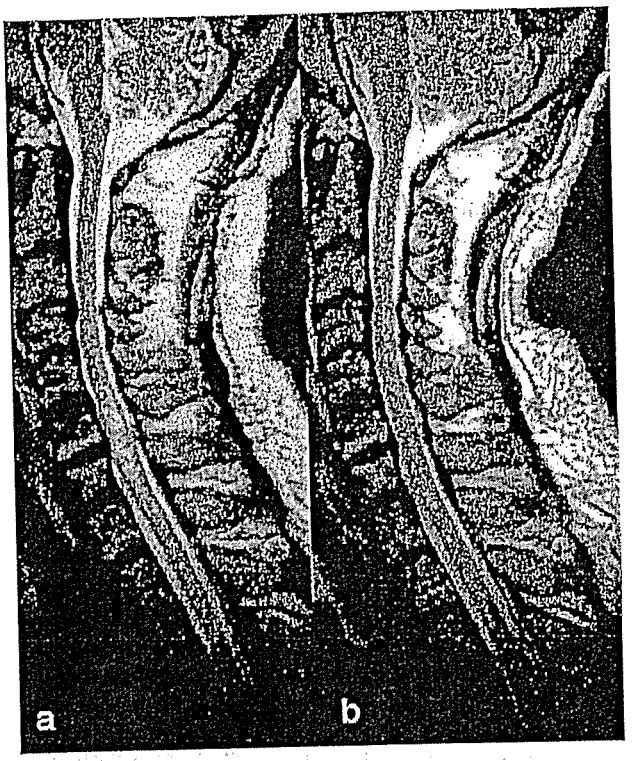

Fig. 4. MRI before (a) and after (b) treatment (patient 3). (a) Sagittal T2WI demonstrates high signal intensity and swelling of the spinal cord from C2 to T3. (b) Sagittal T2WI shows remarkable decrease in high signal intensity.

3.4. Patient 4

A 29-year-old man who lived in Kagoshima prefecture, suddenly noticed numbness in bilateral lower limbs on April 15, 2003. Three days later, he had numbness of the face followed 5 days later by muscle weakness in the lower limbs. He consulted us on April 28, 2003. On neurological examination, he had spastic paraplegia associated with numbness of the face and extremities. He also had urinary retention and bowel dysfunction. Deep tendon reflexes were exaggerated in both upper and lower limbs; however, Babinski signs were negative bilaterally. Spinal and Brain MRI were unremarkable. Biochemical and hematological tests were normal except for eosinophilia (WBC: 4700/mm^3, eosinophil 8%, baso 2%, mono 6%, lympho 38%, neutro 46%) and hyper IgEemia (991 IU/ml). Cerebrospinal fluid examination disclosed the following results: normal cell count 2/mm^3 (mononuclear cell), protein 38 mg/dl, glucose 43 mg/dl. Oligoclonal band was negative, and myelin basic protein was not elevated. EEG showed disorganization of α wave activity, and spikes after hyperventilation. On multiple dot ELISA, both serum and CSF strongly bound to *A. suum* only. Parasite eggs were not found in repeated stool examinations. At this point, she was diagnosed with myelitis caused by *A. suum* infection. She had intravenous methylprednisolone treatment (1000 mg/day for 3 days) followed by oral prednisolone treatment.

Albendazole (600 mg/day) was given for 2 courses of 4 weeks each with a 2-week interval between courses. Thereafter, there was improvement of muscle weakness in the lower limbs.

4. Discussion

The four patients presented were diagnosed with parasitic myelitis. In patients 1, 2, the cause was *T. canis* infection. *T. canis* is an unusual cause of myelopathy, most probably resulting from hematogenous infestation of the spinal cord with *T. canis* larvae. MRI findings in patient 1 revealed swelling of the spinal cord with high intensity lesions involving whole spinal cord. In patient 2, similar abnormalities were found at T1–T7 levels with focal gadolinium enhancement. These changes resolved soon after the starting antihelmintic and corticosteroid therapy. The pathogenesis of myelitis due to larva migrans syndrome is not well understood. Ascarid larvae have been reported to survive longer in the parenchymatous tissue and secrete antigens that cause allergic reactions in hosts. Albendazole was therefore considered to have directly killed the larvae, and albendazole with corticosteroid jointly suppressed the host's allergic reactions.

Patient 1 developed relapsing symptoms associated with re-appearance of spinal MRI abnormalities. Fluctuation of symptoms associated with MRI abnormalities in patients with *T. canis*-myelopathy has never been reported in the literature. During the second attack, antibody for *T. canis* did not increase in CSF, but cell count, protein and MBP were elevated. These findings suggest that allergic reactions in the CNS may have played a major role in the inflammation of the spinal cord in patient 1.

There are only a few reports on MRI findings of myelitis due to *T. canis* (7, 8, 9, 10). Swelling and high signal intensities in the spinal cord, as shown in the present cases, are common. Nevertheless, there were some unusual features in these cases. In patient 1, although the entire spinal cord was swollen, high intensity signals were located both posteriorly and laterally in the cord. Preferential location of residual damage within the posterior columns has been reported in *T. canis*-myelopathy [7]. Clearly symmetrical residual damage confined to both lateral and posterior columns in patient 1 strongly suggests a specific vulnerability of these structures to in this myelopathy. In contrast, high intensity signals were preferentially located in the right lateral columns of the thoracic cord, which are compatible with the neurological symptoms in patient 2. Taken together, both symmetrical and asymmetrical high intensity signals with or without Gd enhancement on spinal MRI could be observed in *T. canis*-myelopathy.

In patients 3, 4, the myelopathy may have been caused by *A. suum* infection. Myelopathy due to *A. suum* infection is rare [6]. In areas where it is endemic, infection with *A. suum* occurs primarily from ingesting vegetables contaminated with pig manure containing parasite eggs. Both patients lived in Kagoshima, an endemic area for *A. suum* infection. In addition, some patients were assumed to be infected by eating raw beef or chicken (liver or meat), contaminated with *A. suum* larvae. Patient 3 had a habit of eating raw beef or chicken meat.

There are two reports describing MRI findings of CNS lesions in patients with *A. suum* infection. In a patient with encephalopathy, there were many Gd-DTPA enhanced lesions in the cerebral cortex on T1WI and diffuse, symmetrical lesions in the cerebral white matter on fluid attenuated inversion recovery (FLAIR) images [12,13]. In a patient with myelopathy, a high signal intensity lesion at the Th_1 spine level on T2WI, which enhanced after gadolinium administration was reported [6]. In the present study, patient 3 showed similar findings from the cervical to the upper thoracic cord levels; however, there were no significant abnormalities in the spinal cord of patient 4. These findings suggest that negative MRI findings do not exclude a possibility of myelopathy caused by *A. suum* or other parasitic infections.

In conclusion, spinal "visceral larva migrans" syndrome caused by *T. canis* or *A. suum* should be considered as one of the differential diagnosis of myelopathy with unknown etiology. Multiple dot ELISA is useful for screening, and semiquantitative analysis by microplate ELISA using antigens *is informative for* evaluation of the treatment.

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Evaluation of Peptidomimetic HTLV-I Protease Inhibitors Containing Hydroxymethylcarbonyl as a Transition-State Isostere

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We have synthesized a series of HTLV-I protease inhibitors containing hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic. From SAR study of the hexapeptide-type inhibitor, we found that phenylglycine (Phg) and tert-leucine (Tle) fitted the S3 and S2 pockets, respectively. Moreover, we found out pentapeptide-type inhibitors, KNI-10247 and -10252 as new lead compounds that show comparative inhibitory activities with hexapeptide-type inhibitors.

Keywords: HTLV-I, aspartic protease inhibitor, hydroxymethylcarbonyl, allophenylnorstatine, transition-state mimic

Introduction

Human T-cell leukemia virus type I (HTLV-I) is a retrovirus that has been associated with adult T-cell leukemia (ATL) [1], tropical spastic paraparesis / HTLV-I associated myelopathy (TSP / HAM) [2, 3] and numerous chronic diseases. Estimations in 1997 revealed that between one and two million people were infected with HTLV-I in Japan where the virus is most prevalent in the world [4]. However, HTLV-I specific chemotherapeutic agents have not been developed. Without a doubt, the discovery of such drugs is urgently needed. HTLV-I encodes a virus-specific aspartic protease responsible for processing the *gag* and *gag-pro-pol* polyproteins, leading to the proliferation of the retrovirus [5]. Since this process is essential for retroviral replication, this protease is one of the major therapeutic targets for developing specific anti-HTLV-I agents.

In our previous study of HIV protease inhibitor, we developed a series of substrate-based peptidomimetic inhibitors containing Apns [allophenylnorstatine; (*2S,3S*)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere as an ideal transition state mimic (Fig. 1) [6]. We performed HTLV-I protease assays on our tripeptide-type HIV-1 protease inhibitors because the sequences of HTLV-I protease shares 28% homology with HIV-1 protease, with a more conserved substrate binding region (45%). Some of the inhibitors containing Apns-Dmt [Dmt; (*R*)-5,5-dimethyl-1,3-thiazolidinecarboxylic acid] at the P1-P1'

positions exhibited inhibitory activities against HTLV-I protease (Fig. 2) [7], which suggests that the Apns-Dmt scaffold is effective for the development of new drugs. Consequently, we synthesized Apns-Dmt containing HTLV-I protease inhibitors based on the amino acid sequence of p19 / p24, and observed that the substrate-mimetic octapeptide **1** possesses high inhibitory activity for HTLV-I protease (Ki = 3.9 ± 0.7 μM) (Fig. 3) [8]. Moreover, from the study of size reduction, we found out hexapeptide-type inhibitor **2** showed enough activity (Fig. 3). Herein, we report further SAR of inhibitors using **2** as a lead compound.

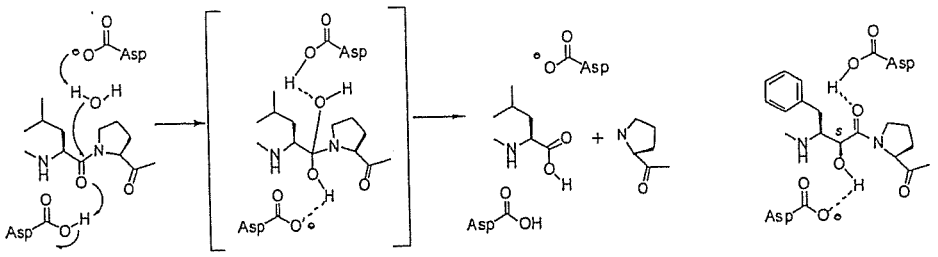


Fig. 1. Hydrolysis by aspartic protease and transition-state analogue containing allophenylnorstatine (Apns) with a hydroxymethylcarbonyl (HMC) isostere.

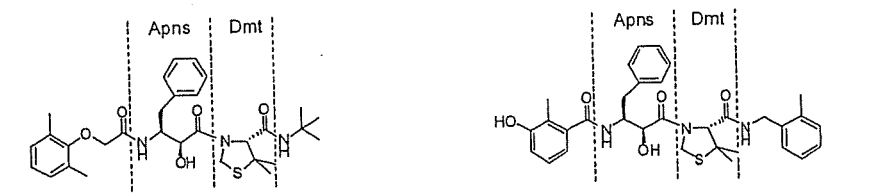


Fig. 2. Structures and inhibitory activities of KNI-727 and KNI-764.

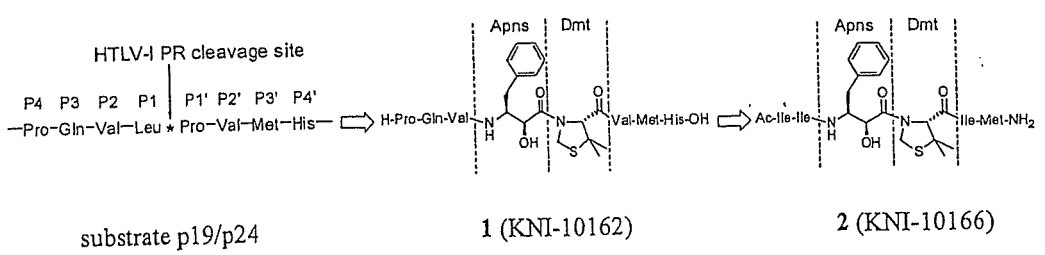


Fig. 3. Structures of substrate-based HTLV-I protease inhibitors.

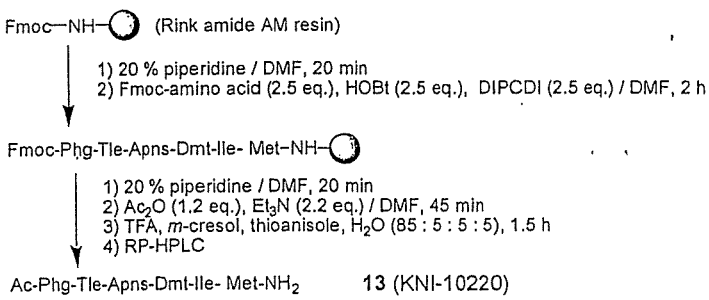
Results and Discussion

We already prepared a series of hexapeptide-type inhibitors by substitution of each position for natural amino acid residues. As the result, compound **2** was the most potent inhibitor. Now we substituted each residue of **2** for unnatural amino acid, Phg (L-α-phenylglycine) or Tle (L-α-*tert*-butylglycine) (Table 1). Under consideration for enzymatic stability, the P3 position of Phg was prefered rather than Ile, however, these modifications at P2' position decreased its activity. The P3' position of **2** was substituted for natural amino acids and we found out that Gln was suitable for this position. Based on these results, we synthesized compound **13** and **14**. These inhibitors showed 99% and 100% inhibitory activities, respectively, and with a IC_{50} value of 0.46 μM for **13**. The synthetic route to **13** is shown in Scheme 1 as a representative example.

In order to obtain smaller-size inhibitor, we synthesized a pentapeptide-type inhibitor **15** (Table 2) by eliminating the P3' moiety of **13**. Since compound **15** maintained relatively high inhibitory activity, we modified P2' position with some benzylamine derivertives as shown in Table 2. Among these compounds, compound **19**, which has a 2-methylbenzylamine moiety, exhibited comparable inhibitory activity with that of compound **15**.

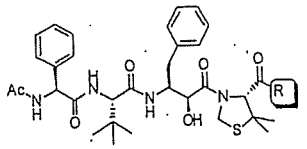
Table 1. SAR of hexapeptide-type inhibitors.

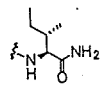
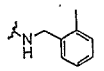
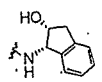
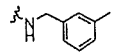
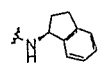
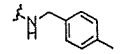
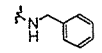
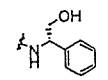
Compound	P3 P2 P1 P1' P2' P3'	Inhibition % HTLV-I (100 μM)	Inhibition % HIV-1 (50 nM)
2 (KNI-10166)	Ac-Ile-Ile-Apns-Dmt-Ile-Met-NH_2	94	98
3	Ac-Tle-Ile-Apns-Dmt-Ile-Met-NH_2	63	98
4	Ac-Phg-Ile-Apns-Dmt-Ile-Met-NH_2	96	98
5	Ac-Ile-Tle-Apns-Dmt-Ile-Met-NH_2	94	97
6	Ac-Ile-Phg-Apns-Dmt-Ile-Met-NH_2	37	88
7	Ac-Ile-Ile-Apns-Dmt-Tle-Met-NH_2	62	79
8	Ac-Ile-Ile-Apns-Dmt-Phg-Met-NH_2	87	86
9	Ac-Ile-Ile-Apns-Dmt-Ile-Gln-NH_2	97	93
10	Ac-Ile-Ile-Apns-Dmt-Ile-Phe-NH_2	90	98
11	Ac-Ile-Ile-Apns-Dmt-Ile-Ala-NH_2	91	82
12	Ac-Ile-Ile-Apns-Dmt-Ile-Phg-NH_2	83	99
	⇩		
13 (KNI-10220)	Ac-Phg-Tle-Apns-Dmt-Ile-Met-NH_2	99	99
14 (KNI-10221)	Ac-Phg-Tle-Apns-Dmt-Ile-Gln-NH_2	100	98



Scheme 1. Synthetic scheme for HTLV-I protease inhibitor 13 using Rink amide AM resin.

Table 2. Modification at P2' position of pentapeptide-type inhibitors.



Compound	R	Inhibition % HTLV-I (100 μM)	Inhibition % HIV-1 (50 nM)	Compound	R	Inhibition % HTLV-I (100 μM)	Inhibition % HIV-1 (50 nM)
15 (KNI-10247)		84	93	19 (KNI-10252)		82	99
16		63	98	20		39	99
17		75	100	21		63	99
18		46	99	22		74	79

In conclusion, we synthesized a series of HTLV-I protease inhibitors containing Apns-Dmt scaffold as transition-state mimic and obtained pentapeptide-type inhibitors KNI-10247 (**15**) and -10252 (**19**) that are small in size and possessed the high inhibitory activities. These results would greatly contribute to the development of small-sized inhibitors with potent inhibitory activity and high cell-permeability.

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