Manuscript #02-0875 REVISED

Different cytokine production in Tax expressing cells between HTLV-I associated myelopathy/tropical spastic paraparesis patients and asymptomatic HTLV-I carriers

Running title; Cytokine production in Tax expressing cells

Yoshitaka Furukawa [1], Mineki Saito [2], Wataru Matsumoto [2], Koichiro Usuku [3], Yuetsu Tanaka[4], Shuji Izumo [5], and Mitsuhiro Osame [2]

1 Division of Blood Transfusion Medicine, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

2 Third Department of Internal Medicine, Faculty of Medicine, Kagoshima University, Japan

3 Department of Medical Informatics, Faculty of Medicine, Kagoshima University, Japan

4 Department of Infectious Disease and Immunology, Okinawa-Asia Research Center of medical Science, Faculty of Medicine, University of The Ryukyu, Uehara-cho 207, Nishihara, Okinawa 903-0215, Japan

5 Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, Japan

All correspondence and reprint request should be addressed to: Yoshitaka Furukawa
Division of Blood Transfusion Medicine, Faculty of Medicine,
Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Tel. No. : 81(Japan)-99-275-5635 Fax No. : 81(Japan)-99-275-5741

E-mail : furukawy@m2.kufm.kagoshima-u.ac.jp

Footnotes to the title page

(1) This study was supported by in part from the Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan.

(2) Presented in part: 43th Conference Annual Meeting of the Japanese Society of Neurology, Sapporo, Japan, 29-31 May 2002.

(3) Informed consent was obtained from all HTLV-I carriers and HAM/TSP pateitns, this research was approved by the institutional review boards of the author's institutions, and human experimentation guidelines of the US Development of Health and Human Services and those of the author's institutions were followed in the conduct of clinical research.

(4) All correspondence and reprint request should be addressed to:

Yoshitaka Furukawa (Dr)

Division of Blood Transfusion Medicine, Faculty of Medicine,

Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Tel. No. : 81(Japan)-99-275-5635

Fax No. : 81(Japan)-99-275-5741

E-mail : furukawy@m2.kufm.kagoshima-u.ac.jp

(This E-mail address can be published with the corresponding address)

(5) The first author (Yoshitaka Furukawa) had moved from "Third Department of Internal Medicine, Faculty of Medicine, Kagoshima University, Japan" to "Division of Blood Transfusion Medicine, Faculty of Medicine, Kagoshima University" since the study, and all the addresses are written as above.

ABSTRACT

HTLV-I provirus load has been reported to be generally higher in HTLV-I associated myelopathy / tropical spastic paraparesis (HAM/TSP) patients than in HTLV-I asymptomatic carriers (ACs). However, there are ACs who have high HTLV-I provirus load as HAM/TSP patients. To examine other factors that influence the outcome of HTLV-I infection, we analyzed spontaneous Tax expression and cytokine production in peripheral blood mononuclear cells using flow-cytometry. The Tax expression in HTLV-I infected cells (% of Tax expressing cells / HTLV-I provirus load when assumed one copy in a cell) and the intensity of Tax expression did not differ between these two groups. However, the production of IFN-γ and TNF-α in Tax expressing cells was significantly lower in ACs with high HTLV-I provirus load than in HAM/TSP patients. This result suggests that the production of inflammatory cytokines in Tax expressing cells is also one of the factors that contribute the development of HAM /TSP.

INTRODUCTION

Human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL) [1, 2] and HTLV-I-associated myelopathy / tropical spastic paraparesis (HAM/TSP) [3, 4]. The main pathological feature of HAM /TSP is chronic inflammation in the spinal cord characterized by perivascular lymphocytic cuffing and parenchymal lymphocytic infiltration [5]. Although the mechanisms by which HTLV-I causes HAM/TSP are not fully elucidated, an increased level of HTLV-I provirus load and cytokines production have been postulated as contributing factors. Firstly, a higher HTLV-I provirus load in peripheral blood mononuclear cells (PBMC) have been observed in HAM / TSP patients than in HTLV-I asymptomatic carriers (ACs) [6, 7, 8] and host genetic factors also appear to influence the risk of development of HAM/TSP [9, 10]. However, there are individuals with a high HTLV-I provirus load who remain asymptomatic [8]. We therefore wondered if there are factors in addition to the HTLV-I virus load that influences the outcome of HTLV-I infection. Secondly, the production of inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) have been examined in HAM/TSP patients [11, 12, 13]. Also, an immunocytochemical study revealed overexpression of IFN-γ and TNF-α by infiltrating lymphocytes in the CNS of patients with HAM/TSP [14]. It was also reported that the adhesion of lymphocytes to cultured cerebral endothelium was increased by IFN-γ and /or TNF-α [15]. Furthermore, IFN-γ induces class II MHC antigens [16] and might elicit a T cell mediated immune response leading to CNS damage [17], and inflammatory cytokines were postulated to play a role in the pathogenesis of HAM/TSP [18]. Expression of these cytokine genes is detected by

RT-PCR method more frequently in HAM/TSP patients than in asymptomatic carriers or seronegative controls [11]. An increased production of these cytokines in HAM /TSP patients was reported in the supernatant of cultured PBMC compared to the non infected normal control [12]. Finally, there is a report that cerebral fluid IFN-γ concentrations are increased in HAM/TSP patients than in ACs [13].

Tax protein coded by the HTLV-I is essential for efficient viral expression [19] and plays an important role via activation of cellular genes such as cytokine genes [20-24] and proto-oncogenes [25]. IFN-γ and TNF-α are also candidate genes transactivated by Tax, because both genes contains nuclear factor of activated T cells (NFAT) binding sites in the promoters [26, 27], and Tax can transactivate genes through NFAT site [28]. A difference in Tax expression would influence the virus load by its transactivational activity on the viral genome. Previously, we reported that the *tax/rex* mRNA expression in HTLV-I infected cells was nearly the same in HAM/TSP patients and ACs [29]. However, another study reported that the *tax* mRNA expression in HTLV-I infected cells was higher in HAM/TSP than ACs [30]. Measurement of the concentration of mRNA or DNA by PCR may have a bias especially at smaller concentrations of RNA or DNA, as is usually the case in HTLV-I infected ACs. To test whether the efficiency of Tax expression in HTLV-I infected cells determines the outcome of HTLV-I infection, it will be more accurate to compare the level of Tax expression in HAM/TSP patients and ACs with the same HTLV-I provirus load. We therefore aimed to compare the Tax expression in HAM/TSP patients and ACs with an HTLV-I provirus load as high as that in the HAM/TSP patients. In addition, the HTLV-I provirus load may influence cytokine production, because HTLV-I infected cells possibly produce greater amounts of cytokines than uninfected cells through the

transactivational activity of Tax on cytokine genes. However, previous studies had not made it clear whether the higher production of these cytokine mRNAs in the PBMCs in HAM /TSP patients compared to the ACs is simply due to the higher HTLV-I provirus load or if the cytokine expression in individual Tax-expressing cells is higher in HAM/TSP patients [11]. Additionally, previous results regarding the cytokine production were obtained in the supernatant of PBMC after 3 to 4 days' culture and it was not determined whether this high cytokine production was produced in HTLV-I infected cells themselves [12]. We therefore compared the intracellular cytokine production produced by T cells naturally infected with HTLV-I in HAM/TSP and healthy carriers who have high HTLV-I provirus load as HAM/TSP patients using a recently described sensitive flow cytometric assay to detect intracellular Tax protein expression [31].

MATERIALS AND METHODS

Study population Fifteen HAM/TSP patients and twenty-three asymptomatic HTLV-I carriers from blood donors (ACs) were studied. We selected asymptomatic carriers from 111 individuals who were notified to be infected with HTLV-I from the Red Cross after blood donation [32]. We initially selected carriers who harbored more than 3% HTLV-I infected cells analyzed by PCR in the peripheral blood mononuclear cells (PBMC) and are also more than 40 years old to match the average age of HAM/TSP patients. There were 14 asymptomatic carriers with high HTLV-I provirus load (more than 3% of HTLV-I infected cells in PBMC) who matched these criteria. We also analyzed nine additional asymptomatic carriers who had lower HTLV-I virus load (less than 3%). No subject in the carrier group with a lower provirus load had an

undetectable provirus load. Samples from HAM/TSP patients were studied only if the patients had never been on therapies or if more than 2 years had passed since the end of immune modulating therapies such as steroid therapy. All HAM/TSP patients and ACs were of Japanese ethnic origin and resided in Kagoshima prefecture, Japan. The diagnosis of HAM/TSP was made according to World Health Organization diagnostic criteria [33]. All of them gave written informed consent before the collection of the blood samples. Six healthy individuals not infected with HTLV-I were also studied as a control.

Cells All of the HAM/TSP patients and ACs visited our clinic and blood was collected. PBMCs were isolated in 2 hours after blood collection by Ficoll-Hypaque centrifugation and stored in liquid nitrogen until use. Cells were cultured in flat bottom 6 well /plate (9.6 cm^2 per one well; Nunc) at 1×10^6 cells/ml in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 2.8mM of L-glutamine, 40U/ml of penicillin and 40μg/ml of streptomycin. Brefeldin A (Sigma) was added to the wells at a final concentration of 10μg/ml at the beginning of culture.

Cell surface staining After incubation for 12 hours at 37°C, cells were washed twice with PBS and fixed in PBS containing 2% paraformaldehyde for 20 minutes, washed with PBS and resuspended in PBS containing 7% of normal goat serum (Sigma). Cells were then incubated for 15 minutes at room temperature with phycoerythrin-cyanin 5.1 (PC5)-labeled anti-CD4 (Beckman Coulter) and energy-coupled dye (ECD)-labeled anti-CD8 (Beckman Coulter).

Detection of Intracellular Tax and cytokines Intracellular Tax and cytokine detection have been described elsewhere [31]. After cell surface staining, cells were resuspended in PBS containing 7% of normal goat serum (Sigma) and 0.2% saponin

(Sigma) (PBS-SAPO) for 10 minutes. Permeabilized cells were washed and resuspended in PBS-SAPO containing an anti-HTLV-I Tax Mab (Lt-4; IgG3) [34] or an Isotype control mAb (Southern Biotechnology) for 20 minutes at room temperature. The cells were then washed twice and resuspended for 20 minutes at room temperature in PBS-SAPO containing fluorescein isothiocyanate (FITC)-labeled goat F(ab')2 antimouse IgG3 serum (Sourthern Biotechnology) and anti-IFN-γ (Beckman Coulter) or anti-tumor necrosis factor α (anti-TNFα) (Beckman Coulter) or anti-interleukin-4 (IL-4) (Beckman Coulter) Mab conjugated with phycoerythrin (PE). Finally, the cells were washed twice and 100,000 events were collected in the flow cytometry assays and analyzed on a Coulter EPICS XL (Beckman Coulter).

Proviral load measurement The HTLV-I provirus load in PBMC was measured by the quantitative PCR reaction using the ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems) as described [8]. The amount of HTLV-I proviral DNA was calculated as follows: copy number of HTLV-1 (*tax*) per 10^4 PBMC = [copy number of *tax* / (copy number of β-actin/2)] x 10^4. Although it is known that there are some cases of multiple copies in single HTLV-I infected cell, most isolates from ATL [35] or HAM/TSP patients [36] harbor one HTLV-I proviral copy in one HTLV-I infected cell, and therefore the percentage of HTLV-I infected cells was shown as the number of HTLV-I infected cells in 100 PBMCs assuming one proviral copy in one HTLV-I infected cell.

Statistical analysis The Mann-Whitney *U* test was used for statistical analysis of HTLV-I provirus load and the variables were treated as continuous. In other cases, Student's t-test was used when the variance of the population was judged as equal by F test. When the variance or the population was judged as not equal by F test, Welch's

t-test was used.

RESULTS

Tax expression and surface marker staining in HAM/TSP patients and asymptomatic carriers with high HTLV-I virus load To ascertain the difference between HAM/TSP and asymptomatic carriers who have an HTLV-I provirus load as high as typical patients with HAM/TSP, we initially selected 14 asymptomatic HTLV-I carriers with a high HTLV-I provirus load (more than 3% of PBMC infected) and compared the Tax and cytokine production. The age of HAM/TSP patients did not differ compared against ACs with high HTLV-I provirus load and non-HTLV-I-infected control. The median HTLV-I provirus load did not differ significantly between HAM /TSP (527 *tax* copies / 10,000 PBMC) and ACs with high HTLV-I provirus load (474 *tax* copies / 10,000 PBMC) ($p = 0.66$ by Mann-Whitney's U test) (Table1). A typical result of intracellular Tax and surface marker staining is shown in Fig 1. The HTLV-I provirus load of the HAM/TSP patient in this figure (upper column) was 7.68 % (768 *tax* copies in 10,000 PBMC) and the fraction of Tax expressing cells was 3.2%. The proportion of Tax expressing cells in HTLV-I infected cells (Tax expressing cells / HTLV-I load) was calculated as 41.7%. The mean intensity of Tax staining in the Tax expressing cells was 33.7 relative units. Similarly, the HTLV-I provirus load of the ACs in this figure (lower column) was 4.67% and the fraction of Tax expressing cells was 2.9 %, the fraction of Tax expressing cells among the HTLV-I infected cells was calculated as 62%. The mean intensity of Tax staining in the Tax expressing cells was 34.8 relative units. Table 1 shows the summary. The mean percentage of Tax expressing cells did not differ between HAM/TSP patients and ACs with high HTLV-I provirus load ($p=0.42$ by Welch's t test). The proportion of Tax expressing cells in HTLV-I infected

cells (Tax positive cells / HTLV-I provirus load) was 47.2% in HAM/TSP and 41.9% in ACs and did not differ significantly (p=0.62 by Student's t-test). The mean fluorescence intensity of Tax expressing cells was 27.5 in HAM/TSP patients and 28.9 in ACs and did not differ significantly (p=0.45 by Student's t-test). The mean % of CD4+ cells, CD4+Tax+ cells, Tax+ cells in CD4+ cell, CD8+ cells, CD8+Tax+ cells, Tax+ cells in CD8+ cell and CD4/CD8 ratio did not differ between HAM/TSP patients and ACs with high HTLV-I provirus load. The age, the mean % of CD4+ cells, CD8+ cells, and CD4/CD8 ratio did not also differ between HAM/TSP patients and non-HTLV-I-infected individuals (Table 1).

Tax expression and cytokine production in HAM/TSP patients and asymptomatic carriers with high HTLV-I provirus load A typical result of intracellular Tax staining and cytokine staining (IL-4, TNF-α, IFN-γ) is shown in Fig. 2. Tax expression and cytokine expression did not differ when examined soon after the separation of PBMC or using cells that were once frozen (data not shown). IFN-γ was partly detected in non-Tax expressing cells in both HAM/TSP patients and ACs with high HTLV-I provirus load. However, interestingly, there was a higher production of IFN-γ in Tax producing cells in HAM/TSP patients than in ACs with high HTLV-I provirus load. TNF-α was detected at a low level, mainly in non-Tax-expressing cells in both HAM/TSP and ACs with high HTLV-I provirus load. IL-4 was nearly exclusively detected in non-Tax expressing cells in both HAM/TSP and ACs with high HTLV-I provirus load. The cytokine production in Tax-expressing and in non-Tax-expressing cells is summarized in Table2.

Total IFN-γ producing cells were 0.20% of PBMCs in HAM/TSP patients and 0.08% in ACs with high HTLV-I provirus load; this difference was statistically significant

(p=0.015 by Student's t-test). This difference of IFN-γ production was not significant in non-Tax-expressing cells (0.08% in HAM/TSP patients and 0.05% in ACs; p=0.187 by Welch's t-test) but was significant in Tax expressing cells (0.119% in HAM/TSP patients and 0.039% in ACs; p=0.023 by Welch's t-test). Also, the proportion of IFN-γ producing cells among the Tax producing cells was significantly higher in HAM/TSP patients (4.48 ± 2.52 %) than in ACs with high HTLV-I provirus load (1.56 ± 1.07 %) (p=0.0006 by Welch's t-test). The proportion of IFN-γ producing cells in non-Tax producing cells did not differ significantly between HAM/TSP patients and ACs with high HTLV-I provirus load. The total frequency of IFN-γ producing cells in non-HTLV-I-infected individuals was 0.04% of PBMCs and was significantly lower than in HAM/TSP patients; however, the proportion of IFN-γ producing cells in non-Tax producing cells did not differ significantly between HAM/TSP patients and non-HTLV-I-infected individuals. The fluorescence intensity of IFN-γ+ cells in Tax- cells did not differ between HAM/TSP patients, ACs with high HTLV-I provirus load and non-HTLV-I infected individuals. Also, the fluorescence intensity of IFN-γ+ cells in Tax+ cells did not differ between HAM/TSP patients and ACs with high HTLV-I provirus load.

TNF-α producing cells did not differ significantly in frequency between HAM/TSP patients and ACs with high HTLV-I provirus load (p=0.11 by Student's t-test). TNF-α+Tax+ cells were significantly less frequent in ACs with high HTLV-I provirus load (0.009 ± 0.007 %) than in HAM/TSP patients (0.025 ± 0.014 %) (p= 0.0016 by Student's t-test), and the proportion of TNF-α producing cells among the Tax expressing cells was significantly lower in ACs with high HTLV-I provirus load (0.462

± 0.367 %) than in HAM/TSP patients (1.511 ± 1.533 %, p=0.02). The proportion of TNF-α producing cells in non-Tax producing cells did not differ between HAM/TSP patients, ACs with high HTLV-I provirus load. Total TNF-α producing cells and the proportion of TNF-α producing cells in non-Tax producing cells in non-HTLV-I-infected individuals did not differ significantly against HAM/TSP patients. The fluorescence intensity of TNF-α+ cells in Tax- cells did not differ between HAM/TSP patients, ACs with high HTLV-I provirus load and non-HTLV-I infected individuals. However, the fluorescence intensity of TNF-α+ cells in Tax+ cells was significantly higher in HAM/TSP patients (8.90 ± 5.67 relative unit) than in ACs with high HTLV-I provirus load (5.15 ± 2.75 relative unit; p = 0.03) (Table 2).

The fraction of total IL-4 producing cells in the PBMCs did not differ significantly between HAM/TSP patients and ACs with high HTLV-I provirus load. (p=0.68 by Student's t-test). However, the fraction of total IL-4 producing cells in the PBMCs in non-HTLV-I infected individuals was significantly lower when compared against HAM/TSP patients (p=0.02) or against ACs with high load (p=0.012). The fraction of IL-4+Tax- cells and IL-4+Tax+ cells among PBMC and the fraction of IL-4 producing cells in Tax+ cells did not differ significantly between HAM/TSP and ACs with high HTLV-I provirus load. However, the fraction of IL-4+Tax- cells among PBMC was significantly lower in non-HTLV-I infected individuals when compared against HAM/TSP patients (p=0.02) or against ACs with high load (p=0.013) (Table2). The fluorescence intensity of IL-4+ cells in Tax- cells was significantly higher in ACs with high HTLV-I load than in HAM/TSP patients (p = 0.002). And the fluorescence intensity of IL-4+ cells in Tax- cells was significantly lower in non-HTLV-I infected individuals than in HAM/TSP patients (p = 0.0001) and was also significantly lower

against ACs with high HTLV-I provirus load ($p = 0.0001$).

Tax and cytokine production in ACs with low HTLV-I provirus load To examine if low inflammatory cytokine expression in Tax producing cells was specific to ACs with high HTLV-I provirus load, we also checked the cytokine production in Tax expressing cells in nine ACs with lower HTLV-I provirus load. The median provirus load in these 9 carriers was 1.84% and was significantly lower than the HTLV-I provirus load of HAM/TSP patients in this study ($p=0.0001$ by Mann-Whitney' s U-test). The mean proportion of Tax expressing cells in the PBMC was 0.85% in ACs with lower HTLV-I load and was significantly lower than in patients with HAM/TSP ($p=0.0089$ by Welch's t test) which reflects the lower HTLV-I provirus load. However, the mean proportion of Tax expressing cells in HTLV-I infected cells (Tax expressing cells / HTLV-I provirus load) was 49.5% in ACs with lower HTLV-I provirus load and there was no significant difference from the value in HAM/TSP patients (47.2%) ($p=0.86$ by Student's t-test). The mean fluorescence intensity of FITC of Tax expressing cells did not differ significantly between HAM/TSP patients and ACs with lower HTLV-I provirus load ($p=0.11$ by Student's t-test). The mean proportion of CD4+ cells, CD8+ cells, and the CD4/CD8 ratio did not differ between HAM/TSP patients and ACs with low HTLV-I provirus load (Table 1). The mean proportion of Tax+CD4+ cells, Tax+ cells in CD4+ cells, and the proportion of Tax+CD8+ cells, Tax+ cells in CD8+ cells, were slightly lower in ACs with low provirus load than in patients with HAM/TSP. However, this was due to the lower number of Tax expressing cells because of the lower HTLV-I provirus load in these ACs (Table 1). Regarding the cytokine production, the proportion of total IFN-γ, TNF-α and IL-4 producing cells in PBMC or in non-Tax-expressing cells did not differ significantly between ACs with lower HTLV-I

provirus load and HAM/TSP patients. Although IFN-γ+Tax+ cells constituted 0.119% of PBMCs in HAM/TSP patients and 0.048% in ACs, and was slightly higher in HAM/TSP patients (p=0.04 by Welch's t-test), the proportion of IFN-γ producing cells in Tax producing cells was 4.48% in HAM/TSP patients and 4.14% in ACs and did not differ significantly (p=0.77 by Student's t-test). The fluorescence intensity of IFN-γ+ cells in Tax- cells did not differ between HAM/TSP patients, ACs with low HTLV-I provirus load and non-HTLV-I infected individuals. Also, the fluorescence intensity of IFN-γ+ cells in Tax+ cells did not differ between HAM/TSP patients and ACs with low HTLV-I provirus load. Although TNF-α+Tax+ cells constituted 0.025% of PBMCs in HAM/TSP patients and 0.009% in ACs, and was significantly higher in HAM/TSP (p=0.0009 by Welch's t-test), the proportion of TNF-α producing cells in Tax expressing cells was 1.511% in HAM/TSP and 1.296% in ACs with lower HTLV-I provirus load and did not differ significantly (p=0.69 by Student's t-test). The fluorescence intensity of TNF-α+ cells in Tax- cells did not differ between HAM/TSP patients, ACs with low HTLV-I provirus load and non-HTLV-I infected individuals. However, the fluorescence intensity of TNF-α+ cells in Tax+ cells was significantly higher in HAM/TSP patients than in ACs with low HTLV-I provirus load. Similarly, although IL-4+Tax+ cells constituted 0.008% of PBMCs in HAM/TSP patients and 0.001 % in ACs, and was significantly higher in HAM/TSP (p=0.028 by Welch's t-test), the proportion of IL-4 producing cells in Tax expressing cells was 0.42 % in HAM/TSP and 0.16 % in ACs with lower HTLV-I provirus load and did not differ significantly (p=0.21 by Student's t-test). The fraction of total IL-4 producing cells in the PBMCs in non-HTLV-I-infected individuals was significantly lower when compared against

ACs with low load (p=0.009). Also, the fraction of IL-4+Tax- cells among PBMC was significantly lower in non-HTLV-I infected individuals when compared against ACs with low load (p=0.009). The fluorescence intensity of IL-4+ cells in Tax- cells did not differ significantly between ACs with low HTLV-I load and HAM/TSP patients (p = 0.32). (Table2).

Correlation between IFN-γ production and IL-4 production Correlation between IL-4 production and either total IFN-γ production, or IFN-γ production in Tax expressing cells were tested. There was a tendency to a negative correlation between IL-4 production and total IFN-γ production (regression coefficient (r) = -0.15), but this was not statistically significant (p = 0.37). There was also a tendency to a negative correlation between IL-4 production and IFN-γ production in Tax expressing cells (r = -0.23), but this was not statistically significant (p = 0.17). (Fig 3)

DISCUSSION

Although HTLV-I provirus load is generally higher in HAM/TSP compared to asymptomatic carriers, there are ACs with an HTLV-I virus load as high as typical cases of HAM/TSP [8]. We aimed to identify other factors than the high HTLV-I provirus load for the development of HAM/TSP by examining the difference between HAM/TSP patients and ACs with high HTLV-I provirus load. Tax is a transactivator protein that enhances HTLV-I expression itself, and also a transactivator protein that enhances host genes including cytokine genes. We firstly examined the efficiency of Tax production in short-term in vitro culture, because Tax may increase the HTLV-I provirus load by its transactivational activity on the viral genome itself, or Tax may also elicit a CTL

response. However, the proportion and the intensity of Tax expressing cells were similar between HAM/TSP patients and ACs with high virus load. There have been conflicting reports where one group found that the *tax/rex* mRNA expression in HTLV-I infected cells was nearly the same in HAM/TSP patients and ACs [29], whereas others reported that the *tax* mRNA expression in HTLV-I infected cells was higher in HAM/TSP than ACs [30]. Because quantitative PCR methods may have a bias especially when very small amounts of DNA or RNA are involved, it will be more accurate to detect protein expression in subjects with the same HTLV-I virus load. Although our result was obtained after short term in vitro culture, and may not reflect the ex-vivo condition, we can say that the capacity of HTLV-I infected cells to produce Tax after 12 hours culture did not differ between HAM/TSP and ACs with high HTLV-I virus load. We also examined if there was a difference in the frequency of HTLV-I infected CD8+ cells between HAM/TSP and ACs, because HTLV-I has been shown to infect not only CD4+ cells but also CD8+ cells ex vivo PBMCs [37, 38]. It has also been shown that CD8+ T cells specific to HTLV-I Tax are infected with HTLV-I [37]. However, Tax expression in CD4+ cells and in CD8+ cells did not differ significantly between HAM/TSP patients and ACs (Table 1).

We next examined the difference of cytokine expression in association with Tax expression. Inflammatory cytokines such as IFN-γ and TNF-α have been postulated to play a role in the pathogenesis of HAM/TSP, and there is a theoretical model that predicted that HAM/TSP patients' T cells would produce more inflammatory cytokines than those from ACs at a given provirus load [39]. This prediction has been verified in the present study. Interestingly, a higher production of IFN-γ was detected in HAM/TSP patients than in ACs with a similar high HTLV-I provirus load and this

difference was mainly observed in Tax expressing cells. (Fig 2 and Table 2). One may argue that the proportion of IFN-γ producing cells is lower compared to the previously published data [31]. This may be due partly to the small number of samples tested in the previous study, and differences in the HTLV-I genotype between the U.K. and Japan [40]. Secretion of IFN-γ by T cells is usually the result of antigen stimulation [41]. Indeed, IFN-γ production in CD8 cells is also observed in HTLV-I infected individuals as a result of HTLV-I associated antigen stimulation [42]. When blood cells were not stimulated, there is little or faint intra-cellular cytokine expression [43]. In our present study, HTLV-I-associated antigen stimulation appeared not to contribute to cytokine production: Brefeldin A was added at the beginning of culture, and IFN-γ production in Tax expressing cells was not inhibited by anti-MHC class II mAb in experiments using the same protocol [31]. These findings suggest that IFN-γ production is increased in HAM/TSP patients not only in CD8 positive cells that respond to HTLV-I associated antigen stimulation [42], but also in Tax expressing cells without HTLV-I associated antigen stimulation. CD4+ cells as well as CD8+ positive cells are observed in the spinal cord in an active early stage of HAM/TSP, whereas in late stable stage, CD8+ cells are mainly observed [44]. It is possible that CD4+ cells which are main reservoir of HTLV-I are playing an important role in the pathogenesis of HAM/TSP.

TNF-α producing cells in Tax expressing cells after 12 hours' culture were more frequent in HAM/TSP patients than in ACs with a high virus load. Although the main production of TNF-α was observed in non-Tax expressing cells, the fluorescence intensity of TNF-α positive cells in Tax expressing cells was significantly higher in HAM/TSP patients than in ACs regardless of their provirus load. This suggests that

the difference of TNF-α production in Tax expressing cell is also one of the factors that influences the development of HAM/TSP.

To examine if low inflammatory cytokine expression in Tax producing cells is specific to ACs with high HTLV-I provirus load, we also measured the cytokine production in Tax expressing cells in ACs with a lower HTLV-I provirus load. Interestingly, the proportion of IFN-γ and TNF-α producing cells among Tax expressing cells in ACs with low HTLV-I provirus load was nearly the same that was observed in HAM/TSP (Table 2). This result suggests that although the cytokine expression in Tax expressing cells in ACs with low provirus load is as high as HAM/TSP, the total amount of cytokine production is less in ACs than HAM/TSP due to the low HTLV-I provirus load: the lower cytokine production may help to keep these carriers asymptomatic. In contrast, ACs with high HTLV-I provirus load remains asymptomatic, due to the low production of inflammatory cytokines in Tax expressing cells. In conclusion, although a higher viral load is a very important factor for the development of HTLV-I associated inflammatory diseases, higher production of IFN-γ and TNF-α in Tax expressing cells are additional factors that influence the outcome of HTLV-I infection.

Regarding the mechanism of difference in the production of IFN-γ, we tested if IL-4 production was suppressing the IFN-γ production. IFN-γ is a Th1 type cytokine and IL-4 is secreted by Th2 cells. It has been shown that as a regulatory mechanism of the immune response, cytokines secreted by Th2 cells may downregulate Th1 cells and vice versa [45]. In our study, there was a tendency to a negative correlation between IL-4 production and either total IFN-γ production, or IFN-γ production in Tax

expressing cells, but this was not statistically significant in either case (Fig 3). However, interestingly, fluorescence intensity of IL-4 was significantly higher in ACs with high HTLV-I provirus load than in patients with HAM/TSP. Also, the fluorescence intensity of IL-4 in ACs with low HTLV-I provirus load did not differ against HAM/TSP. These findings suggest that high production of IL-4 in ACs with high HTLV-I provirus load is suppressing IFN-γ production in Tax expressing cells and keeping them asymptomatic. There is also a report that exogenous addition of interleukin-10 (IL-10), which is another Th2 cytokine, decreased IFN-γ production in vitro in HTLV-I infected blood donors [46]. These findings suggest that the amount of proinflammatory cytokines produced in Tax expressing cells is not only influenced by the viral load, which influences the total amount of cytokines in Tax expressing cells, but is also regulated by Th2 cytokines. We are currently examining the polymorphisms in IFN-γ gene [47] that can be transactivated by Tax [48]. We are also examining whether there is a difference in polymorphism in IL-4, IL-10, interleukin-18 (IL-18), interleukin-12 (IL-12) [49], IFN-γ regulatory factors (*IRF-1*, *IRF-2*) [50], *IFN-α*, *IFN-β* [51], *IFN-α receptor*, *IFN-β receptor* genes that can influence IFN-γ production.

ACKNOWLEDGMENTS

This study was supported by in part from the Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan.

We thank Ms. T. Muramoto and Ms. Y. Nishino (Third department of Internal Medicine, Kagoshima University, Japan) for their excellent technical assistance. We also thank

Professor Charles R. M. Bangham (Immunology Department, Imperial College Faculty of Medicine, United Kingdom) for critical reading of the manuscript.

REFERENCES

1. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci U S A **1980**; 77:7415-7419.
2. Yoshida M, Miyoshi I, Hinuma Y: Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A **1982**;79:2031-2035.
3. Gessain A, Barin F, Vernant JC, et al: Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet **1985**;2:407-410.
4. Osame M, Usuku K, Izumo S, et al: HTLV-I associated myelopathy, a new clinical entity. Lancet **1986**;1:1031-1032.
5. Akizuki S, Nakazato O, Higuchi Y, et al: Necropsy findings in HTLV-I associated myelopathy. Lancet **1987**;1:156-157.
6. Yoshida M, Osame M, Kawai H, et al: Increased replication of HTLV-I in HTLV-I-associated myelopathy. Ann Neurol **1989**;26:331-335.
7. Kira J, Koyanagi Y, Yamada T, et al: Increased HTLV-I proviral DNA in HTLV-I-associated myelopathy: a quantitative polymerase chain reaction study. Ann Neurol **1991**;29:194-201.
8. Nagai M, Usuku K, Matsumoto W, et al: Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. J Neurovirol **1998**;4:586-593.
9. Jeffery KJ, Siddiqui AA, Bunce M, et al: The influence of HLA class I alleles and

heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J Immunol **2000**;165:7278-7284.

10. Vine AM, Witkover AD, Lloyd AL, et al.: Polygenic control of HTLV-I infection outcome: HTLV-I associated myelopathy (HAM) risk quantified by host genetic and viral factors. J Infect Dis. **2002**;186:932-939.
11. Watanabe H, Nakamura T, Nagasato K, et al: Exaggerated messenger RNA expression of inflammatory cytokines in human T-cell lymphotropic virus type I-associated myelopathy. Arch Neurol **1995**;52:276-280.
12. Nishiura Y, Nakamura T, Ichinose K, et al: Increased production of inflammatory cytokines in cultured CD4+ cells from patients with HTLV-I-associated myelopathy. Tohoku J Exp Med **1996**;179:227-233.
13. Kuroda Y, Matsui M: Cerebrospinal fluid interferon-gamma is increased in HTLV-I-associated myelopathy. J Neuroimmunol **1993**;42:223-226.
14. Umehara F, Izumo S, Ronquillo AT, Matsumuro K, Sato E, Osame M: Cytokine expression in the spinal cord lesions in HTLV-I-associated myelopathy. J Neuropathol Exp Neurol **1994**;53:72-77.
15. Hughes CC, Male DK, Lantos PL: Adhesion of lymphocytes to cerebral microvascular cells: effects of interferon-gamma, tumour necrosis factor and interleukin-1. Immunology **1988**;64:677-681.
16. Sztein MB, Steeg PS, Johnson HM, Oppenheim JJ: Regulation of human peripheral blood monocyte DR antigen expression in vitro by lymphokines and recombinant interferons. J Clin Invest **1984**;73:556-565.
17. Moore GR, Traugott U, Scheinberg LC, Raine CS: Tropical spastic paraparesis: a model of virus-induced, cytotoxic T-cell-mediated demyelination?. Ann Neurol

1989;26:523-530.

18. Biddison WE, Kubota R, Kawanishi T, et al: Human T cell leukemia virus type I (HTLV-I)-specific CD8+ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase. J Immunol **1997**;159:2018-2025.
19. Sodroski JG, Rosen CA, Haseltine WA. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **1984**; 225:381-385.
20. Siekevitz M, Feinberg MB, Holbrook N, Wong Staal F, Greene WC. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. Proc. Natl. Acad. Sci. U. S. A. **1987**; 84:5389-5393.
21. Kim SJ, Kehrl JH, Burton J, et al. Transactivation of the transforming growth factor beta 1 (TGF-beta 1) gene by human T lymphotropic virus type 1 tax: a potential mechanism for the increased production of TGF-beta 1 in adult T cell leukemia. J. Exp. Med. **1990**; 172:121-129.
22. Lindholm PF, Reid RL, Brady JN. Extracellular Tax1 protein stimulates tumor necrosis factor-beta and immunoglobulin kappa light chain expression in lymphoid cells. J. Virol. **1992**; 66:1294-1302.
23. Mori N, Prager D. Transactivation of the interleukin-1 alpha promoter by human T-cell leukemia virus. Leuk. Lymphoma. **1997**; 26:421-433.
24. Mori N, Mukaida N, Ballard DW, Matsushima K, Yamamoto N. Human T-cell leukemia virus type I Tax transactivates human interleukin 8 gene through acting concurrently on AP-1 and nuclear factor-kappaB-like sites. Cancer Res. **1998**;

58:3993-4000.

25. Fujii M, Sassone Corsi P, Verma IM. c-fos promoter trans-activation by the tax1 protein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. U. S. A. **1988**; 85:8526-8530.

26. Oum JH, Han J, Myung H, Hleb M, Sharma S, Park J.: Molecular mechanism of NFAT family proteins for differential regulation of the IL-2 and TNF-alpha promoters. Mol Cells **2002**;13:77-84.

27. Kiani A, Garcia-Cozar FJ, Habermann I, et al: Regulation of interferon-gamma gene expression by nuclear factor of activated T cells. Blood **2001**;98:1480-1488.

28. Rivera I, Harhaj EW, Sun SC.: Involvement of NF-AT in type I human T-cell leukemia virus Tax-mediated Fas ligand promoter transactivation. J Biol Chem. **1998**;273:22382-22388.

29. Furukawa Y, Osame M, Kubota R, Tara M, Yoshida M: Human T-cell leukemia virus type-1 (HTLV-1) Tax is expressed at the same level in infected cells of HTLV-1-associated myelopathy or tropical spastic paraparesis patients as in asymptomatic carriers but at a lower level in adult T-cell leukemia cells. Blood **1995**;85:1865-1870.

30. Yamano Y, Nagai M, Brennan M, et al: Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood **2002**;99:88-94.

31. Hanon E, Goon P, Taylor GP, et al: High production of interferon gamma but not interleukin-2 by human T-lymphotropic virus type I-infected peripheral blood mononuclear cells. Blood **2001**;98:721-726.

32. Furukawa Y, Kubota R, Eiraku N et al.: Human T-cell lymphotropic virus type I (HTLV-I) related clinical and laboratory findings in HTLV-I infected blood donors. J AIDS. In press.

33. Osame M. HTLV. In: Blattner (ed.). Human Retrovirology. New York: Raven Press, **1990**:191-197.

34. Lee B, Tanaka Y, Tozawa H: Monoclonal antibody defining tax protein of human T-cell leukemia virus type-I. Tohoku J Exp Med **1989**;157:1-11.

35. Shimamoto Y, Miyahara M, Yamada H, Shibata K, Matsuzaki M, Ono K.: Adult T-cell leukaemia/lymphoma with multiple integrations of human T-cell lymphotropic virus type I proviral DNA: differing clinical features are linked to varied proviral integration. Br J Haematol. **1996**; 92:632-638.

36. Furukawa Y, Fujisawa J, Osame M, et al.: Frequent clonal proliferation of human T-cell leukemia virus type 1 (HTLV-1)-infected T cells in HTLV-1-associated myelopathy (HAM-TSP). Blood **1992**;80:1012-1016.

37. Hanon E, Stinchcombe JC, Saito M, et al: Fratricide among CD8(+) T lymphocytes naturally infected with human T cell lymphotropic virus type I. Immunity **2000**;13:657-664.

38. Nagai M, Brennan MB, Sakai JA, Mora CA, Jacobson S: CD8(+) T cells are an in vivo reservoir for human T-cell lymphotropic virus type I. Blood **2001**;98:1858-1861.

39. Asquith B, Bangham CRM: The role of Cytotoxic T lymphocytes in human T-cell lymphotropic virus type 1 infection. J Theor. Biol. **2000**;207:65-79.

40. Furukawa Y, Yamashita M, Usuku K, Izumo S, Nakagawa M, Osame M: Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax

gene and their association with different risks for HTLV-I-associated myelpathy/tropical spastic paraparesis. J Infect Dis. **2000**;182:1343-1349.

41. Yang J, Zhu H, Murphy TL, Ouyang W, Murphy KM: IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR-induced, IFN-gamma production. Nat Immunol **2001**;2:157-164.

42. Kubota R, Kawanishi T, Matsubara H, Manns A, Jacobson S: Demonstration of human T lymphotropic virus type I (HTLV-I) tax-specific CD8+ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection. J Immunol **1998**;161:482-488.

43. Westby M, Marriott JB, Guckian M, Cookson S, Hay P, Dalgleish AG: Abnormal intracellular IL-2 and interferon-gamma (IFN-gamma) production as HIV-1-assocated markers of immune dysfunction. Clin Exp Immunol **1998**;111:257-263.

44. Umehara F, Izumo S, Nakagawa M, et al.: Immunocytochemical analysis of the cellular infiltrate in the spinal cord lesions in HTLV-I-associated myelopathy. J Neuropathol Exp Neurol **1993**;52:424-430.

45. Fiorentino DF, Bond MW, Mosmann TR: Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med **1989**;170:2081-2095.

46. Carvalho EM, Bacellar O, Porto AF, Braga S, Galvao-Castro B, Neva F: Cytokine profile and immunomodulation in asymptomatic human T-lymphotropic virus type 1-infected blood donors. J Acquir Immune Defic Syndr **2001**;27:1-6.

47. Bream JH, Carrington M, O'Toole S, et al: Polymorphisms of the human IFNG

gene noncoding regions. Immunogenetics **2000**;51:50-58.

48. Brown DA, Nelson FB, Reinherz EL, Diamond DJ: The human interferon-gamma gene contains an inducible promoter that can be transactivated by tax I and II. Eur J Immunol **1991**;21:1879-1885.

49. Yoshimoto T, Takeda K, Tanaka T, et al: IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. J Immunol **1998**;161:3400-3407.

50. Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T: Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. Mol Cell Biol **1994**;14:1500-1509.

51. Sareneva T, Matikainen S, Kurimoto M, Julkunen I: Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. J Immunol **1998**;160:6032-6038.

LEGENDS TO FIGURES

Figure 1. **Expression of surface markers (CD4 and CD8) in HTLV-I Tax+ PBMCs.** PBMCs were isolated and harvested after 12 hours of cultivation in vitro. Representative study of a HAM/TSP patient (upper panel) and an asymptomatic carrier with high HTLV-I provirus load (lower panel) are shown.

Figure 2. **Detection of IFN-γ, TNF-α and IL-4 expression in Tax expressing PBMCs.** Dot plots showing both cytokine and HTLV-I Tax expression in PBMCs after 12 hours cultivation in vitro. Representative study of a HAM/TSP patient (upper column) and of an asymptomatic carrier with high HTLV-I provirus load (lower column).

Figure 3. **Correlation between IL-4 and IFN-γ production.** Correlation between the percentage of total IL-4 producing cells and the percentage of total IFN-γ producing cells (left column) or the proportion of IFN-γ producing cells in Tax positive cells (right column) are shown. Closed circles represent HAM / TSP patients, closed triangles represent ACs with high HTLV-I load and open triangles represent ACs with low HTLV-I provirus load. Lines in each figure represent regression line. "r" represent regression coefficient.