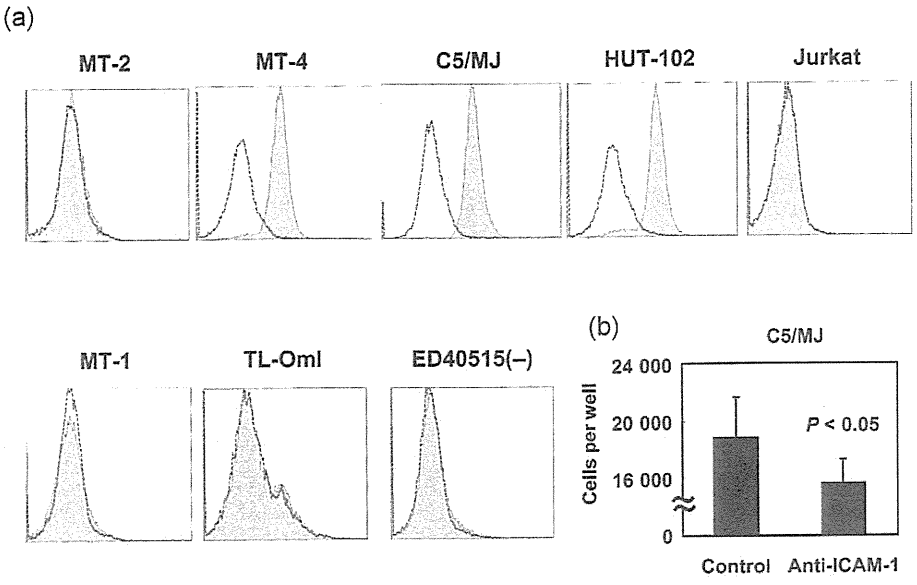

Fig. 3. ICAM-1 is involved in HTLV-I-infected T-cell adhesion to A549 cells. (a) Flow cytometry analysis of surface expression of the LFA-1 α-chain in human T-cell lines. Cells were reacted with FITC-labelled anti-LFA-1 α-chain or an isotype control antibody. (b) An anti-human ICAM-1 pAb was used to block ICAM-1 on the surface of A549 cells. After 1 h, fluorescently labelled C5/MJ cells were added to A549 cells that had been exposed to MT-2 supernatant for 6 h to determine adhesion. After cell lysis, the fluorescence intensity was measured. Data are means ± SD of three experiments.

time of RNA isolation. To examine whether HTLV-I infection had occurred in the infected A549 cells, expression of viral mRNA for Tax and the HTLV-I basic leucine zipper domain protein (HBZ) was assessed by RT-PCR. As shown in Fig. 6(a), A549 cells co-cultured with MT-2 cells showed strong expression of Tax and HBZ mRNA in an MT-2 dose-dependent manner. To further exclude the possibility that viral gene amplification was due to contamination from residual MT-2 cells, we used RT-PCR to amplify MT-2-specific human CD4. As shown in Fig. 6(a), human CD4 product was amplified from MT-2 cells but not from A549 cells co-cultured with MT-2 cells. These results suggested that A549 cells were infected by HTLV-I after co-culture.

To determine whether viral antigens were produced in infected A549 cells, immunofluorescence assays for Tax were performed by indirect immunofluorescence staining. Expression of Tax was detected in the cytoplasm and nuclei of A549 cells at 3 days after HTLV-I infection (Fig. 6b), whereas no Tax expression was observed when a control IgG was used or in control A549 cells that were not co-cultured with MT-2 (data not shown). These observations again demonstrated that HTLV-I infected the A549 cells.

Next, we examined the expression of ICAM-1 mRNA in co-cultured A549 cells by RT-PCR. As shown in Fig. 6(a), ICAM-1 mRNA expression in A549 cells increased substantially 2 days after co-culture with MT-2 cells in an MT-2 dose-dependent manner. However, transcripts of IL-1α were not detected in any of the samples. The surface expression of ICAM-1 on co-cultured A549 cells was also examined by flow cytometry. As shown in Fig. 6(c), ICAM-1 expression on A549 cells increased 1 day after co-culture with MT-2 cells, reached a peak level on day 2 and plateaued on day 3. However, the levels of HTLV-I-upregulated ICAM-1 expression were less than those following exposure to supernatant of HTLV-I-infected T-cell lines. Upregulation of ICAM-1 expression was not observed in A549 cells co-cultured with MMC-treated Jurkat cells or in untreated A549 cells (Fig. 6c).

Tax is the primary viral transactivator protein, modulating the expression of both viral and cellular genes. To examine the effect of Tax on ICAM-1 expression at the transcriptional level, we performed luciferase reporter assays in A549 cells using an ICAM-1 promoter/luciferase reporter plasmid (pGL1.3). As shown in Fig. 6(d), co-transfection of an expression vector for Tax activated the ICAM-1 promoter in A549 cells, indicating that Tax can directly activate the ICAM-1 promoter.

To determine the precise regions of the ICAM-1 promoter necessary for Tax-mediated activation, an NF-κB site mutant (pGL1.3κB$^-$) was co-transfected. Mutation of the NF-κB site abolished the Tax-mediated activation of the ICAM-1 promoter (Fig. 6d). In addition, we confirmed that Tax activated the NF-κB site using the luciferase reporter plasmid regulated by NF-κB elements (κB-LUC; Fig. 6d). These results suggested that Tax transactivates the ICAM-1 gene via the NF-κB-binding site.

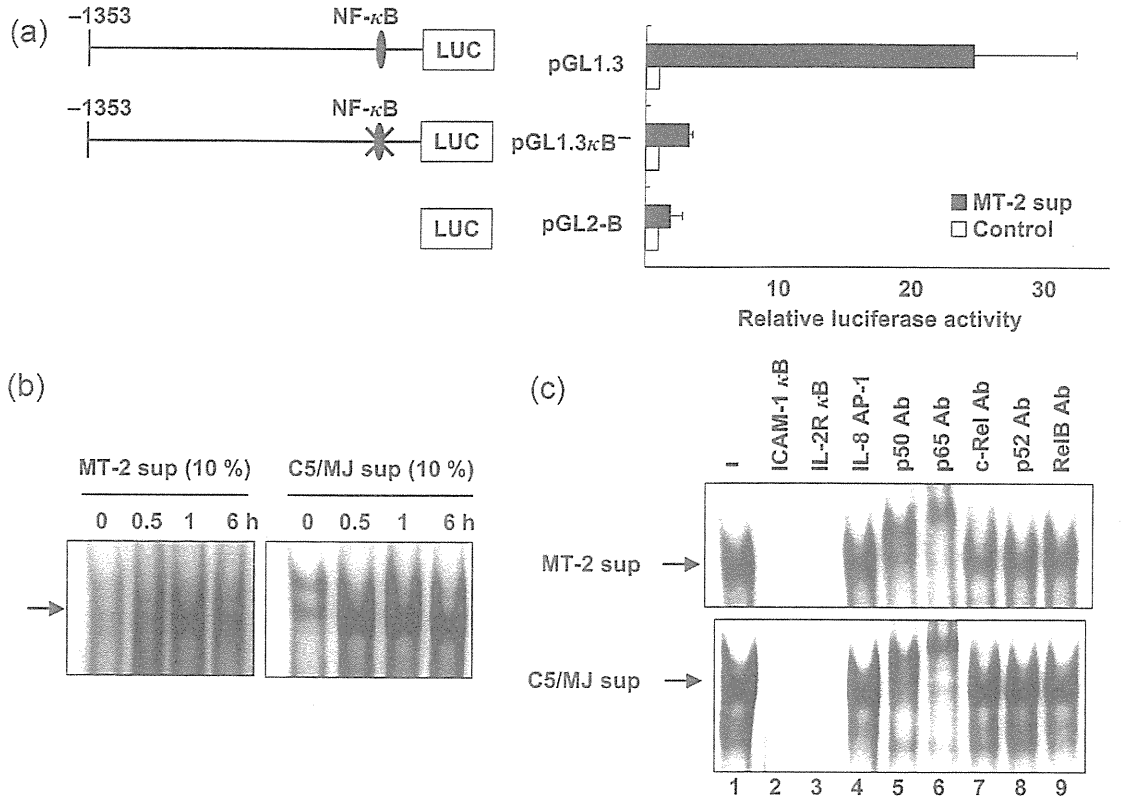

Fig. 4. Identification of a critical supernatant-responsive region of the ICAM-1 promoter. (a) Mutation of the NF-κB site suppresses supernatant-induced ICAM-1 promoter activity. The indicated constructs (left) were transfected into A549 cells and the cells subsequently exposed to MT-2 supernatant for 6 h. The activities (right) of each construct are expressed relative to that of cells transfected with pGL2-Basic (pGL2-B) without further treatment, which was defined as 1. Data are means ± SD of three experiments. (b) Time course of NF-κB activation in A549 cells exposed to MT-2 and C5/MJ culture supernatants was evaluated using EMSA. Nuclear extracts prepared at the indicated time points from A549 cells exposed to the different supernatants were mixed with ICAM-1 NF-κB-binding site labelled probe. The complex is indicated by an arrow. (c) Sequence specificity of NF-κB-binding activity and characterization of NF-κB proteins that bind to the NF-κB-binding site of the ICAM-1 gene. Competition assays were carried out with nuclear extracts from A549 cells exposed to MT-2 supernatant for 0.5 h or C5/MJ supernatant for 1 h. Lane 1 shows the complex formed in the absence of competing oligonucleotide or antibody (indicated by an arrow). Where indicated, a 100-fold excess of a specific competitor oligonucleotide (lanes 2–4) was added to the reaction mixture with the labelled probe. A supershift assay of the NF-κB DNA-binding complex in the same nuclear extracts was also carried out where appropriate antibodies were added to the reaction mixture (lanes 5–9) as indicated.

Detection of IL-1α and ICAM-1 in the lungs of patients with HTLV-I-related pulmonary diseases and in Tax transgenic mice

RT-PCR was used to determine the expression of viral mRNAs of Tax and HBZ in BALF cells from patients with HTLV-I-related pulmonary diseases. In all six patients, Tax and HBZ mRNAs were detected in BALF cells (Fig. 7a). IL-1α mRNA was also detectable in BALF cells. Fig. 7(b) shows the concentrations of IL-1α in BALF samples obtained from HTLV-I carriers and non-infected volunteers. The concentration of IL-1α was higher in four of the HTLV-I carriers than in non-infected controls.

Finally, we immunostained lung tissues obtained from patients with HTLV-I-related pulmonary diseases. The expression of IL-1α and ICAM-1 was noted in the epithelial cells, lymphocytes and macrophages of these patients (Fig. 7c). We also immunostained the lung tissues of transgenic mice to assess the expression of IL-1α and ICAM-1. We examined the distribution of IL-1α and ICAM-1 proteins in the lungs of transgenic mice. Strong immunostaining for IL-1α and ICAM-1 was observed in epithelial cells, lymphocytes and macrophages in the lungs of transgenic mice, but not in the lungs of littermate mice (Fig. 7d).

DISCUSSION

We have been interested in the role of humoral factors released from HTLV-I-infected T cells in the development of the characteristic pathological changes of HTLV-I-associated diseases. In this study, we hypothesized that

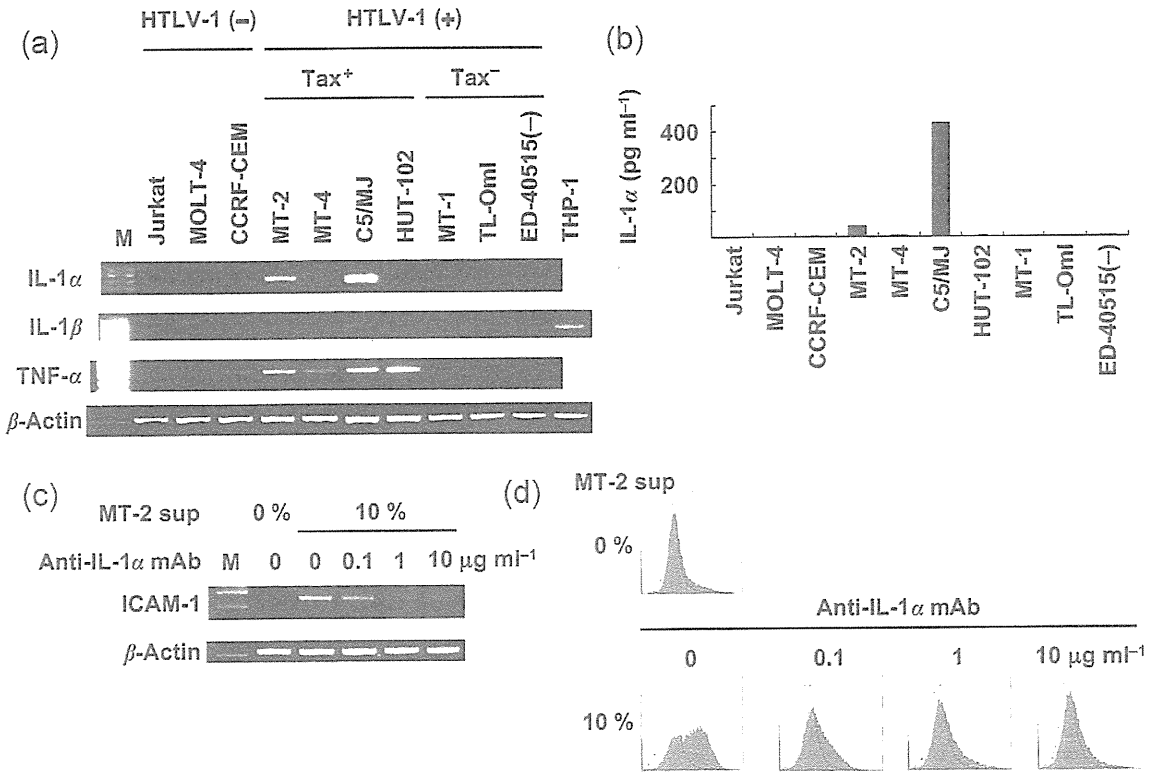

Fig. 5. IL-1α mediates most of the supernatant-induced ICAM-1 expression. (a) Expression of IL-1α, IL-1β and TNF-α mRNAs in various human T-cell lines was examined by RT-PCR. RNA prepared from THP-1 cells was used as a positive control for IL-1β. Human β-actin mRNA was used as a control. M, 100 bp ladder (Toyobo Life Science). (b) Levels of IL-1α secreted by human T-cell lines. Cells were cultured for 72 h and the culture supernatants were collected. The level of IL-1α was determined by ELISA. (c) Effect of neutralizing anti-IL-1α mAb on MT-2 supernatant-induced ICAM-1 mRNA expression in A549 cells. A549 cells were left untreated or were exposed to 10% MT-2 supernatant, cultured with or without the indicated concentrations of neutralizing mAb to IL-1α for 3 h. Human β-actin mRNA was used as a control. (d) The effect of neutralizing anti-IL-1α mAb on MT-2 supernatant-induced ICAM-1 expression in A549 cells was measured by flow cytometry. A549 cells were left untreated or exposed to 10% MT-2 supernatant, cultured with or without the indicated concentrations of neutralizing mAb to IL-1α for 3 h and assessed for cell-surface expression of ICAM-1.

ICAM-1 is strongly expressed on lung epithelial cells of patients with HTLV-I-related pulmonary diseases. In testing this hypothesis, we also examined whether the upregulation of ICAM-1 was mediated by paracrine signalling (i.e. by cytokines secreted from HTLV-I-infected T cells) or directly as a result of HTLV-I infection. The results demonstrated that the culture supernatants of HTLV-I-infected T-cell lines induced the expression of cell-surface ICAM-1 on A549 cells. The culture supernatants activated the ICAM-1 promoter via the NF-κB-binding site. Furthermore, HTLV-I infection of A549 cells also upregulated ICAM-1. However, the levels of ICAM-1 upregulated by HTLV-I infection were less than those following exposure to the supernatants of HTLV-I-infected T-cell lines. HTLV-I Tax alone could also activate the ICAM-1 promoter via the NF-κB-binding site.

It is well known that HTLV-I-infected T cells produce various cytokines, such as IL-1 and tumour necrosis factor (Tschachler *et al.*, 1989; Wano *et al.*, 1987). Our results showed that the main molecule responsible for the biological activity of the culture supernatants was IL-1α for the following reasons: (i) the levels of IL-1α were remarkably high in the MT-2 and C5/MJ supernatants; (ii) adding mAb to IL-1α reduced ICAM-1 expression almost completely; and (iii) the effects of recombinant IL-1α on A549 cells were similar to those of the culture supernatants from HTLV-I-infected T cells. Although MT-2 and C5/MJ cells expressed TNF-α mRNA, HTLV-I-infected T-cell lines did not produce biological TNF-α (Tschachler *et al.*, 1989). These findings suggest that IL-1α was responsible for the biological activities of the supernatants observed in the present study.

With regard to pulmonary lesions, the results demonstrated the presence of high levels of IL-1α in BALF and IL-1α mRNA expression in BALF cells in HTLV-I carriers. We are currently investigating the relationship between IL-1α concentrations and percentages of lymphocytes in the BALF of HTLV-I carriers. In this study, ICAM-1 and IL-1α were detected in lung epithelial cells and lymphocytes,

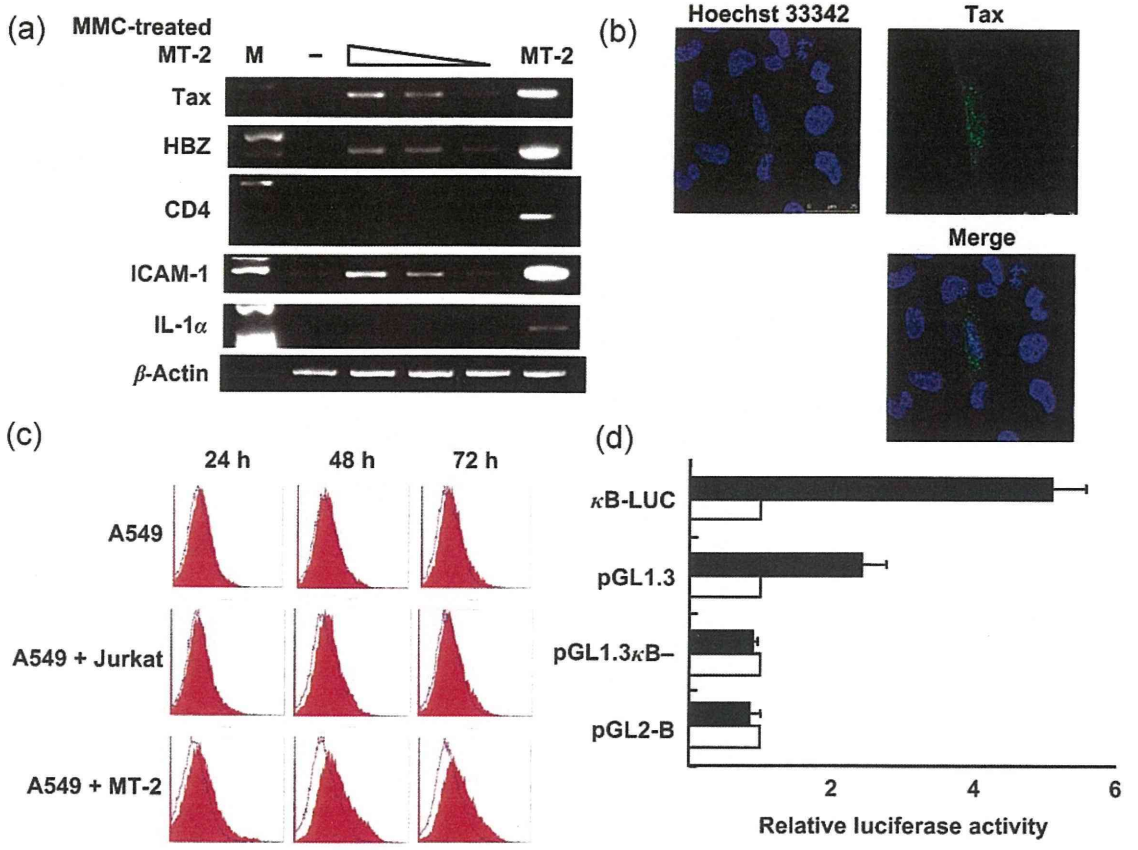

Fig. 6. HTLV-I infection induces ICAM-1 expression in A549 cells. (a) Detection of HTLV-I Tax, HBZ and ICAM-1 mRNAs in A549 cells by RT-PCR. A549 cells were co-cultured with MMC-treated MT-2 cells. After co-cultivation for 2 days, the A549 cells were harvested and expression of the indicated genes was analysed. Human β-actin mRNA was used as a control. M, 100 bp ladder (Toyobo Life Science). (b) Detection of HTLV-I Tax by immunofluorescence. A549 cells were co-cultured with MT-2 cells, fixed and incubated with an anti-Tax antibody, followed by an Alexa Fluor 488-labelled anti-mouse secondary antibody, together with Hoechst 33342 staining of the nuclei. (c) ICAM-1 expression on A549 cells is upregulated after co-culture with MT-2 cells. A549 cells were co-cultured with or without MMC-treated MT-2 or Jurkat cells for 1, 2 and 3 days. Cells were collected and flow cytometry was performed to detect ICAM-1 expression on the cell surface. (d) Tax activates the ICAM-1 promoter via NF-κB. A549 cells were transfected with the indicated constructs together with a Tax expression vector or empty vector. Cells were harvested at 24 h post-transfection and luciferase activity was measured. Activity is expressed relative to that of cells transfected with the reporter construct together with empty vector, which was defined as 1. Data are means ± SD of three experiments. Black bars, Tax; white bars, vector.

respectively, from patients with HTLV-I-related pulmonary diseases. However, ICAM-1 was also detected in lymphocytes and macrophages, and IL-1α was also detected in lung epithelial cells and macrophages. In the lungs of patients with pulmonary disorders associated with HTLV-I, HTLV-I-infected T cells and lung epithelial cells may produce various types of pro-inflammatory cytokines, resulting in upregulation of ICAM-1 and IL-1α in these cells. The concentration of soluble ICAM-1 in the BALF of patients with HTLV-I-associated pulmonary disorders has been reported to be significantly higher than that in non-infected healthy control subjects (Seki *et al.*, 2000). In accordance with our hypothesis, the concentration of soluble ICAM-1 correlated well with the percentage of activated T cells (Seki *et al.*, 2000).

Because ICAM-1 is an attractive target for therapeutic intervention based on its involvement in the inflammatory and viral infectious processes, understanding the precise mechanisms by which its expression is regulated is important. The present study found an HTLV-I-infected cell supernatant-responsive and Tax-responsive region in the ICAM-1 gene containing an NF-κB site. NF-κB regulates the expression of IL-1α (Mori & Prager, 1996). Accordingly, NF-κB is an attractive target for treatment of HTLV-I-associated pulmonary disorders. However, the addition of ICAM-1-blocking pAb inhibited, at least in part, HTLV-I-infected T-cell adhesion to lung epithelial cells. The partial blockade of cell-to-cell adhesion by ICAM-1-blocking pAb suggests the possible effects of another adhesion molecule, in addition to ICAM-1, that

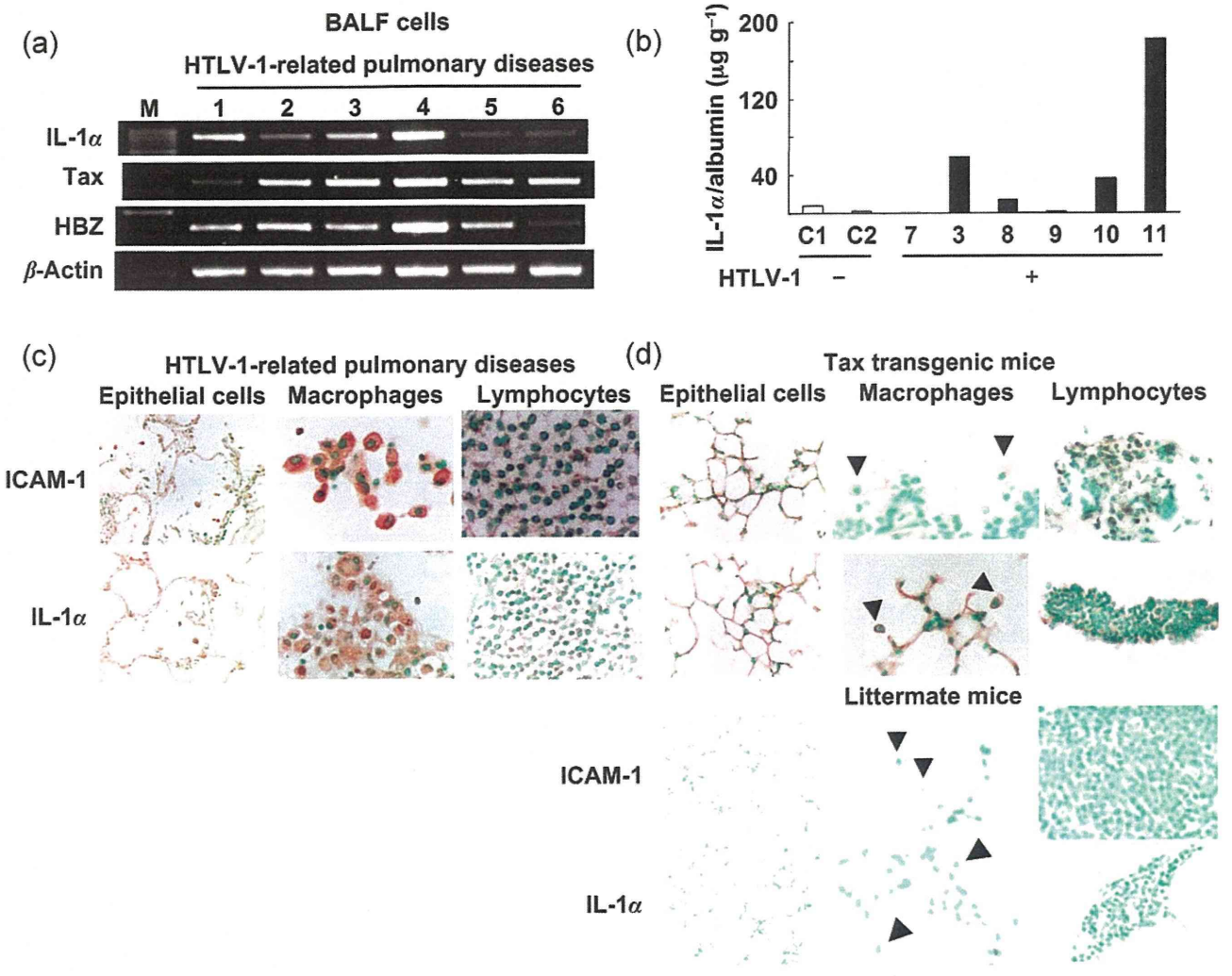

Fig. 7. Detection of IL-1α and ICAM-1 in the lungs of patients with HTLV-I-related pulmonary diseases and in Tax transgenic mice. (a) Detection of Tax, HBZ and IL-1α mRNAs in BALF cells obtained from six patients with HTLV-I-related pulmonary diseases. Human β-actin mRNA was used as a control. Lanes 1–6 denote patients 1–6. M, 100 bp ladder (Toyobo Life Science). (b) Concentration of IL-1α in BALF from patients with HTLV-I-related pulmonary diseases and non-infected controls. Case 3 is the same as patient 3 in (a). C1 and C2 were control subjects who were seronegative for HTLV-I. (c, d) Detection of IL-1α and ICAM-1 proteins by immunohistochemistry. In the lung tissues of patients with HTLV-I-related pulmonary diseases (c) and Tax transgenic mice (d), immunohistochemical staining showed a definite brownish staining for IL-1α and ICAM-1 proteins in the membrane and cytoplasm of epithelial cells, and in macrophages and infiltrated lymphocytes. Arrowheads indicate the surfaces of macrophages in lung tissues of mice. Cells were counterstained with methyl green.

mediates HTLV-I-infected T-cell adhesion to lung epithelial cells.

Taken together, the results of this study suggest that IL-1α may play a role in the upregulation of ICAM-1 expression on lung epithelial cells in patients with HTLV-I-associated pulmonary disorders. Such a process may be involved in the pathogenesis of HTLV-I-associated pulmonary disorders. To assess the possible action of IL-1α, further studies will be needed to investigate the effects of neutralizing antibody against IL-1α on the development of HTLV-I-related lung lesions.

In summary, the findings of the present study allow the construction of a hypothesis in which IL-1α, produced by HTLV-I-infected Tax^+ T cells, is involved in the upregulation of ICAM-1 on lung epithelial cells. We found upregulation of LFA-1, the counter-receptor for ICAM-1, in HTLV-I-infected Tax^+ T cells. The detection of Tax mRNA expression in BALF cells of patients with HTLV-I-related pulmonary diseases suggests that the lung is a preferential site for its expression. Indeed, Tax-expressing transgenic mice exhibited inflammatory changes with infiltration of lymphocytes in the lung (Miyazato *et al.*, 2000). Tax has been reported to upregulate IL-1α in T cells (Mori & Prager, 1996). Tax may be

involved in the development of lung inflammation caused by HTLV-I through the induction of local production of IL-1α. The adhesion of T cells to the lung epithelial cells may play an important role in the pathogenesis of HTLV-I-related pulmonary diseases.

ACKNOWLEDGEMENTS

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特集(1)：HTLV-1感染の検査と臨床

2. HTLV-1感染の疫学

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〔Key Words〕Human T-lymphotropic virus type-1：HTLV-1(ヒトTリンパ向性ウイルス1型)，adult T-cell leukemia：ATL(成人T細胞白血病)，natural history(自然史)

はじめに

ヒトTリンパ向性ウイルス1型(human T-lymphotropic virus type-1：HTLV-1)は，成人T細胞白血病・リンパ腫(adult T-cell leukemia/lymphoma：ATL)，さらにHTLV-1関連脊髄症(HTLV-1 associated myelopathy：HAM)や眼疾患であるHTLV-1関連ブドウ膜炎(HTLV-1 associated uveitis：HU)などの疾患を引き起こす[1)~3)]。また一部の膠原病や小児の皮膚疾患などとの関連も報告されている[4)5)]。ATLは1977年に高月らにより，新しい疾患概念として最初に報告された[1)]。1981年にはこのウイルスがATLの原因ウイルスであることが日沼らにより明らかにされた[6)]。

HTLV-1感染者(キャリア)の大部分はHTLV-1関連疾患を発症せずに無症状であるが，その一部(約5%)が感染後数十年の長い潜伏期を経てATLを発症する。HAMやHUの頻度はATLよりも少ないと報告されている。本邦では現在でも約108万人のキャリアが存在し，その分布は人口の移動により従来の西南日本のみならず大都市圏にも拡大し，1年間に1000人以上がATLを発症すると推定されている。

HTLV-1キャリアにおいてウイルス粒子そのものは血液や体液中より検出することはできず，主にCD4陽性リンパ球中にプロウイルスの形で存在している。このためHTLV-1感染でいうウイルス量とは多くの場合感染細胞数を意味している。HTLV-1感染は感染細胞(プロウイルス保有細胞)が他者の体内に入り細胞－細胞接触により成立する。新規感染成立後は体内でも細胞間感染により感染が拡大し，HTLV-1に対する免疫の発動後は感染細胞のクローン増殖が感染の維持に大きな役割を果たすと考えられている。感染は母児間ないし配偶者間感染で成立するが，医原的には輸血や臓器移植で起こりうる。感染が成立すると終生持続感染が維持され，自然にウイルスが消失することはないと考えられている。本項ではHTLV-1感染の疫学について述べる。

I. 世界におけるHTLV-1キャリアの地理的分布

世界におけるHTLV-1感染者は約1000～2000万人と推測されるが正確な数は明らかでない。ヒトからヒトに感染するウイルスであるにもかかわらず，HTLV-1自体の感染力が非常に

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弱いため浸淫地域は限られている。主な浸淫地域は日本，カリブ海沿岸諸国，南米，南－中央アフリカ，メラネシア，パプアニューギニアなどであり(図1)，そのほかではアジアや中東の一部に抗体陽性者の報告がある[7]。HTLV-1は分子生物学的手法によって3つの大きな系統(1a: コスモポリタン型，1b: 中央アフリカ型，1c: メラネシア型)に分けられる。コスモポリタン型はさらにA(トランスコンチネンタル)，B(日本)，C(西アフリカ)，D(北アフリカ)，E(ペルー)のサブタイプが報告されている[8]。これらのサブグループはおおよそ，そのウイルスキャリアの居住地と一致しており，民族やヒトの移動といった人類学的背景を反映したものと考えられる。HTLV-1のサブグループの違いによる関連疾患の違いは報告されていない。

II. 本邦における疫学

本邦におけるHTLV-1に関する疫学調査は，1980年代に日沼，田島らによって行われ，推定キャリア数120万人，ATL患者数700人/年間と報告された[9]。それ以降本邦におけるHTLV-1キャリア数の変化の詳細については不明であったが，2008～2010年度に厚生労働科学研究班「本邦におけるHTLV-1感染及び関連疾患の実態調査と総合対策」(山口班)が組織され，全国的なHTLV-1キャリアおよび関連疾患の実態調査が行われた。その中で，初回献血者の抗体陽性者の調査から，全国のキャリア数は約108万人と推定され，依然として全国民の1%に相当する多数のキャリアが存在していることが判明し，またキャリアの高齢化が明らかとなっ

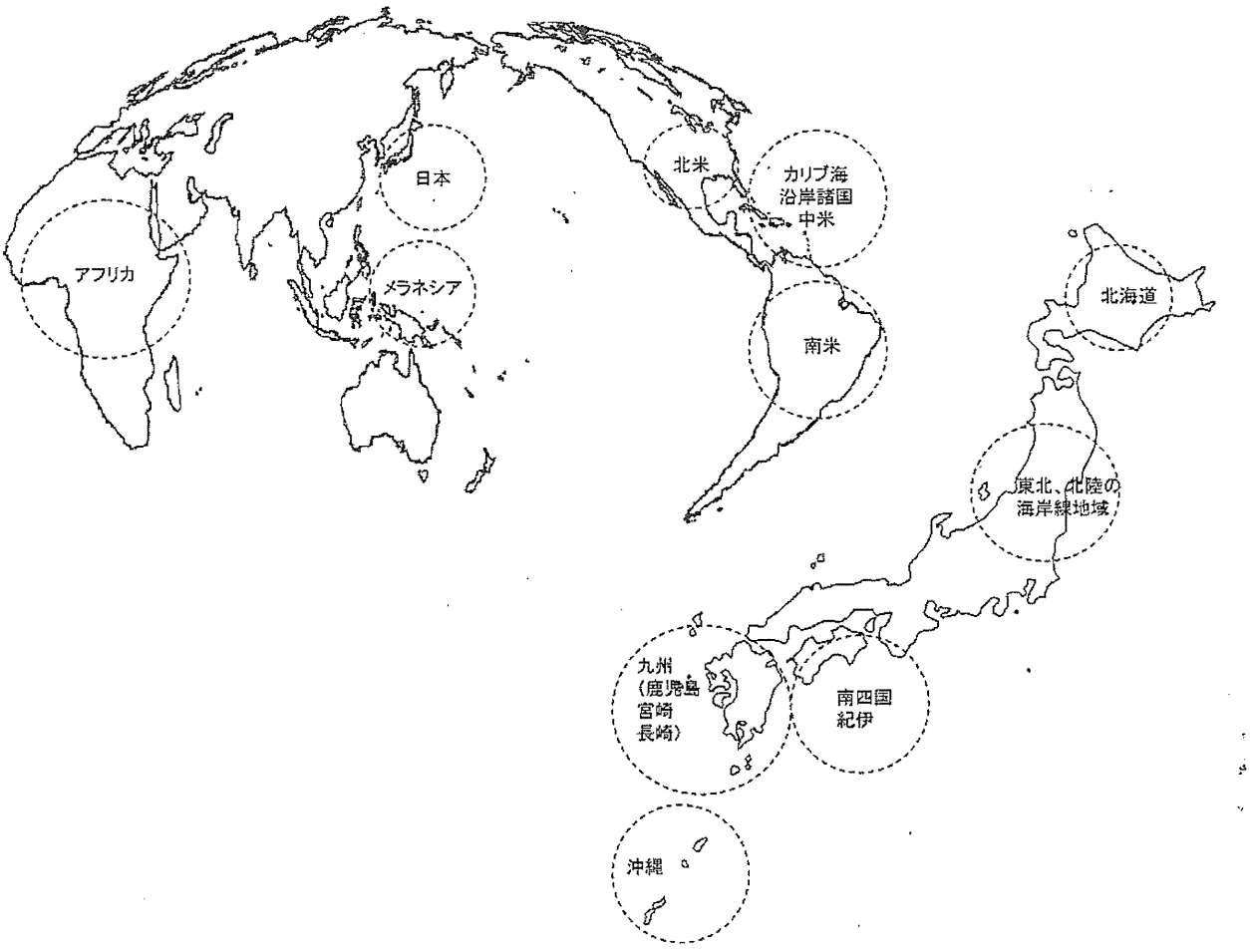

図1　世界におけるHTLV-1の分布と日本国内におけるHTLV-1高浸淫地域

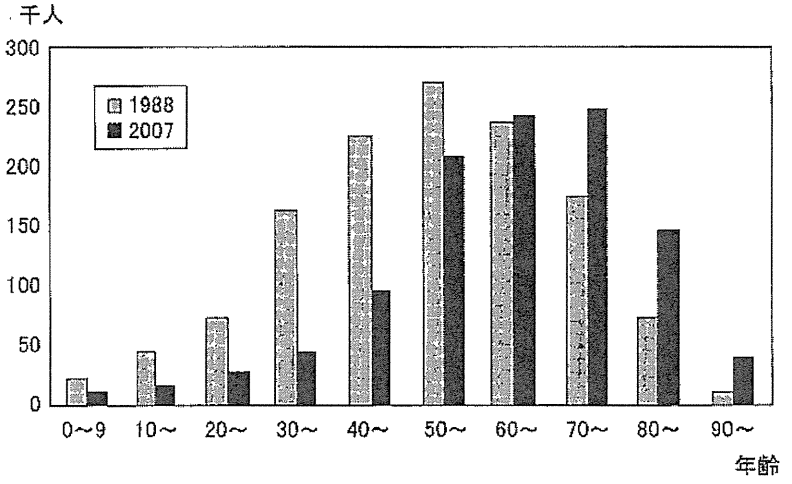

図2　推定されるキャリアの年齢別分布の推移(文献[10]より改変)

た(図2)[10]。ATL の実態調査から ATL 患者数はむしろ増加傾向(1000 人/年間超)にあり，人口の高齢化に伴い，今後も持続的に ATL は発症すると考えられた。1980 年代の疫学調査からは国内においても HTLV-1 キャリアの分布には地域差があり，感染率の高い地域は九州，四国，沖縄などの西南日本が主であるとされた。このほかに東北や北陸地方の一部，特に海岸線地域，また北海道に感染者の割合の比較的高い地域が報告された(図1)[9]。今回の調査で九州・沖縄地方のキャリアの割合が減少している一方，関東地方と近畿地方の大都市圏では増加が認められ，感染が全国に拡散する傾向があることが示された(図3)[10]。

HTLV-1 高浸淫地方において，地域における感染率は隣接する地域，市町村単位であっても大きく異なる[11]。これは HTLV-1 の自然感染がおもに家族内に限られることによると思われる。このため周囲との交通の不便な離島や集落単位で陽性率の高い地域がある。このような地域における 1980 年代の研究では，感染率は年齢が高いほど高く，また特に中年以降において女性が男性よりも高いことが示されてきた[12]。年齢が高くなるほど感染率が高くなる理由としては 2 つ考えられた。ひとつは出生コホート効果とよばれるもので，年齢が高い人たちの陽性率の高さは，その人々が出生した年における感染率(おそらく母児感染の頻度)が高かったことを反

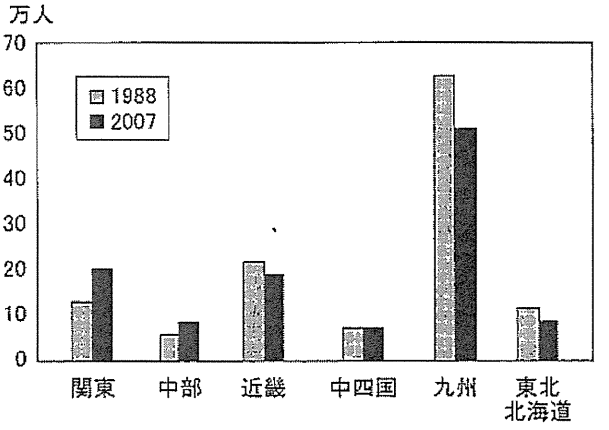

図3　推定される地域別キャリア数の推移
(文献[10]より改変)

映しているというものである[13]。もうひとつの理由は配偶者間感染のため一定の年齢以降感染率が高くなるというものである。実際に HTLV-1 感染者の配偶者を長期にフォローすると一定の割合で抗体の陽転が生じる[14]。

III. HTLV-1 の感染経路

HTLV-1 の感染経路としては，母児間感染，配偶者間感染，輸血による感染が知られている[15]。本邦では輸血によるものは献血者の抗体スクリーニングにより 1984 年以降阻止されており，母児間感染が主要な感染経路と考えられている。

母児間感染は主に母乳を介して成立する。キャリアの母親が母乳を与えた場合の児への感染

率は16～30％といわれており，母乳中の感染リンパ球が児への感染に関与していると考えられている[16)17)]。大部分のキャリアはATLを発症しないが，ATLが発症した場合の予後が不良であることを考慮し，将来のATL発症予防のためHTLV-1キャリア妊婦に対しては母乳で哺育しないことが推奨されてきた。長崎県において1987年よりHTLV-1陽性の母親への介入が開始され，母乳哺育を遮断することにより母児間感染の大部分を回避することが可能となった[18)]。その他の対策として人工乳哺育，3ヵ月以内の短期母乳哺育，凍結哺育などの方法があり，児へのHTLV-1感染リスクが減少することが報告されてきた。ただし母乳による哺育を行わない場合でも，児への感染は数％あり，母乳以外の感染経路(子宮内感染あるいは産道感染)があると推定されている。

このようなHTLV-1キャリア妊婦に対する対策は従来，九州・沖縄などHTLV-1キャリアの多い地域を中心に推進されてきたが，山口班の研究によりHTLV-1キャリアが全国に拡散していることが報告された。また平成21年度厚労科研費補助金厚労科学特別研究事業「HTLV-1の母子感染予防に関する研究」(齋藤班)からも全国の妊婦に対してHTLV-1抗体スクリーニング検査の必要性が提起された。このような状況をうけて，2010年秋より母子感染予防の組織的な取り組みが全国的なレベルで行われることとなった。しかし，齋藤班により，特にHTLV-1キャリアが少ない地域でHTLV-1スクリーニング検査(粒子凝集法もしくは化学発光法)を行うと，陽性者の中に偽陽性者が少なからず存在し，確認検査(Western blot法)が必要であること，また確認検査を行っても判定保留(2～20％)となる場合もあることが報告された。このため検査の性質について検査前にキャリア妊婦に説明とカウンセリングを行っておくことの必要性が示されている。また確認検査については，血清学的検査以外の方法の導入をふくめて，検査法のさらなる改善が試みられつつある。

HTLV-1のもうひとつの感染経路は性交渉による配偶者間感染である。配偶者間感染はプロウイルスの遺伝子配列の比較によっても証明されており，男女どちらからでも起こりうるが，夫がキャリアである場合は妻がキャリアである場合に比して4～5倍感染が成立しやすい[19)]。このことは中年以降において女性の抗体陽性率が男性よりも高いことも説明する。配偶者間感染は必ずしも結婚の直後に成立するわけではなく，50歳代，60歳代になって感染することもまれではない。感染してからATLを発症するまでには通常数十年の長い年月がかかるため，配偶者間で感染してもATLが発症することはないと考えられている。しかし，HAMやHUは低率ではあるが発症することがありうる。性交渉による感染は理論的にはコンドーム使用により防ぐことができると考えられるが，挙児希望の場合には確実に感染を予防できる方法はなく，また感染を防ぐワクチンも開発されていない。そのため，将来的には配偶者間感染に関してもなんらかの予防法が検討される必要があると思われる。

配偶者間感染の初期像の解析から，ヒト免疫不全ウイルス同様HTLV-1でも感染後抗体陰性の時期があり，その時期にウイルス量がいったん増加すること，その後の抗体産生後にウイルス量が低下し，一定のレベル(セットポイント)に安定すること，またセットポイントはキャリアごとに異なり，長期間安定の状態にあると考えられている[14)]。さらにジャマイカの母児感染の研究から母児間感染についてもセットポイントは各小児において異なること，10歳前後ですでにHTLV-1感染細胞のクローン増殖をきたす例があり，皮膚病変などの症状を呈することがあることも判明している[20)]。また，キャリアの長期フォロー研究から，感染ウイルス量が多いことはATL発症をきたす危険因子であることも明らかとなっている[21)22)]。これらの知見よりHTLV-1感染の防止の必要性は明らかであるが，感染成立後においてもセットポイントのウイルス量を減少させることが可能であればHTLV-1関連疾患の予防に有効と考えられる。しかし，

現在のところヒト HTLV-1 新規感染に対して母乳遮断以外にワクチンなどの有効な感染予防策は知られておらず，また感染ウイルス量を抑える方法も明らかでない。今後の検討課題であると思われる。

IV. ま と め

本邦において HTLV-1 キャリア数は自然減あるいは母児感染対策により減少してきている。しかし，ATL を含めた HTLV-1 関連疾患を発症するキャリア数は未だに決して少なくなく，解決されるべき問題は多い。今後，HTLV-1 感染を駆逐するような抗ウイルス療法，あるいはウイルス量を低下させる方法論の開発により，関連疾患の発症予防が可能となることが望まれる。

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Proviral loads of human T-lymphotropic virus Type 1 in asymptomatic carriers with different infection routes

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High human T-lymphotropic virus Type 1 (HTLV-1) proviral DNA load (PVL) has been reported to be one risk factor for the development of adult T-cell leukemia/lymphoma (ATL). ATL is also believed to develop in HTLV-1 carriers who acquire infection perinatally. ATL cells have been reported to frequently harbor defective provirus. In our study, PVLs for three different regions of HTLV-1 provirus (5′LTR-*gag*, *gag* and *pX*) were measured in 309 asymptomatic carriers with different infection routes. PVLs for the *pX* region in 21 asymptomatic carriers with maternal infection was significantly higher than in 24 carriers with spousal infection. Among 161 carriers with relatively high *pX* PVLs (equal to or greater than 1 copy per 100 peripheral blood mononuclear cells), 26 carriers (16%) had low *gag* PVL/*pX* PVL (less than 0.5) and four (2%) had low 5′LTR-*gag* PVL/*pX* PVL (less than 0.5). Low *gag* PVL/*pX* PVL ratio, which reflects deficiency and/or polymorphism of HTLV-1 proviral DNA sequences for the *gag* region, was also associated with maternal infection. These data suggest that HTLV-1 carriers with maternal infection tend to have high PVLs, which may be related to provirus with deficiency and/or the polymorphism of proviral DNA sequences. In addition, there is a possibility that this ratio may be used as a tool to differentiate the infection routes of asymptomatic HTLV-1 carriers, which supports the need for a large scale study.

Human T-lymphotropic virus Type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and a progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] Major routes of HTLV-1 infection have been reported as mother to child infection at infancy, sexual contact between spouses and blood transfusion.[5–7] The majority of HTLV-1 carriers are asymptomatic, and only a fraction of carriers develop ATL after a long latent period.[8,9] It has been reported that approximately 4% of HTLV-1 carriers develop ATL eventually.[10] Studies of the mothers of patients with ATL have reported most of them to be HTLV-1 carriers.[11,12] Therefore, ATL is believed to develop in HTLV-1 carriers who acquire infection perinatally. However, there has been no method of identifying the infection route of HTLV-1 positive individuals without information on family HTLV-1 status.

When an individual is infected by HTLV-1, the virus randomly integrates into the genome of affected T-cells in the form of provirus.[13] HTLV-1 infection drives the proliferation of T-cells, leading to the clonal expansion of HTLV-1 infected cells.[14–16] Recently, it was reported that HTLV-1 clonal expansion *in vivo* is favored by orientation of the provirus in the same sense as the nearest host gene.[17] We have reported that the clonality of HTLV-1 infected cells in adult seroconverters who were newly infected from HTLV-1 carrier spouses is more heterogeneous and less stable than that of long-term carriers who acquired infection from their mothers at infancy.[18] The selective maintenance of certain clones is supposed in the latter. Recently, we reported that clonal expansion of HTLV-1 infected cells was found in a certain population of asymptomatic carriers and that these carriers had high proviral DNA loads (PVLs).[19] High PVLs have been reported to be a risk factor for developing ATL.[20,21] In another study, we analyzed the PVLs of 13 pairs of HTLV-1 seroconverters and their spouses.[22] Although seroconverters and their spouses shared the same HTLV-1, PVLs in both individuals in a couple were not always equivalent. These findings suggested that host-related factors play an important role to determining the PVL in each carrier. However, it was

Key words: HTLV-1, defective virus, infection route, proviral DNA loads
Abbreviations: ATL: adult T-cell leukemia/lymphoma; HTLV-1: human T-lymphotropic virus type 1, LTR: long-terminal repeat, PBMCs: peripheral blood mononuclear cells, PCR: polymerase chain reaction; PVLs: proviral DNA loads
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not clear in that study whether HTLV-1 carriers who acquired infection from their mothers at infancy have more PVLs than the carriers who acquired infection from their spouses in adulthood.

Defective provirus has frequently been detectable in patients with ATL.[23–27] The complete HTLV-1 provirus is approximately 9 kb and contains the coding regions for core protein (*gag*), protease (*pro*), polymerase (*pol*), envelope protein (*env*), regulatory proteins, such as Tax and Rex, and some accessory molecules between 5′ and 3′ long-terminal repeats (LTRs).[8,28] Tamiya *et al.*[23] reported two types of genome deletion in defective provirus. One form retains both LTRs and lacks internal sequences, such as the *gag* and *pol* regions. The other form has the 3′ LTR, and the 5′ LTR and its flanking internal sequences are preferentially deleted. HTLV-1 infected cells harboring the latter defective virus were frequently found in patients with ATL.[26] Both types of defective provirus were suspected of being harbored by the clonally expanded HTLV-1 infected cells in asymptomatic carriers.[19] The polymorphism of the proviral genome was also found in asymptomatic carriers in that study; however, we could not show how commonly the deficiency or polymorphism of the proviral genome was detectable.

These questions prompted us to investigate HTLV-1 PVLs in asymptomatic carriers with different infection routes. In addition, to clarify whether the defective provirus and/or polymorphism of the proviral genome affected PVLs, we tested PVLs for three different regions (5′LTR-*gag*, *gag* and *pX*) of provirus in each individual and compared them among the carriers with different infection routes in our study.

Material and Methods

Samples

Samples of peripheral blood mononuclear cells (PBMCs) were obtained from 309 HTLV-1 carriers (103 men and 206 women, median age: 67 years), who had no symptoms or laboratory data suggesting HTLV-1 related disease, in the Miyazaki Cohort Study.[29] Infection routes were investigated by family HTLV-1 status and history of HTLV-1 seroconversion.[18,22] An HTLV-1 carrier with HTLV-1 positive mother/HTLV-1 negative spouse or with HTLV-1 positive siblings/HTLV-1 negative spouse or with HTLV-1 seroconverter was defined as infected by his/her mother. An HTLV-1 carrier who was a HTLV-1 seroconverter with HTLV-1 positive spouse or with HTLV-1 negative mother/HTLV-1 positive spouse was defined as infected by his/her spouse. Carriers with history of blood transfusion were excluded from the analysis of family status. As a result, 21 and 24 carriers were defined as infected by their mothers and by their spouses, respectively. Infection routes could not be determined in 264 carriers. Informed consent was obtained from the study participants and the study protocol was approved by the institutional review board at University of Miyazaki.

Real-time polymerase chain reaction

PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) were determined by real-time polymerase chain reaction (PCR) using Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). Genomic DNA was isolated from PBMCs of asymptomatic HTLV-1 carriers by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Approximately 100 ng genomic DNA was used as the template. The nucleotide position number of HTLV-1 provirus was according to Seiki *et al.*[30] (accession no. J02029). The primers and probes for real-time PCR were designed to minimize the differences of the melting points 5′LTR-*gag*, *gag* and *pX* and were as follows: 5′LTR-*gag*: the forward primer (5′LTR-SDS-F 5′-AAGTACCGGCGACTCCGTTG-3′: positions 700–719), the reverse primer (HTLV-gag-LTR-R2 5′-GGCTAGCGCTACGGGAAAAG-3′: positions 854–835) and the FAM-labeled probe (5′-FAM-CGTCCGGGATACGAGCGCCCCTT-TAMRA-3′: positions 788–810); *gag*: the forward primer (HTLV-gag-F5 5′-ACCCTTCCTGGGCCTCTATC-3′: positions 1,602–1,621), the reverse primer (HTLV-gag-R5 5′-TCTGGCAGCCCATTGTCAAG-3′: positions 1,695–1,676) and the FAM-labeled probe (HTLV-gag-P5 5′-FAM-ACCACGCCTTCGTAGAACGCCTCAAC-TAMRA-3′: positions 1,644–1,669); *pX*: the forward primer (HTLV-pX2-S 5′-CGGATACCCAGTCTACGTGTT-3′: positions 7,359–7,379), the reverse primer (HTLV-pX2-AS 5′-CAGTAGGGCGTGACGATGTA-3′: positions 7,458–7,439) and the FAM-labeled probe (HTLV-pX2-Probe 5′-FAM-CTGTGTACAAGGCGACTGGTGCC-TAMRA-3′: positions 7,386–7,408).[18,26] A coding region for albumin (*Alb*) was used to measure the copy number of human genome. The primers and the probe for the *Alb* were as follows: The forward primer (Alb-S2 5′-TGTCATCTCTTGTGGGCTGT-3′), the reverse primer (Alb-AS2 5′-GGTTCTCTTTCACTGACATCTGC-3′) and the FAM-labeled probe (Alb-probe 5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA-3′). A plasmid containing PCR products for HTLV-1 5′LTR-*gag*, *gag*, *pX* regions and *Alb* was constructed using pGEM T-Easy Vector (Promega Corporation, Madison, WI) and was used as a control template for real-time PCR. PVLs of each region of HTLV-1 provirus were measured in a duplicate manner and were shown as copies per 100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To detect the provirus with large deletion of HTLV-1 internal sequence, long PCR, which amplifies provirus maintaining both 5′ and 3′ LTR, was performed as described previously.[19] The primers were as follows: 5′LTR (HTLV-0647F 5′-GTTCCACCCCTTTCCCTTTCATTCACGACTGACTGC-3′: positions 647–682) and 3′LTR (HTLV-8345R 5′-GGCTCT AAGCCCCCGGGGGATATTTGGGGCTCATGG-3′: positions

8,345–8,310).[26] Long PCR was performed using LA Taq Hot start version (Takara Bio, Shiga, Japan). Genomic DNA containing 200 copies of HTLV-1 provirus for the *pX* region was used for this assay. To ensure that the same amount of provirus was used in each reaction, PCR for the *pX* region was performed as an internal control. Primers for this PCR were as follows: the forward primer (HTLV-7396F 5′-GGCGACTGGTGCCC-CATCTCTGGGGGACTATGTTCG-3′: positions 7,396–7,431) and the reverse primer described above (HTLV-8345R). The PCR products were electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by inverse long PCR

As described in results, both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in two carriers (C20 and 21) and they were suspected of having provirus with deletion of 5′LTR and its flanking internal sequence. Inverse long PCR (IL-PCR) was used to amplify the genomic DNA adjacent to the 3′LTR of HTLV-1 provirus according to the method described previously with slight modifications.[15] In brief, the genomic DNA was digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and then self-ligated by T4 ligase following digestion with *Mlu* I. Amplification of the resultant DNA was performed using the LA Taq Hot start version. The primers used in this analysis were as follows; a forward primer in the U5 region of the LTR (5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′: positions 8,856–8,889) and a reverse primer, HTLV-7002R (5′-AGTATTTGAAAAGGAAGGAAGAGGAGAAGGCA-3′: positions 7,002–6,971). Subcloning of the amplified fragments of IL-PCR were subjected to sequencing assay according to the protocol of the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using ABI Prism 310 DNA Sequencer (Applied Biosystems) and the human genomic sequence downstream of the HTLV-1 provirus was obtained. The human genomic sequence upstream of the provirus was assumed based on this information by BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat).[31] The primers for human genomic sequence upstream of the provirus were designed and long PCR was performed using a forward primer (5′-GTGATC-CATGGTGTTTGTCCACCTGAAAGC-3′) and a reverse primer HTLV-7002R in C20, and a forward primer (5′-TCCAAGTGGGATGTCACGGCCACTTCTC-3′) and a reverse primer HTLV-7002R in C21. To determine the upstream junction sequence between host genome and provirus, the PCR products were subjected to direct sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit.

Statistical Analysis

Mann-Whitney's U test was used to compare *pX* PVLs, *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios among the groups of asymptomatic HTLV-1 carriers with different infection routes. Spearman's correlation coefficient by rank was used to determine the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratio.

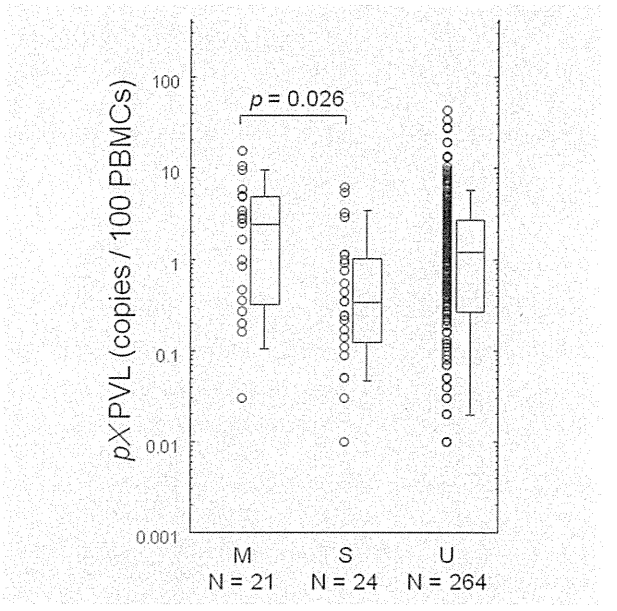

Figure 1. *PX* PVLs in HTLV-1 carriers with different infection routes M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

Results

pX PVLs in HTLV-1 carriers with different infectious routes

PVLs for the 5′LTR-*gag*, *gag* and *pX* regions in each individual were measured in 309 asymptomatic HTLV-1 carriers. Because the *pX* region has been reported to be conserved in the HTLV-1 provirus, *pX* PVL was considered to represent total PVLs.[23,25] As shown in Figure 1, median *pX* PVL (2.49 copies/100 PBMCs) in 21 asymptomatic carriers, who were infected by their mothers, was significantly higher than that (0.34 copies/100 PBMCs) in 24 carriers who were infected by their spouses ($p = 0.026$). Median *pX* PVL in 264 asymptomatic carriers, whose infection routes were undetermined, was between these values (1.24 copies/100 PBMCs).

PVLs for 3 different proviral regions (5′LTR-gag, gag and pX) of HTLV-1

To determine whether PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) of HTLV-1 were equal in asymptomatic carriers, PVLs for the 5′LTR-*gag* and *gag* regions were measured and compared to PVLs for the *pX* region. Because 100 ng of genomic DNA, which is derived approximately 15,000 PBMCs, was used for the template for real time-PCR, 148 carriers with *pX* PVL, which was less than 1 copy/100 PBMCs, were not provided for further analysis to avoid unstable result due to the small number of proviral copies in each reaction. The results of our study were

shown as the ratio of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in each individual (Fig. 2). The median 5′LTR-*gag* PVL/*pX* PVL ratio of 161 HTLV-1 carriers tested was 0.97. Therefore, HTLV-1 proviral sequence for 5′LTR-*gag* PVL was considered to be conserved in the majority of asymptomatic carriers. The median *gag* PVL/*pX* PVL ratio, however, was 0.61.

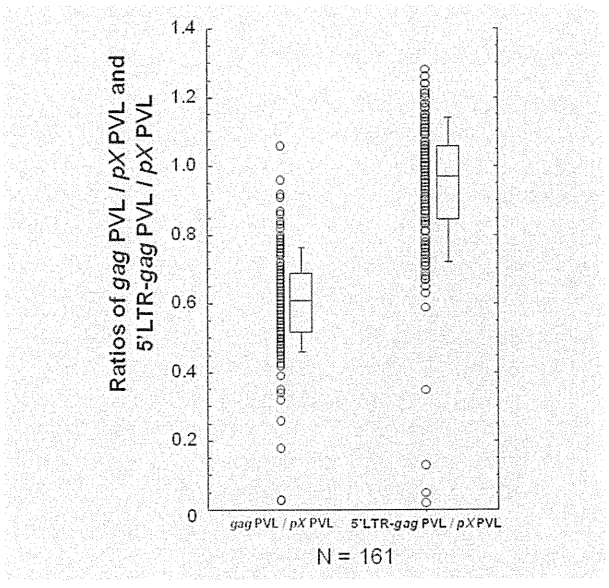

Figure 2. The ratios of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in 161 asymptomatic HTLV-1 carriers, whose *pX* PVLs were equal to or greater than 1 copy/100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To determine whether the provirus with deletion of HTLV-1 internal sequence accounted for low *gag* PVL/*pX* PVL ratio, long PCR was performed. For this analysis, we chose 26 carriers with low *gag* PVL/*pX* PVL ratios of less than 0.5; however, adequate DNA sample for long PCR was available in only 17 of the 26 subjects. All subjects except C1 showed a band of 7.7 kb, which was considered to be derived from complete provirus, and some additional smaller bands suggesting defective provirus (Fig. 3*a*). C1 showed only a dense band of 4.5 kb. C1 was analyzed in our previous study and a large deficiency (3.2 kb, positions 1,203–4,368) of internal sequence was shown.[19] Additional four carriers (C3, 4, 11 and 13) showed dense bands equal to or stronger than the band for complete provirus (arrows in Fig. 3*a*). Cloning and DNA sequencing of these dense bands showed large deficiencies of internal sequences (4.9 kb, positions 1,368–6,286 in C3; 0.9 kb, positions 1,413–2,284 in C4; 4.8 kb, positions 1,009–5,763 in C11 and 4.8 kb, positions 1,133–5,974 in C13).

Four carriers (C18–21) had low 5′LTR-*gag* PVL/*pX* PVL ratios of less than 0.5. Long PCR of C18 and 19 showed dense bands of 7.7 kb, which were considered to be derived from complete provirus, and some additional smaller bands (Fig. 3*b*). Polymorphism of proviral DNA sequence of the sites for primers and/or probe for 5′LTR-*gag* PVL was suspected in these two cases, and cloning and DNA sequencing of the PCR products were performed. The polymorphisms of DNA sequence for the annealing site of the forward primer (708 G > A and 709 C > G in C18; 712 C > T in C19) were consistently found, and these polymorphisms were

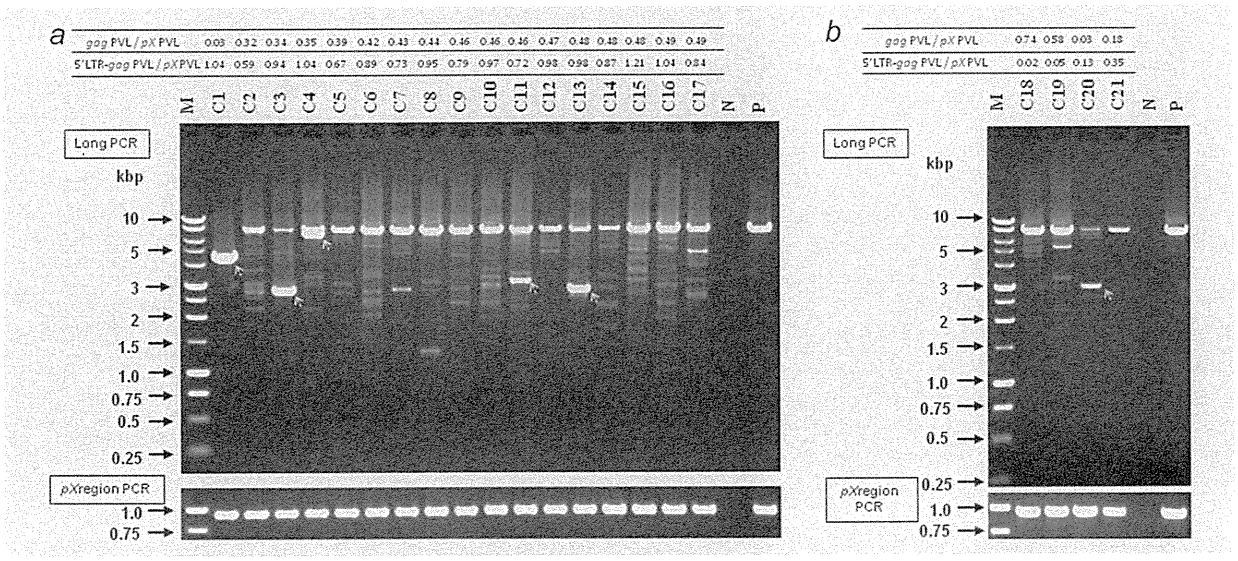

Figure 3. Detection of defective provirus by long PCR. (*a*) Asymptomatic HTLV-1 carriers with low *gag* PVL/*pX* PVL ratios less than 0.5. (*b*) Asymptomatic HTLV-1 carriers with low 5′LTR-*gag* PVL/*pX* PVL ratios less than 0.5. Arrows indicate PCR products for HTLV-1 provirus lacking large internal sequence. M: Molecular weight marker; N: HTLV-1-negative subject; P: HTLV-1-positive cell line, ED-40515(-).

considered to account for the decreased efficacy of real time-PCR for 5′LTR-*gag* PVL.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by IL-PCR

Both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in the additional two carriers (C20 and 21). Long PCR showed a weak band of 7.7 kb for complete provirus and a stronger band of 2.9 kb in C20 (Fig. 3*b*). In the case of C21, only a weak band for complete band was observed (Fig 3*b*). These data suggested defective provirus, which had not been detected by long PCR, existed in C20 and C21. Because these proviruses were suspected of lacking 5′LTR and its flanking internal sequence, we attempted to identify them by IL-PCR. First, the genomic DNA of C20 and C21 were digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and resultant DNA was provided for IL-PCR as a template. In C20, approximately 1.1 kb of PCR product was obtained in digestion with *Kpn* I alone (Fig. 4*a-1*). No IL-PCR product was obtained using other restriction enzymes (data not shown). When this PCR product was digested with *Kpn* I, two major bands appeared, as expected (Fig. 4*a-1*). Cloning and sequencing revealed that this product consisted of HTLV-1 provirus (*Kpn* I site at position: 6,141 to the end of 3′LTR) and its flanking genomic DNA of human chromosome 2 (2q13). Based on the information obtained, a forward primer to anneal the upstream human genome adjuncted to the provirus was prepared and clone-specific PCR was performed. Cloning and sequencing of this clone-specific PCR product revealed that it lacked 5′LTR and its internal flanking sequence (until position 5,999; Fig. 4*a-2*). In the case of C21, IL-PCR product was obtained in digestion with *Hind* III alone. Following the same procedure as in C20, it was revealed that a provirus integrated in human chromosome 18 (18p11.32), and that it lacked 5′LTR and its internal flanking sequence (until position 4,976) (Figs. 4*b-1* and 4*b-2*).

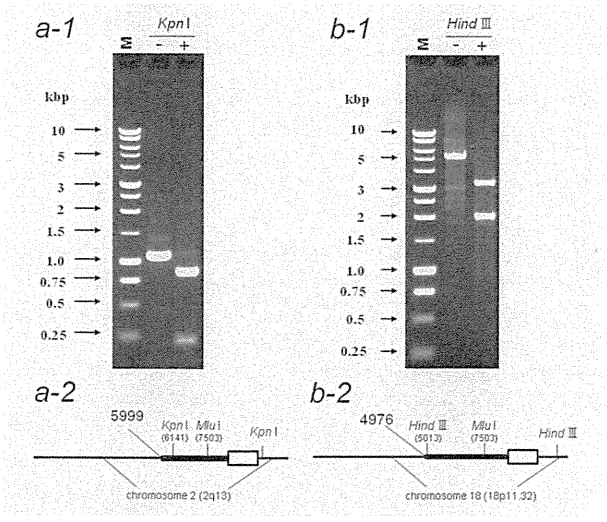

Figure 4. Detection of provirus with deletion of 5′LTR and its internal flanking sequence by IL-PCR. (*a-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C20, with or without *Kpn* I digestion. (*a-2*) Scheme of the structure of defective provirus in C20. (*b-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C21, with or without Hind III digestion. (*b-2*) Scheme of the structure of defective provirus in C21.

Relationship between pX PVL and gag PVL/pX PVL or 5′LTR-gag/pX PVL ratios

To determine whether the HTLV-1 PVLs correlated with the number of provirus with deficiency and/or polymorphism of the *gag* or 5′LTR-*gag* regions, the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios was analyzed. As shown in Figure 5*a*, there was a negative

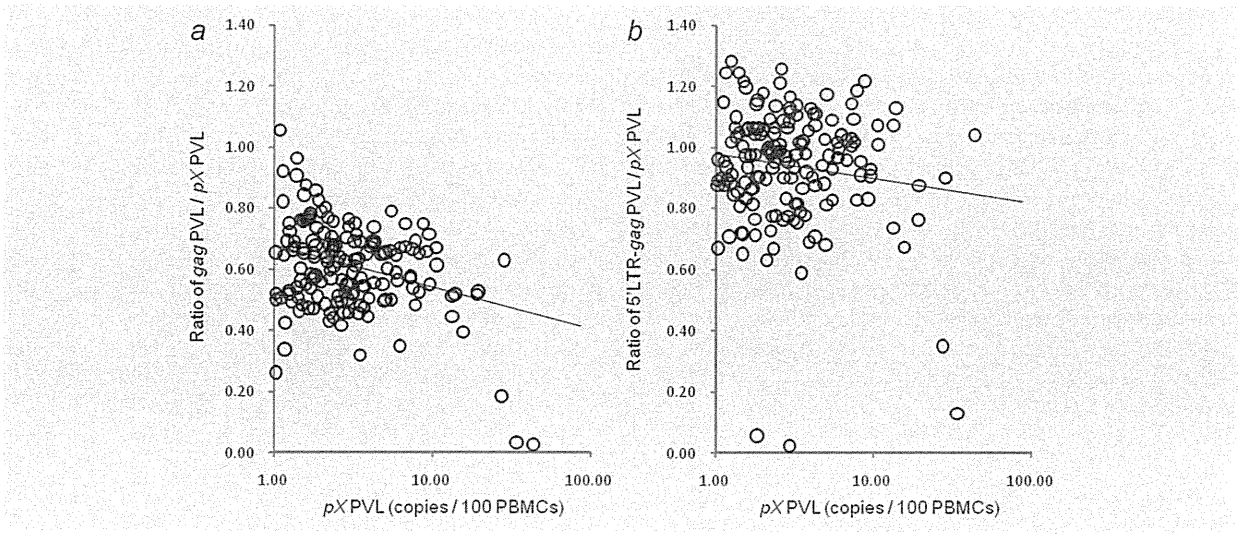

Figure 5. Relations of *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratios in 161 asymptomatic carriers. (*a*) Relation of *pX* PVL and *gag* PVL/*pX* PVL. (*b*) Relation of *pX* PVL and 5′LTR-*gag* PVL/*pX* PVL.

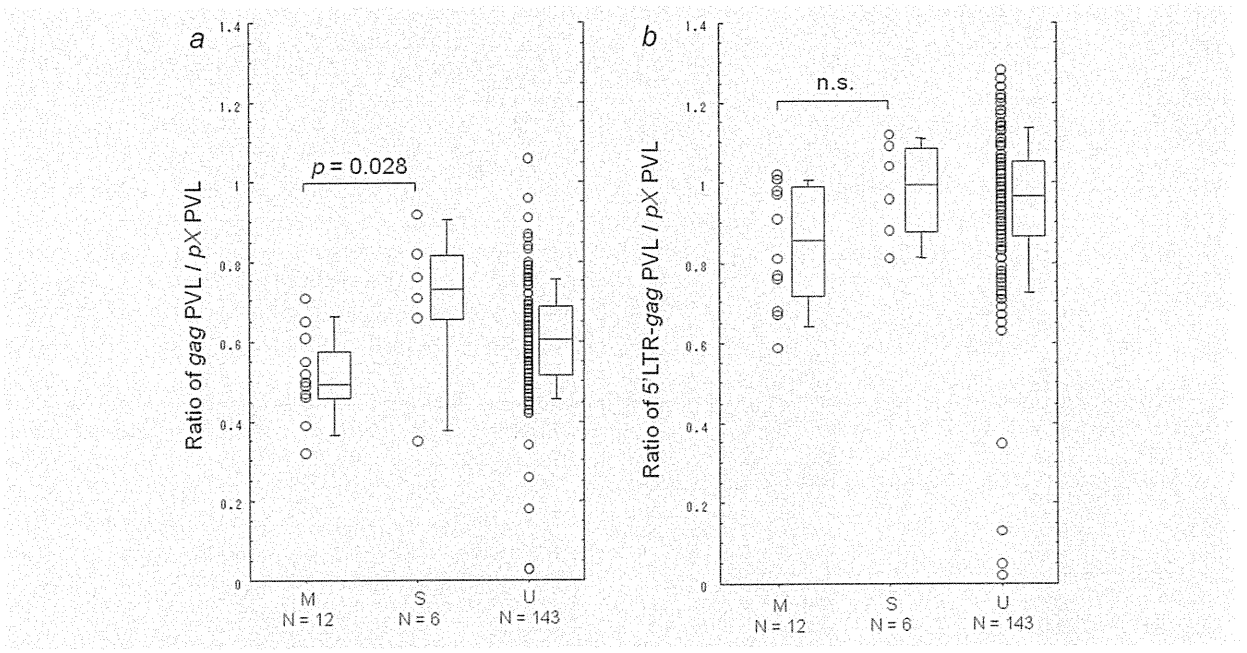

Figure 6. The ratios of *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL in HTLV-1 carriers with different infection routes in 161 asymptomatic carriers. (*a*) The ratio of *gag* PVL/*pX* PVL. (*b*) The ratio of 5′LTR-*gag* PVL/*pX* PVL. M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

correlation between *pX* PVL and the *gag* PVL/*pX* PVL ratio ($r = -0.46$, $p = 0.02$). Therefore, HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region were considered to be more prevalent in asymptomatic carriers with high PVL. In the case of 5′LTR-*gag*/*pX* PVL ratio, the trend was not obvious (Fig. 5*b*) ($r = -0.20$, $p = 0.94$). However, variability of the 5′LTR-*gag*/*pX* PVL ratio was greater than that of *gag* PVL/*pX* PVL ratio. This may have been the result of technical inadequacies in the measurement of 5′LTR-*gag* PVL.

The ratios of gag PVL/pX PVL and 5′LTR-gag PVL/pX PVL in HTLV-1 carriers with different infection routes

Next, the relationships between infection routes and the *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios were analyzed. The median ratio of *gag* PVL/*pX* PVL in 12 HTLV-1 carriers with maternal infection (0.50) was significantly lower than that in six carriers with spousal infection (0.74) ($p = 0.028$) (Fig. 6*a*). The median *gag* PVL/*pX* PVL ratio of 143 carriers with undetermined infection route (0.62) was between these. The 5′LTR-*gag* PVL/*pX* PVL ratio did not reveal a significant difference between the carriers with maternal infection and spousal infection (Fig. 6*b*). Therefore, the carriers with maternal infection were considered to have a greater number of HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region. In addition, when a *gag* PVL/*pX* PVL ratio of 0.65 was used as cut-off value, 11 of 12 (92%) carriers with maternal infection, against only one of six (17%) carriers with spousal infection, showed lower values.

Discussion

First, HTLV-1 PVLs in asymptomatic carriers with different infection routes were analyzed. *PX* PVL in 21 asymptomatic carriers with maternal infection was significantly higher than that in 24 carriers with spousal infection. These results agreed with data reported by Roucoux *et al.*[32] showing that PVLs in index HTLV-1 positive carriers were higher than those of their newly infected partners. Asymptomatic carriers whose infection routes were undetermined showed values between these. Previously, we analyzed the PVLs of HTLV-1 seroconverters and their spouses and showed that PVLs were not equivalent between them.[22] Because HTLV-1 in a seroconverter and in his/her spouse is identical, the host factor was considered important in the determination of HTLV-1 PVL. The results of our study suggest that infection route and/or time of infection are factors in the determination of PVL in HTLV-1 carriers. We also reported that HTLV-1 carriers who developed ATL had high PVLs even before they developed the disease.[20] Recently, Iwanaga *et al.*[21] also tested the PVLs of 1,218 HTLV-1 carriers and found that HTLV-1 carriers that developed ATL had high PVLs. These data suggest that high HTLV-1 PVL is a risk factor for developing ATL. In our study, HTLV-1 carriers with maternal infection tended to have high PVLs. This may account for why perinatal infection is a risk factor of ATL at least in part.

Because the frequent detection of defective provirus in patients with ATL has been reported, we examined provirus with deficiencies and/or polymorphism of proviral sequence in asymptomatic HTLV-1 carriers. The *pX* region has been

reported to be conserved in HTLV-1 provirus, and PCR for this region was used to measure total PVL.[23,25] Ohshima *et al.*[25] reported that variation of DNA sequence is frequently detected in the *gag* region of HTLV-1 provirus in patients with ATL. Kamihira *et al.*[24] also reported that most of deficient provirus in patients with ATL lacked part of the *gag* region in the proviral regions of HTLV-1 tested. HTLV-1 provirus with deletion of the 5′LTR, and its flanking internal sequences was also found in patients with ATL.[26] In our study, therefore, we tried to find provirus with deficiencies and/or polymorphism of DNA sequence in the asymptomatic carriers by measuring PVLs for the *gag* and 5′LTR-*gag* regions as ratios to *pX* region PVLs. As a result, median 5′LTR-*gag* PVL/*pX* PVL and *gag* PVL/*pX* PVL ratios of 161 HTLV-1 carriers with relatively high *pX* PVL (equal to or greater than one copy per 100 PBMCs) were 0.97 and 0.61, respectively. Our interpretation of this result was that many HTLV-1 infected cells in asymptomatic carriers harbor provirus with deficiency and/or polymorphism of DNA sequences for the sites of primers and/or probe for *gag* real time-PCR.

Long PCR analysis was performed on 17 carriers with low *gag* PVL/*pX* PVL ratios. Five of 17 carriers (29%) were shown to have the provirus with large deletions of internal DNA sequence including the *gag* region. The clonal expansion of HTLV-1 infected cells harboring defective provirus in these five carriers was most likely. In fact, clonal expansion of HTLV-1 infected cells in C1 was already shown in our previous study.[19] The reason for the low *gag* PVL/*pX* PVL ratios in the other 12 carriers was not clear. Contribution of the sum total of HTLV-1 infected cells with defective provirus, which did not reveal dense bands, was possible. Alternatively, polymorphism of the proviral DNA sequence for the *gag* region may have decreased the efficiency of real time-PCR for *gag* PVL. However, cloning and DNA sequencing of the sites for primers and probes for real time-PCR for *gag* PVL in these carriers did not show consistent polymorphism of the proviral DNA (data not shown). This may be because there is high diversity of proviral DNA sequence in the *gag* region of HTLV-1 and it was not possible to prepare cloning primers to work for all of them.

The other two (C20 and 21) showed low ratios not only of 5′LTR-*gag* PVL/*pX* PVL but also of *gag* PVL/*pX* PVL. Our previous study showed that they had high PVLs and clonal expansion of HTLV-1 infected cells with defective provirus.[19] We could not identify the type of defective provirus in the previous study. In our study, however, we found provirus lacking 5′LTR and its internal flanking region existed in these carriers.

In our study, the provirus with deficiency and/or polymorphism of the *gag* region was commonly found in asymptomatic HTLV-1 carriers. Few carriers had provirus lacking 5′LTR and its flanking sequence. Carriers with provirus with deficiency and/or polymorphism of the *gag* region were found frequently among asymptomatic carriers with high PVLs. These infected cells may not express certain HTLV-1 proteins. This change may make it possible for the HTLV-1 infected cells to avoid attack by cytotoxic T-lymphocytes.[33] Therefore, there is a possibility that provirus with deficiency and/or polymorphism of HTLV-1 provirus contributes to the survival of HTLV-1 infected cells. Indeed, our previous study showed that C1, 20 and 21 had clonal expansion of HTLV-1 infected cells.[19]

Low *gag* PVL/*pX* PVL ratio was found to be associated with maternal infection. The reason carriers with maternal infection have a greater number of HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region was not clear in our study. The replication of HTLV-1 infected cells in long-term infected carriers may account for this. Alternatively, a low level of new cell to cell infection *in vivo* can contribute to the creation of deficiency and/or polymorphism in proviral genome.

Maternal infection has been considered to be a risk factor for the development of ATL in asymptomatic carriers. However, there has been no method to identify infection route in the absence of information on family HTLV-1 status. The results of our study suggest the possibility that *gag* PVL/*pX* PVL ratio can be used as a tool to differentiate the infection routes of asymptomatic HTLV-1 carriers. Due to the fact that only a small number of HTLV-1 carriers with known infectious routes were analyzed in our study, further study with a larger number of subjects is necessary.

A major limitation of our study is that the subjects were elderly individuals, whose median age was 67 years old. The average age at onset of ATL was reported as 60 years.[34] Therefore, it is not clear whether the same result would be obtained from an analysis of younger HTLV-1 asymptomatic carriers. In addition, carriers with low *pX* PVL (less than 1 copy/100 PBMCs) were not provided for the analysis of deficiency and/or polymorphism of HTLV-1 proviral sequence because of technical limitations. Further analysis of carriers with low PVLs using improved methodology is necessary.

In conclusion, our study showed that *pX* PVL in carriers with maternal infection was significantly higher than that in carriers with spousal infection. Low *gag* PVL/*pX* PVL ratio reflecting deficiency and/or polymorphism in proviral genome was associated with high PVLs and maternal infection. These data suggest that development of ATL in carriers with maternal infection may be due in part to high PVL, which can be related to provirus with deficiency and/or polymorphism in proviral genome. In addition, *gag* PVL/*pX* PVL ratio has potential for use as a tool to differentiate infection routes of asymptomatic HTLV-1 carriers. Further study is necessary to clarify the mechanism of deficiency and/or polymorphism in HTLV-1 proviral genome and its implications in ATL development.

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