Materials and Methods

Subjects

Twenty-eight HAM/TSP patients (6 men and 22 women; mean age, 58.5 years; age range, 29-78 years) were included in the study. The diagnosis of HAM/TSP was based on previously described criteria (Osame 1990). Control subjects comprised of 9 HTLV-I-infected individuals without HAM/TSP (2 men and 7 women; mean age, 60.4 years; age range, 26-69 years) and 22 HTLV-I-uninfected individuals (8 men and 14 women; mean age, 54.5 years; age range, 26-75 years). The 9 HTLV-I-infected individuals without HAM/TSP included patients with cerebrovascular accident (n = 2), essential tremor (n = 2), tension-type headache (n = 1), spinocerebellar degeneration (n = 1), amyotrophic lateral sclerosis (n = 1), cervical spondylosis (n = 1), diabetic polyneuropathy (n = 1), whereas the 22 HTLV-I-uninfected individuals included patients with cerebrovascular accident (n = 5), tension-type headache (n = 3), Parkinson disease (n = 2), spinocerebellar degeneration (n = 2), multiple sclerosis (remission phase, n = 2), migraine (n = 2), cervical spondylosis (n = 2), essential tremor (n = 1), epilepsy (n = 1), Bell's palsy (n = 1), and brain tumor (n = 1). None of the patients had been treated with immunomodulatory drugs, including corticosteroids and interferon-α during the last one year. Informed written consent was obtained from all patients in the study. This research was approved by the review boards of Graduate School of Biomedical Sciences, Nagasaki University.

RNA isolation and quantitative RT-PCR analysis

PBMC were separated by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Total cellular RNA was extracted from PBMC by Sepasol-RNA I super (NACALAI TESQUE, INC., Kyoto). cDNA was synthesized from 2.0 μg RNA treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) using Oligo (dT) 12-18 primer (Promega). M-MLV reverse transcriptase (1 μl) (Promega) and 25 units of Ribonuclease inhibitor (Promega) were used in a total volume of 25 μl. For quantitative analysis of IL-12Rβ2, T-bet, GATA-3, SOCS1, SOCS3, HTLV-I Tax and β2-microglobulin (β2m) in

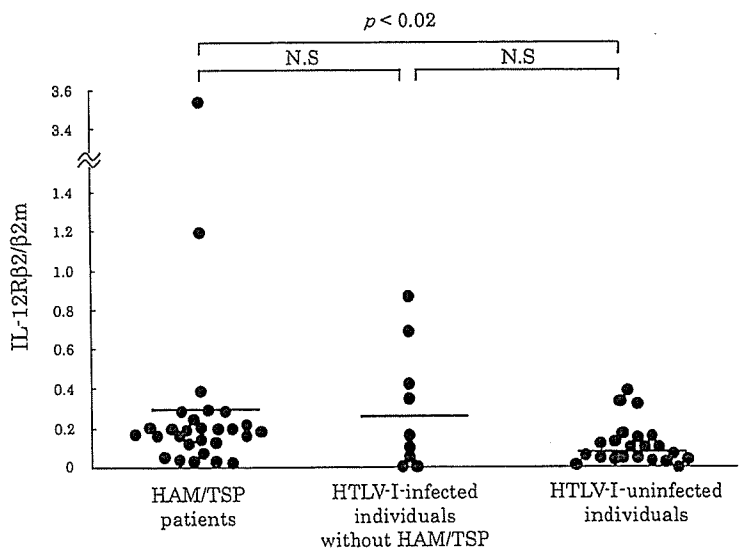

Fig. 2. Comparison of mRNA expression of IL-12Rβ2 in PBMC. mRNA expression of IL-12Rβ2 in PBMC was significantly higher in HAM/TSP patients than in HTLV-I-uninfected individuals. Although mRNA expression of IL-12Rβ2 seemed to be higher in HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals, this difference was not statistically significant. Mann-Whitney's U-test was used for statistical analysis.

PBMC, real-time quantitative RT-PCR was performed in a Light-Cycler FastStart DNA Master (Roche Diagnostics, Mannheim, Germany) based on general fluorescence detection with SYBR Green. The primer sequences and the sizes of the PCR products are shown in Table 1; and PCR conditions are shown in Table 2. For quantitative RT-PCR, bulk cDNA derived from cell lines were used as standards. HCT-1, derived from CSF cells from a patient with HAM/TSP, was used for IL-12Rβ2, SOCS1 and SOCS3; MT-2 was used for β2m, T-bet and HTLV-I Tax; and Jurkat was used for GATA-3. Each mRNA expression was evaluated by normalization to β2m.

Statistical analysis

Results were expressed as mean ± standard error of the mean. Differences between groups were tested for statistical significance by use of the nonparametric Mann-Whitney's U-test. Correlation analyses were performed by use of nonparametric Spearman's rank correlation test. The level of significance was set at $p = 0.05$.

Results

mRNA expression of each cytokine signaling molecules in PBMC was measured by real-time quantitative RT-PCR. Each mRNA expression was evaluated by normalization to β2m. The value of each mRNA expression was shown in Table 3. As shown in Fig. 1A and 1B, mRNA expression of both T-bet and GATA-3 was significantly increased in PBMC of HAM/TSP patients compared to HTLV-I-uninfected individuals. In addition, mRNA expression of IL-12Rβ2 was significantly higher in HAM/TSP patients than in HTLV-I-uninfected individuals (Fig. 2). Although mRNA expression of T-bet, GATA-3 and IL-12Rβ2 seemed to be higher in HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals, these differences were not statistically significant. In addition, no significant differences in mRNA expression were found among HTLV-I-infected individuals with or without HAM/TSP.

mRNA expression of SOCS1 in PBMC was significantly increased in HAM/TSP patients and

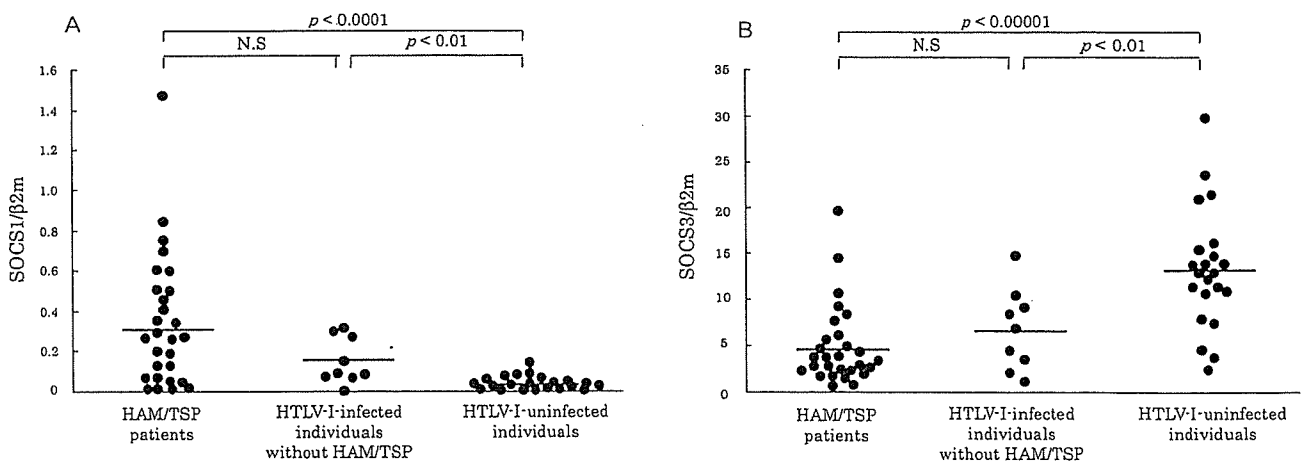

Fig. 3. Comparison of mRNA expression of SOCS1 and SOCS3 in PBMC. mRNA expression of SOCS1 in PBMC (A) was significantly higher in HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals. Although mRNA expression of SOCS1 seemed to be higher in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP, this difference was not statistically significant. Conversely, mRNA expression of SOCS3 in PBMC (B) was significantly decreased in both groups of HTLV-I-infected individuals compared to HTLV-I-uninfected individuals. There was no significant difference in mRNA expression of SOCS3 between HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP. Mann-Whitney's U-test was used for statistical analysis.

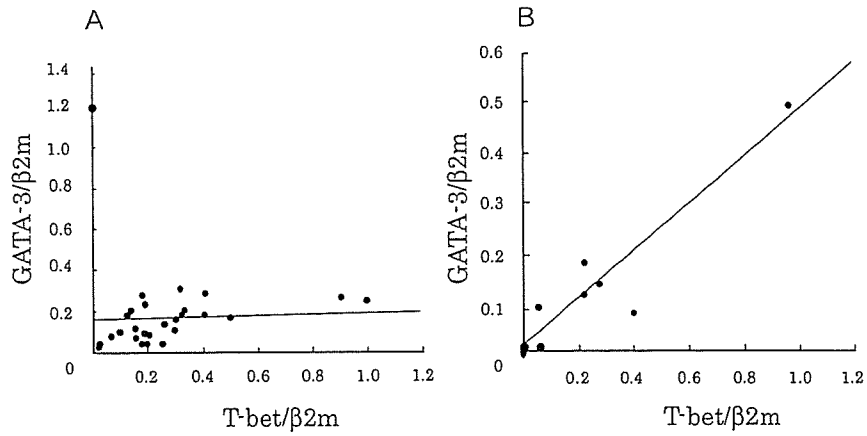

Fig. 4. Correlation between T-bet and GATA-3 mRNA expression among HTLV-I-infected individuals. mRNA expression of both transcription factors showed moderately positive correlation in HAM/TSP patients and strongly positive correlation in HTLV-I-infected individuals without HAM/TSP, respectively. Nonparametric Spearman's rank correlation test was used for statistical analysis.
A: HAM/TSP patients (rs = 0.393, p = 0.041), B: HTLV-I-infected individuals without HAM/TSP (rs = 0.754, p = 0.033).

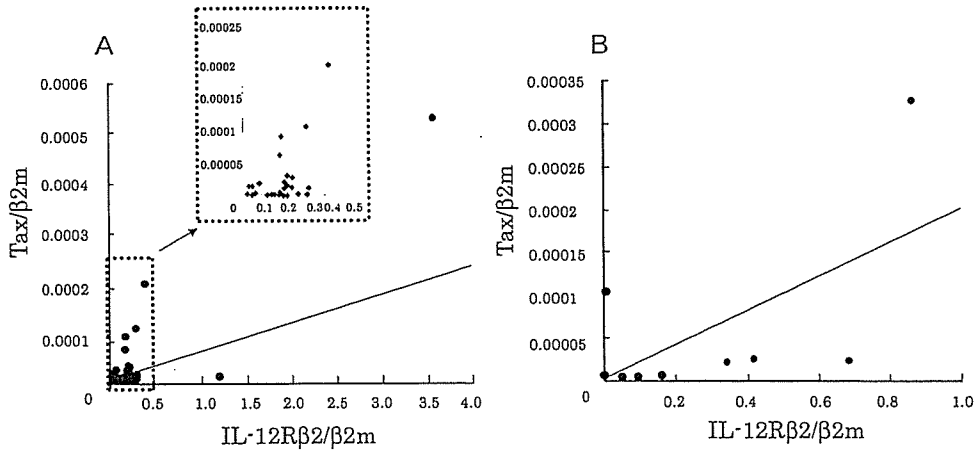

Fig. 5. Correlation between IL-12Rβ2 and HTLV-I tax mRNA expression among HTLV-I-infected individuals. There was moderately positive correlation in only HAM/TSP patients, but not HTLV-I-infected individuals without HAM/TSP. Nonparametric Spearman's rank correlation test was used for statistical analysis.
A: HAM/TSP patients (rs = 0.380, p = 0.048), B: HTLV-I-infected individuals without HAM/TSP (rs = 0.197, p = 0.367).

HTLV-I-infected individuals without HAM/TSP, compared to HTLV-I-uninfected individuals (Fig. 3A). Conversely, mRNA expression of SOCS3 was significantly decreased in HTLV-I-infected individuals with HAM/TSP and without HAM/TSP, compared to HTLV-I-uninfected individuals (Fig. 3B). However, no significant difference in mRNA expression of SOCS3 was found between HTLV-I-infected individuals with or without HAM/TSP.

In HTLV-I-infected individuals, increased mRNA expression of SOCS1 in PBMC indicated up-regulation of IFN-γ signaling, and decreased mRNA expression of SOCS3 in PBMC indicated down-regulation of IL-4 signaling. Moreover, GATA-3 mRNA expression seemed to also be up-

regulated in HTLV-I-infected individuals. Therefore, we analyzed the correlation between T-bet and GATA-3 mRNA expression in HTLV-I-infected individuals. As shown in Fig. 4, mRNA expression of both transcription factors showed moderately positive and strongly positive correlation in HAM/TSP patients (rs = 0.393; $p = 0.041$) and in HTLV-I-infected individuals without HAM/TSP (rs = 0.754; $p = 0.033$), respectively.

Next, we analyzed the correlation between either T-bet, SOCS1 or IL-12Rβ2 and HTLV-I tax mRNA expression. There were no correlations between either T-bet or SOCS1 and HTLV-I tax mRNA expression in both HAM/TSP patients (T-bet vs. HTLV-I tax: rs = 0.050; $p = 0.398$, SOCS1 vs. HTLV-I tax: rs = 0.080; $p = 0.678$) and HTLV-I-infected individuals without HAM/TSP (T-bet vs. HTLV-I tax: rs = 0.029; $p = 0.467$, SOCS1 vs. HTLV-I tax: rs = −0.067; $p = 0.850$) (data not shown). However, as shown in Fig. 5, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients (rs = 0.380; $p = 0.048$), but not in HTLV-I-infected individuals without HAM/TSP (rs = 0.197; $p = 0.367$).

Discussion

This is the first ex vivo analysis of Th1/Th2-related cytokine signaling molecules in HTLV-I-infected individuals. Firstly, to clarify the molecular mechanism of Th1 activation in HTLV-I-infected individuals, we focused mRNA expression of T-bet which are master switches in Th1 differentiation (Grogan and Locksley 2002; O'Shea and Paul 2002). Expression of T-bet mRNA in PBMC was increased in HAM/TSP patients than in HTLV-I-uninfected individuals.

Secondly, we showed significantly increased SOCS1 mRNA expression concomitant with the decreased SOCS3 mRNA expression in PBMC of HTLV-I-infected individuals compared to HTLV-I-uninfected controls. Increased SOCS1 mRNA expression strongly suggests that both IFN-γ and IL-12 signaling are up-regulated, and conversely, decreased SOCS3 mRNA expression suggests IL-4 signaling is down-regulated in HTLV-I-infected individuals. Alternatively, there might exist more Th1 cells and fewer Th2 cells in PBMC of HTLV-I-infected individuals compared to HTLV-I-uninfected individuals, if the distinct pattern of SOCS expression indicates Th lineage (Egwuagu et al. 2002; Seki et al. 2003). From the point of view of SOCS expression, our data revealed that the Th1/Th2 balance is toward Th1 immune activation in HTLV-I-infected individuals. We previously reported the importance of Th1 immune deviation in the development of HAM/TSP (Nakamura et al. 2000b). However, as far as the pattern of SOCS mRNA expression is concerned, there was no significant difference between HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP. Nevertheless, the pattern of SOCS1 or SOCS3 mRNA expression showed the tendency that both IFN-γ and IL-12 signaling are more up-regulated with more down-regulated IL-4 signaling in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP.

From these results, we thought that the expression of GATA-3, located downstream of IL-4R, might be down-regulated in HTLV-I-infected individuals because of down-regulation of IL-4 signaling. However, unexpectedly, GATA-3 mRNA expression was also up-regulated in HAM/TSP patients than in HTLV-I-uninfected individuals. We are unable to precisely explain the reasons for this phenomenon. One possible explanation, however, is that auto-activation of GATA-3 itself might be involved (Ouyang et al. 2000). In this regard, there was moderate to strong correlation between T-bet and GATA-3 mRNA expression among HTLV-I-infected individuals with and without HAM/TSP. Therefore, Th1/Th2 balance in HTLV-I-infected individuals is under a compensatory control.

Thirdly, we analyzed mRNA expression of IL-12Rβ2, which is located up-stream of STAT4 signaling and plays an important role in Th1 differentiation (Grogan and Locksley 2002). IL-12Rβ2 mRNA expression in PBMC was significantly

higher in HAM/TSP patients than in HTLV-I-uninfected individuals, strongly indicating that Th1 cells are increased in HAM/TSP patients. IL-12Rβ2 is induced by STAT4 signaling following the stimulation of IL-12 itself (Rogge et al. 1997). Therefore, down-regulation of SOCS3 mRNA expression in HAM/TSP patients might be involved in this phenomenon although the precise mechanisms are unclear.

Although mRNA expression of T-bet, GATA-3, IL-12Rβ2 and SOCS1 seemed to be higher, and mRNA expression of SOCS3 seemed to be lower in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP, the differences were not statistically significant. Therefore, we, next, analyzed the correlation between these Th1-related cytokine signaling molecules and HTLV-I tax mRNA expression in both groups. Neither T-bet nor SOCS1 mRNA expression correlated with HTLV-I tax mRNA expression in HTLV-I-infected individuals with or without HAM/TSP, suggesting that HTLV-I tax expression is not directly related to expression of these signaling molecules. Interestingly, however, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients. Therefore, in patients with HAM/TSP, HTLV-I tax might be cooperatively involved in IL-12R/STAT4 signaling in HTLV-I-infected cells and contribute to Th1 activation. Recently, Furukawa et al. (2003) demonstrated that IFN-γ producing cells in the population of HTLV-I tax-expressing cells are increased in HAM/TSP patients, compared to HTLV-I asymptomatic carriers with a high HTLV-I proviral load comparable to that in HAM/TSP patients. Further studies are needed to investigate at which step HTLV-I tax up-regulates IL-12R/STAT4 signaling pathway in patients with HAM/TSP.

Acknowledgments

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Involvement of p38 MAPK signaling pathway in IFN-γ and HTLV-I expression in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis

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Abstract

We analyzed the relationship between the expression of interferon (IFN)-γ and HTLV-I p19 antigen and activation of p38 mitogen-activated protein kinase (p38 MAPK) in two HTLV-I-infected T cell lines derived from two patients (HCT-1 and HCT-4) with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and three HTLV-I-infected T cell lines derived from three patients with adult T cell leukemia (ATL). Expression of phosphorylated (activated)-p38 MAPK was markedly increased concomitant with high levels of both IFN-γ and HTLV-I p19 antigen expression in both HCT-1 and HCT-4 compared with cell lines derived from ATL patients. Treatment with SB203580, a specific inhibitor of p38 MAPK, suppressed IFN-γ and HTLV-I p19 antigen expression levels in HCT-1, HCT-4 and peripheral blood $CD4^+$ T cells of HAM/TSP patients. These findings strongly suggest that activation of p38 MAPK signaling pathway is involved in the up-regulation of IFN-γ expression with high HTLV-I proviral load in HAM/TSP patients.

Keywords: HAM/TSP; HTLV-I; Th1; IFN-γ; p38 MAPK

1. Introduction

Human T lymphotropic virus type I (HTLV-I) is the causative agent for both adult T cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al., 1986; Yoshida, 2001). However, the exact mechanisms underlying the entirely different clinical conditions caused by HTLV-I, such as aggressive lymphoproliferative malignancy and chronic myelitis, are still unknown. In addition, it is still not clear why only a small proportion of HTLV-I-infected individuals develop both HTLV-I-associated diseases.

The main pathological feature of HAM/TSP is chronic inflammation of the spinal cords characterized by perivascular cuffing and parenchymal infiltration of lymphocytes (Iwasaki, 1990). Although the interaction between HTLV-I-infected cells and HTLV-I-specific cytotoxic T cells play an important role in the etiopathomechanism of HAM/TSP (Jacobson, 2002; Osame, 2002), we previously proposed the importance of Th1 immune activation, such as increased expression of interferon (IFN)-γ, in the development of HAM/TSP (Nakamura et al., 2000). Indeed, high HTLV-I proviral load is an important factor in the development of HAM/TSP (Jeffery et al., 1999). Recently, however, a comparative study of intracellular cytokine expression levels in HAM/TSP patients and HTLV-I asymptomatic carriers with a high HTLV-I proviral load equivalent to those of HAM/TSP patients revealed abundance of IFN-γ and

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tumor necrosis factor (TNF)-α producing cells in the population of HTLV-I tax-expressing cells, but not tax-non-expressing cells, in HAM/TSP patients (Furukawa et al., 2003). These findings suggested that the expression levels of both cytokines in HTLV-I-infected cells influence the development of HAM/TSP with Th1 activation in addition to high HTLV-I proviral load.

Numerous signaling molecules are involved in the regulation of expression of IFN-γ, a well known representative Th1 cytokine (Murphy et al., 2000; Seder and Paul, 1994). The p38 mitogen-activated protein kinase (p38 MAPK) is one of them and is involved in Th1 differentiation with IFN-γ induction through the downstream target, activation transcription factor (ATF)-2 (Dong et al., 2002; Rincón and Pedraza-Alva, 2003; Szabo et al., 2003). The p38 MAPK, which is phosphorylated by activated MAPK kinase, phosphorylates ATF-2 in turn and the phosphorylated-ATF-2 induces transcription of the gene encoding IFN-γ. In addition, the importance of p38 MAPK signaling pathway in the Th1 differentiation, also in signal transducers and activators of transcription-4 or T cell receptor (TCR)-independent manner, has been reported (Yang et al., 2001; Zhang and Kaplan, 2000).

On the other hand, HTLV-I tax protein, which is the gene product of pX region in the 3′ terminal region of HTLV-I genome, plays an important role in transcription of HTLV-I proviral genome from HTLV-I long terminal repeat (LTR) (Yoshida, 2001). However, it is well known that tax binds HTLV-I LTR not directly but through binding of the cAMP response element binding protein and ATF-1 or -2 (CREB/ATF family) (Franklin et al., 1993; Xu et al., 1996; Yoshida, 2001). This implies that the efficient recruitment of CREB and ATF to LTR of HTLV-I provirus is necessary for efficient HTLV-I replication. ATF-2 is the downstream target of p38 MAPK as mentioned above. In addition, it is reported that CREB is also activated by the downstream target of p38 MAPK, MAPKAP kinase-2 (Tan et al., 1996).

Although spontaneous IFN-γ expression is up-regulated with high HTLV-I proviral load in HAM/TSP patients as mentioned above, it is still not clear which mechanisms induces such a status. However, if p38 MAPK signaling is activated in HTLV-I-infected T cells of HAM/TSP patients, such status might be linked to both up-regulated spontaneous IFN-γ expression and high HTLV-I proviral load in HAM/TSP patients. In the present study, we first analyzed activated (phosphorylated)-p38 MAPK expression with regard to both spontaneous IFN-γ production and HTLV-I p19 antigen expression in HTLV-I-infected T cell lines derived from HAM/TSP patients, compared with same cells derived from ATL patients. Second, we analyzed the suppressive effect of a specific pyrinidyl imidazole inhibitor SB203580, which is p38 MAPK specific inhibitor (Cuenda et al., 1995), on both spontaneous IFN-γ production and HTLV-I p19 antigen expression in HTLV-I-infected T cell lines and peripheral blood $CD4^+$ T cells derived from HAM/TSP patients.

2. Subjects and methods

2.1. Cell lines

HTLV-I-infected T cell lines derived from the cerebrospinal fluid of two different HAM/TSP patients (HCT-1 and HCT-4) (Nakamura et al., 1989) and from peripheral blood of three ATL patients (KK-1, SO-4, and KOB) (Yamada et al., 1996) were used. The cell lines were all interleukin (IL)-2-dependent. All cell lines were maintained in RPMI 1640 containing 20% fetal bovine serum (FBS) (HCT-1 and HCT-4) or 10% FBS (KK-1, KOB, and SO-4) supplemented with 100 units/ml of recombinant human IL-2 (kindly provided by Shionogi, Japan).

2.2. Patients

Six patients with HAM/TSP (all women; mean age, 62 years; range, 40–73 years) were recruited for the study. The diagnosis of HAM/TSP was based on the criteria described previously (Osame, 1990). Control subjects comprised 3 anti-HTLV-I-seropositive individuals (1 man and 2 women; average age, 61 years; range, 50–78 years) and 4 anti-HTLV-I-seronegative healthy individuals (1 man and 3 women; average age, 50 years; range, 40–71 years). The three anti-HTLV-I-seropositive individuals comprised patients with spinocerebellar degeneration, lumbar polyradiculopathy, and tension-type headache. None of the patients had been treated with immunomodulatory drugs, including corticosteroids and IFN-α. Informed written consent was obtained from all patients and healthy volunteers.

2.3. Separation of peripheral blood $CD4^+$ T cells

Peripheral blood mononuclear cells were separated from heparinized venous blood by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). The peripheral blood $CD4^+$-enriched T cells were separated in the negative selection by depletion of $CD8^+$ T cells from macrophage/B cell depleted mononuclear cells using magnetic beads coated with anti-CD8 monoclonal antibody, according to the instructions provided by the manufacturer (Dynal Biotech, Oslo, Norway). $CD4^+$-enriched T cells were washed with phosphate-buffered saline (PBS) and used as $CD4^+$ T cells. Analysis of the purity of $CD4^+$ T cells using anti-CD4, anti-CD20, and anti-CD14 monoclonal antibodies showed more than 90% purity with the presence of less than 1% B cells, and less than 1% monocytes as assessed by flow cytometry (Epics XL system II, Beckman Coulter, Fullerton, CA).

2.4. Cell culture and treatment with SB203580 for HCT-1, HCT-4, and peripheral blood $CD4^+$ T cells

To study IFN-γ production by and HTLV-I p19 antigen expression on HTLV-I-infected T cell lines derived from

HAM/TSP and ATL patients, each cell line was cultured at 1×10^5 cells/ml in the presence of 100 units/ml of IL-2 in 24-well culture plate for 48 h. To study the suppression of both IFN-γ production and HTLV-I p19 antigen expression by p38 MAPK inhibitor SB203580, each cell line was cultured in the presence of dimethyl sulfoxide (DMSO) only or different concentrations of SB203580 (Cuenda et al., 1995; Zhang and Kaplan, 2000) in the same culture condition above. Next, peripheral blood $CD4^+$ T cells were cultured at concentration of 1×10^6 cells/ml in the presence of DMSO only or 10 μM of SB203580 in RPMI 1640 supplemented with 20% FBS in 24-well culture plates for 72 h. The culture medium was then centrifuged at 2500 rpm for 10 min and the supernatant was stored at −40 °C until use.

2.5. Western blot analysis of p38 MAPK expression

Each culture of HTLV-I-infected T cells was collected and lysed by the addition of TNE buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1% Nonidet P-40, and 50 mM NaF) supplemented with 1 mM Na_3VO_4 and complete EDTA-free (Roche, Mannheim, Germany). Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 °C and the supernatant was used for western blotting. An identical amount of protein for each lysate (10 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ATTO, Tokyo). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After overnight blocking with PVDF buffer, the PVDF membrane was incubated at room temperature for 2 h in the presence of rabbit polyclonal anti-phosphorylated p38 MAPK antibody (1:2000 dilution, Promega, Madison, WI) or rabbit polyclonal anti-p38 MAPK antibody (1:1000 dilution, Cell Signaling, Beverly, MA). After 1 h incubation with donkey anti-rabbit IgG coupled with horseradish peroxidase (Promega) or sheep anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences, UK), proteins were visualized by using an enhanced chemiluminescence kit (Amersham Biosciences). The blots were stripped and reproved with monoclonal mouse IgG against to β-actin (Sigma) to ensure equal protein loading.

2.6. Measurement of IFN-γ and HTLV-I p19 antigen levels in culture supernatants

IFN-γ level in the culture supernatants was measured using an enzyme-amplified sensitivity immunoassay (EASIA) kit according to the instruction provided by the manufacturer (Medgenix, Fleurus, Belgium). The amount of IFN-γ was determined in duplicate. The minimum measurable level of IFN-γ was 0.03 IU/ml. The suppression ratio of IFN-γ production (SR) in the culture of peripheral blood $CD4^+$ T cells was determined as follows: SR=[(IFN-γ level in culture supernatant of cultured $CD4^+$ T cells in the presence of DMSO only-IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of 10 μM SB203580)/(IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of DMSO only)]. The level of HTLV-I p19 antigen in the culture supernatants was measured by using the RETROtek HTLV p19 antigen enzyme-linked immunosorbent assay (ELISA) kit using the instructions provided by the manufacturer (Zeptometrix, Buffalo, NY). The amount of HTLV-I p19 antigen was determined in duplicate. The minimum measurable level of HTLV-I p19 antigen was 25.0 pg/ml.

2.7. Proliferation assay

Each HTLV-I-infected T cell line (2.5×10^3 cells/well) was cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well U-bottom plates. Cells were pulsed for the last 12 h of a 48-h incubation at each HTLV-I-infected T cell line with 0.8 μCi/well of [^{3}H]TdR (Perkin Elmer, Boston, MA) and harvested onto glass-fiber filters. Radioactivity (cpm) was recorded using a liquid scintillation counter (LSC-5100, Aloka, Tokyo). Cultures were studied in triplicate and results were expressed as mean counts per minute.

2.8. MTS assay

HTLV-I-infected T cell line (2×10^4 cells/well) was cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well flat-bottom plates for 48 h. Peripheral blood $CD4^+$ T cells (1×10^5 cells/well) were cultured in the presence of DMSO only or 10 μM SB203580 in 96-well flat-bottom plates for 72 h. The number of viable cells was determined by a modified MTT assay, MTS (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) nonradioactive cell proliferation assay (Promega). Briefly, 20 μl of MTS mixture was added to each individual well, and after 1 h incubation, absorbance at 490 nm was measured using a multiscan plate reader. Cultures were

Table 1
Levels of IFN-γ and HTLV-I p19 antigen in culture supernatants of HTLV-I-infected T cell lines

Cell lines	IFN-γ (IU/ml)	HTLV-I p19 (pg/ml)
HAM		
HCT-1	65.30	16450.0
HCT-4	148.80	43485.0
ATL		
KK-1	0.79	409.0
KOB	0.66	1456.0
SO-4	0.77	<25.0

Each cell line was cultured at 1×10^5 cells/ml in the presence of 100 units/ml of IL-2 for 48 h. Each culture supernatant was collected and the levels of IFN-γ and HTLV-I p19 antigen were measured by ELISA. The minimum measurable level of IFN-γ was 0.03 IU/ml and that of HTLV-I p19 antigen was 25.0 pg/ml.

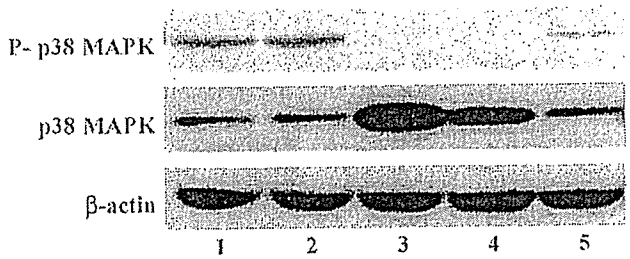

Fig. 1. Western blot analysis of p38 MAPK expression in HTLV-I-infected T cell lines. Lanes 1 and 2: HTLV-I-infected T cell lines derived from HAM patients; HCT-1 and HCT-4, respectively. Lanes 3, 4, and 5: HTLV-I-infected T cell lines derived from ATL patients; KK-1, KOB, and SO-4, respectively. P-p38 MAPK: phosphorylated-p38 MAPK, β-actin: internal control.

studied in triplicate and results were expressed as the mean optical density (OD).

2.9. Statistical analysis

The sign test was used for the statistical analysis. Differences were considered as statistically significant at $p<0.05$.

3. Results

3.1. IFN-γ/HTLV-I p19 antigen expression correlates with phosphorylated-p38 MAPK expression in HTLV-I-infected T cell lines

As shown in Table 1, both IFN-γ and HTLV-I p19 antigen levels were significantly higher in two HTLV-I-infected T cell lines from HAM/TSP patients (HCT-1 and HCT-4) than in three HTLV-I-infected T cell lines from ATL patients (KK-1, KOB, and SO-4). Based on this finding, we next analyzed the expression of phosphorylated-p38 MAPK in each cell line, using western blot. As shown in Fig. 1, the expression of phosphorylated-p38 MAPK was significantly increased in both HCT-1 and HCT-4. On the other hand, although the expression of phosphorylated-p38 MAPK was faintly detected in SO-4, it was not detected in both KK-1 and KOB at all (Fig. 1). These results suggest that high IFN-γ and HTLV-I p19 antigen expression in HTLV-I-infected T cells from HAM/TSP patients depend on activation of p38 MAPK signaling pathway.

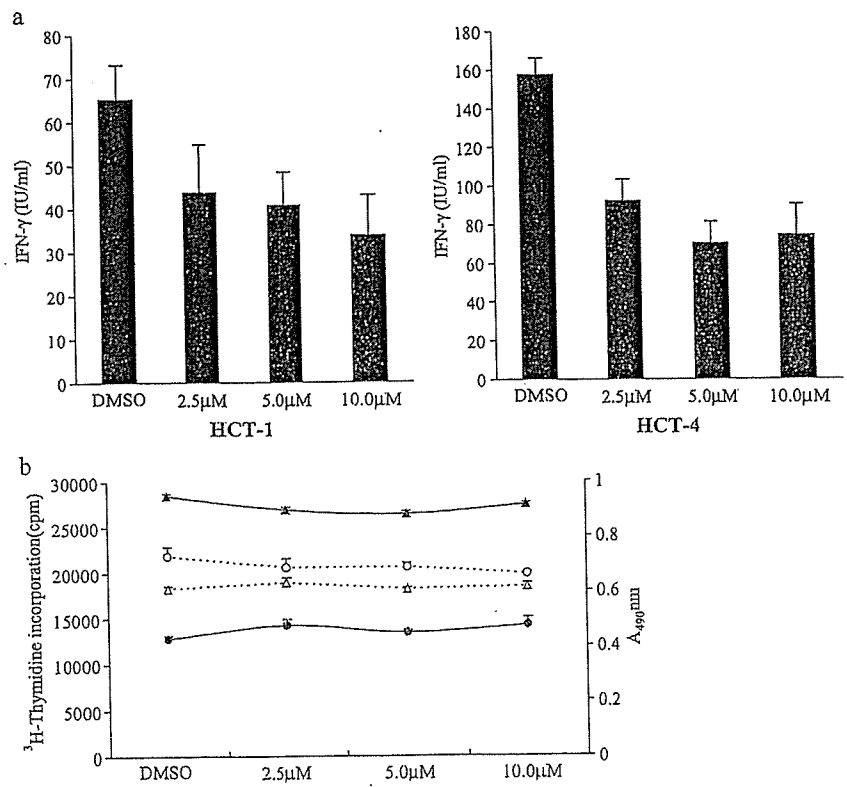

Fig. 2. Effects of SB203580 on HTLV-I-infected T cell lines derived from HAM/TSP patients (HCT-1 and HCT-4). (a) SB203580 suppresses spontaneous IFN-γ production by HCT-1 and HCT-4. HCT-1 or HCT-4 cells were cultured at concentration of 1×10^5 cells/ml in the presence of DMSO only or different concentrations (2.5, 5.0, and 10.0 μM) of SB203580 with 100 units/ml of IL-2 for 48 h, and then the culture supernatants were collected. Levels of IFN-γ in each culture supernatant were measured by ELISA. Data are mean±SEM of triplicate cultures. (b) Cell proliferation and cell viability assays. HCT-1 or HCT-4 cells were cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well plates. For cell proliferation assay, cells were pulsed for the last 12 h of a 48-h incubation with [^{3}H]TdR. Data are mean±SEM of triplicate cultures. HCT-1: ●-●, HCT-4: O···O. For cell viability assay, MTS assay was performed according to the instructions provided by the manufacturer. Data represent OD titer at wavelength of 490 nm. Data are mean±SEM of triplicate cultures. HCT-1: ▲-▲, HCT-4: △···△.

3.2. SB203580 suppresses IFN-γ production by HCT-1 and HCT-4

To confirm whether this p38 MAPK signaling pathway is functionally activated for IFN-γ induction in HCT-1 and HCT-4, we analyzed the effect of p38 MAPK specific inhibitor, SB203580, on spontaneous IFN-γ production by both cell lines. As shown in Fig. 2a, SB203580 suppressed dose-dependently, by up to about 50%, IFN-γ production by both cell lines. To determine whether the SB203580-induced suppression of IFN-γ production was dependent on inhibition of cell proliferation, we checked the changes in cell proliferation of both HCT-1 and HCT-4 treated with SB203580. However, as shown in Fig. 2b, SB203580 did not affect cell proliferation. In addition, MTS assay revealed that this treatment also did not affect cell viability (Fig. 2b). These results indicate that the suppression of spontaneous IFN-γ production by treatment with SB203580 was based on inhibition of p38 MAPK signaling pathway in IFN-γ induction, but not suppression of cell proliferation.

3.3. SB203580 suppresses IFN-γ production by peripheral blood CD4$^+$ T cells

SB203580 induced about 24–79% suppression of IFN-γ production by peripheral blood CD4$^+$ T cells of all HAM/TSP patients (Fig. 3, p=0.0156). However, SB203580 produced only about 20% suppression of IFN-γ production by peripheral blood CD4$^+$ T cells of all HTLV-I carriers and HTLV-I-seronegative controls (Fig. 3). Similar to HTLV-I-infected T cell lines, SB203580 did not affect the viability of peripheral blood CD4$^+$ T cells (data not shown). These findings suggest that p38 MAPK signaling pathway is preferentially involved in spontaneous IFN-γ production by peripheral blood CD4$^+$ T cells in HAM/TSP patients.

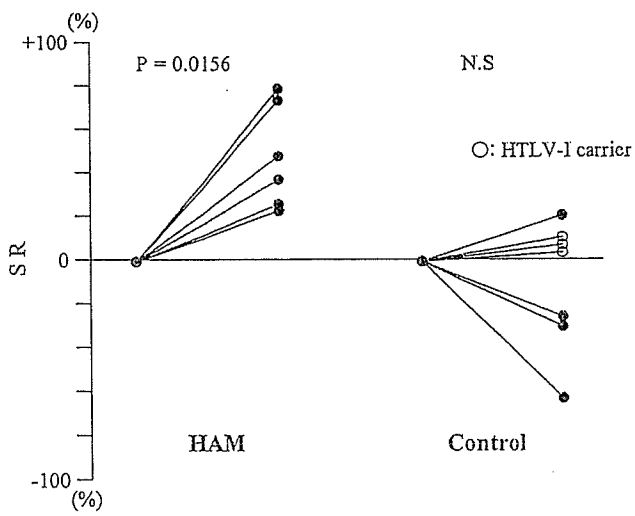

Fig. 3. SB203580 suppresses IFN-γ production by peripheral blood CD4$^+$ T cells. Peripheral blood CD4$^+$ T cells were cultured at concentration of 1×10^6 cells/ml in the presence of DMSO only or 10 μM SB203580 for 72 h, and the culture supernatants were collected. Levels of IFN-γ in each culture supernatant were measured by ELISA. The suppression ratio of IFN-γ production (SR) was determined as described in the text. Open circle: an HTLV-I-seropositive carrier, N.S.: not significant.

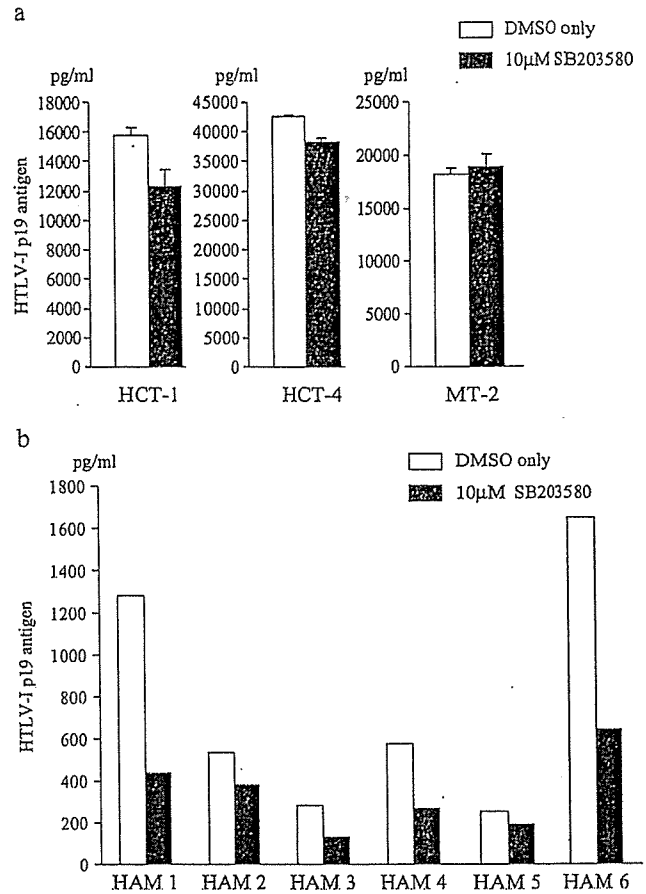

Fig. 4. SB203580 reduces HTLV-I p19 antigen expression in HCT-1, HCT-4, and peripheral blood CD4+ T cells of HAM/TSP patients. (a) HCT-1 or HCT-4 cells were cultured at concentration of 1×10^5 cells/ml in the presence of DMSO only or 10 μM SB203580 with 100 units/ml of IL-2 for 48 h. As control, MT-2 cells were cultured without IL-2 for 48 h in the same condition. The culture supernatants were collected and the levels of HTLV-I p19 antigen were measured by ELISA. Data are mean±SEM of triplicate cultures. Open bars: DMSO only, solid bars: 10 μM SB203580. (b) Peripheral blood CD4$^+$ T cells of HAM/TSP patients were cultured at 1×10^6 cells/ml in the presence of DMSO only or 10 μM SB203580 for 72 h. The culture supernatants were collected and the levels of HTLV-I p19 antigen in the culture supernatant were measured by ELISA. Open bars: DMSO only, solid bars: 10 μM SB203580.

3.4. SB203580 inhibits HTLV-I p19 antigen expression in HCT-1, HCT-4, and CD4$^+$ T cells of HAM/TSP patients

Treatment of HCT-1 and HCT-4 with SB203580 suppressed HTLV-1 p19 antigen expression by about 22% and 10%, respectively (Fig. 4a). However, the same treatment did not reduce HTLV-I p19 antigen expression in HTLV-I-transformed T cell line, MT-2 cells (Miyoshi et al., 1981) (Fig. 4a), which produce low amounts of IFN-γ without the activation of p38 MAPK expression (data not shown). SB203580 produced about 24–66% reduction of HTLV-I

p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients (Fig. 4b).

4. Discussion

This is the first report that examined the involvement of p38 MAPK signaling pathway in both IFN-γ induction and HTLV-I expression in HTLV-I infection. Spontaneous IFN-γ expression is up-regulated by the high HTLV-I proviral load in HAM/TSP patients. However, it is not clear how such status is induced in patients with HAM/TSP. Although it is well known that cytokine expression is regulated by various signaling molecules, we focussed on the involvement of p38 MAPK signaling pathway for IFN-γ expression in HAM/TSP patients because p38 MAPK signaling plays an important role in IFN-γ induction in TCR-independent condition (Yang et al., 2001). Analysis of activated p38 MAPK expression in relation to IFN-γ production in HTLV-I-infected T cell lines derived from HAM/TSP patients, compared with same cells derived from ATL patients, revealed that spontaneous IFN-γ production correlated with activation of p38 MAPK. We also confirmed the activation of p38 MAPK signaling pathway in IFN-γ expression in HTLV-I-infected T cell lines of HAM/TSP patients by treatment of these cells with SB203580, a p38 MAPK specific inhibitor. Since SB203580 did not completely inhibit IFN-γ production by these cells, other signaling pathways such as nuclear factor kappaB (NF-κB) and c-Jun N-terminal kinase (Murphy et al., 2000; Rincón and Pedraza-Alva, 2003) or IFN-γ promoter activation by HTLV-I tax itself (Brown et al., 1991), might also contribute to IFN-γ induction in these cell lines. Although these signaling pathways were not analyzed in our experiment, activation of p38 MAPK pathway seems to be certainly an important signaling pathway involved in the up-regulation of spontaneous IFN-γ expression in these cell lines.

To confirm the involvement of the p38 MAPK signaling pathway, we also investigated the effect of SB203580 on IFN-γ production by peripheral blood $CD4^+$ T cells of HAM/TSP patients. SB203580 significantly reduced spontaneous IFN-γ production by peripheral blood $CD4^+$ T cells of HAM/TSP patients but not those of control patients including HTLV-I carriers. These results suggest that up-regulation of spontaneous IFN-γ expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients is also based on the activation of p38 MAPK pathway in TCR-independent manner. Although it is not clear in our experiment whether increased IFN-γ production from peripheral blood $CD4^+$ T cells of HAM/TSP patients is derived from HTLV-I-infected T cells only, TCR-independent IFN-γ induction occurs over a relatively long period compared with TCR-dependent IFN-γ induction (Yang et al., 2001). Therefore, activation of p38 MAPK signaling and the related up-regulation of IFN-γ expression seem consistent with the clinical course of HAM/TSP with chronic inflammatory status, such as slow progression.

On the other hand, HTLV-I p19 antigen expression also correlated with activation of p38 MAPK expression in our study using HTLV-I-infected T cell lines. It is already known that HTLV-I tax binds HTLV-I LTR, which is the promoter of HTLV-I itself, not directly, but in concert with CREB/ATF family (Franklin et al., 1993; Yoshida, 2001). These transcription factors are the downstream target of activated p38 MAPK (Dong et al., 2002; Tan et al., 1996). Therefore, we postulate that activation of p38 MAPK is also involved in HTLV-I expression in HTLV-I-infected T cell lines derived from HAM/TSP patients. Indeed, SB203580 treatment down-regulated production of HTLV-I p19 antigen from HTLV-I-infected T cell lines of HAM/TSP patients. This action was probably mediated by interruption of efficient recruitment of CREB/ATF-2 to HTLV-I LTR. Interestingly, HTLV-I p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients was also suppressed about 24–66% by SB203580, suggesting that activation of p38 MAPK signaling pathway plays a crucial role in HTLV-I expression in HAM/TSP patients. These facts imply that high HTLV-I proviral load in HAM/TSP patients might be based on activated p38 MAPK signaling in HTLV-I-infected T cells.

In conclusion, we have demonstrated in the present study that activation of p38 MAPK signaling pathway is involved in the up-regulation of IFN-γ expression in HAM/TSP patients with high HTLV-I proviral load. Although it is not clear how p38 MAPK signaling is activated in HAM/TSP patients, perpetuation of activation of p38 MAPK signaling pathway in HTLV-I-infected state might strongly contribute to the development of HAM/TSP. In addition, it is also still obscure why only a minor proportion of HTLV-I-infected individuals develop HAM/TSP. However, p38 MAPK might be a potential target in the treatment of HAM/TSP.

Acknowledgements

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AIRWAY BIOLOGY

Bronchoalveolar lymphocytosis correlates with human T lymphotropic virus type I (HTLV-I) proviral DNA load in HTLV-I carriers

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Background: A study was undertaken to investigate the pathogenesis of pulmonary involvement in human T lymphotropic virus type I (HTLV-I) carriers.

Methods: The bronchoalveolar lavage (BAL) cell profile of 30 HTLV-I carriers (15 asymptomatic HTLV-I carriers (AHCs) and 15 symptomatic HTLV-I carriers (SHCs)) with chronic inflammatory diseases of respiratory tract and eight patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) was investigated. The HTLV-I proviral deoxyribonucleic acid (DNA) load in peripheral blood mononuclear cells (PBMCs) and BAL fluid from HTLV-I carriers was estimated using the quantitative polymerase chain reaction method and the correlation between the lymphocyte number in BAL fluid and the HTLV-I proviral DNA load in PBMCs and BAL fluid was examined.

Results: The percentage of lymphocytes in BAL fluid was increased (>18%) in 11 of 30 HTLV-I carriers although there was no significant difference compared with control subjects. In HTLV-I carriers the lymphocyte number in BAL fluid correlated well with the copy number of HTLV-I proviral DNA in PBMCs. In addition, the copy number of HTLV-I proviral DNA in BAL fluid correlated well with the number of lymphocytes (both CD4+ and CD8+ cells) in BAL fluid.

Conclusions: These findings suggest that pulmonary lymphocytosis can occur in a subset of HTLV-I carriers without HAM/TSP and that the increased HTLV-I proviral DNA load may be implicated in the pathogenesis of pulmonary involvement in HTLV-I carriers.

Human T lymphotropic virus type I (HTLV-I) is a type C retrovirus that is aetiologically associated with adult T cell leukaemia[1 2] and with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP).[3 4] In addition to these diseases, a number of inflammatory disorders have also been described in association with HTLV-I including HTLV-I uveitis,[5] arthropathy,[6] and Sjögren's syndrome.[7] Pulmonary involvement is also associated with HTLV-I—for example, in patients with HAM/TSP and HTLV-I uveitis pulmonary involvement may be characterised by bronchoalveolar lymphocytosis.[8–11] Furthermore, a few preliminary studies have shown that similar pulmonary involvement is observed in HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis.[10 12]

Many kinds of immunological abnormalities and an increased HTLV-I proviral deoxyribonucleic acid (DNA) load in peripheral blood, cerebrospinal fluid, and bronchoalveolar lavage (BAL) fluid from patients with HAM/TSP[13 14] and HTLV-I uveitis[11] have been reported, suggesting that immunological mechanisms related to an increased amount of HTLV-I proviral DNA may be implicated in the pathogenesis of these diseases. However, despite advances in elucidating the pathophysiology of these diseases, much of the information on the pathogenesis is confined to HAM/TSP and HTLV-I uveitis. There is little information available regarding pulmonary involvement and pathophysiology in HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis.

To examine the incidence and pathogenesis of pulmonary lymphocytosis in HTLV-I carriers, we have analysed BAL cell profiles in HTLV-I carriers including asymptomatic HTLV-I carriers (AHCs). We also estimated the HTLV-I proviral DNA load in peripheral blood mononuclear cells (PBMCs) and BAL cells from HTLV-I carriers by the quantitative polymerase chain reaction (PCR) method and examined the correlation between the HTLV-I proviral DNA load and pulmonary lymphocytosis.

METHODS

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research.

Study subjects

The study subjects consisted of 30 HTLV-I carriers and eight patients with HAM/TSP consecutively presenting to our department between 1989 and 2000. The 30 HTLV-I carriers consisted of 15 AHCs (three men and 12 women) and 15 symptomatic HTLV-I carriers (SHCs; five men and 10 women) as shown in table 1. There were no significant differences in age between each of the groups and the control subjects. All subjects were seronegative for human immunodeficiency virus (HIV) 1.

To assess the cellular characteristics of BAL fluid in AHCs, the serum anti-HTLV-I antibody was checked in individuals consulting our department for an annual chest radiograph. The anti-HTLV-I antibody was measured by the gelatin particle agglutination method (Fujirebio, Tokyo, Japan). After obtaining informed consent, further examinations including fibreoptic bronchoscopy were performed on HTLV-I seropositive individuals along with careful history taking including occupational history. Individuals who

Abbreviations: AHC, asymptomatic HTLV-I carrier; BAL, bronchoalveolar lavage; HAM/TSP, HTLV-I associated myelopathy/tropical spastic paraparesis; HTLV-I, human T lymphotropic virus type I; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; SHC, symptomatic HTLV-I carrier

Table 1 Clinical background and bronchoalveolar lavage (BAL) findings of HTLV-I carriers and patients with HAM/TSP

Patient no. and clinical diagnosis/symptoms	Age/sex	Peripheral blood: WBC (/mm³)	HTLV-I Ab‡ (×)	Cell count (×10⁶/ml)	Bronchoalveolar lavage fluid: AM (%)	Ly (%)	Neu (%)	Eo (%)	CD4 (%)	CD8 (%)	CD4/CD8
1 AHC	64/F	4300	2048	1.4	94.3	5.0	0.8	0.0	ND	ND	ND
2 AHC†	77/M	7500	2048	1.0	96.4	3.2	0.4	0.0	52.0	32.9	1.6
3 AHC	72/F	5800	256	2.0	90.2	8.3	1.3	0.2	47.1	27.3	1.7
4 AHC	48/F	2600	512	0.4	86.9	12.3	0.6	0.1	48.9	29.2	1.7
5 AHC	57/F	6500	1024	0.7	74.9	24.6	0.5	0.0	53.5	25.0	2.1
6 AHC	78/F	6300	8192	1.9	88.2	10.5	0.8	0.0	53.0	28.3	1.9
7 AHC	58/F	3600	8192	0.9	84.4	14.3	1.2	0.0	ND	ND	ND
8 AHC†	64/M	4300	512	0.7	96.2	3.3	0.5	0.0	ND	ND	ND
9 AHC	71/M	5400	4096	2.8	70.5	28.6	0.9	0.1	75.6	23.2	3.3
10 AHC	56/F	7000	128	1.0	80.5	18.7	0.6	0.1	44.5	39.5	1.1
11 AHC	64/F	4000	128	1.3	89.2	9.7	0.8	0.3	58.8	22.8	2.6
12 AHC	55/F	3700	2048	0.6	95.3	4.0	0.3	0.5	ND	ND	ND
13 AHC	77/F	4300	16384	1.5	76.1	23.4	0.4	0.1	75.3	13.4	5.6
14 AHC	71/F	5300	4096	0.6	84.3	13.5	1.6	0.5	64.6	26.9	2.4
15 AHC	67/F	4700	1024	0.8	91.5	5.5	1.2	0.0	42.8	17.1	2.5
16 Chronic cough	76/F	5100	256	1.0	93.6	5.7	0.7	0.0	37.1	34.8	1.1
17 Chronic cough	67/F	3900	1024	0.9	68.5	30.1	1.4	0.0	71.7	25.9	2.8
18 Chronic cough*	82/F	7000	128	1.7	95.2	4.7	0.2	0.0	ND	ND	ND
19 Middle lobe syndrome	59/F	5200	4096	0.7	61.0	34.6	3.4	0.0	55.8	21.1	2.6
20 SBS	71/F	3400	4096	0.5	92.0	7.1	0.8	0.2	48.4	31.7	1.5
21 Bronchiectasis	63/F	2800	2048	0.6	94.9	4.9	0.1	0.0	40.4	42.1	1.0
22 Inactive Tbc	57/M	3900	1024	1.3	72.7	27.3	0.0	0.0	57.1	40.5	1.4
23 Inactive Tbc†	73/M	7000	2048	3.0	94.9	4.7	0.4	0.0	60.0	30.8	2.0
24 Chronic bronchitis	47/M	4700	256	0.6	91.6	8.1	0.2	0.0	ND	ND	ND
25 Chronic bronchitis*	57/M	5200	256	1.8	87.0	11.4	1.4	0.2	ND	ND	ND
26 Bronchiectasis	72/F	5000	512	1.9	97.4	2.6	0.0	0.0	ND	ND	ND
27 Bronchiectasis	68/F	6000	128	1.5	55.1	29.6	13.1	1.2	57.5	22.9	2.5
28 SBS	46/M	6900	2048	5.8	42.5	20.3	25.0	0.6	41.1	49.7	0.8
29 Middle lobe syndrome	66/F	6300	512	2.5	44.4	55.3	0.1	0.2	61.9	29.6	2.1
30 SBS	18/F	7900	4096	2.7	47.6	45.5	7.1	0.3	41.6	55.2	0.8
31 HAM/TSP	33/F	4100	2048	2.0	42.7	56.8	0.2	0.2	43.1	51.5	0.8
32 HAM/TSP	50/F	4900	2048	2.3	65.6	21.0	13.4	0.0	46.4	49.2	0.9
33 HAM/TSP	54/F	2800	512	1.4	63.0	36.0	1.0	0.0	69.1	28.5	2.4
34 HAM/TSP	60/M	5100	512	2.4	73.4	24.5	2.1	0.0	47.1	48.2	1.0
35 HAM/TSP	65/F	5200	2048	1.7	40.8	58.9	0.3	0.0	33.1	61.0	0.5
36 HAM/TSP	59/F	4900	32768	4.0	27.0	71.9	1.1	0.0	76.2	20.4	3.7
37 HAM/TSP	34/M	5600	8192	1.6	73.2	25.7	1.2	0.0	50.2	42.1	1.2
38 HAM/TSP	50/M	6100	8192	3.9	31.2	68.3	0.0	0.5	52.0	46.9	1.1

WBC, white blood cell; M, male; F, female; HTLV-I, human T lymphotropic virus type I; AHC, asymptomatic HTLV-I carrier; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; SBS, sinobronchial syndrome; Tbc, tuberculosis; AM, alveolar macrophage; Ly, lymphocyte; Neu, neutrophil; Eo, eosinophil; ND, not determined.
*Current smoker.
†Recent/ex-smoker.
‡HTLV-I antibody (Ab) was measured by the gelatin particle agglutination method.

worked in environments known to cause allergic lung diseases were excluded. After the diagnostic procedure, 15 AHCs were recruited to the study (table 1); the chest radiographic findings were normal in 13 individuals and two had minimal inactive tuberous lesions.

The 15 SHCs (subjects 16–30) were recruited from the outpatient clinic of our department for chronic inflammatory diseases of the respiratory tract (three sinobronchial syndrome, three bronchiectasis, two middle lobe syndrome, and two chronic bronchitis); two had an inactive tuberculous lesion and three (subjects 16–18) complained of a slight cough for 1–3 months during the study. Eight patients with HAM/TSP (three men and five women) had been diagnosed according to the criteria proposed by Osame *et al*.[15] Three HTLV-I carriers (subjects 9, 18 and 25) were current smokers, three HTLV-I carriers (subjects 2, 8 and 23) were ex-smokers with intervals ranging from 3 months to 5 years since smoking cessation, and the others had never smoked.

White blood cell counts in peripheral blood were within the normal range in all subjects. The serum anti-HTLV-I antibody titre ranged from 128 to 16384× in HTLV-I carriers and from 512 to 32768× in patients with HAM/TSP. The median (range) % vital capacity of AHCs, SHCs, and patients with HAM/TSP was 104.9% (77.2–131.8), 85.8% (75.9–131.3), and 96.7% (76.7–108.0), respectively, and the % forced expiratory volume in 1 second in the three groups was 82.2% (79.3–91.7), 70.7% (51.6–86.2), and 79.7% (70.0–88.3%), respectively. There were no significant differences in pulmonary function between the three groups.

Nine healthy individuals (three men and six women) of median age 53 years, all non-smokers, who were seronegative for HTLV-I acted as controls. They included four healthy volunteers and five healthy individuals undergoing an annual chest radiographic examination. These latter five healthy individuals were finally diagnosed as having a small solitary lung nodule without signs of pulmonary disease (n = 3) or minimal inactive tuberculous lesions (n = 2). BAL was performed to diagnose the small lung nodule and the cellular characteristics of the BAL fluid from these subjects served as

Table 2 Oligonucleotides for PCR detection of HTLV-I proviral DNA

Function	Nucleotide sequence (5′ to 3′)	Position*
Primer	GGC TCC GTT GTC TGC ATG TA	7765–7784
Primer	AAT CAT AGG CGT GCC ATC GG	8091–8072
Probe	CCT AAT AAT TCT ACC CGA AGA CTG TTT GCC	7932–7961

*GenBank Accession No. J02029.
PCR, polymerase chain reaction; HTLV-I, human T lymphotropic virus type I.

control values for this study. The chest radiographic findings of the five healthy volunteers were normal.

Bronchoalveolar lavage

Informed consent was obtained from all individuals before they underwent BAL which was performed before interventions including corticosteroid administration. Under local anaesthesia with 2% lidocaine, a fibreoptic bronchoscope was placed in the subsegment of the right middle lobe or lingua and 160 ml sterile saline was infused in four aliquots through the bronchoscope and aspirated by gentle hand suction. The lavage fluid obtained was passed through two sheets of sterile gauze and a 10 ml aliquot was centrifuged at 400*g* for 10 minutes, stained with Wright-Giemsa stain, and the cell differentials were determined (at least 500 cells were counted). The lavage fluid was then washed twice and the total number of cells counted.

An aliquot of BAL cells was used for identification of T lymphocyte subsets. The cells were washed twice with phosphate buffered saline and incubated with an optimal concentration of fluorescence conjugated monoclonal antibodies (OKT4 (CD4), OKT8 (CD8); Ortho Diagnostics, Raritan, NJ, USA). The cells were then analysed for surface fluorescence using flow cytometry (FCMID, Nihon Bunko, Tokyo, Japan).

Isolation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated from 30 ml heparinised peripheral blood by Ficoll-Hypaque density gradient centrifugation (Pharmatica, Uppsala, Sweden). Blood samples were obtained before performing BAL in all subjects. These samples were stored in liquid nitrogen until use.

Quantitative PCR of PBMCs and BAL cells

Quantitative PCR assay was performed as previously described.[16] The amount of HTLV-I proviral DNA was calculated using the following formula: copy number of HTLV-I (pX) per 10^4 PBMCs and per 10^4 BAL cells = [(copy number of pX)/(copy number of β-actin/2)] × 10^4

Detection of HTLV-I proviral DNA from BAL cells by PCR

To examine the presence of HTLV-I proviral DNA in BAL cells, isolated BAL cells from 10 initial AHCs (nos 1–9 and 11) were analysed. As negative and positive controls, BAL cells and HTLV-I infected TCL-Kan cells[17] from HTLV-I seronegative controls were also assayed. 1 µg of DNA extracted from BAL cells was used for the PCR. The amplification reaction of PCR was performed for 35 cycles and consisted of denaturation at 94°C for 1 minute, annealing at 65°C for 2 minutes, and primer extension at 72°C for 3 minutes. 10 µl of the amplified products was blotted onto a nylon membrane and hybridised with a biotin labelled probe for HTLV-I pX. After incubation with streptavidin-alkaline phosphate conjugate, disodium 3-(4-methoxyspirol [1,2-dioxetane-3-2'-tricyclo-[3.3.1.1.3.7] decan]-4-yl)phenyl phosphate (AMPPD)[18] (Southern Light Kit, Troix) was added to the blot to a final concentration of 0.25 mM and the immersed blot was then slowly agitated for 5 minutes. Finally, the chemiluminescent signal was detected by exposing the radiographic film.

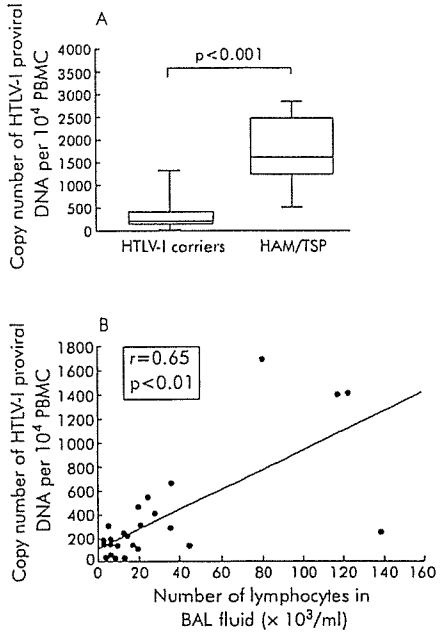

Figure 1 (A) Copy number of HTLV-I proviral DNA per 10^4 PBMCs in HTLV-I carriers and patients with HAM/TSP. The whisker box plots represent the 25–75th percentile of results inside the box, the median is shown by the horizontal bar across the box, and whiskers on the box represent the 10–90th percentiles. (B) Correlation between the number of lymphocytes in bronchoalveolar lavage (BAL) fluid and the copy number of HTLV-I proviral DNA per 10^4 PBMCs in HTLV-I carriers. HTLV-I, human T lymphotropic virus type I; PBMCs, peripheral blood mononuclear cells; HAM/TSP = HTLV-I associated myelopathy/tropical spastic paraparesis.

Table 3 Bronchoalveolar lavage findings in HTLV-I carriers and HAM/TSP patients

Subjects	Cell count (×10^5/ml)	AM (%)	Ly (%)	Neu (%)	Eo (%)	CD4+ cells (%)	CD8+ cells (%)	CD4/CD8
HTLV-I carriers	1.2**	87.6	11.0	0.8	0.0	53.3	28.8	1.9
N=30 (22)†	(0.4–5.8)	(42.5–97.4)	(2.6–55.3)	(0.0–25.0)	(0.0–1.2)	(37.1–75.6)	(13.4–55.2)	(0.8–5.6)
AHCs	1.0*	88.2	10.5	0.8	0.1	53.0	26.9	2.1*
N=15 (11)†	(0.4–2.8)	(70.5–96.4)	(3.2–28.6)	(0.3–1.6)	(0.0–0.5)	(42.8–75.6)	(13.4–39.5)	(1.1–5.6)
SHCs	1.5**	87.0	11.4	0.7	0.0	55.8	31.7	1.5
N=15 (11)†	(0.5–5.8)	(42.5–97.4)	(2.6–55.3)	(0.0–25.0)	(0.0–1.2)	(37.1–71.7)	(21.1–55.2)	(0.8–2.8)
HAM/TSP	2.2**	52.9**	46.4**	1.1	0.0	48.7	47.6*	1.0
N=8	(1.4–4.0)	(27.0–73.4)	(21.0–71.9)	(0.0–13.4)	(0.0–0.5)	(33.1–76.2)	(20.4–61.0)	(0.5–3.7)
Controls	0.6	91.2	8.4	0.6	0.1	48.7	31.4	1.5
N=9	(0.3–1.0)	(87.9–95.8)	(3.8–10.5)	(0.2–1.3)	(0.0–0.4)	(33.4–59.5)	(22.7–45.1)	(0.9–2.1)

Data are shown as median (range).
N, number of subjects; AM, alveolar macrophage; Ly, lymphocyte; Neu, neutrophil; Eo, eosinophil; HTLV-I, human T lymphotropic virus type I; AHCs, asymptomatic HTLV-I carriers; SHCs, symptomatic HTLV-I carriers with chronic inflammatory diseases of respiratory tract; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis.
*p<0.05, **p<0.01 compared with control subjects.
†The cell differential in BAL fluid was determined in 30 HTLV-I carriers (15 AHCs and 15 SHCs) and T lymphocyte subsets were determined in 22 HTLV-I carriers (11 AHCs and 11 SHCs).

Table 4 Quantification of HTLV-I proviral DNA in PBMCs and BAL fluid from HTLV-I carriers and HAM/TSP patients

	PBMCs			BAL fluid		
Subjects	N	Median	Range	N	Median	Range
HTLV-I carriers	26	199	ND–1704	14	126	22–1268
AHCs	11	138	ND–1704	3	75	70–132
SHCs	15	232	30–1390	11	141	22–1268
HAM/TSP	8	1611*	261–2857	8	601**	294–3495

HTLV-I copy number per 10^4 PBMCs and per 10^4 BAL cells are presented.
HTLV-I, human T lymphotropic virus type I; AHCs, asymptomatic HTLV-I carriers; SHCs, symptomatic HTLV-I carriers with chronic inflammatory diseases of respiratory tract; HAM/TSP, HTLV-I associated myelopathy/tropical spastic paraparesis; PBMCs, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; N, number of subjects; ND, not detected.
*$p<0.01$ compared with HTLV-I carriers, AHCs and SHCs; **$p<0.01$ compared with HTLV-I carriers and SHCs.

The location and sequences of the primers and probe are summarised in table 2.

Statistical analysis

All values are shown as median (range). Statistical analysis was performed using the Mann-Whitney U test and Spearman rank correlation. p values of <0.05 were considered significant.

RESULTS

Cellular characteristics and T lymphocyte subset of BAL fluid

The recovery ratios of BAL fluid in HTLV-I carriers, AHCs, SHCs, and patients with HAM/TSP did not differ significantly from those of control subjects (data not shown). As shown in table 3, the BAL fluid cell count was increased in HTLV-I carriers, AHCs, SHCs, and patients with HAM/TSP compared with control subjects. The differential cell count showed an increased percentage of lymphocytes and a decreased percentage of macrophages in BAL fluid from patients with HAM/TSP compared with control subjects. The percentage of lymphocytes in BAL fluid was increased (>18%) in four AHCs and seven SHCs but there was no significant difference from that in control subjects.

Analysis of T lymphocyte subsets in BAL fluid showed an increased percentage of CD8+ cells in patients with HAM/TSP and an increased ratio of CD4/CD8 in AHCs.

HTLV-I proviral DNA in PBMCs and correlation with lymphocytes in BAL fluid

The copy number of HTLV-I proviral DNA in PBMCs was determined in 26 HTLV-I carriers (11 AHCs and 15 SHCs) and eight patients with HAM/TSP. As shown in table 4 and fig 1A, the copy number of HTLV-I proviral DNA per 10^4 PBMCs was significantly increased in patients with HAM/TSP compared with those of HTLV-I carriers ($p<0.001$), AHCs ($p<0.01$), and SHCs ($p<0.01$). There was no significant difference in the copy number of HTLV-I proviral DNA of PBMCs between AHCs and SHCs.

The relationship between the HTLV-I proviral load in PBMCs and the lymphocyte number in BAL fluid was examined in 26 HTLV-I carriers. As shown in fig 1B, the number of lymphocytes in BAL fluid correlated well with the copy number of HTLV-I proviral DNA in PBMCs of HTLV-I carriers ($r = 0.65$, $p<0.05$).

HTLV-I proviral DNA in BAL fluid and correlation with lymphocytes and T lymphocyte subsets in BAL fluid

HTLV-I proviral DNA was detected by PCR in BAL cells from all 10 AHCs examined and was not detected in BAL cells from HTLV-I seronegative controls.

The copy number of HTLV-I proviral DNA in BAL fluid was determined in 14 HTLV-I carriers (three AHCs and 11 SHCs) and in eight patients with HAM/TSP. As shown in table 4, the copy number of HTLV-I proviral DNA per 10^4 BAL cells was significantly increased in patients with HAM/TSP compared with those of HTLV-I carriers ($p<0.001$) and SHCs ($p<0.01$).

The correlation between HTLV-I proviral DNA load, lymphocyte number, and T cell subsets in BAL fluid was examined in 14 HTLV-I carriers and 10 HTLV-I carriers. The copy number of HTLV-I proviral DNA in BAL fluid correlated well with the number of lymphocytes in BAL fluid from HTLV-I carriers ($r = 0.58$, $p<0.05$, fig 2A), CD4+ cells ($r = 0.77$, $p<0.05$, fig 2B), and CD8+ cells ($r = 0.83$, $p<0.05$, fig 2C).

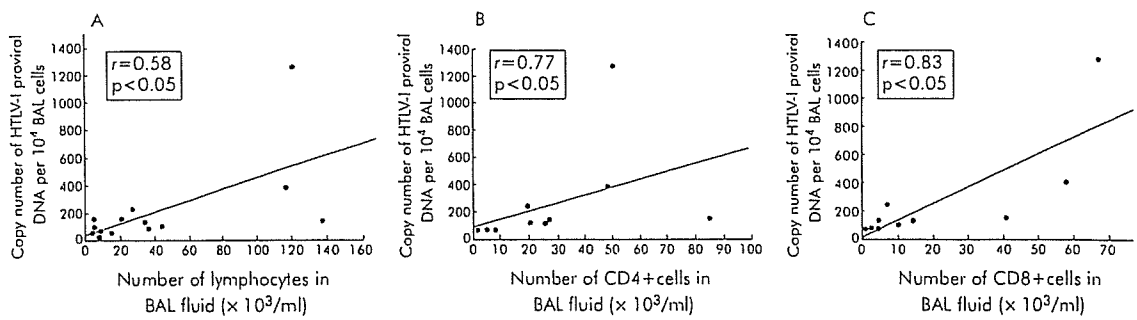

Figure 2 Correlation between (A) the number of lymphocytes in bronchoalveolar lavage (BAL) fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers; (B) the number of CD4+ cells in BAL fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers; and (C) the number of CD8+ cells in BAL fluid and the copy number of HTLV-I proviral DNA per 10^4 BAL cells from HTLV-I carriers. Statistical analyses were performed using Spearman rank correlation. HTLV-I, human T lymphotropic virus type I.

Follow up of asymptomatic HTLV-I carriers with bronchoalveolar lymphocytosis

Three AHCs (subjects 5, 10 and 13) with bronchoalveolar lymphocytosis (>18% lymphocytes in BAL fluid) were available for follow up evaluation of clinical and radiographic variables for 5–10 years. A further individual (subject 9) dropped out 1 year after the study. None of these four individuals showed overt respiratory illness and chest radiographic findings remained normal during the follow up period. They did not develop adult T cell leukaemia, HAM/TSP, or other HTLV-I associated disorders during this time.

DISCUSSION

The major findings of this study are: (1) bronchoalveolar lymphocytosis (>18% lymphocytes in BAL fluid) was observed in 11 of 30 HTLV-I carriers without HAM/TSP or HTLV-I uveitis (36.7%), although there was no significant difference between HTLV-I carriers and control subjects; (2) the number of lymphocytes in the BAL fluid of HTLV-I carriers was significantly correlated with the HTLV-I proviral DNA load in PBMCs; and (3) the HTLV-I proviral DNA load in the BAL fluid was significantly correlated with the number of lymphocytes, CD4+ cells and CD8+ cells in the BAL fluid of HTLV-I carriers.

Recent studies have indicated that immunological dysfunction related to the increased HTLV-I proviral load may be involved in the pathogenesis of HAM/TSP and HTLV-I uveitis.[13 16 19–23] However, some studies have shown that these immunological and virological findings are also present in some HTLV-I carriers including relatives of subjects with HAM/TSP and AHCs who have not developed HAM/TSP or HTLV-I uveitis.[13 16 20] The genetic background may be implicated in the HTLV-I proviral load and immunological dysfunction in HTLV-I carriers.[21 24] These reports suggest that HTLV-I carriers consist of groups of individuals of different genetic backgrounds with various amounts of HTLV-I proviral DNA in PBMCs. Our results showed that the HTLV-I proviral load is high in a subset of HTLV-I carriers without HAM/TSP or HTLV-I uveitis and that the increased HTLV-I proviral load correlates well with bronchoalveolar lymphocytosis in HTLV-I carriers. The present findings, together with those of previous studies, suggest that an increased HTLV-I proviral load may lead to certain systemic conditions including bronchoalveolar lymphocytosis in HTLV-I carriers.

In addition to the genetic background, direct or indirect mechanisms induced by inflammatory conditions may have influenced the HTLV-I proviral load in PBMCs of some SHCs with chronic inflammatory diseases of the respiratory tract in this study, as occurs in HIV-1 infection.[25]

The HTLV-I proviral load in BAL fluid appeared to be related to the proportion of lymphocytes in the BAL fluid of HTLV-I carriers, as reported previously.[26] Interestingly, the HTLV-I proviral load in the BAL fluid correlated with the number of CD8+ cells as well as with the number of CD4+ cells in HTLV-I carriers, even though CD4+ cells are thought to be preferentially infected by HTLV-I.[27] Our findings may be consistent with more recent observations of the tropism of HTLV-I to CD8+ lymphocytes.[28 29] Further investigations are needed to determine which T cell subsets are predominantly infected with HTLV-I in the lungs of HTLV-I carriers.

A few studies have described some AHCs with bronchoalveolar lymphocytosis who did not develop HAM/TSP and HTLV-I uveitis.[30 31] In the present study pulmonary involvement was subclinical in AHCs with bronchoalveolar lymphocytosis, similar to the findings of these previous studies. Furthermore, follow up studies suggested that the clinical development of bronchoalveolar lymphocytosis may be delayed in HTLV-I carriers.

HTLV-I infected lymphocyte(s) commonly exist in the lower respiratory tract of HTLV-I seropositive individuals, as shown in the PCR study of BAL cells. This suggests that factors other than the presence of HTLV-I in the lung—such as a systemic increase in the HTLV-I proviral load, as found in this study—may be necessary for excessive accumulation of lymphocytes in the lung. The mechanisms by which an increased HTLV-I proviral load affects pulmonary involvement in HTLV-I carriers remains to be fully clarified. One possible mechanism is that the increased number of HTLV-I infected cells enhances the probability that infected cells will enter the target organs,[31 32] resulting in a local inflammatory response. However, the frequency of HTLV-I specific cytotoxic T lymphocytes[22 23 33] related to the increased amount of the virus might be involved in the pathogenesis, as has been shown in lung disorders of patients with HIV infection.[25 34]

In conclusion, we have shown that pulmonary lymphocytic infiltration can occur in a subset of HTLV-I carriers who have not developed HAM/TSP or HTLV-I uveitis. This pulmonary involvement may be associated with an increased amount of HTLV-I proviral DNA in peripheral blood.

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LUNG ALERT

Some asthma genotypes may not respond to β_2 agonists

▲ Israel E, Chinchilli VM, Ford JG, *et al.* Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet* 2004;364:1505–12

This was a prospective crossover trial comparing the use of salbutamol with placebo in 78 mild asthmatics (diagnosed by chest physician, only using inhaled β agonist <56 puffs/week, FEV_1 >70%) aged 18–55 years. 50% had a genetic polymorphism resulting in homozygosity for arginine (Arg/Arg) at amino acid residue number 16 of the β_2 agonist receptor instead of glycine (Gly/Gly), as in the other half. Each patient was matched with a patient from the other group by FEV_1.

Following a 6 week run in period using a placebo metered dose inhaler (two puffs qds; rescue medication ipratropium inhaler), each pair was randomised to receive either active salbutamol (90 µg) or placebo (two puffs qds) for 16 weeks followed by an 8 week run out period using placebo and then crossed over. In the Gly/Gly group there was no change in pre-inhaler morning peak expiratory flow rate (PEFR) with placebo but an increase in PEFR with salbutamol producing a difference of 14 l/min ($p<0.05$). In the Arg/Arg group the reverse occurred with a difference of −10 l/min ($p<0.05$). This group also needed to use their ipratropium inhaler more, which did produce an increase in PEFR. Similar results were seen in FEV_1, symptom scores, and rescue inhaler use.

It appears that Gly/Gly patients respond to salbutamol while those with Arg/Arg seem to get better when salbutamol is withdrawn. It may be that the latter group actually responds to ipratropium. A longer treatment trial is needed with more patients with more severe asthma and with other genetic polymorphisms, using other β_2 agonists, to determine if reliever strategies excluding salbutamol are more suitable for Arg/Arg patients.

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Differences in viral and host genetic risk factors for development of human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis between Iranian and Japanese HTLV-1-infected individuals

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Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a neurological disease observed only in 1–2 % of infected individuals. HTLV-1 provirus load, certain HLA alleles and HTLV-1 *tax* subgroups are reported to be associated with different levels of risk for HAM/TSP in Kagoshima, Japan. Here, it was determined whether these risk factors were also valid for HTLV-1-infected individuals in Mashhad in northeastern Iran, another region of endemic HTLV-1 infection. In Iranian HTLV-1-infected individuals ($n=132$, 58 HAM/TSP patients and 74 seropositive asymptomatic carriers), although HLA-DRB1*0101 was associated with disease susceptibility in the absence of HLA-A*02 ($P=0{\cdot}038$; odds ratio $=2{\cdot}71$) as observed in Kagoshima, HLA-A*02 and HLA-Cw*08 had no effect on either the risk of developing HAM/TSP or HTLV-1 provirus load. All Iranian subjects possessed *tax* subgroup A sequences, and the protective effects of HLA-A*02 were observed only in Kagoshima subjects with *tax* subgroup B but not in those with *tax* subgroup A. Both the prevalence of HTLV-1 subgroups and the host genetic background may explain the different risks levels for HAM/TSP development in these two populations.

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INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982) is a causative agent of adult T-cell leukaemia (Hinuma *et al.*, 1981; Yoshida *et al.*, 1984) and the chronic neurodegenerative disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.*, 1985; Osame *et al.*, 1986). Only a minority of HTLV-1-infected individuals develop HAM/TSP, and most infected individuals remain healthy throughout their lives. A previous seroepidemiological survey in Kyushu Island, in southwestern Japan, where Kagoshima prefecture is located, estimated the incidence of HAM/TSP among HTLV-1-infected persons at $3{\cdot}1 \times 10^{-5}$ cases per year; assuming a lifespan of 75 years, the lifetime incidence is therefore approximately 0·25 % (Kaplan *et al.*, 1990). In HAM/TSP patients from Kagoshima, the median provirus load in peripheral blood mononuclear cells (PBMCs) is more than ten times higher than HTLV-1-seropositive asymptomatic carriers (HCs) and high provirus load is also associated with an increased risk of progression to disease (Nagai *et al.*, 1998). HTLV-1 provirus load has been correlated with progression of motor disability (Takenouchi *et al.*, 2003) and the risk of sexual transmission of HTLV-1 (Kaplan *et al.*, 1996). Thus, HTLV-1 provirus load is an important correlate of virus transmission as well as disease progression. A previous study