Ⅲ どんな検体を採取するか？

- 神経系感染症が疑われる場合には検体を採取し，病原診断を行う．
- 血液検査（血算，血沈，CRP，血液生化学検査など）と咽頭ぬぐい液・喀痰・尿・血液培養を行う．
- 各種髄膜炎・脳炎ではルーチンの髄液検査でも起因する病原体の種類を推察するにあたってある程度のところまで絞り込める．その際，大切なポイントは増加している白血球の種類（多核球か，単核球か）と糖の低下の有無である（必ず同時に血糖も測定しておくことが必要である）．ただし，すでに抗生物質などによる治療が開始されている例では典型的な所見にならないこともありうるので注意を要する．
- 採取された髄液は遠沈し，グラム染色，抗酸菌染色，墨汁染色を行うとともに，培養に提出する．すでに抗生物質が投与されている例や結核性あるいは真菌性の症例では必ずしも検出率は高くないということを認識しておくべきである．
- 細菌の菌体抗原を同定することができるキット（肺炎球菌，髄膜炎菌，インフルエンザ菌，B 群溶連菌など）やクリプトコッカスの莢膜多糖抗原を検出するキットが市販されていて，髄液にても検査可能である．
- 病原体の遺伝子を増幅して検出する感度と特異性の高い検査法として polymerase chain reaction（PCR）法がある．最近では，感度と特異性の高い nested PCR 法も開発されている．しかし，プライマーの設定，検出感度，検査時期などの問題点がある．また，false negative や false positive の結果に注意が必要である．
- PCR 法において髄液でも結核菌，クリプトコッカス，単純ヘルペスウイルス（1 型，2 型），水痘・帯状疱疹ウイルス，EB ウイルス，サトメガロウイルス，日本脳炎ウイルス，エンテロウイルスなどで検査可能である．
- 多くの subtype を有するエンテロウイルスではウイルス RNA の共通の配列の部分を増幅する RT－PCR 法によって検出することが可能になっている．
- ウイルス性髄膜炎・脳炎の場合，血清ウイルス抗体価が 2 週間間隔で 4 倍以上の上昇があれば，起因ウイルスの可能性が高い．ただし，エンテ

ロウイルスの場合，多種の血清型があり同定不能の例が多い．

- 種々のウイルス抗体の検査法として，従来の補体結合抗体法や中和抗体法よりも非常に感度の高いELISA法が開発され，発病初期の診断に有用である．
- 髄液ウイルス抗体価のELISA法による測定は病原診断に大いに有用である．本来，髄腔内抗体産生を反映するものであるが，血液脳関門の破綻による血清抗体の髄腔内へのleakageの可能性を考えて，抗体価比（血清抗体価/髄液抗体価）あるいは抗体価指数（髄液抗体価/血清抗体価÷髄液アルブミン濃度/血清アルブミン濃度）を算出しなければならない．これらの値はすべてのウイルスで一定ではないが，ちなみに単純ヘルペス脳炎では前者が20以下，後者が1.91以上とされている．
- SSPE，PMLの髄液では特徴的所見はなく正常のことが多い．しかし，SSPEの場合，IgGが著明に高く麻疹ウイルス抗体価も高値であるとともに，オリゴクローナルバンドが出現することがある．いずれの疾患でも麻疹ウイルス，JCウイルスの検出のためのPCR法が開発されている．
- プリオン病の髄液も特徴的所見はなく，まず異常がないと考えてよい．しかし，14-3-3蛋白やニューロン特異的エノラーゼ（NSE）あるいは最近の報告では総tau蛋白が発病初期より増加することがあり，早期診断の参考になる．
- 遺伝性プリオン病が疑われる場合，末梢血白血球のプリオン蛋白遺伝子解析によって点変異などの遺伝子異常の有無を検索する．
- マイコプラズマによる髄膜炎・脳炎が疑われる場合はマイコプラズマ血球凝集抗体価および寒冷凝集素価の上昇が参考になる．

Ⅳ 診断がつかない場合に考えること

- 髄膜炎・脳炎の場合，治療開始の遅れは死亡率，後遺症など予後に大きく影響する．したがって，病原診断に至らなくても早期に可能性の考えられるものに対する治療を開始することが望ましい．特に，ウイルス性脳炎が疑われる場合，散発性脳炎の中では単純ヘルペス脳炎の頻度が最も高いこと，また本脳炎に対する治療薬（アシクロビル，ビラダビン）の安全性の高さなどより考えて早期に治療を開始することが望ましい．

後で病原診断ができる可能性があるため，血清，髄液は必ず来院時と回復期のペアで保存しておくことが重要である．

感染病原体以外で惹起される髄膜炎・脳炎様の病態があり，急性散在性脳脊髄炎，神経ベーチェット病，各種膠原病あるいはサルコイドーシスによる脳障害，癌性髄膜炎，ライ症候群なども考慮する必要がある．

補足説明 HTLV-I関連脊髄症について

ヒトレトロウイルスの一つであるHTLV-I（human T-lymphotropic virus type-I）感染によってひき起こされる慢性脊髄炎である．臨床的には排尿障害を伴った緩徐進行性の痙性対麻痺を示す．本疾患は1986年Osameらによって発見された．西南日本を中心に多発しているが，原因不明の痙性対麻痺の症例では抗HTLV-I抗体を測定してみる必要がある．

（中村龍文）

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Chapter II

The Role of HTLV-I-Infected CD4$^+$ T Cells as Activated Th1 Cells in the Immunopathogenesis of HTLV-I-Associated Myelopathy /Tropical Spastic Paraparesis

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Abstract

Chronic myelitis, characterized by perivascular cuffing and parenchymal infiltration of lymphocytes, is the primary neuropathological feature of human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the exact cellular and molecular events underlying the induction of chronic inflammation in the spinal cord by HTLV-I are still unclear, long-standing bystander mechanisms, such as the destruction of surrounding nervous tissue by the interaction between HTLV-I-infected CD4$^+$ T cells and HTLV-I-specific cytotoxic T cells in the spinal cord, are believed to play an important role in the immunopathogenesis of HAM/TSP. As the first step in this scenario, exaggerated transmigration activity of HTLV-I-infected CD4$^+$ T cells to the spinal cord is a most important prerequisite. In this regard, we demonstrated that HTLV-I-infected CD4$^+$ T cells of HAM/TSP patients,

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compared to anti-HTLV-I-seropositive carriers, have heightened transmigrating activity to tissues based on the up-regulation of matrix metalloproteinase expression. Is Th1 or Th2 activation involved in increased transmigrating activity of HTLV-I-infected $CD4^+$ T cells in HAM/TSP patients? The facts, such as increased levels of Th1 cytokines expression with Th1 cytokine-related signaling molecules in the peripheral blood T cells of HAM/TSP patients, indicated that the immunological status of HAM/TSP is undoubtedly based on Th1 activation. Most importantly, HTLV-I-infected $CD4^+$ T cells are concentrated in the cell population having transmigration potential to the tissues with Th1 characters. In studying the signaling pathways in the regulation of Th1 activation, we found that p38 mitogen-activated protein kinase (p38 MAPK) was involved. In addition, our data indicated that activation of this signaling might be involved in the high HTLV-I proviral load in HAM/TSP patients. However, the transmigrated HTLV-I-infected $CD4^+$ Th1 cells must settle locally over a long period for the establishment of long-standing perpetuation of a chronic inflammatory state. Our study revealed that the peripheral blood $CD4^+$ T cells of HAM/TSP patients are resistant to apoptosis triggered through mitochondrial death pathway by up-regulation of Bcl-xL expression. Collectively, these findings strongly suggest that HTLV-I-infected $CD4^+$ T cells of HAM/TSP patients, having the characteristics of activated Th1 based on activation of p38 MAPK signaling, concomitant with up-regulated Bcl-xL expression, have the potential to trigger the neuropathological process in the spinal cord.

Introduction

Human T lymphotropic virus type I (HTLV-I), a member of the Oncovirinae subfamily in the family Retroviridae, is the causative agent of two clinically disparate diseases, namely adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1, 2]. With the discovery of HAM/TSP, it has become evident that HTLV-I has the remarkable capacity for aggressive lymphoproliferation and profound chronic inflammation. However, the precise pathophysiologic mechanisms underlying these entirely different clinical conditions caused by HTLV-I are poorly understood. Although high HTLV-I proviral load in the peripheral blood is an important factor in the development of HAM/TSP [3, 4], it is unclear how such a situation is induced. In addition, it is not clear why only a very small proportion of HTLV-I-infected individuals develop either of these HTLV-I-associated diseases.

As the name indicates, the principal clinical manifestation of HAM/TSP is spastic paraplegia or paraparesis, characterized by a slowly progressive course of prominent upper motor neuron involvement and mild sensory deficit with sphincteric disturbance [5-7]. Not unexpectedly, the primary neuropathological feature of HAM/TSP is chronic inflammation of the spinal cord, mainly the lower thoracic cord, characterized by perivascular cuffing and parenchymal infiltration of mononuclear cells [8, 9]. Immunohistochemical studies have revealed that $CD4^+$ and $CD8^+$ T cells and macrophages infiltrate active, chronically inflammed lesions in the spinal cords of HAM/TSP patients with short-lived illness. Also, apoptosis was found in $CD45RO^+$ T cells, the main target of HTLV-I infection, with distribution of $TIA\text{-}1^+$ $CD8^+$ T cells in the lesions [10, 11]. These findings suggest that interactions between HTLV-I-infected $CD4^+$ T cells and HTLV-I-specific $CD8^+$ cytotoxic T cells in the spinal cord are operative in the immunopathological process leading to HAM/TSP. Moreover, bystander mechanisms, such as destruction of the surrounding nervous tissue induced by the interactions

between both T-cell populations, are probably critical [12]. Finally, the fact that anti-apoptotic proteins are also expressed in both CD4$^+$ and CD8$^+$ T-cell populations [11] might explain the long-standing perpetuation of a chronic inflammatory state in the spinal cord lesions of HAM/TSP patients.

HTLV-I-infected CD4$^+$ T cells are the first responders in the immunopathogenesis of HAM/TSP. That is, exaggerated transmigration activity of HTLV-I-infected CD4$^+$ T cells to the spinal cord is a most important prerequisite for the establishment of chronic myelitis. In this review, we will analyze the immunological abnormalities in the peripheral blood CD4$^+$ T cells of HAM/TSP patients, such as transmigration, cytokine expression, anti-apoptotic protein expression, in comparison with anti-HTLV-I-seropositive carriers, and discuss the importance of HTLV-I-infected CD4$^+$ T cells as activated Th1 cells in the development of HAM/TSP.

1. Exaggerated Transmigrating Activity of Peripheral Blood HTLV-I-Infected CD4$^+$ T Cells in HAM/TSP Patients

We previously reported significantly increased adherence of peripheral blood T cells to human endothelial cells (EC) in HAM/TSP patients, compared to T cells from anti-HTLV-I-seropositive carriers and anti-HTLV-I-seronegative controls [13], as evidenced by the adherence of activated CD4$^+$ T cells (rather than CD8$^+$ T cells) to EC with heightened lymphocyte function antigen-1 (LFA-1) expression [14]. Subsequently, we identified the cells transmigrating through EC in HAM/TSP patients as activated CD4$^+$ T cells [15]. Although these findings indicate that the peripheral blood CD4$^+$ T cells of HAM/TSP patients have a prominent potential to transmigrate through EC, theses cells must penetrate and transverse the subendothelial basement membrane after trasmigrating through the EC for the invasion of tissues. Therefore, we investigated the transmigrating activity of peripheral blood CD4$^+$ T cells of HAM/TSP patients through a reconstituted basement membrane (RBM) [16], using Transwell cell-culture chambers [17]. Either peripheral blood CD4$^+$ or CD8$^+$ T cells were applied to the upper compartment of the Transwell inserts, which were polyvinylpyrrolidone-free polycarbonate filters of 8-μm pore size pre-coated with laminin on the lower surface and RBM (Matrigel) on the upper surface, respectively. After a 6-hour incubation period, the transmigrating cells in the lower chamber were counted by trypan blue staining. As shown in figure 1a, the percentage of transmigrating CD4$^+$ T cells was significantly higher than that of CD8$^+$ T cells in patients with HAM/TSP (n = 18). By contrast, no significant difference was observed in the percentage of transmigrating CD4$^+$ and CD8$^+$ T cells in controls (n = 16, including 8 anti-HTLV-I-seropositive carriers and 8 anti-HTLV-I-seronegative individuals) (figure 1b). When only transmigrated CD4$^+$ T cells were compared, the percentage of transmigrating cells in HAM/TSP patients (range, 3.0% - 40.1%; mean ± SD, 16.5% ± 12.0%) was significantly higher than that in either anti-HTLV-I-seropositive carriers (range, 0.5% - 5.0%; mean ± SD, 2.8% ± 1.8%) or anti-HTLV-I-seronegative controls (range, 0.3% - 6.9%; mean ± SD, 2.8% ± 2.6%), respectively (figure 2). Since the lower surface of the polycarbonate filters of the Transwell inserts was coated with a high concentration of laminin, the ligand of very late activating antigen-6 (VLA-6) [18], there was some possibility that the

increased transmigration activity of $CD4^+$ T cells in HAM/TSP patients might be related to the increased numbers of VLA-6^+ $CD4^+$ T cells. However, no significant differences were noted in the percentage of VLA-6^+ $CD4^+$ T cells between HAM/TSP patients and controls (data not shown).

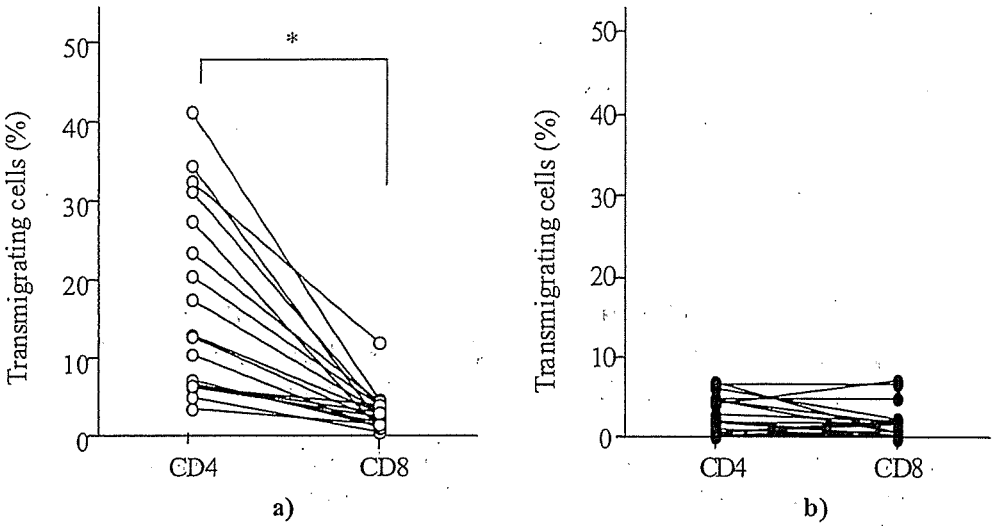


Figure 1. Percentage of transmigrating $CD4^+$ and $CD8^+$ T cells. The percentage of transmigrating cells was calculated using the formula, transmigrating cells/applied cells x 100 (%). a) The percentage of transmigrating $CD4^+$ T cells, compared to transmigrating $CD8^+$ T cells, was significantly higher in 18 HAM/TSP patients. b) In 16 controls (including 8 anti-HTLV-I-seropositive carriers), there was no significant difference between the percentage of transmigrating $CD4^+$ and $CD8^+$ T cells. $*p < 0.01$. Wilcoxon single-rank test was used for statistical analysis. Quotation from Ref. 16.

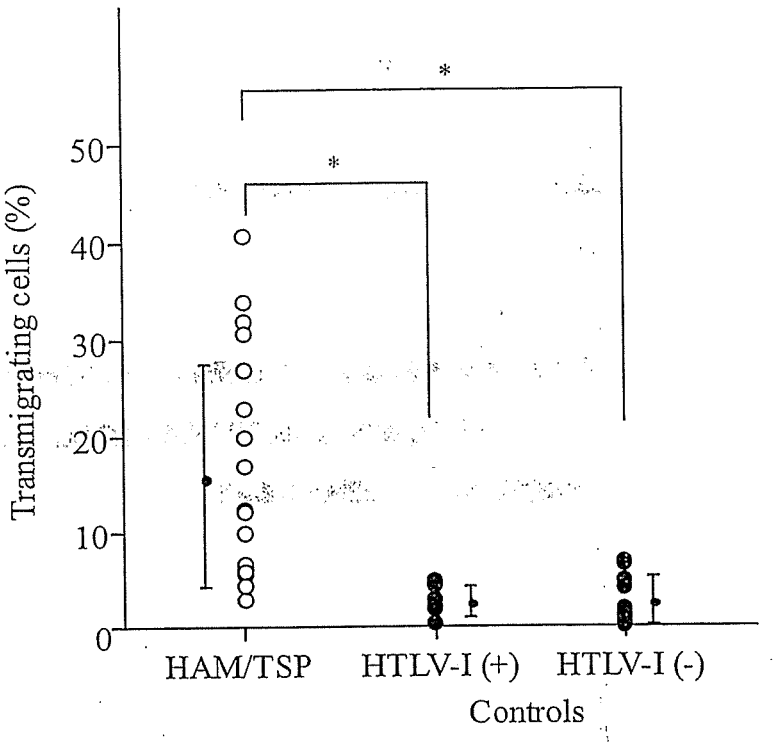


Figure 2. Comparison of the percentage of transmigrating CD4$^+$ T cells between HAM/TSP patients and controls. CD4$^+$ T cells of HAM/TSP patients (open circles) were significantly increased in comparison with either anti-HTLV-I-seropositive carriers (HTLV-I (+)) or anti-HTLV-I-seronegative controls (HTLV-I (-)) (closed circles). *$p < 0.01$; closed circle and bar, mean ± SD. Mann-Whitney U-test was used for statistical analysis. Quotation from Ref. 16.

Next, in analyzing the HTLV-I proviral load in transmigrated and non-transmigrated CD4$^+$ T cells by competitive PCR, using oligonucleotide primers of the HTLV-I pX gene, in 9 HAM/TSP patients and in 5 anti-HTLV-I-seropositive carriers, we found that the HTLV-I proviral load in transmigrated CD4$^+$ T cells from HAM/TSP patients was two- to eight-fold higher than in non-transmigrated CD4$^+$ T cells (figure 3). By contrast, no significant difference was found in HTLV-I proviral load in transmigrated and non-transmigrated CD4$^+$ T cells from anti-HTLV-I-seropositive carriers, although the copy numbers of HTLV-I provirus were very low (figure 3).

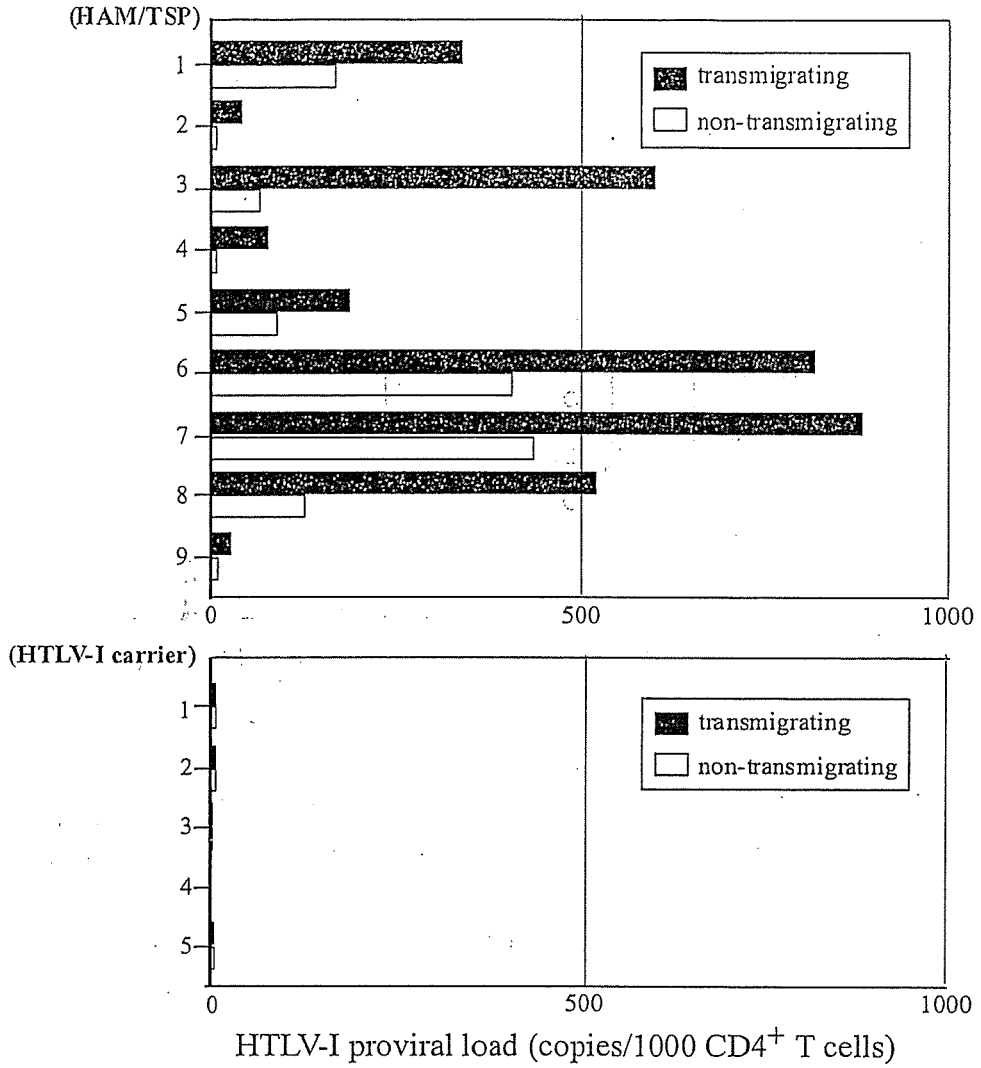


Figure 3. Quantitative analysis of HTLV-I proviral DNA in $CD4^+$ T cells. HTLV-I proviral load was compared between transmigrating and non-transmigrating $CD4^+$ T cells. HTLV-I proviral DNA, quantitated in the transmigrating $CD4^+$ T cells from the peripheral blood of 9 HAM/TSP patients, was higher than in non-transmigrating $CD4^+$ T cells. No significant difference in HTLV-I proviral load was found among 5 anti-HTLV-I-seropositive carriers (HTLV-I carrier). Quotation from Ref. 16.

Our data clearly demonstrated that $CD4^+$ T cells of HAM/TSP patients have significantly increased transmigrating activity through RBM, compared to that of anti-HTLV-I-seropositive carriers and HTLV-I-seronegative individuals. Moreover, the HTLV-I proviral load in transmigrated $CD4^+$ T cells of HAM/TSP patients was increased significantly as compared to that in non-transmigrated $CD4^+$ T cells. These findings strongly suggest that HTLV-I-infected $CD4^+$ T cells in HAM/TSP patients have exaggerated transmigration activity to extravasate from the blood to central nervous system tissues.

The findings mentioned above, such as the heightened adherence to EC and exaggerated transmigrating activity through EC and RBM of peripheral blood $CD4^+$ T cells, particularly

HTLV-I-infected CD4$^+$ T cells, from HAM/TSP patients giving rise to the phenomenon derive from *in vitro* studies. In this regard, although the rolling phenomenon is necessary *in vivo* for the initial attachment of T cells to EC before the adhesion of T cells to EC [19], the selectin family of adhesion molecules, including E-selectin and L-selectin, plays an important role [20]. We previously reported that serum levels of both soluble forms of E- and L-selectin were significantly elevated in HAM/TSP patients [21]. The degree of lymphocyte adherence to EC and the activated state of EC *in vivo* are reflected in a soluble form of both selectins in sera because soluble forms of E- and L-selectin are derived from activated EC membrane and cell surface of lymphocytes following attachment to EC, respectively [22, 23]. Therefore, elevated levels of soluble forms of selectins in HAM/TSP patients suggest that the peripheral blood CD4$^+$ T cells actively adhere to EC and transmigrate through EC and BM to the tissues *in vivo* in HAM/TSP patients. In addition, the possibility of active transmigration of peripheral blood CD4$^+$ T cells of HAM/TSP patients to the tissues *in vivo* might be also supported by the existence of other HTLV-I-associated diseases including Sjögren's syndrome [24], alveolitis [25] and uveitis [26], each of which occasionally occurs in conjunction with HAM/TSP. Indeed, we previously reported T- lymphocytic infiltration in labial salivary glands of all HAM/TSP patients examined and a very high frequency of Sjögren's syndrome in HAM/TSP patients [27]. We also reported a HAM/TSP patient with multi-organ inflammatory diseases, including Sjögren's syndrome, uveitis and interstitial cystitis, who had a high HTLV-I proviral load in the peripheral blood, suggesting that increased numbers of HTLV-I-infected cells might potentially induce systemic inflammation in several organs [28].

Overall, our findings indicate that peripheral blood HTLV-I-infected CD4$^+$ T cells from HAM/TSP patients exhibit highly invasive activities to extravasate from the blood to tissues. These activities presumably serve to trigger the first steps of the pathogenetic process in the spinal cords of HAM/TSP patients.

2. Mediators that Induce the Exaggerated Transmigrating Activities of Peripheral Blood HTLV-I-Infected CD4$^+$ T Cells in HAM/TSP Patients

Matrix metalloproteinases (MMPs) are important mediators, which play a critical role in the transmigration of T cells to tissues and the degradation of the extracellular matrix [29]. Among these MMPs, gelatinase, such as MMP-2 and MMP-9, cleave native collagen types IV and V, gelatine, fibronectin and laminin [30], which are the main components of the vascular endothelium and basement membrane. In the first step of T-cell transmigration into tissues, the interaction between each adhesion molecule, such as the integrin family, and the vascular EC or the vascular BM, subsequently followed by the induction of MMPs, is a very important process. To clarify the role of mediators in the induction of activated transmigrating activity of HTLV-I-infected CD4$^+$ T cells of HAM/TSP patients, we investigated MMP-2 in the peripheral blood T cells of HAM/TSP patients after their contact to vascular cell adhesion molucule-1 (VCAM-1), which is a ligand for the $\alpha 4\beta 1$ integrin known as very late activating antigen-4 (VLA-4) [31]. After the peripheral blood T cells of HAM/TSP patients were co-

cultured with or without VCAM-1 (+) cells for 12 hrs, only T cells were collected. After that, the collected T cells were cultured in serum-free conditions for 6 hrs and the culture supernatant was collected and designated as T sup. As shown in gelatin zymography (figure 4a), the gelatinolytic activity of 72-kDa gelatinase (MMP-2) in T sup was higher when T cells were co-cultured with VCAM-1 (+) cells compared to T sup of cultured T cells alone in 5 HAM/TSP patients than 5 control patients. As shown in figure 4a, 67-kDa gelatinase, which is the active form of 72-kDa gelatinase (MMP-2), was detected in only T sup derived from T cells of 4 of 5 HAM/TSP patients co-cultured with VCAM-1 (+) cells but not in T sup derived from T cells of all 5 control patients. In addition, anti-MMP-2 immunoblot analysis of samples from 3 HAM/TSP patients showed that the high gelatinolytic activity of MMP-2 was due to increased production of MMP-2 protein (figure 4b). For analysis of gelatinolytic activity of MMP-2, gels were photographed and scanned, then imported into Adobe Photoshop software and densitometric analysis was performed using the NIH Image Analysis software. The incremental ratio (IR) of gelatinolytic activity of MMP-2 was determined as follows: IR = densitometric counts in each T sup when co-cultured with VCAM-1 (+) cells / densitometric counts in each T sup when T cells were cultured alone. When we compared the IR among HAM/TSP patients, anti-HTLV-I-seropositive carriers and anti-HTLV-I-negative controls, the IR in HAM/TSP patients was significantly higher than that in anti-HTLV-I-seropositive carriers and in anti-HTLV-I-negative controls (figure 5). Next, in order to investigate whether the mechanisms of the induction of MMP-2 in T cells are really based on VCAM-1/VLA-4 interaction, we pretreated VCAM-1 (+) cells with anti-VCAM-1 blocking monoclonal antibody before co-culture with T cells in 5 HAM/TSP patients. In this case, the IR was determined as follows: IR = densitometric counts in each T sup when cultured with VCAM-1 (+) cells pretreated with anti-VCAM-1 blocking monoclonal antibody or control (irrelevant) monoclonal antibody / densitometric counts in each T sup when T cells were cultured alone. As shown in figure 6, although the IR of T sup of T cells co-cultured with VCAM-1 (+) cells pretreated by control monoclonal antibody were similar to the IR of T sup derived from T cells co-cultured with non-pretreated VCAM-1 (+) cells, the IR of T sup from T cells co-cultured with VCAM-1 (+) cells pretreated by anti-VCAM-1 blocking monoclonal antibody were significantly reduced, indicating that VCAM-1/VLA-4 interaction plays an important role in up-regulation of gelatinolytic activity of MMP-2 in T cells of HAM/TSP patients. As far as we compared the percentage of VLA-4^+ cells in cultured T cells from HAM/TSP patients with those of control patients, no significant differences were found between both groups.

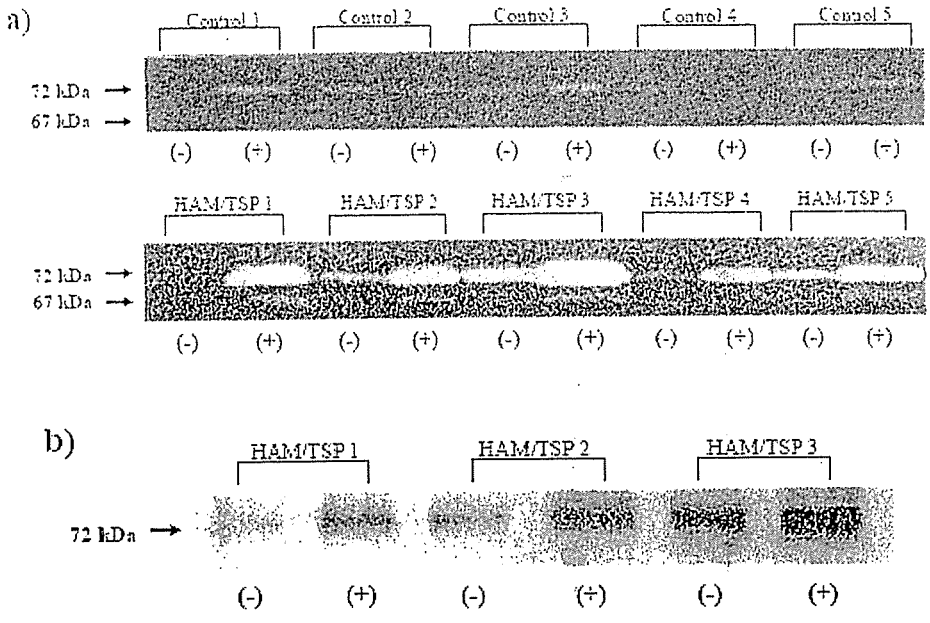


Figure 4. Representative gelatin zymography of gelatinolytic activity of MMP-2 (a) and immunoblot of MMP-2 protein (b) in T sup after co-culture with VCAM-1 (+) or after culture of T cells alone (-). (a) Gelatinolytic activity of MMP-2 (72-kDa gelatinase) in T sup was higher when T cells were co-cultured with VCAM-1 (+) cells compared to T sup from cultures of T cells alone in five HAM/TSP patients than in five controls. Gelatinolytic activity of 67-kDa gelatinase, which is the active form of 72-kDa gelatinase (MMP-2), was detected in only T sup derived from T cells of four of five HAM/TSP patients co-cultured with VCAM-1 (+) cells. (b) Immunoblot analysis of immunoprecipitates of T sup of three HAM/TSP patients indicating an up-regulated state of MMP-2 protein production. Arrow indicates MMP-2 (72-kDa gelatinase). Quotation from Ref. 31.

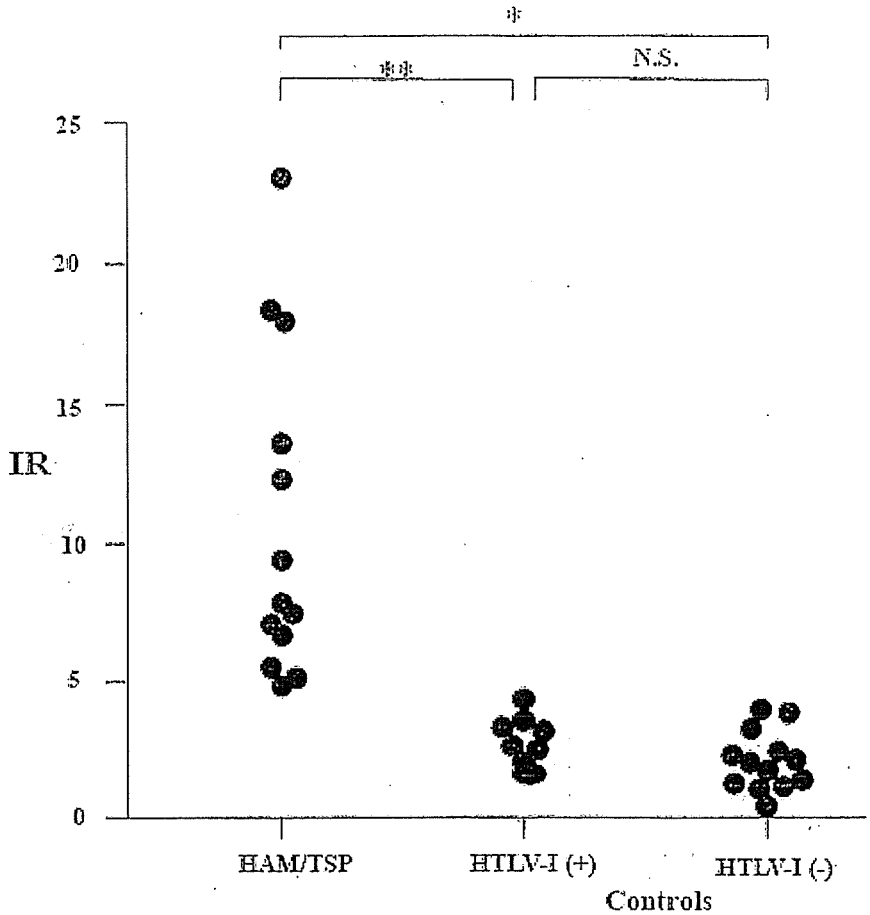


Figure 5. Comparison of IR of gelationolytic activity of MMP-2 (72-kDa gelatinase) in T sup of co-cultured T cells with VCAM-1 (+) cells or T sup derived from cultures of T cells alone between HAM/TSP and controls. IR of gelatinolytic activity of MMP-2 was determined as follows: IR = densitometric counts in each T sup when cultured withVCAM-1 (+) cells / densitometric counts in each T sup when cultured T cell alone. IR is significantly higher in HAM/TSP patients than in controls including anti-HTLV-I-seropositive carriers. HTLV-I (+): anti-HTLV-I-seropositive carriers; HTLV-I (-): anti-HTLV-I-seronegative controls. $*p < 0.0001$, $**p = 0.0002$, N.S.; not significant. Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 31.

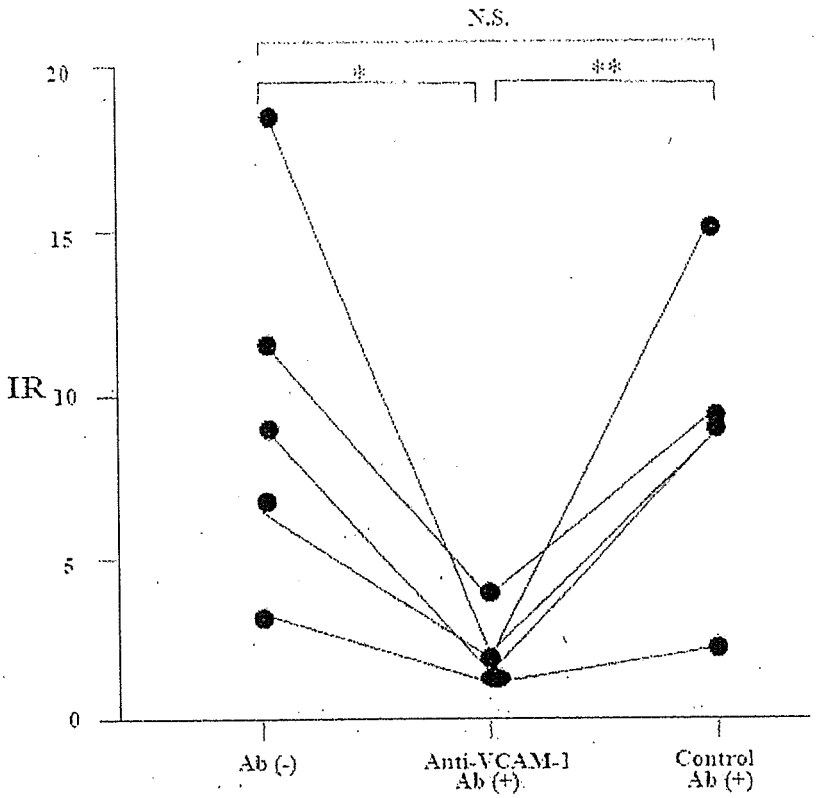


Figure 6. Effect of pretreatment of VCAM-1 (+) cells with anti-VCAM-1 monoclonal antibody on gelatinolytic activity of MMP-2 (72-kDa gelatinase) in T sup. IR of gelatinolytic activity of MMP-2 was determined as follows: IR = densitometric counts in each T sup of T cells co-cultured with VCAM-1 (+) cells or VCAM-1 (+) cells pretreated with anti-VCAM-1 monoclonal antibody or control monoclonal antibody / densitometric counts in each T sup of cultured T cell alone. IR in T sup of T cells co-cultured with VCAM-1 (+) cells pretreated with anti-VCAM-1 monoclonal antibody was significantly reduced, compared to IR of T sup derived from T cells co-cultured with VCAM-1 (+) cells pretreated with control antibody or non-pretreated VCAM-1 (+) cells. Ab (-): no treatment; Anti-VCAM-1 Ab (+): pretreatment with anti-VCAM-1 antibody; Control Ab (+): pretreatment with control antibody. *p = 0.0324, **p = 0.0237, N.S.; not significant. Wilcoxon single-rank test was used for statistical analysis. Quotation from Ref. 31.

In this study, we demonstrated that VCAM-1/VLA-4 interaction can induce up-regulation of MMP-2 activity in the peripheral blood T cells of HAM/TSP patients based on up-regulation of its expression. We previously reported increased adherence of peripheral blood CD4$^+$ T cells to human EC in HAM/TSP patients, as described in section 1 [14]. Therefore, it is suggested that the adherence of the peripheral blood CD4$^+$ T cells to EC itself triggers MMPs activation in these cells for the transmigration to the tissues in HAM/TSP patients. Although our data presented in this section are based on adhesion molecule/ligand interactions, we previously showed that the gelatinolytic activity of MMP-9 in the peripheral blood CD4$^+$ T cell of HAM/TSP patients, treated with phorbol myristate acetate (PMA), was more up-regulated than that of control patients despite no significant difference in the condition without the treatment with PMA between both groups (unpublished data). Thus, the peripheral blood CD4$^+$ T cells of

HAM/TSP patients might have the potential to be able to easily induce MMPs activation by triggering lymphocyte activation.

The immunopathological analysis of spinal cord lesions in HAM/TSP patients revealed that infiltrating mononuclear cells expressed VLA-4 concomitantly with highly expressed VCAM-1 on vascular endothelium, and MMP-2 and MMP-9 are also expressed on infiltrating mononuclear cells with disruption of vascular endothelium in chronic active lesions [32, 33]. Therefore, our *in vitro* data are supported by these histopathological findings in spinal cords of HAM/TSP patients. In addition, the importance of MMP in the transmigration of T cells, by the degradation of the extracellular matrix, is also supported by the finding that the transmigration of the peripheral blood $CD4^+$ T cells of HAM/TSP patients was significantly inhibited by the selective MMP inhibitor, *N*-biphenyl sulfonyl-phenylalanine hydroxamic acid (BPHA), in an *in vitro* transmigration assay system [34].

The exact mechanisms by which such an activated status of peripheral blood $CD4^+$ T cells of HAM/TSP patients [35, 36], induces an up-regulated state of MMP expression after activation, such as the cell adherence by adhesion molecule/ligand interaction and T-cell stimulator, remain unknown. At least the MMP-2 gene promoter seems not to be influenced by HTLV-I tax gene, since it contains neither TPA-responsive elements or NF-κB elements nor other tax-responsive elements [37]. Therefore, up-regulation of MMP-2 expression by VCAM-1/VLA-4 interaction might not be directly based on HTLV-I infection itself in HAM/TSP patients but on the altered intracellular signal transduction or cytokine expression in the peripheral blood $CD4^+$ T cells, particularly HTLV-I-infected T cells, of HAM/TSP patients. On the other hand, the MMP-9 gene promoter contains NF-kB, SP-1 and AP-1 responsive elements [38]. Therefore, HTLV-I tax can activate the MMP-9 promoter and induce MMP-9 expression in HTLV-I-infected T cells through the action of these transcription factors [39]. In this regard, it has been reported recently that the longer d(CA) repeat alleles in MMP-9 promoter polymorphism were more frequently observed in HAM/TSP patients than in HTLV-I seropositve asymptomatic carriers, suggesting that the longer alleles are involved in the up-regulated MMP-9 expression observed in HAM/TSP patients [40].

Aminopeptidase-N (AP-N), which is a widely distributed transmembrane ectoenzyme in mammalian cells [41, 42], is another proteases that degradates extracellular matrix. We previously reported that AP-N activity in peripheral blood T cells of HAM/TSP patients was increased significantly, as compared to that of controls [16]. AP-N, like MMP, has the ability to degrade type IV collagen, which is one of the main components of the basement membrane. As such, both proteinases might be involved in not only the transmigration to the tissues but also the tissue destruction, such as the damage of the blood-brain barrier [43, 44].

Our findings indicate that VCAM-1/VLA-4 interaction can induce up-regulation of MMP-2 in peripheral blood T cells of HAM/TSP patients, compared to control patients including anti-HTLV-I-seropositive carriers. However, in considering the increased transmigrating activity of peripheral blood $CD4^+$ T cells of HAM/TSP patients, HTLV-I-infected $CD4^+$ T cells of HAM/TSP patients seem to easily transmigrate to the spinal cords by using increased activity of MMP-2, MMP-9 and AP-N as the mediators

3. Th1 or Th2 Activation Status in the Increased Transmigrating Activity of HTLV-I-Infected CD4$^+$ T Cells in HAM/TSP Patients

Helper T cells are generally divided into two distinct populations, Th1 and Th2, based on their cytokine-production profiles [45, 46]. The former produce, among others, interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin, while the latter produce interleukin-4, interleukin-5, interleukin-6 and interleukin-10 (IL-4, IL-5, IL-6 and IL-10). Although interleukin-12 (IL-12) is secreted, as both the biologically active form (p70 heterodimer) and the biologically inactive form (p40 chains) [47], mainly from antigen-presenting cells, such as macrophages and dendritic cells, IL-12 induces IFN-γ production from Th1 cells [48]. Thus, IL-12 and IFN-γ are very important cytokines that regulate the differentiation to the Th1 type and induce cell-mediated immunity [45]. On the other hand, the differentiation to the Th2 type that induces humoral immunity is regulated by IL-4 and IL-10 [49, 50]. Of these cytokines, IL-4 is very important for the differentiation to the Th2 type [46]. The importance of an imbalance between Th1 and Th2 in the pathogenesis of autoimmune or inflammatory diseases has been reported [49-51].

We have previously reported simultaneous up-regulation of mRNA expression of inflammatory cytokines, such as IFN-γ, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-1α in the peripheral blood lymphocytes of HAM/TSP patients [52]. Subsequently, we demonstrated that the spontaneous production of inflammatory cytokines, such as IFN-γ, TNF-α, and GM-CSF, but not IL-4, increased simultaneously in cultured the peripheral blood CD4$^+$, but not CD8$^+$, T cells [53]. These findings suggested that the Th1 cell population rather than the Th2 cell population predominates in the peripheral blood CD4$^+$ T cells of HAM/TSP patients. Therefore, to clarify the immunological balance in HAM/TSP patients, we measured IL-12 and IFN-γ which play an important role in the Th1 state, and IL-4 and IL-10 as Th2 cytokine, in both the sera and cerebrospinal fluid (CSF) and compared the results to those for the controls including anti-HTLV-I-seropositive carriers [54].

As shown in figure 7a, serum levels of total IL-12 in 22 HAM/TSP patients (mean ± SE; 128.68 ± 12.21 pg/mL) were significantly higher than in 22 controls including 9 anti-HTLV-I-seropositive carriers (mean ± SE; 72.36 ± 8.80 pg/mL). In addition, serum p70 heterodimer, which is a biologically active form of IL-12, was detected in 9 and 2 of 22 HAM/TSP patients and 22 controls, respectively. Serum levels of p70 heterodimer in HAM/TSP patients (mean ± SE; 3.20 ± 0.98 pg/mL) were also significantly higher than in controls (mean ± SE; 0.36 ± 0.25 pg/mL) (figure 7b). However, CSF levels of total IL-12 in 15 HAM/TSP patients were the same as in 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 16.53 ± 4.41 and 9.46 ± 3.92 pg/mL, respectively) (figure 7c). In addition, CSF p70 heterodimer was detected in only one of 15 HAM/TSP patients and not detected in 13 controls (data not shown). On the other hand, serum IFN-γ levels in 22 HAM/TSP patients (mean ± SE; 0.33 ± 0.09 IU/mL) were significantly higher than in 22 controls, including 9 anti-HTLV-I-seropositive carriers (mean ± SE; 0.20 ± 0.10 IU/mL) (figure 8a). Finally, as shown in figure 8b, CSF IFN-γ levels were also significantly higher in 15 HAM/TSP patients (mean ± SE; 0.40 ± 0.07

IU/mL) than in 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 0.15 ± 0.04 IU/mL).

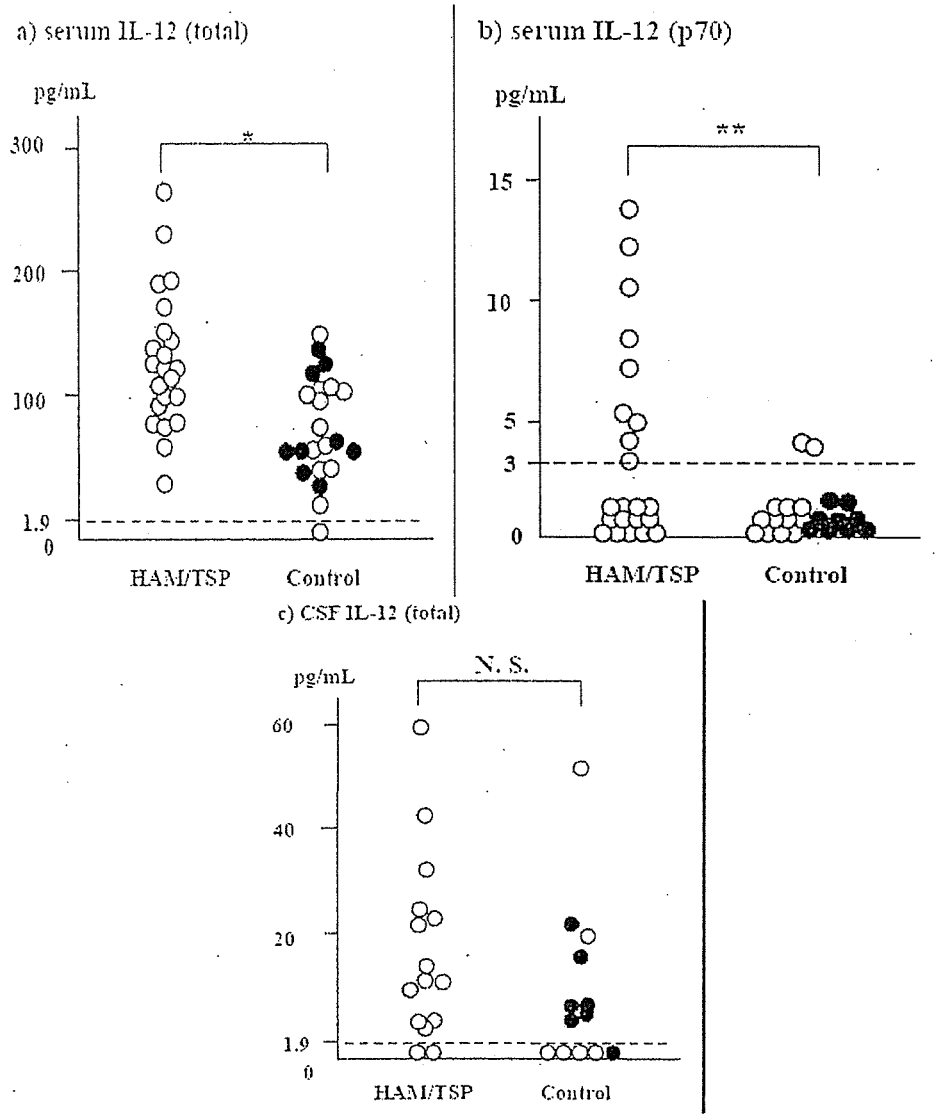


Figure 7. Serum levels of total IL-12 (p70 heterodimer plus p40 chains) and IL-12 (p70 heterodimer) and cerebrospinal fluid (CSF) levels of total IL-12. a) Serum total IL-12: serum levels of total IL-12 in HAM/TSP patients were higher than in the controls (mean ± SE; 128.68 ± 12.21 and 72.36 ± 8.80 pg/mL) (*p = 0.0011). b) Serum p70 heterodimer: serum levels of p70 heterodimer in HAM/TSP patients were higher than in the controls (mean ± SE; 3.20 ± 0.98 and 0.36 ± 0.25 pg/mL) (**p = 0.0095). c) CSF total IL-12: CSF levels of total IL-12 of HAM/TSP patients were the same as those of the controls (mean ± SE; 16.53 ± 4.41 and 9.46 ± 3.92 pg/mL) (N.S.: not significant). Minimal measurable levels (indicated by dotted lines): 1.9 pg/mL for total IL-12, 3.0 pg/mL for p70 heterodimer. HAM: HAM/TSP patients (serum: n=22; CSF: n=15), Controls: Anti-HTLV-I-seropositive carriers (closed circle) (serum: n=9; CSF: n=7) and anti-HTLV-I-seronegative controls (serum: n=13; CSF: n=6). Mann-Whitney U-test was used for statistical analysis. Quotation from Ref. 54.

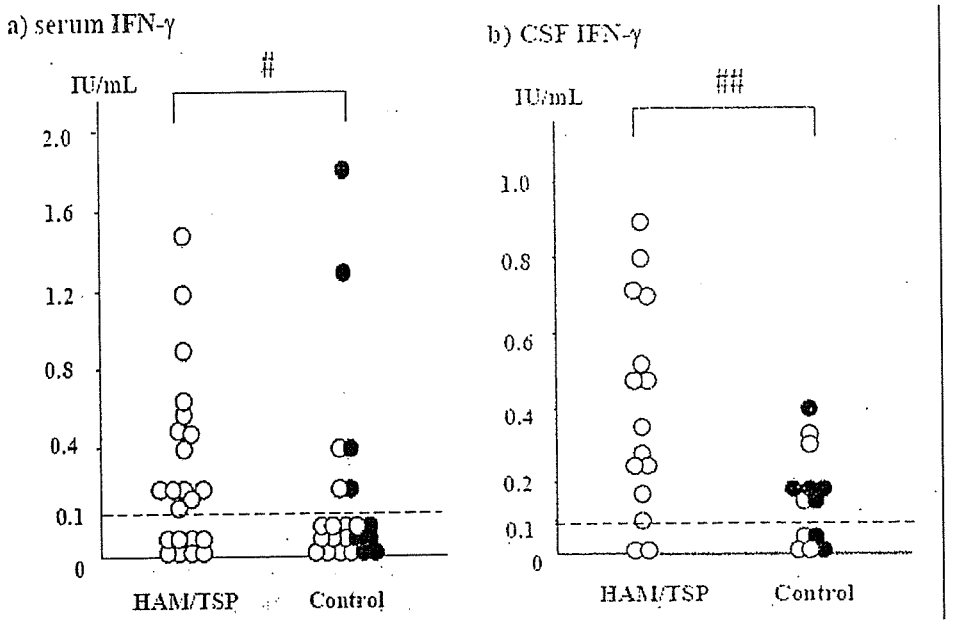


Figure 8. Serum and CSF levels of IFN-γ. a) Serum IFN-γ: serum levels of IFN-γ of HAM/TSP patients were higher than in the controls (mean ± SE; 0.33 ± 0.09 and 0.20 ± 0.10 IU/mL) (#p = 0.0035). b) CSF IFN-γ: CSF levels of IFN-γ of HAM/TSP patients were also higher than in the controls (mean ± SE; 0.40 ± 0.07 and 0.15 ± 0.04 IU/mL) (##p = 0.015). Minimal measurable levels (indicated by dotted lines): 0.1 IU/mL for IFN-γ. HAM: HAM/TSP patients (serum: n=22; CSF: n=15), Controls: Anti-HTLV-I-seropositive carriers (closed circle) (serum: n=9; CSF: n=7) and anti-HTLV-I-seronegative controls (serum: n=13; CSF: n=6). Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 54.

Next, we measured IL-4 and IL-10 as Th2 cytokines. Serum IL-4 was present in only 4 of 22 HAM/TSP patients and in 3 of 22 controls. Serum IL-4 levels in HAM/TSP patients (mean ± SE; 1.55 ± 0.73 pg/mL) were the same as in controls (mean ± SE; 2.22 ± 1.27 pg/mL). In addition, although serum IL-10 was detected in 7 of 22 HAM/TSP patients and in 10 of 22 controls, there were no significant differences in the levels between both groups (mean ± SE; 3.28 ± 1.52 pg/ml, mean ± SE; 3.06 ± 0.90 pg/mL, respectively). On the other hand, although CSF IL-4 was present in only 4 anti-HTLV-I-seropositive carriers of 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 3.00 ± 1.32 pg/mL), CSF IL-4 was not detected in 15 HAM/TSP patients (p = 0.023). CSF IL-10 was present in 5 of 15 HAM/TSP patients and in 7 or 13 controls, including 7 anti-HTLV-I-seropositive carriers. The CSF IL-10 levels in HAM/TSP patients (mean ± SE; 7.20 ± 3.96 pg/mL) was the same as in controls (mean ± SE; 5.39 ± 2.23 pg/mL).

These findings indicate that the immunological balance of helper T cells between Th1 and Th2 is toward Th1 in both the peripheral blood and the central nervous system (CNS) in HAM/TSP patients. Increased serum but not CSF levels of IL-12 suggest that differentiation of the helper T cells population to Th1 is induced outside of the CNS in HAM/TSP patients and these Th1 cells migrate to the CNS. In addition, in considering that IL-4 in the CSF was not detected in any of HAM/TSP patients although all 4 of the controls in whom IL-4 in the CSF was detected were anti-HTLV-I-seropositive carriers, these findings are a strong indication that

the balance of the helper T cells which function in the CNS of HAM/TSP patients tends toward Th1, rather than Th2.

How IL-12 production is up-regualted in HAM/TSP patients is unclear. The increased CD40 ligand expression on activated $CD4^+$ T cells can induce increased IL-12 production in monocytes [55]. In addition, the combination of CD40 ligand and INF-γ can generate large amounts of IL-12 in some population of human monocyte-derived dendritic cells [56]. However, as far as we determined by comparing the percentage of CD40 ligand-positive cells in the peripheral blood $CD4^+$ T cells of HAM/TSP patients and controls, there was no significant difference between the two groups [57]. Therefore, although elevated serum IL-12 levels in HAM/TSP patients might be based on different mechanisms from CD40/CD40 ligand interaction, it is conceivable that it is strongly involved in Th1 activation or maturation in HAM/TSP patients through the IL-12/IL-12 receptor axis.

In differentiation of naive T cells into Th1 or Th2 helper T cells, cytokine signaling plays an important role. IL-12 receptor/signal transducers and activators of transcription 4 (IL-12R/STAT4) and IL-4R/STAT6 signaling are involved in Th1 and Th2 differentiation, respectively [58]. In addition, T-bet, which is a member of the T-box family, has been cloned as a Th1-specific transcription factor [59]. Therefore, both IL-12R/STAT4 signaling and T-bet work cooperatively in Th1 differentiation, and IL-4R/STAT6 signaling induces GATA-3 activation, one of the Th2-specific transcription factors in Th2 differentiation [60]. Recently, the importance of negative regulation of cytokine signal transduction in the differentiation and homeostasis of the immune system has become somewhat clearer [61]. The suppressor of cytokine signaling (SOCS) family is a representative of the negative regulators which act through a feedback mechanism or an inhibitory signal to the Janus kinases (JAK)/STAT systems in cytokine signaling [62]. In this system, SOCS1 is induced by IFN-γ receptor (IFN-γR)/STAT1 signaling and inhibits IL-4R/STAT6 signaling concomitant with the negative feedback to its own JAK/STAT1 signaling [61, 63]. Although SOCS3 is induced by a number of humoral factors, such as IL-2, IL-6, IL-10, growth hormone, prolactin and leptin, with simultaneous negative feed back to their own JAK/STATs [64], it was reported that SOCS3 also inhibits IL-12R/STAT4 signaling [65]. These findings suggest that up-regulation of SOCS1 expression, based on activation of IFN-γ signaling, inhibits Th2 differentiation by blocking the IL-4R/STAT6 signaling pathway, and that down-regulation of SOCS3 expression leads to Th1 differentiation. Indeed, it has been reported that Th1 cells express significantly higher levels of SOCS1 than SOCS3 and expression levels of each SOCS protein are reversed in Th2 cells, indicating that either SOCS1 or SOCS3 protein is a marker for Th1 cells or Th2 cells, respectively [65, 66]. To clarify the Th1 activation state in HAM/TSP patients from the standpoint of cytokine signaling, we analyzed mRNA expression of T-bet, GATA-3, IL-12Rβ2, SOCS1 and SOCS3 in the peripheral blood mononuclear cells (PBMC) of HAM/TSP patients, as compared to either anti-HTLV-I-seropositive carriers or anti-HTLV-I-seronegative controls, by quantitative real-time RT-PCR [67].

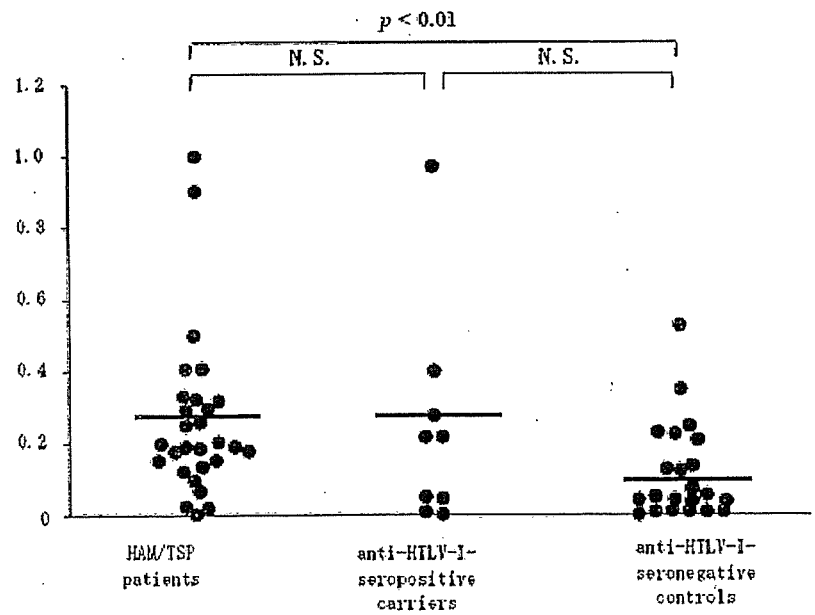
After each mRNA expression was evaluated by normalization to β2-microglobulin (β2m), the value of each mRNA expression was determined (table 1). As shown in figure 9a and 9b, mRNA expression of both T-bet and GATA-3 was significantly increased in PBMC of 28 HAM/TSP patients, compared to 22 anti-HTLV-I-seronegative controls. However, there was no significant difference between HAM/TSP patients and anti-HTLV-I-seropositive carriers

(figure 9a and 9b). In addition, mRNA expression of IL-12Rβ2 was significantly higher in HAM/TSP patients than in anti-HTLV-I-seronegative controls (figure 10). Although mRNA expression of T-bet, GATA-3 and IL-12Rβ2 seemed to be higher in anti-HTLV-I-seropositive carriers without HAM/TSP than in anti-HTLV-I-seronegative controls, 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.

Table 1. mRNA expression of cytokine signaling molecules.

	HAM/TSP patients	anti-HTLV-I-seropositive carriers without HAM/TSP	anti-HTLV-I seronegative controls
T-bet	0.260 ± 0.043	0.241 ± 0.100	0.118 ± 0.028
GATA-3	0.167 ± 0.040	0.115 ± 0.051	0.022 ± 0.005
IL-12Rβ2	0.321 ± 0.126	0.288 ± 0.104	0.114 ± 0.023
SOCS1	0.338 ± 0.062	0.147 ± 0.038	0.035 ± 0.007
SOCS3	4.860 ± 0.818	6.714 ± 1.467	13.234 ± 1.385

After each mRNA expression was evaluated by normalization to β2m, the value of each cytokine; signaling molecule mRNA expression was presented as mean ± SE. Quotation from Ref. 67.



a)