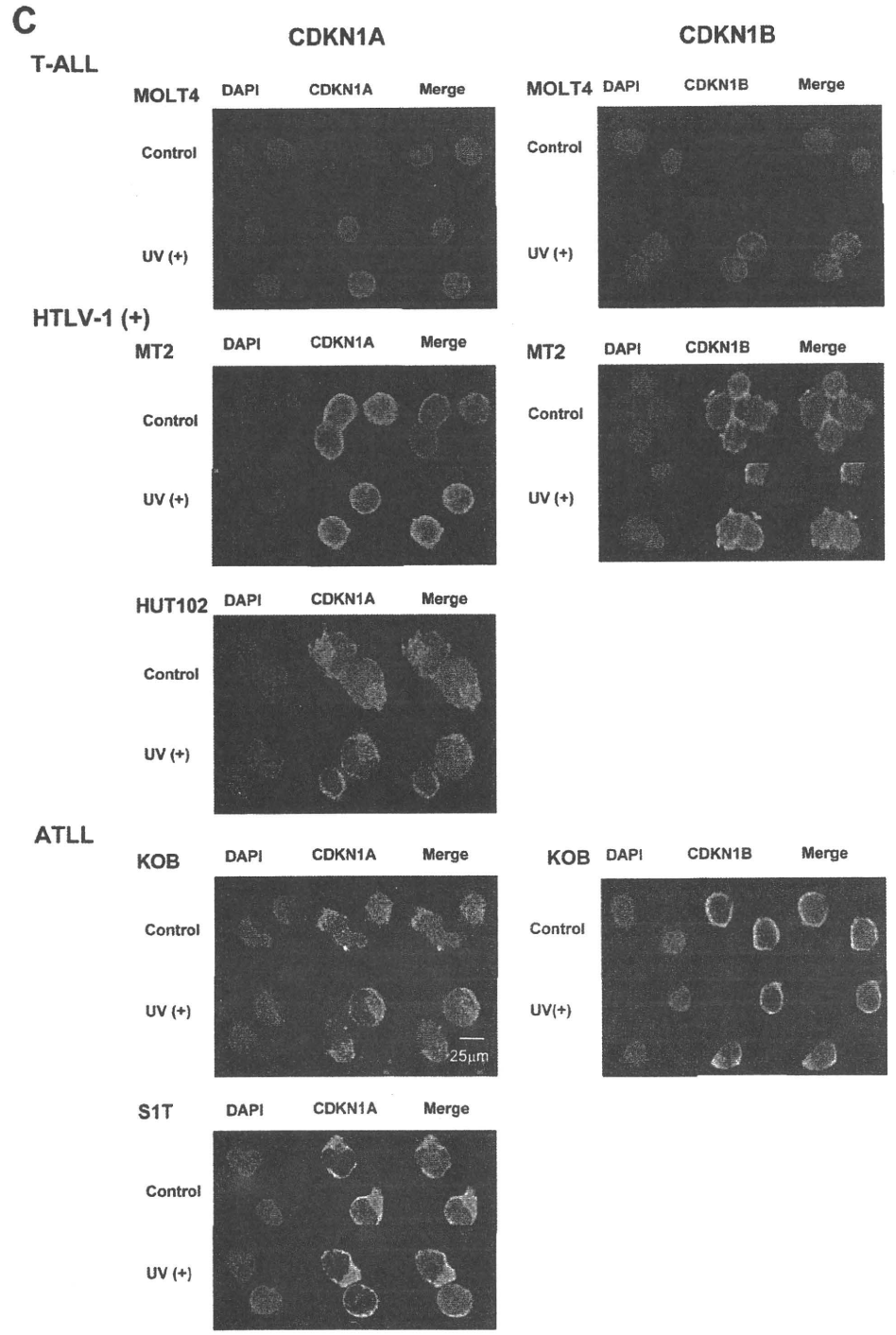


FIG. 3—*Continued.*

CDKN1B were not different between three samples of peripheral blood lymphocytes and three HTLV-1-infected cell lines (Fig. 4A). Moreover, the expression levels of CDKN1B were not significantly different between $CD4^+$ lymphocytes and acute-type ATLL cells (data not shown). On the other hand, recent findings from several labs have also shown that HTLV-1 Tax and HTLV-1 infection drastically upregulate expression levels of both CDKN1A and CDKN1B to cause cell cycle arrest or cellular senescence just after the infection (15, 33). However, many reports also showed that the HTLV-1-infected cell lines could grow well and proliferate intensely, despite abundant *CDKN1A* expression (5). Notably, we observed the cytoplasmic localization of CDKN1A protein phosphorylated at T145 by Akt in HTLV-1-infected and ATLL cell

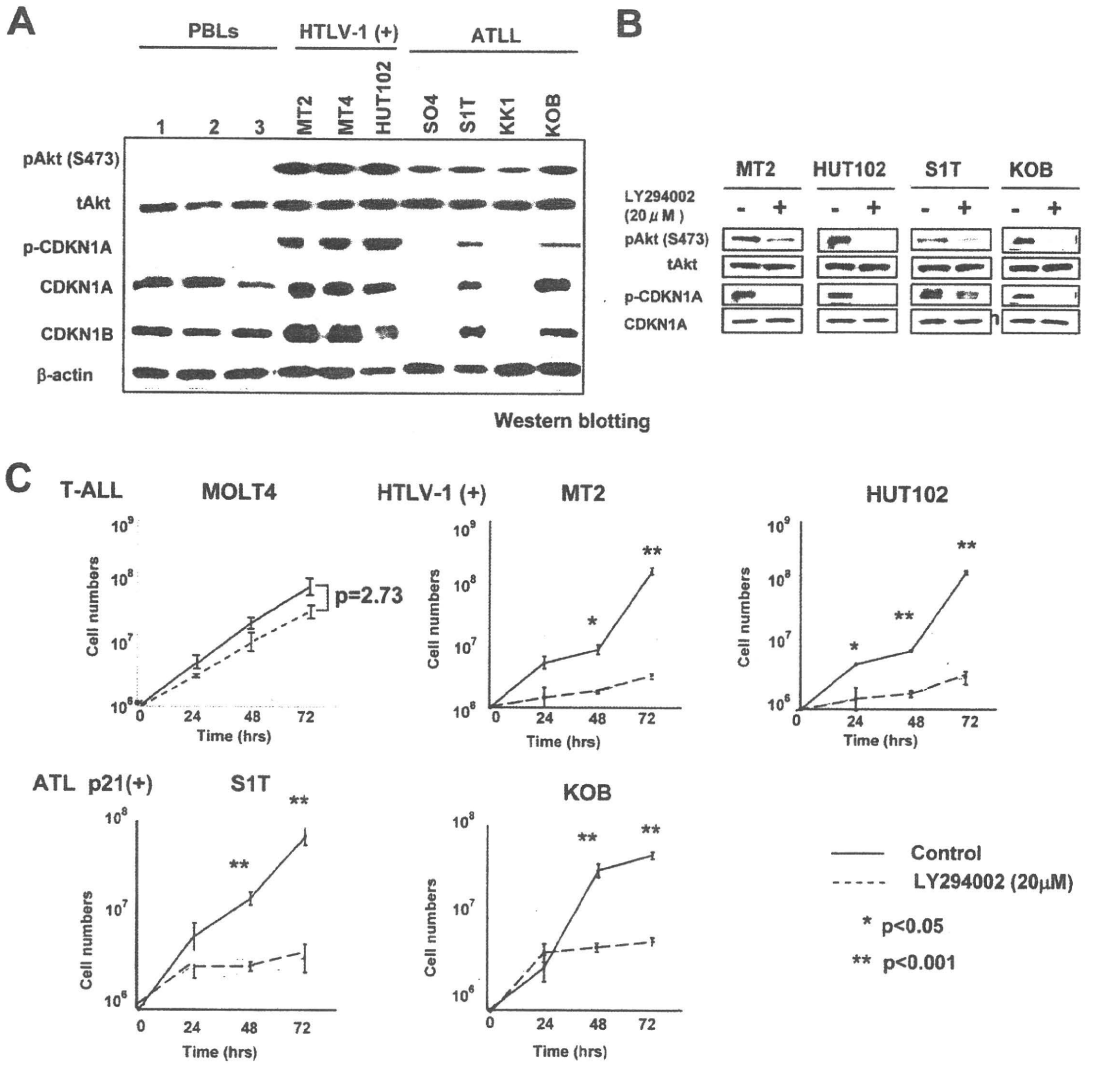


FIG. 4. Cytoplasmic sequestration and functional inactivation of CDKN1A in HTLV-1-infected and ATLL cell lines. (A) Expression of Akt, phospho-Akt (Ser473), CDKN1A, phospho-CDKN1A (Thr145), and CDKN1B in PBLs and HTLV-1-infected and ATLL cell lines was examined by Western blotting. (B) HTLV-1-infected cell lines and ATLL cell lines were treated with 20 μM LY294002 for 2 h, and Western blot analyses were performed for phospho-Akt, Akt, phospho-CDKN1A, and CDKN1A. (C) Survival curves of various types of T-cell leukemia cell lines after treatment with the PI3K inhibitor LY294002. Cells were treated with 20 μM LY294002 for each indicated time point, and their cell numbers were measured by trypan blue exclusion method (0 h represents the untreated controls). A Student's *t* test was used for statistical analysis. (D) The subcellular localization of CDKN1A and CDKN1B with or without LY294002 treatment was detected by immunofluorescence in various types of T-cell leukemia cell lines. DAPI stain was used to visualize the nuclei. Magnification, ×400; bar, 25 μm. (E) MT-2 and KOB cells were grown under serum-free conditions and either left untreated (control) or treated with UV radiation (20 J/m^2), LY294002 (20 μM), or both UV radiation and LY294002 and cultured for the indicated times. Cell cycle progression and apoptosis were analyzed by FACS analysis upon PI staining. The percentage of cells in each fraction at 0 h and 48 h is presented. (F) KOB ATLL cells were treated with UV radiation and/or LY294002 as described for panel E. The percentage of cells undergoing apoptosis was quantitated by staining with annexin V-FITC and PI, and the distribution of cells is presented in each quadrant by flow cytometric analysis.

lines. It is reported that activation of PI3K/Akt signaling contributes to the phosphorylation of CDKN1A at T145 and of CDKN1B at T157 with their cytoplasmic localization (15, 26, 34, 35, 36, 39). In HTLV-1-infected cell lines, Tax is reported to promote Akt phosphorylation by directly binding to PI3K (18) or by downregulating *PTEN* transcription through NF-κB signaling (8). In this study, LY294002-mediated dephosphorylation of Akt induced relocation of CDKN1A and CDKN1B into the nucleus, with accumulation of cells in the G_1 phase, suggesting that cytoplasmic localization of CDKN1A is an important factor in the antiapoptotic effect in HTLV-1-infected cell lines. However, most of the CDKN1A and CDKN1B still remained in the cytoplasm of ATLL cells after LY294002 treatment, suggesting that mechanisms other than phosphorylation may contribute to the subcellular localization of CDKN1A in ATLL cells, and we further speculate that the nuclear import system of CDKN1A may be disturbed in ATLL cells for unknown reasons.

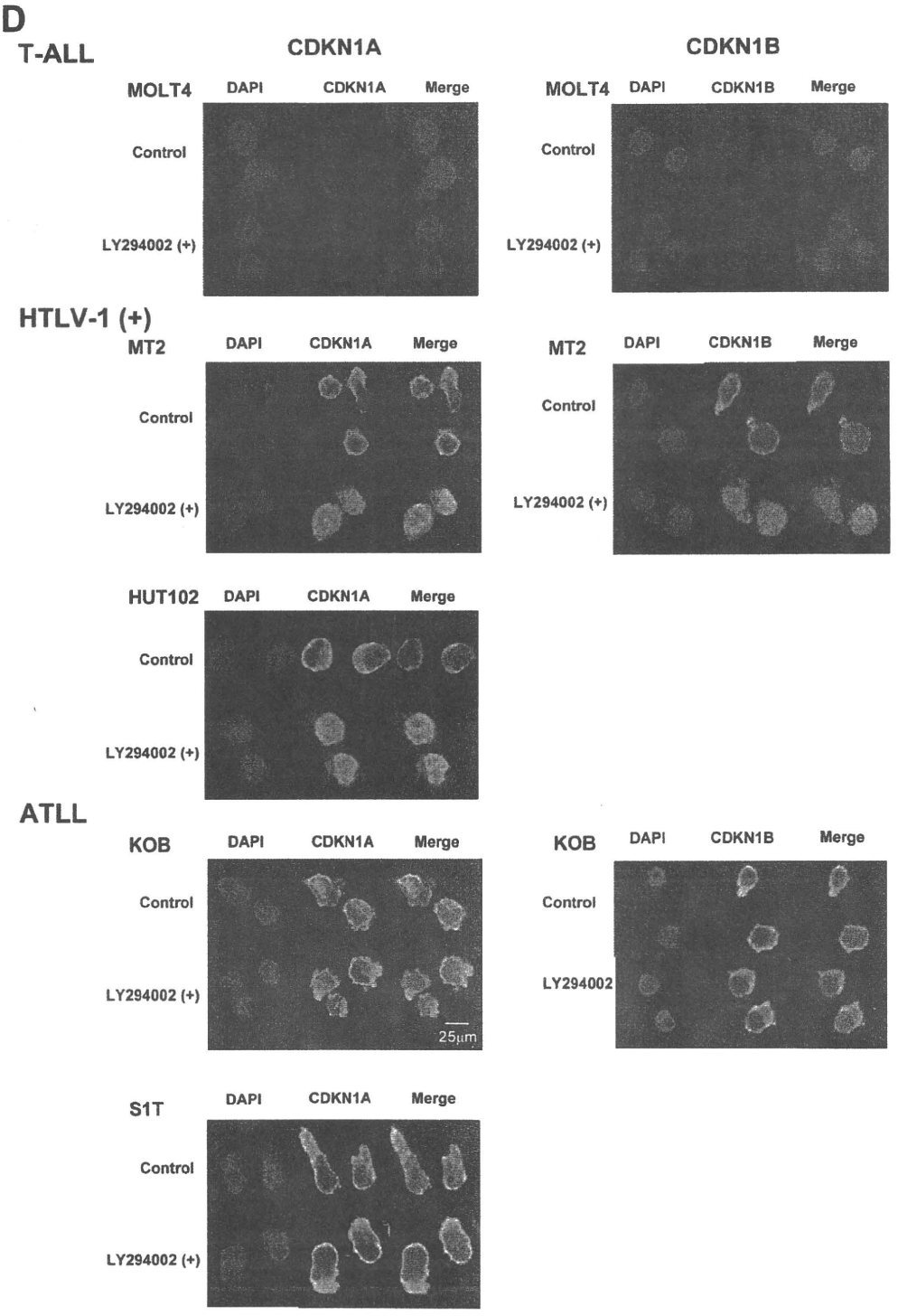


FIG. 4—*Continued.*

Neither UV irradiation nor LY294002 treatment induced apoptotic cell death in HTLV-1-infected cell lines. Tax could account, at least in part, for the antiapoptotic property of HTLV-1-infected cells, through, for instance, activation of the PI3K/Akt pathway, costimulatory receptor signaling (OX40/OX40L), and enhanced expression of various antiapoptotic proteins (c-Flip, Bcl-xL, Bfl-1, and Hiap-1) (12). On the other hand, apoptotic cell death was induced in ATLL cells after UV irradiation or LY294002 treatment. Since inactivation of Tax was observed in over 70% of ATLL cells because of genetic

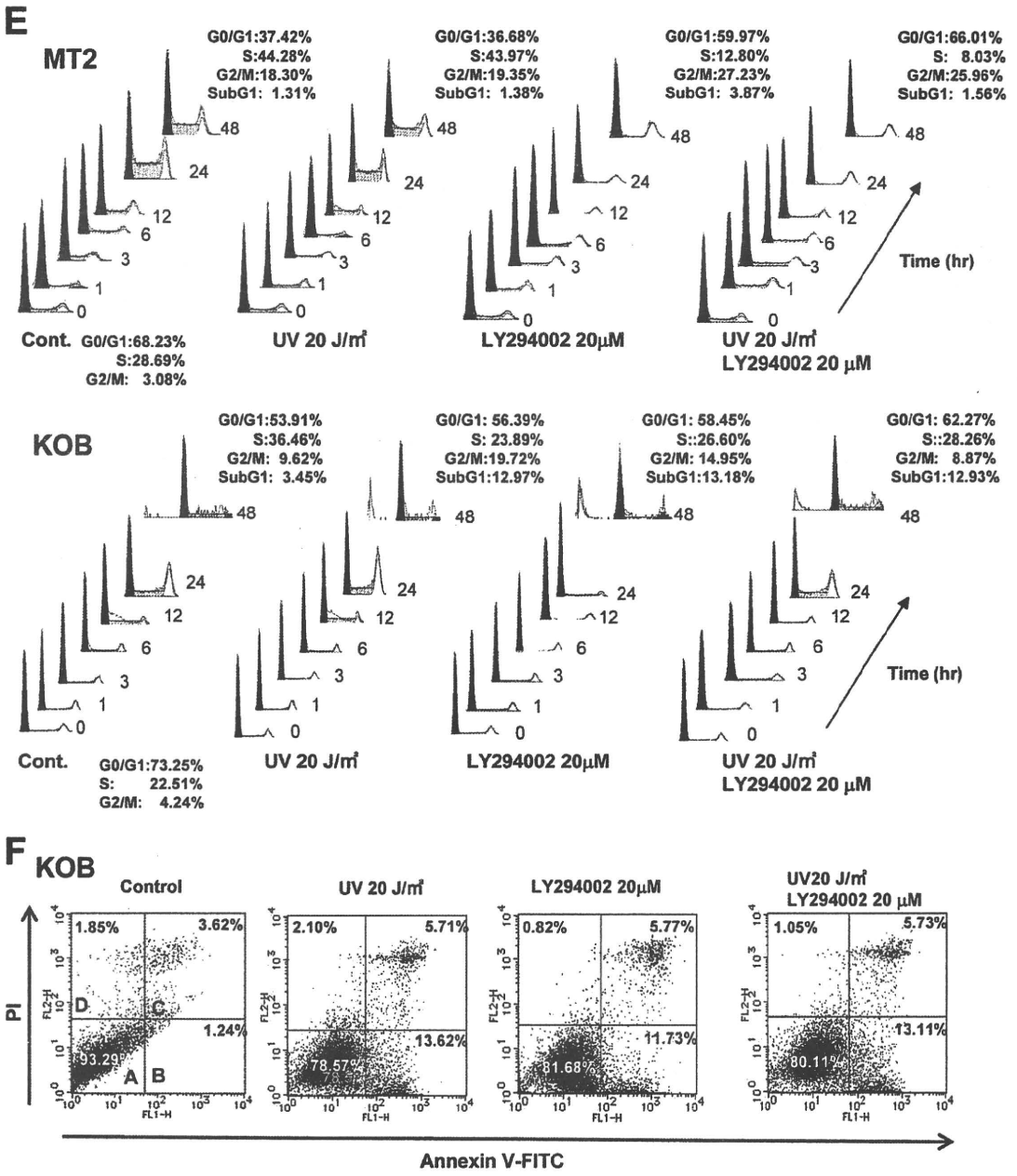


FIG. 4—*Continued.*

abnormalities and DNA methylation in the HTLV-1 genome, apoptotic cell death induced after UV irradiation or LY294002 treatment may be due to a lack of Tax-mediated antiapoptotic effects. As the promoter region of *CDKN1A* was also methylated in the majority of the ATLL patients, the accumulation of abnormal DNA methylation in ATLL cells may be involved in leukemogenesis by the downregulation of *Tax* and *CDKN1A* during the progression from HTLV-1-infected cells to ATLL cells. Recently, the HTLV-1 bZIP factor gene (*HBZ*) within unmethylated or undeleted regions of the 3′ long terminal repeat (LTR) has been shown to be consistently expressed and to promote the cell growth of ATLL cells, suggesting that the *HBZ* gene likely is critical for pathogenesis of ATL cells along with the downregulation of *Tax* and *CDKN1A*.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research of Priority Area from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a research fund from the Miyazaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST.

REFERENCES

1. **Akagi, T., H. Ono, and K. Shimotohno.** 1995. Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. Blood **86**:4243–4249.

2. **Cereseto, A., R. Washington Parks, E. Rivadeneira, and G. Franchini.** 1999. Limiting amounts of p27Kip1 correlates with constitutive activation of cyclin E-CDK2 complex in HTLV-I-transformed T-cells. Oncogene **18:**2441–2450.
3. **Claus, R., and M. Lubbert.** 2003. Epigenetic targets in hematopoietic malignancies. Oncogene **22:**6489–6496.
4. **de La Fuente, C., L. Deng, F. Santiago, L. Arce, L. Wang, and F. Kashanchi.** 2000. Gene expression array of HTLV type 1-infected T cells: Up-regulation of transcription factors and cell cycle genes. AIDS Res. Hum. Retroviruses **16:**1695–1700.
5. **de La Fuente, C., F. Santiago, S. Y. Chong, L. Deng, T. Mayhood, P. Fu, D. Stein, T. Denny, F. Coffman, N. Azimi, R. Mahieux, and F. Kashanchi.** 2000. Overexpression of $p21^{waf1}$ in human T-cell lymphotropic virus type 1-infected cells and its association with cyclin A/Cdk2. J. Virol. **74:**7270–7283.
6. **Dewan, M. Z., N. Takamatsu, T. Hidaka, K. Hatakeyama, S. Nakahata, J. Fujisawa, H. Katano, N. Yamamoto, and K. Morishita.** 2008. Critical role for TSLC1 expression in the growth and organ infiltration of adult T-cell leukemia cells in vivo. J. Virol. **82:**11958–11963.
7. **Fang, J. Y., and Y. Y. Lu.** 2002. Effects of histone acetylation and DNA methylation on p21 (WAF1) regulation. World J. Gastroenterol. **8:**400–405.
8. **Fukuda, R. I., K. Tsuchiya, K. Suzuki, K. Itoh, J. Fujita, A. Utsunomiya, and T. Tsuji.** 2009. Human T-cell leukemia virus type I Tax down-regulates the expression of phosphatidylinositol 3,4,5-trisphosphate inositol phosphatases via the NF-κB pathway. J. Biol. Chem. **284:**2680–2689.
9. **Harhaj, E. W., L. Good, G. Xiao, and S. C. Sun.** 1999. Gene expression profiles in HTLV-I-immortalized T cells: deregulated expression of genes involved in apoptosis regulation. Oncogene **18:**1341–1349.
10. **Hidaka, T., S. Nakahata, K. Hatakeyama, M. Hamasaki, K. Yamashita, T. Kohno, Y. Arai, T. Taki, K. Nishida, A. Okayama, Y. Asada, R. Yamaguchi, H. Tsubouchi, J. Yokota, M. Taniwaki, Y. Higashi, and K. Morishita.** 2008. Down-regulation of TCF8 is involved in the leukemogenesis of adult T-cell leukemia/lymphoma. Blood **112:**383–393.
11. **Jiemjit, A., T. E. Fandy, H. Carraway, K. A. Bailey, S. Baylin, J. G. Herman, and S. D. Gore.** 2008. p21(WAF1/CIP1) induction by 5-azacytosine nucleosides requires DNA damage. Oncogene **27:**3615–3623.
12. **Katrin, S., and G. Ralph.** 2007. Human T cell leukemia virus type 1 Tax-induced signals in cell survival, proliferation, and transformation. Signal Transduct. **7:**34–52.
13. **Kehn, K., L. Deng, C. de la Fuente, K. Strouss, K. Wu, A. Maddukuri, S. Baylor, R. Rufner, A. Pumfery, M. E. Bottazzi, and F. Kashanchi.** 2004. The role of cyclin D2 and p21/waf1 in human T-cell leukemia virus type 1 infected cells. Retrovirology **1:**6.
14. **Koiwa, T., A. Hamano-Usami, T. Ishida, A. Okayama, K. Yamaguchi, S. Kamihira, and T. Watanabe.** 2002. 5′-Long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. J. Virol. **76:**9389–9397.
15. **Liang, J., J. Zubovitz, T. Petrocelli, R. Kotchetkov, M. K. Connor, K. Han, J. H. Lee, S. Ciarallo, C. Catzavelos, R. Beniston, E. Franssen, and J. M. Slingerland.** 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G_1 arrest. Nat. Med. **8:**1153–1160.
16. Reference deleted.
17. **Matsuoka, M., and K. T. Jeang.** 2007. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat. Rev. Cancer. **7:**270–280.
18. **Peloponese, J. M., Jr., and K. T. Jeang.** 2006. Role for Akt/protein kinase B and activator protein-1 in cellular proliferation induced by the human T-cell leukemia virus type 1 tax oncoprotein. J. Biol. Chem. **281:**8927–8938.
19. **Pise-Masison, C. A., M. Radonovich, R. Mahieux, P. Chatterjee, C. Whiteford, J. Duvall, C. Guillerm, A. Gessain, and J. N. Brady.** 2002. Transcription profile of cells infected with human T-cell leukemia virus type I compared with activated lymphocytes. Cancer Res. **62:**3562–3571.
20. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. U. S. A. **77:**7415–7419.
21. **Proietti, F. A., A. B. Carneiro-Proietti, B. C. Catalan-Soares, and E. L. Murphy.** 2005. Global epidemiology of HTLV-I infection and associated diseases. Oncogene **24:**6058–6068.
22. **Raizis, A. M., F. Schmitt, and J. P. Jost.** 1995. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. Anal. Biochem. **226:**161–166.
23. **Roman-Gomez, J., J. A. Castillejo, A. Jimenez, M. G. Gonzalez, F. Moreno, C. Rodriguez Mdel, M. Barrios, J. Maldonado, and A. Torres.** 2002. 5′ CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. Blood **99:**2291–2296.
24. **Sasaki, H., I. Nishikata, T. Shiraga, E. Akamatsu, T. Fukami, T. Hidaka, Y. Kubuki, A. Okayama, K. Hamada, H. Okabe, Y. Murakami, H. Tsubouchi, and K. Morishita.** 2005. Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. Blood **105:**1204–1213.
25. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. U. S. A. **80:**3618–3622.
26. **Shin, I., F. M. Yakes, F. Rojo, N. Y. Shin, A. V. Bakin, J. Baselga, and C. L. Arteaga.** 2002. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat. Med. **8:**1145–1152.
27. **Shin, J. Y., H. S. Kim, J. Park, J. B. Park, and J. Y. Lee.** 2000. Mechanism for inactivation of the KIP family cyclin-dependent kinase inhibitor genes in gastric cancer cells. Cancer Res. **60:**262–265.
28. **Siegel, R. S., R. B. Gartenhaus, and T. M. Kuzel.** 2001. Human T-cell lymphotropic-I-associated leukemia/lymphoma. Curr. Treat. Options Oncol. **2:**291–300.
29. **Tajima, K.** 1990. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of ATL and its geographical and clinical features. The T- and B-cell Malignancy Study Group. Int. J. Cancer **45:**237–243.
30. **Takeda, S., M. Maeda, S. Morikawa, Y. Taniguchi, J. Yasunaga, K. Nosaka, Y. Tanaka, and M. Matsuoka.** 2004. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. Int. J. Cancer. **109:**559–567.
31. **Tanaka, G., A. Okayama, T. Watanabe, S. Aizawa, S. Stuver, N. Mueller, C. C. Hsieh, and H. Tsubouchi.** 2005. The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. J. Infect. Dis. **191:**1140–1147.
32. **Taniguchi, Y., K. Nosaka, J. Yasunaga, M. Maeda, N. Mueller, A. Okayama, and M. Matsuoka.** 2005. Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. Retrovirology **2:**64.
33. **Tripp, A., P. Banerjee, M. Sieburg, V. Planelles, F. Li, and G. Feuer.** 2005. Induction of cell cycle arrest by human T-cell lymphotropic virus type 1 Tax in hematopoietic progenitor ($CD34^+$) cells: modulation of $p21^{cip1/waf1}$ and $p27^{kip1}$ expression. J. Virol. **79:**14069–14078.
34. **Viglietto, G., M. L. Motti, P. Bruni, R. M. Melillo, A. D'Alessio, D. Califano, F. Vinci, G. Chiappetta, P. Tsichlis, A. Bellacosa, A. Fusco, and M. Santoro.** 2002. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat. Med. **8:**1136–1144.
35. **Winters, Z. E., R. D. Leek, M. J. Bradburn, C. J. Norbury, and A. L. Harris.** 2003. Cytoplasmic p21WAF1/CIP1 expression is correlated with HER-2/neu in breast cancer and is an independent predictor of prognosis. Breast Cancer Res. **5:**R242–R249.
36. **Xia, W., J. S. Chen, X. Zhou, P. R. Sun, D. F. Lee, Y. Liao, B. P. Zhou, and M. C. Hung.** 2004. Phosphorylation/cytoplasmic localization of p21Cip1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients. Clin. Cancer Res. **10:**3815–3824.
37. **Yamada, Y., M. Tomonaga, H. Fukuda, S. Hanada, A. Utsunomiya, M. Tara, M. Sano, S. Ikeda, K. Takatsuki, M. Kozuru, K. Araki, F. Kawano, M. Niimi, K. Tobinai, T. Hotta, and M. Shimoyama.** 2001. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. Br. J. Haematol. **113:**375–382.
38. **Yoshida, M., I. Miyoshi, and Y. Hinuma.** 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. U. S. A. **79:**2031–2035.
39. **Zhou, B. P., Y. Liao, W. Xia, B. Spohn, M. H. Lee, and M. C. Hung.** 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat. Cell Biol. **3:**245–252.
40. **Zhu, W. G., K. Srinivasan, Z. Dai, W. Duan, L. J. Druhan, H. Ding, L. Yee, M. A. Villalona-Calero, C. Plass, and G. A. Otterson.** 2003. Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21 (Cip1) promoter. Mol. Cell. Biol. **23:**4056–4065.

Original Paper

Intervirology

Intervirology 2010;53:229–239
DOI: 10.1159/000302760

Received: September 2, 2009
Accepted after revision: December 9, 2009
Published online: March 30, 2010

Multiple Integrations of Human T-Lymphotropic Virus Type 1 Proviruses in the Engrafted Cells from the Asymptomatic Carriers in NOD/SCID/γc^{null} Mice

Ikuo Yamamoto[a] Ichiro Takajo[a] Kazumi Umeki[a] Kazuhiro Morishita[b]
Kinta Hatakeyama[c] Hiroaki Kataoka[d] Hajime Nomura[a] Akihiko Okayama[a]

[a]Department of Rheumatology, Infectious Diseases and Laboratory Medicine, [b]Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Departments of [c]Pathophysiology and [d]Oncopathology and Regenerative Biology, University of Miyazaki, Miyazaki, Japan

Key Words
Human T-lymphotropic virus type 1 · NOG mouse · Multiple proviral integration

Abstract
Objectives: Successful engraftment of human T-lymphotropic virus type 1 (HTLV-1)-infected cells and a marked increase of proviral DNA loads (PVLs) in non-obese diabetic/severe combined immunodeficient (NOD/SCID)/γc^{null} (NOG) mice have been reported. Whether the increased PVL in transplanted mice is due to the new infection of HTLV-1 was examined. ***Methods:*** Mononuclear cells from 3 NOG mice with primary engraftment from asymptomatic HTLV-1 carriers were transplanted into a second group of NOG mice. HTLV-1 PVL, proviral integration by fluorescence in situ hybridization assay, expression of viral antigen, and T-cell clonality were analyzed. ***Results:*** The PVLs in the secondarily transplanted NOG mice were significantly higher than those of primarily transplanted NOG mice. Multiple signals of HTLV-1 proviruses in the nucleus of the infected cells were revealed by fluorescence in situ hybridization analysis. Expression of HTLV-1 *tax/rex* mRNA and antigen was observed. The variety of T-cell clones was limited in the transplanted NOG mice. ***Conclusions:*** Multiple proviral integrations were considered to be due to the new infection from HTLV-1-infected cells to the other cells. Only a certain fraction of T cells seemed to have selectively survived in NOG mice after engraftment.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL), and a progressive demyelinating disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis [1–4]. As HTLV-1 infection is associated with cell-to-cell contact, the major routes of transmission of HTLV-1 are via breast-feeding, sexual contact, and blood transfusion [5–7]. It has been postulated that the clonal proliferation of HTLV-1 infected T cells likely plays an important role in the maintenance of HTLV-1 infection in carriers over long periods of time [8, 9].

A variety of animal models of HTLV-1 infection have provided available information about HTLV-1 infection in vivo [10]. Recently, genetically modified mice, including both transgenic and knockout mice, have become important models for testing the behavior of human cells in

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/10/0534–0229$26.00/0

Accessible online at:
www.karger.com/int

Akihiko Okayama, MD, PhD
Department of Rheumatology, Infectious Diseases and Laboratory Medicine
Faculty of Medicine, University of Miyazaki
5200 Kihara, Kiyotake, Miyazaki 889-1692 (Japan)
Tel. +81 985 85 7284, Fax +81 985 85 4709, E-Mail okayama@med.miyazaki-u.ac.jp

vivo. Non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice, such as the NOD/LtSz-*scid* and the NOD/Shi-*scid* mouse, have been considered appropriate models for this purpose [11–14]. We have shown the successful engraftment of HTLV-1-infected cells from asymptomatic carriers in NOD/SCID/γc^{null} (NOG) mice [15]. In this previous study, HTLV-1 provirus was recognized only in the human mononuclear cells, which infiltrated into the various organs of transplanted NOG mice. The increased proviral DNA loads ≥100,000 copies per 100,000 human cells were shown. This proviral DNA load increasing may be due to clonal expansion of HTLV-1-infected cells directly derived from the peripheral blood of asymptomatic carriers. Alternatively, transmission of HTLV-1 from the infected human cells to other human cells might occur in vivo. To clarify whether these speculations are true or not, we isolated the human cells in NOG mice with primary engraftment from HTLV-1 asymptomatic carriers, and inoculated them into the other NOG mice. The integration of HTLV-1 provirus into the chromosome of human cells was examined by fluorescence in situ hybridization (FISH) assay. To evaluate whether there is an active replication of HTLV-1 or not, the expression of *tax/rex* mRNA and HTLV-1 antigen was also tested. In addition, the rearrangement of the T-cell receptor-γ (TCR-γ) gene that reflects the clonality of T cells [16] was compared between mice with primary and secondary engraftment. The results suggested that the transmission of HTLV-1 from infected cells to other cells occurred efficiently in NOG mice and only limited fraction of T cells seemed to have selectively survived.

Materials and Methods

Subjects

Three asymptomatic HTLV-1 carriers (carrier A, B, and C) were recruited into this study after obtaining written informed consent. The white blood cell counts of the HTLV-1-infected carriers were within the reference value without any abnormal cells. The peripheral blood mononuclear cells (PBMCs) were isolated from carriers A, B, and C by density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, St. Louis, Mo., USA), washed three times with phosphate-buffered saline (PBS) and preserved with Cell banker® (JUJI Inc., Tokyo, Japan) at −80 ° until use. The study protocol was approved by the Institutional Review Board of University of Miyazaki.

Inoculation of Human PBMCs from HTLV-1 Carriers into the NOG Mice

NOG mice were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan). All mice were bred and maintained under specific pathogen-free conditions in the Department of Bioresources, Division of Biotechnology, Frontier Science Research Center, University of Miyazaki (Miyazaki, Japan). Female NOG mice aged from 8 to 10 weeks were used for the experiments. A total of 5×10^6 human PBMCs from carriers A, B, and C were injected intraperitoneally into one half of them and intravenously into the other half (primary transplantation, NOG A-1, B-1, and C-1, respectively). The mice were sacrificed 4 weeks after inoculation. Mononuclear cells were isolated from the livers of NOG A-1, B-1, and C-1 using density gradient centrifugation. More than 99% of these mononuclear cells were T cells (data not shown). A total of 3×10^6 cells were inoculated into new NOG mice (secondary transplantation, NOG A-2, B-2, and C-2, respectively). The secondarily transplanted NOG mice were sacrificed 4 weeks after inoculation. The experimental protocol was approved by the University of Miyazaki ethics review committee for animal experimentation.

Quantification of Human Cells and HTLV-1 Provirus

Chromosomal DNA was isolated from the PBMCs of carriers and mononuclear cells obtained from the livers of NOG mice by sodium dodecyl sulfate proteinase K digestion at 56°, followed by phenol-chloroform extraction and ethanol precipitation. HTLV-1 proviral copy number (i.e. proviral DNA load) was measured by real-time polymerase chain reaction (PCR) using LightCycler® DX 400 (Roche Diagnostics, Mannheim, Germany) as described previously [15]. In brief, quantitative real-time PCR was performed for human *albumin* DNA, mouse *GAPDH* DNA, and HTLV-1 provirus. The primers and probe for the human *albumin* gene were as follows: the forward primer Alb-S (5′-GCTGTCATCTCTTGTGGGCTGT-3′), the reverse primer Alb-AS (5′-AAACTCATGGGAGCTGCTGGT-3′), and the FAM-labeled albumin TaqMan® probe (5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA-3′). TaqMan Gene Expression Assays (Applied Biosystems Japan, Tokyo, Japan) were used for the primers and the probe for the mouse *GAPDH* gene. The primers and the probe for the *pX* region of HTLV-1 were as follows: the forward primer pX2-S (5′-CGGATACCCAGTCTACGTGTT-3′: positions 2359–2379), the reverse primer pX2-AS (5′-CAGTAGGGCGTGACGATGTA-3′: positions 7458–7439), and the FAM-labeled pX2 probe (FAM-CTGTGTACAAGGCGACTGGTGCC-TAMRA′: positions 7386–7408). Quantitative PCR was performed in a duplicate manner.

FISH Analysis of HTLV-1-Infected Cells

FISH analysis was performed to detect HTLV-1 proviral signals in the mononuclear cells isolated from the livers of NOG mice according to a slightly modified version of the method reported by Taniwaki et al. [17]. For FISH assay probes, PCR products prepared using four different primer sets for HTLV-1, which cover almost the entire genome of HTLV-1, were used. Primer sets used for PCR were as follows: region 1: the forward primer (HTLV23F 5′-TGACAATGACCATGAGCCCCAA-3′: positions 23–44), and the reverse primer (HTLV2060R 5′-CTAATAGGAGGGCATCTTCCTC-3′: positions 2060–2039), region 2: the forward primer (HTLV2013F 5′-AGCCCACTATCCCAGAACCAGA-3′: positions 2013–2034), and the reverse primer (HTLV4741R 5′-CGAATAGCAAGGGAGGTACACA-3′: positions 4741–4720), region 3: the forward primer (HTLV4562F 5′-AGACACCTTTTCAGGAGCCATC-3′: positions 4562–4583), and the reverse primer (HTLV7292R 5′-AGGGCTGTTTCGAT-

GCTTGCCT-3′: positions 7292–7271), and region 4: the forward primer (HTLV7150F 5′-CTTCTAAGGATAGCAAACCGTCA-3′: positions 7150–7172), and the reverse primer (HTLV9041R 5′-CTCTCCTGAGAGTGCTATAGGA-3′: positions 9041–9020). Subsequently, the PCR products were subcloned by pGEM®-T Easy vector system (Promega, Madison, Wisc., USA) and were supplied for the probe preparation. The Nick Translation System® (Invitrogen, Carlsbad, Calif., USA) and Biotin-16-dUTP® (Roche) were used for biotinylating probes. Cell suspensions on glass slides were fixed in methanol/acetic acid (3:1). Biotinylated probe DNA was denatured, allowed to preanneal with unlabeled human Cot-1 DNA (15–30 pg) at 37° for 20 min, and hybridized with treated chromosomal samples on glass slide at 37° for 20 h. Biotinylated probes were detected with Fluorescein Avidin DCS® (Vector Laboratories, Inc., Burlingame, Calif., USA), Biotinylated Anti-Avidin D® (Vector Laboratories, Inc.), and a second layer of Fluorescein Avidin DCS. Cells were counterstained with 4′,6-diamino-2-phenylindole (DAPI) and mounted in VECTASHIELD® (Vector Laboratories, Inc.). Results were analyzed on a conventional fluorescence microscope DMRA (Leica, Wetzlar, Germany) and photographed by a Leica Q550CW system (Leica, Cambridge, UK). Hybridization signals were evaluated in approximately 100 nuclei.

Detection of HTLV-1 mRNA by Reverse Transcriptase-PCR

The detailed procedure for reverse transcriptase (RT)-PCR was described elsewhere [15]. In brief, total cellular RNA was extracted from the liver of transplanted NOG C mice using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Five μg of total RNA was reverse transcribed using M-MCV RT (Invitrogen) using random primers. The following primers were used for RT-PCR: PX-1 (5′-AGGGTTTGGACAGAGTCTTC-3′: positions 7335–7354) and PX-2 (5′-AAGGACCTTGAGGGT-CTTAG-3′: positions 7590–7571) for *tax/rex* mRNA; *GAPDH*-118F (5′-GTCGGAGTCAACGGATTTGGTCG-3′: positions 76–98) and *GAPDH*-640R (5′-CATGGACTGTGGTCATGAGTCC-3′: positions 598–577) for *GAPDH* mRNA (Sigma-Aldrich Japan). The PCR products were electrophoresed on 1.2% agarose gel, and visualized by ethidium bromide staining. The RNA sample from an HTLV-1-positive cell line, HUT102, was used as a positive control at a 1:100 dilution.

Detection of HTLV-1 Antigen by Indirect Immunofluorescence Assay

Mononuclear cells isolated from the liver of transplanted NOG C mice were deposited on the glass slides using Cytospine 3 (Shandon Scientific, London, UK). Cell suspensions on glass slides were fixed in cold acetone for 1 min, air dried, and treated with 10% bovine serum albumin in PBS for 30 min. Then, they were reacted with 1:100 diluted human serum with or without anti-HTLV-1 antibody at 37° for 30 min, washed with PBS, and treated with fluorescein isothiocyanate-conjugated goat anti-human IgG/F(ab′)$_2$ (Medical & Biological Laboratories, Nagano, Japan) at 37° for 30 min. Cells were counterstained with DAPI and were analyzed on a conventional fluorescence microscope DMRA and photographed by a Leica Q550CW system.

Analysis of Rearranged TCR-γ Gene

The rearrangement of TCR-γ gene of mononuclear cells in transplanted NOG mice was detected by PCR based on the report by Benhattar et al. [16], using forward primer (TVG 5′-AGGGTT-GTGTTGGAATCAGG-3′) and reverse primer (TJG 5′-CGTCGA-CAACAAGTGTTGTTCCAC-3′). The expected size of the PCR products was approximately 170–200 base pairs. Amplified products were electrophoresed on 10% nondenaturing polyacrylamide gel, and visualized by ethidium bromide staining. Then, PCR products were subcloned by pGEM®-T Easy vector system (Promega) and transfected into *Escherichia coli* JM109 (TAKARA Biosystems, Shiga, Japan). The reaction mixture was applied to a Luria-Bertani (LB) agar plate containing ampicillin and incubated overnight at 37°. From each subject, 30–40 colonies were randomly obtained and cultured by LB broth containing ampicillin overnight at 37°. DNA sequences of the cloned PCR products were analyzed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif., USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

The Kruskal-Wallis test was used to compare the proviral DNA loads among the PBMCs of the carriers, and the engrafted cells in NOG mice with primary and secondary transplantation. One-tailed t test was used to compare the percentage of human cells among total cells in the livers of NOG mice with primary and secondary transplantation. The χ^2 test was used to compare frequencies of clones with unique rearrangement of the V-J region of TCR-γ among tested PCR products in carriers and NOG mice with primary engraftment and secondary engraftment. p values <0.05 were considered as statistically significant.

Results

HTLV-1 Proviral Loads in NOG Mice with Primary and Secondary Transplantation

All mice grew normally without piloerection or weight loss for 1 month after transplantation. No tumors or lymph node swelling were found when the mice were sacrificed. Infiltration of mononuclear cells was seen in various organs of all tested NOG mice on microscopic examination, as described previously [15]. In order to measure the number of human cells, mouse cells, and HTLV-1 provirus present in the tissues of NOG mice, human *albumin* DNA, mouse *GAPDH* DNA, and HTLV-1 provirus were quantified using real-time PCR. HTLV-1 proviral DNA copy numbers in 100 human cells and the percentages of human cells among total cells in the livers of transplanted NOG mice are shown in figure 1. HTLV-1 proviral copies in 100 PBMCs of carriers A, B, and C were 12.3, 3.2, and 0.2, respectively. The proviral loads in the livers of primarily transplanted NOG mice (NOG A-1, B-1, and C-1) were higher (82.6, 134.7, and 11.1 copies per 100 human cells, respectively) than those of carriers A, B and C. Moreover, the proviral loads in the livers of secondarily transplanted NOG mice (NOG A-2, B-2, and C-2) were significantly higher (1,428.6, 445.3, and 812.0

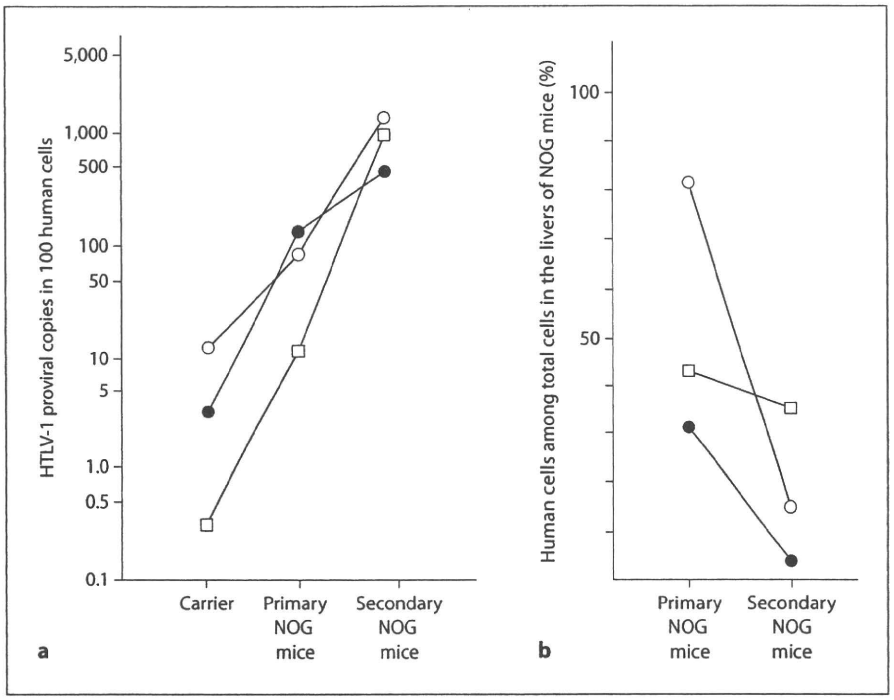


Fig. 1. HTLV-1 proviral DNA loads and percentage of human cells in the livers of transplanted NOG mice. **a** HTLV-1 proviral copies in 100 mononuclear cells. **b** Percentage of human cells among total cells in the liver of NOG mice. Open circles, closed circles and squares indicate NOG A series, NOG B series and NOG C series, respectively.

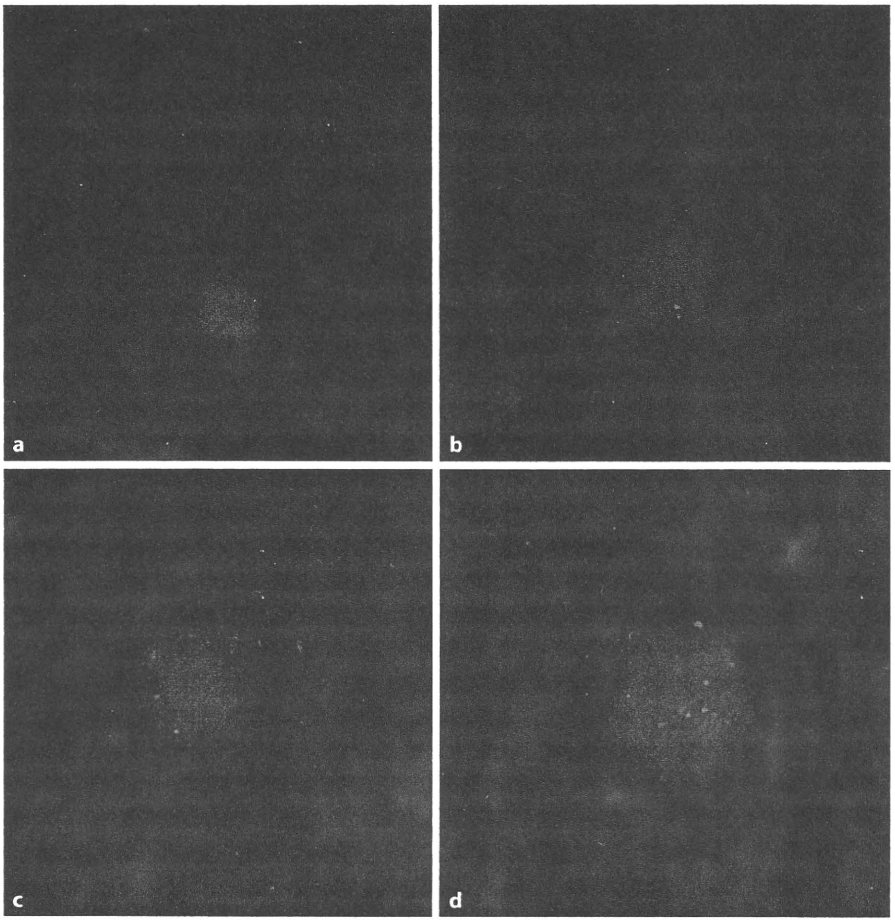


Fig. 2. Representative data of FISH assay of HTLV-1 proviruses integrated into the genome of the mononuclear cells in the liver of NOG mice with secondary engraftment. Green signals of HTLV-1 proviruses by fluorescein isothiocyanate can be detected in the nucleus of a cell dyed in blue by diamidino phenylindole. **a** No signal. **b** Two signals. **c** Four signals. **d** Thirteen signals. ×1,000.

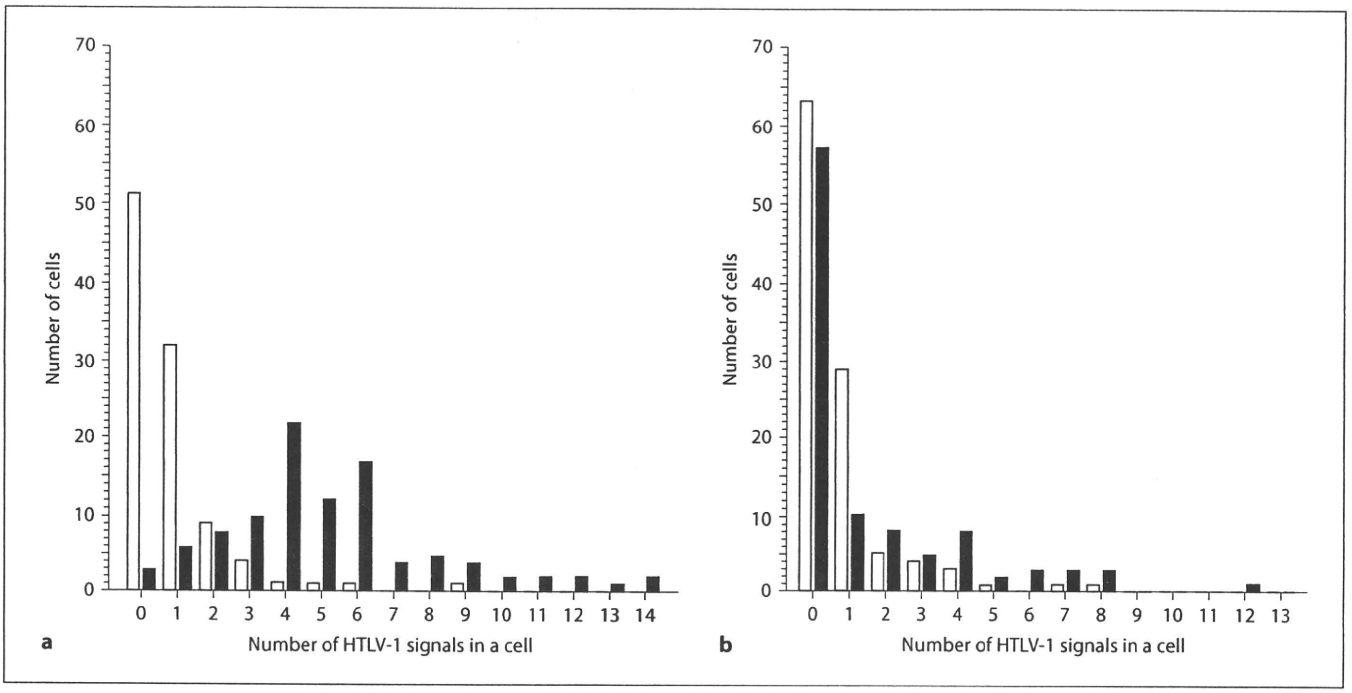


Fig. 3. Distributions of the numbers of cells based on the number of signals of HTLV-1 proviruses by FISH assay in the transplanted NOG mice. **a** NOG A series. **b** NOG B series. Open and closed columns represent the primarily and secondarily transplanted NOG mice, respectively.

copies per 100 human cells, respectively) than those of primarily transplanted NOG mice (NOG A-1, B-1, and C-1; $p = 0.039$, Kruskal-Wallis test). The percentage of human cells among total cells in the livers of secondarily transplanted NOG mice (NOG A-2: 16.5%, B-2: 4.6%, and C-2: 35.6%) were significantly lower than those of primarily transplanted NOG mice (NOG A-1: 81.9%, B-1: 30.2%, and C-1: 43.3%; $p = 0.035$, one-tailed t test). These results suggested that HTLV-1-infected cells at least in the secondarily transplanted NOG mice harbor multiple copies of HTLV-1 proviruses; however, the number of the human cells in the secondarily transplanted NOG mice decreased compared to the primarily transplanted NOG mice. Therefore, it was assumed that more than several copies of HTLV-1 proviruses were integrated into the genome of infected cells.

FISH Analysis of HTLV-1-Infected Cells Infiltrated in the Livers of Primarily and Secondarily Transplanted NOG Mice

Because the proviral loads especially increased in secondarily transplanted NOG mice suggested multiple integrations of proviruses in HTLV-1-infected cells, HTLV-1-infected cells in the livers of NOG A and B mice with primary and secondary engraftment were analyzed by FISH assay. NOG C mice could not be analyzed due to insufficient samples for FISH analysis. When the PBMCs of HTLV-1 carriers were analyzed by FISH, cells with only one signal, which represented one HTLV-1 provirus, were observed occasionally among cells with no signal. No cells with multiple signals were observed (data not shown). When cells isolated from the liver of primarily and secondarily transplanted NOG mice were analyzed, the numbers of signals per cell varied from 0 to 14 (fig. 2). The distributions of numbers of cells based on the number of signals by FISH in the primarily and secondarily transplanted NOG mice are shown in figure 3. The fraction of cells with no signal were most prevalent (approximately 50% of all cells) in the primarily transplanted mouse, NOG A-1. Approximately 30% and fewer than 20% of all cells were found to have one signal and multiple signals in one cell, respectively. In the secondarily transplanted mouse, NOG A-2, the fraction of cells with no signal was only 3% and greater than 90% of cells had multiple signals of HTLV-1 proviruses. The primarily and secondarily transplanted mice, NOG B-1 and B-2, respectively, showed the same trend, although the trend was less evident compared to that of NOG A-1 and A-2.

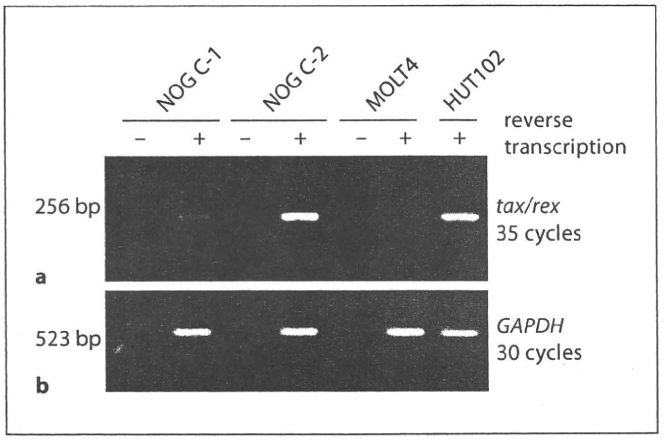


Fig. 4. Detection of the HTLV-1 *tax/rex* mRNA in the livers of transplanted NOG mice (C-1 and C-2) by RT-PCR. Upper panel: HTLV-1 *tax/rex* mRNA; lower panel: *GAPDH* mRNA (normalizing RNA samples). MOLT 4 and HUT102 cells were used as the HTLV-negative and -positive control, respectively.

Detection of HTLV-1 mRNA in Liver of NOG Mice by RT-PCR

To evaluate whether there is an active replication of HTLV-1 in the transplanted NOG mice, HTLV-1 *tax/rex* mRNA was tested by RT-PCR. As shown in figure 4, HTLV-1 *tax/rex* mRNA was shown in the livers of transplanted NOG C-1 and C-2. The signal from the secondary transplanted mouse, C-2, looked stronger than that from the firstly transplanted mouse, C-1.

Detection of HTLV-1 Antigen by IF Assay

Because the transcription of HTLV-1 gene in the transplanted NOG mice was shown in the previous experiment (fig. 4), HTLV-1 antigen (viral protein) on the mononuclear cells from the livers in the transplanted NOG mice was tested. As shown in figure 5, HTLV-1 antigen was positive for mononuclear cells both in NOG C-1 and C-2 mice by IF assay. Thirteen cells (6.5%) and 104 cells (52%) among 200 cells counted tested positive for HTLV-1 antigen in NOG C-1 and C-2 mice, respectively. These results met the results of HTLV-1 *tax/rex* RNA (fig. 4), which showed stronger signal in C-2 mouse than that in C-1 mouse.

Analysis of the Clonality of Human T Cells Using Detection of Rearrangement of TCR-γ Gene in Primarily and Secondarily Transplanted NOG Mice

Whether the human T cells, which survived in the transplanted NOG mice, are polyclonal or oligoclonal is the next question. The clonality of HTLV-1-infected cells in the carriers has been commonly analyzed by methods that identify cells with identical HTLV-1 provirus integration sites using Southern blot or the techniques based on PCR. Because multiple proviruses were shown to be integrated into the genome of HTLV-1-infected cells in NOG mice with engraftment, we could not analyze T-cell clonality using the method based on the HTLV-1 provirus integration sites in this case. Therefore, we used the method to analyze the rearrangement of the V-J region of TCR-γ gene of T cells. As shown in figure 6, the products of PCR to amplify the V-J region of TCR-γ gene both from the HTLV-1-positive and -negative PBMCs showed a smear pattern in electrophoresis assay, which reflected the polyclonality of T cells. On the other hand, secondarily transplanted NOG mice (NOG A-2, B-2, and C-2) showed several dense bands, which indicated the clear oligoclonal pattern. The primarily transplanted NOG mice, especially NOG B-1 and C-1, showed a moderate pattern between HTLV-1 carriers and the secondarily transplanted NOG mice. Therefore, only restricted clones of human T cells seemed to have survived in the transplanted NOG mice.

The frequencies of T cell clones with a unique (different) V-J region of TCR-γ gene in each NOG mouse with primary and secondary engraftments were assessed by cloning and DNA sequencing of the PCR products (fig. 7). Only two clones with unique rearrangement of the V-J region of TCR-γ gene were detectable more than twice among 31 tested PCR products in the PBMCs of carrier A (fig. 7a). In the primarily transplanted NOG mouse (NOG A-1), five clones with unique rearrangement of the V-J region of TCR-γ gene were detectable more than twice among 35 tested PCR products, and the frequencies of these clones were greater than those of carrier A. In the secondarily transplanted NOG mouse (NOG A-2), four clones with unique rearrangement of the V-J region of TCR-γ gene were detectable more than twice among 33 tested PCR products, and the frequencies of these clones were greater than those of NOG A-1. This tendency was also evident in cases of the NOG mice with primary and secondary engraftments from carrier B and C (fig. 7b, c, respectively). The proportions of clones with unique rearrangement of the V-J region of TCR-γ gene among tested cloned PCR products were 28/31 (90%), 25/35 (71%), and 16/33 (49%) in carrier A, NOG A-1, and NOG A-2, respectively. The rates of unique clone in the case of carrier B, NOG B-1, and NOG B-2 were 27/28 (96%), 21/33 (64%), and 14/38 (37%), respectively, and those in carrier C, NOG C-1, and NOG C-2 were 31/32 (97%), 27/34 (79%),

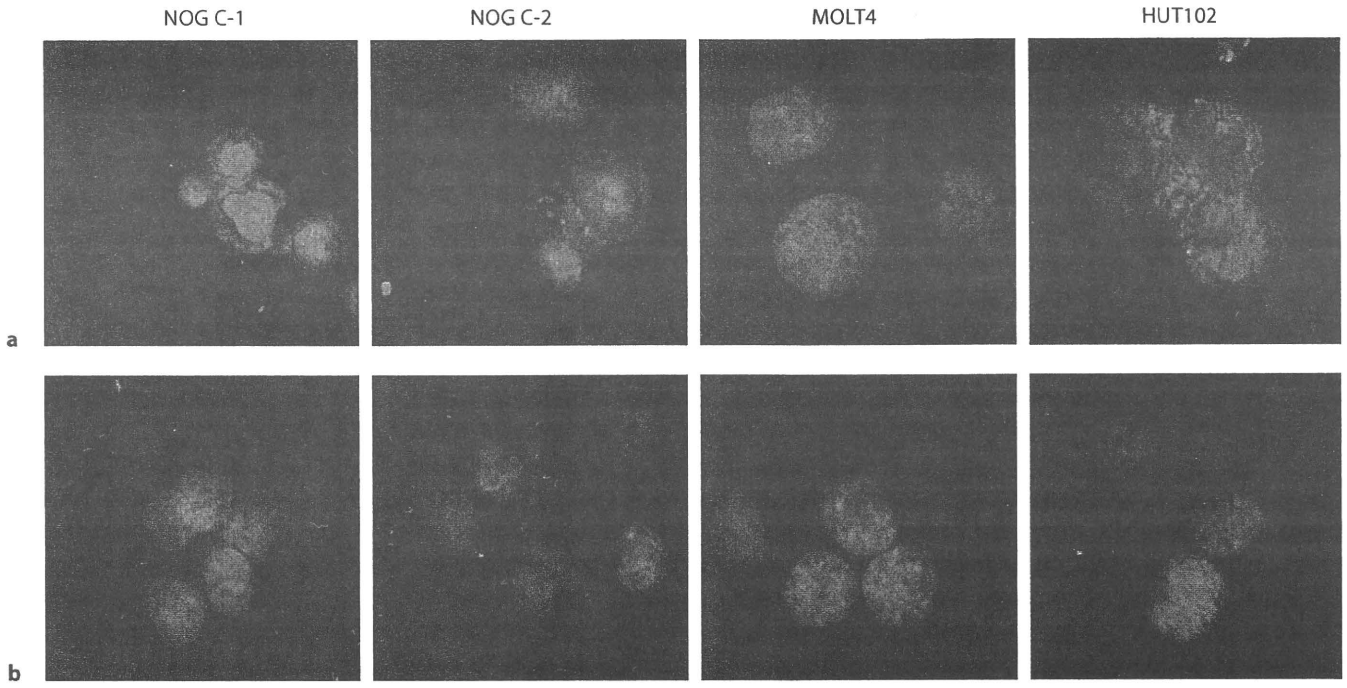


Fig. 5. Detection of HTLV-1 antigen in the mononuclear cells from the livers of transplanted NOG mice by indirect immunofluorescence assay. Mononuclear cells from the livers of transplanted NOG C-1 and NOG C-2 mice were reacted with the sera from HTLV-1-positive and -negative subjects. MOLT4 and HUT102 cells were used as the HTLV-negative and -positive control, respectively. Magnification: NOG C-1 and NOG C-2 ×1,000, MOLT4 and HUT102 ×640. **a** Anti-HTLV-1 antibody-positive serum used as 1st antibody. **b** Anti-HTLV-1 antibody-negative serum as control.

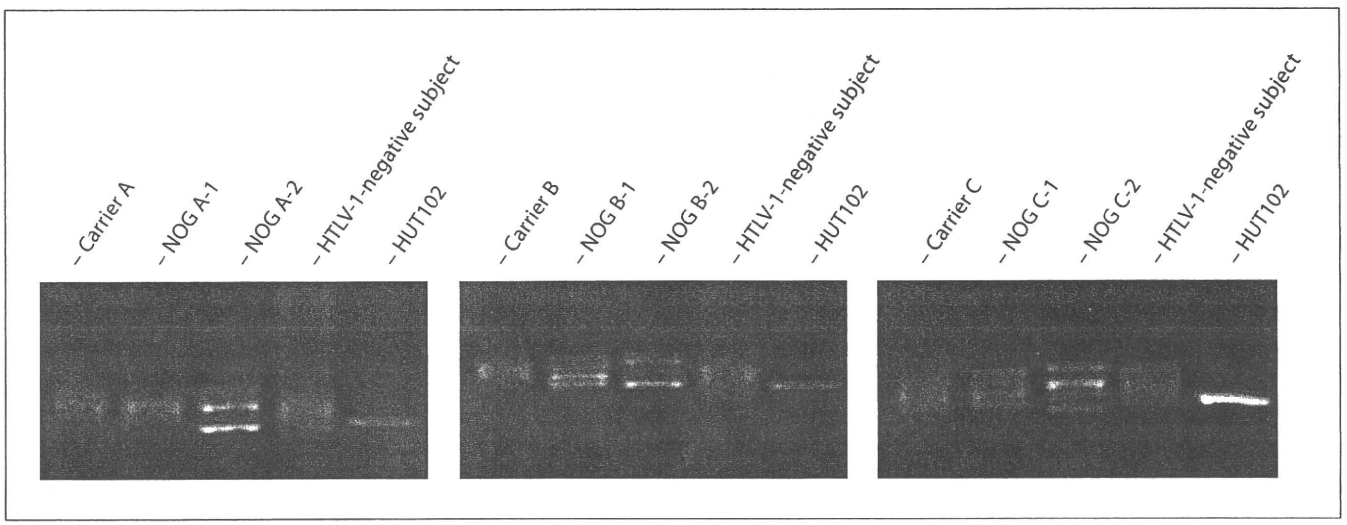
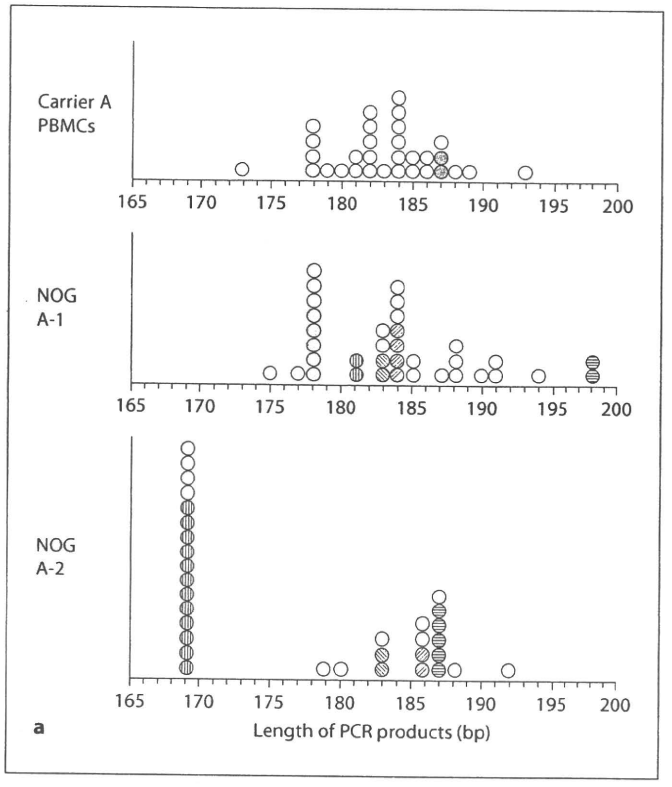
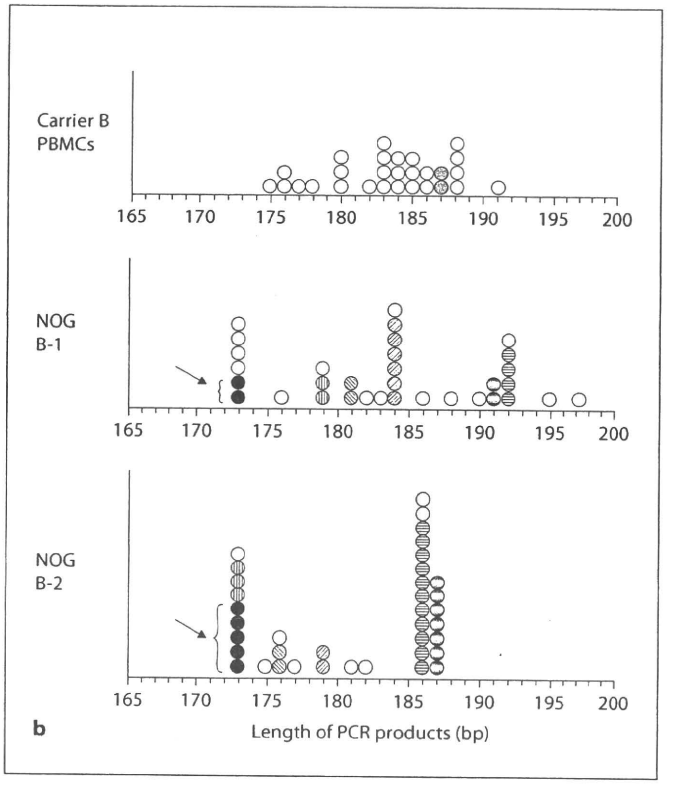


Fig. 6. PCR analysis of the rearrangement of V-J region of TCR-γ gene. Panels represent 10% polyacrylamide gel electrophoresis of PCR products from the PBMCs of carriers and the human cells in the livers of primarily and secondarily transplanted NOG mice. PBMCs from an HTLV-1-negative subject and HTLV-1-positive cell line, HUT102 were used as negative and positive controls, respectively.





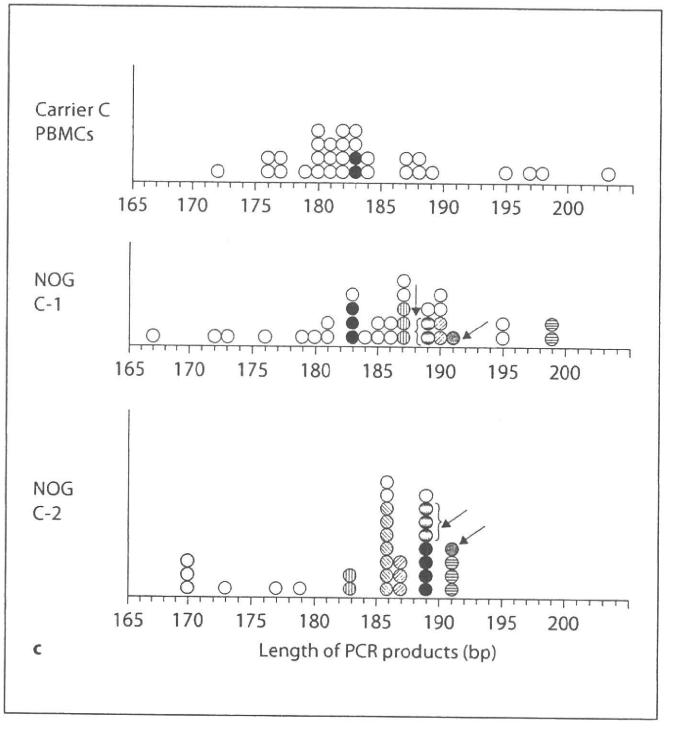


Fig. 7. Frequencies of T cells with unique (different) V-J region of TCR-γ gene in the carriers' PBMC and the NOG mice with the primary and secondary engraftments. **a–c** Results from NOG mice A, B and C, respectively. The circles with the same pattern indicate cloned PCR products with identical V-J region of TCR-γ gene and open circles indicate cloned PCR products with unique V-J region of TCR-γ gene. Arrows indicate clones with identical V-J region of TCR-γ gene, which were detected both in the NOG mice with the primary and the secondary engraftment.

Table 1. Frequencies of clones with unique rearrangement of V-J region of T-cell receptor-γ gene among tested PCR products in carriers, NOG mice with primary engraftment and secondary engraftment

	Carriers' PBMCs	NOG mice with primary engraftment	NOG mice with secondary engraftment
NOG A series	28/31 (90%)	25/35 (71%)	16/33 (49%)
NOG B series	27/28 (96%)	21/33 (64%)	14/38 (37%)
NOG C series	31/32 (97%)	27/34 (79%)	14/32 (44%)
Total	86/91 (95%)	73/102 (72%)	44/103 (43%)
		c	
	a		b

[a] $p < 0.0001$ for carriers' PBMCs vs. NOG mice with primary engraftment; [b] $p < 0.0001$ for NOG mice with primary engraftment vs. NOG mice with secondary engraftment; [c] $p < 0.0001$ for carriers' PBMCs vs. NOG mice with secondary engraftment.

and 14/32 (44%), respectively. The differences in the frequencies of clones with unique rearrangement of the V-J region of TCR-γ gene among tested cloned PCR products from carriers and NOG mice with primary and secondary engraftments were statistically significant ($p<0.0001$, χ^2 test; table 1). Therefore, the clonality of T cells in the NOG mice was less heterogeneous than that of HTLV-1 carriers, especially in the secondarily transplanted NOG mice. In addition, none of the clones were consistently detected among carrier A, NOG A-1, and NOG A-2. Only one clone in the NOG B series and two clones in the NOG C series were detected both in the primarily and secondarily transplanted mice (arrows in fig. 7b, c).

Discussion

In the present study, successful engraftment of HTLV-1-infected cells, which were isolated from the infiltrated organs of NOG mice with primary transplantation of PBMCs from asymptomatic HTLV-1 carriers, was shown in the NOG mice with secondary transplantation. HTLV-1 proviral copies per cell significantly increased in the secondarily transplanted NOG mice when compared with those in the PBMCs of original carriers and, even with those in the primarily transplanted NOG mice. The HTLV-1 proviruses were confirmed to be integrated into the human chromosome only and not into the mouse chromosome in our previous study [15]. The integration of more than one copy of HTLV-1 provirus per human cell in the transplanted mice was expected based on the numbers of HTLV-1 proviruses obtained by real-time PCR. Indeed, FISH analysis showed that more than one signal, which represented the integrated HTLV-1 proviruses in nuclei, were detectable in the cells of the transplanted NOG mice in the present study. A greater number of cells with multiple signals were found in the NOG mice with secondary engraftment than in the NOG mice with primary engraftment. Because only one signal per cell was detectable in the PBMCs of the HTLV-1 carriers, the cells with multiple proviruses might be caused by the new infection of HTLV-1 from infected cells to the other cells in NOG mice. Indeed, HTLV-1 *tax/rex* mRNA and HTLV-1 antigen were detected in these cells, indicating an active replication of HTLV-1. The signal of HTLV-1 *tax/rex* mRNA in NOG C-2 mouse was stronger than that in C-1 mouse. Moreover, the number of HTLV-1 antigen-positive cells in NOG C-2 mouse was more than that in NOG C-1 mouse. These results suggested HTLV-1 replication in the secondarily transplanted mouse was more active than that in the firstly transplanted mouse.

Alternatively, there is a possibility that there are small numbers of HTLV-1-infected cells with multiple integrations of proviruses, undetectable by FISH assay, in PBMCs of HTLV-1 carriers, and these cells expanded in the NOG mice after the transplantation. The integration of two or three copies of proviruses in the leukemic cells of some patients with ATL has been reported [18]. However, integration of more than 10 copies of proviruses into the genome of human cells in vivo has not been reported to date. Moreover, with the exception of one or two clones, same T-cell clones were not found both in the mice with primary and secondary engraftments. Therefore, the cells with multiple proviruses found in the transplanted NOG mice were less likely due to the expansion of the HTLV-1 cells, which were directly derived from the original carriers.

The reason why the new infection from HTLV-1-infected cells to the other cells occurred after transplantation in the NOG mice was not clear. There is no antibody to HTLV-1 or effective cytotoxic T cells in NOG mice. The inhibition of HTLV-1 infection to the offspring by the sera of HTLV-1 carriers was reported in the rabbit model [19]. The addition of antibody to HTLV-1, anti-gp-46, to the coculture of HTLV-1-infected and -uninfected cells also inhibits new infection [20]. These data suggest that immune responses to HTLV-1 may prevent active infection between noninfected cells and infected cells in

carriers. Therefore, loss of normal immune response in the NOG mice may account for the new infection of HTLV-1. Alternatively, immune cells in the PBMCs from HTLV-1 carriers, which were inoculated into the NOG mice, recognized the tissue of NOG mice as a foreign antigen and were activated. HTLV-1-infected T cells among these cells were also activated during this process and might have been triggered for the production of the viral antigen. The results in the present study suggest that the new cell-to-cell infection of HTLV-1 is able to occur even in humans when the HTLV-1 carriers are under strong immunosuppression. Indeed, HTLV-1 infection from the recipient cells to the donor cells was reported in the case of bone marrow transplantation to patients with ATL [21].

The human cells which survived in the NOG mice were decreased in number, especially in the mice with secondary transplantation. When the rearrangement of TCR-γ gene of human cells was analyzed in the present study, the variety of T-cell clones was limited in the NOG mice. These data suggest that only a certain fraction of T cells selectively survived in NOG mice after the transplantation. The majority of the mononuclear cells isolated from the NOG A-2 were shown to harbor HTLV-1 provirus by FISH. HTLV-1 Tax protein has been shown to promote the proliferation of infected cells [22–25]. Therefore, it was possible that the HTLV-1-infected T cells had a growth advantage due to Tax protein in the NOG mice because the transcription of HTLV-1 *pX* genes was shown in the present study. However, half of the cells from the NOG B-2 were shown not to harbor HTLV-1 provirus by FISH assay. The direct activation of T cells as an immune response to the mouse antigens is also possible. Alternatively, activated HTLV-1-infected cells due to Tax protein may produce cytokines to induce the activation of HTLV-1-noninfected cells (bystander effect) in NOG mice.

Whether the selectively surviving T cells in NOG mice have characteristics similar to leukemic cells is an interesting question. When smoldering-type ATL cells were transplanted into NOD/SCID/beta$_2$-microglobulin^{null} mice, expansion of single HTLV-I-infected clone was reported [22]. However, based on the analysis of the rearrangement of TCR-γ gene in the present study, the majority of the T-cell clones found in the primarily transplanted NOG mice disappeared in the secondarily transplanted NOG mice. Further study is necessary to clarify whether the surviving T cells in NOG mice have the potential to develop leukemic cells and whether this engraftment model is useful in the identification of carriers with higher risk of developing ATL.

In conclusion, HTLV-1-infected cells derived from NOG mice with primary engraftment were successfully transplanted into other NOG mice. An increased number of HTLV-1 proviral copies per cell was observed in the secondarily transplanted NOG mice, and this was considered due to the new infection from HTLV-1-infected cells to the other cells. The human cells which survived in NOG mice were decreased in number, especially in mice with secondary engraftment. Only a fraction of T cells seemed to have selectively survived in NOG mice after the transplantation. This engraftment model using NOG mice could be useful in clarifying the mechanism of HTLV-1 infection in vivo and the development of the reagents to inhibit HTLV-1 infection.

Acknowledgements

We thank Ms. Y. Motoyoshi (University of Miyazaki) for her technical assistance. This study was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan, and Miyazaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST.

References

1 Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H: Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 1977;50:481–492.

2 Yoshida M, Miyoshi I, Hinuma Y: Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci USA 1982;79:2031–2035.

3 Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M: HTLV-1 associated myelopathy, a new clinical entity. Lancet 1986;327:1031–1032.

4 Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de Thé G: Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 1985;24:407–410.

5 Kusuhara K, Sonoda S, Takahashi K, Tokugawa K, Fukushige J, Ueda K: Mother-to-child transmission of human T-cell leukemia virus type 1 (HTLV-1): a fifteen-year follow-up study in Okinawa, Japan. Int J Cancer 1987; 40:755–757.

6 Tajima K, Tominaga S, Suchi T, Kawagoe T, Komoda H, Hinuma Y, Oda T, Fujita K: Epidemiological analysis of the distribution of antibody to adult T-cell leukemia-virus-associated antigen: possible horizontal transmission of adult T-cell leukemia virus. Gann 1982;73:893–901.

7 Okochi K, Sato H, Hinuma Y: A retrospective study on transmission of adult T-cell leukemia virus by blood transfusion: seroconversion in recipients. Vox Sang 1984;46: 245–253.

8 Etoh K, Tamiya S, Yamaguchi K, Okayama A, Tsubouchi H, Ideta T, Mueller N, Takatsuki K, Matsuoka M: Persistent clonal proliferation of human T-lymphotropic virus type 1-infected cells in vivo. Cancer Res 1997;57:4862–4867.
9 Cavrois M, Leclercq I, Gout O, Gessain A, Wain-Hobson S, Wattel E: Persistent oligoclonal expansion of human T-cell leukemia virus type 1-infected circulating cells in patients with tropical spastic paraparesis/HTLV-1 associated myelopathy. Oncogene 1998;17:77–82.
10 Lairmore MD, Silverman L, Ratner L: Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. Oncogene 2005;24:6005–6015.
11 Greiner DL, Shultz LD, Yates J, Appel MC, Perdrizet G, Hesselton RM, Schweitzer I, Beamer WG, Shultz KL, Pelsue SC, Leif JH, RajanTV: Improved engraftment of human spleen cells in NOD/LtSz-scid/scid mice as compared with C.B-17-scid/scid mice. Am J Pathol 1995;146:888–902.
12 Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB, Tennent B, McKenna S, Mobraaten L, Rajan TV, Greiner DL, Leiter EH: Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 1995;154:180–191.
13 Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, Mitsui T, Manabe A, Tanaka R, Kobayashi K, Ito M, Yasukawa K, Nakahara T: Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest 2000;105:1013–1021.
14 Koyanagi Y, Tanaka Y, Tanaka R, Misawa N, Kawano Y, Tanaka T, Miyasaka M, Ito M, Ueyama Y, Yamamoto N: High levels of viremia in hu-PBL-NOD-scid mice with HIV-1 infection. Leukemia 1997;11(suppl 3):109–112.
15 Takajo I, Umeki K, Morishita K, Yamamoto I, Kubuki Y, Hatakeyama K, Kataoka H, Okayama A: Engraftment of peripheral blood mononuclear cells from human T-lymphotropic virus type 1 carriers in NOD/SCID/γc^{null} (NOG) mice. Int J Cancer 2007;121:2205–2211.
16 Benhattar J, Delacretaz F, Martin P, Chaubert P, Costa J: Improved polymerase chain reaction detection of clonal T-cell lymphoid neoplasms. Diagn Mol Pathol 1995;4:108–112.
17 Taniwaki M, Speicher MR, Lengauer C, Jauch A, Popp S, Cremer T: Characterization of two marker chromosomes in a patient with acute nonlymphocytic leukemia by two-color fluorescence in situ hybridization. Cancer Genet Cytogenet 1993;70:99–102.
18 Kamihira S, Dateki N, Sugahara K, Yamada Y, Tomonaga M, Maeda T, Tahara M: Real-time polymerase chain reaction for quantification of HTLV-1 proviral load: application for analyzing aberrant integration of the proviral DNA in adult T-cell leukemia. Int J Hematol 2000;72:79–84.
19 Miyoshi I, Takehara N, Sawada T, Iwahara Y, Kataoka R, Yang D, Hoshino H: Immunoglobulin prophylaxis against HTLV-I in a rabbit model. Leukemia 1992;6(suppl 1):24–26.
20 Ishizaki J, Okayama A, Kuroki M, Tsubouchi H: Detection of human T-lymphotropic virus type I (HTLV-I) infection during coculture of HTLV-I infected and uninfected cells using inverse long PCR. Intervirology 2002;45:164–171.
21 Ljungman P, Lawler M, Åsjo B, Bogdanovic G, Karlsson K, Malm C, McCann SR, Ringdén O, Gahrton G: Infection of donor lymphocytes with human T lymphotrophic virus type 1 (HTLV-I) following allogeneic bone marrow transplantation for HTLV-I positive adult T-cell leukaemia. Br J Haematol 1994;88:403–405.
22 Kawano N, Ishikawa F, Shimoda K, Yasukawa M, Nagafuji K, Miyamoto T, Baba E, Tanaka T, Yamasaki S, Gondo H, Otsuka T, Ohshima K, Shultz LD, Akashi K, Harada M; Efficient engraftment of primary adult T-cell leukemia cells in newborn NOD/SCID/β2-microglobulin^{null} mice. Leukemia 2005;19:1384–1390.
23 Franchini G, Fukumoto R, Fullen JR: T-cell control by human T-cell leukemia/lymphoma virus type 1. Int J Hematol 2003;78:280–296.
24 Grassmann R, Aboud M, Jeang KT: Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene 2005;24:5976–5985.
25 Yoshida M: Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. Annu Rev Immunol 2001;19:475–496.

Mini Review

Natural history of human T-lymphotropic virus type 1 infection and immune system imbalances

Akihiko Okayama

Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Miyazaki, Japan

The human T-lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). In addition, increased incidences of infectious diseases, autoimmune diseases, chronic inflammatory diseases and virus-associated malignancies have been reported in HTLV-1 carriers. HTLV-1 infection causes the continuous activation and clonal expansion of T cells. The levels of regulatory T cells and naïve T-cells are decreased in the peripheral blood of asymptomatic carriers. Spontaneous proliferation of peripheral blood mononuclear cells and subclinical deficiencies in both type 1 and type 2 immunity are also observed. It is possible that the immune system imbalances seen in HTLV-1 infection account for the disease manifestations described above, although the precise mechanism remains unclear.

Rec.1/29/2009, Acc.11/4/2009, pp103-108

* Correspondence should be addressed to:
Akihiko Okayama, Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Phone: +81-985-85-7284, Fax : +81-985-85-4709, e-mail: okayama@med.miyazaki-u.ac.jp

Key words HTLV-1 infection, asymptomatic carriers, immune system imbalances

Introduction

The human T-lymphotropic virus type 1 (HTLV-1) has been shown to be a causative agent of adult T-cell leukemia/lymphoma (ATL), one of the most aggressive hematological malignant diseases[1,2]. The mechanism of leukemogenesis has not yet been determined; however, monoclonal expansion of T-cells with the provirus integrated into the genome is essential. HTLV-1 also causes the progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[3]. Infiltration of mononuclear cells into the spinal cord with an increased level of proviral loads in the peripheral load is observed in patients with HAM/TSP. Polyclonal expansion of HTLV-1 positive cells in the peripheral blood is also seen. Therefore, HTLV-1 causes two different types of disease in humans; namely, lymphocytic malignancy and non-malignant disease. In addition, there is evidence that HTLV-1 infection contributes to a broader spectrum of diseases, such as uveitis, arthropathy, polymyositis, pneumonitis, and infective dermatitis in children

Table 1 Diseases reported to be associated with HTLV-1 infection

Adult T-cell leukemia/lymphoma
HTLV-1-associated myelopathy/tropical spastic paraparesis
HTLV-1-associated uveitis
Infectious diseases
Infective dermatitis (children)
Strongyloidiasis
Autoimmune diseases/Chronic inflammatory diseases
Polymiositis
Sjogren's syndrome
Arthropathy
Pneumonitis
Virus-associated malignancies
Hepatitis C virus infection-associated hepatocellular carcinoma

(Table 1)[4-9]. HTLV-1 infection also increases the risk of Strongyloides stercoralis infection and hepatocellular carcinoma due to hepatitis C virus infection[10,11]. In this mini-review, immune abnormality due to HTLV-1 infection and its possible connection to the development of HTLV-1-associated diseases among asymptomatic carriers are discussed.

HTLV-1 infection

HTLV-1 transmission is mediated by live cells and not via cell-free body fluids[12]. The RNA genome undergoes reverse transcription into a DNA provirus that is integrated into the host genome[13]. HTLV-1 growth and propagation are supported primarily by CD4+ cells. One of the key regulatory elements of HTLV-1 replication is the Tax protein, which activates transcription of the viral genome[14]. HTLV-1 Tax protein has also been shown to promote the proliferation of infected cells and to be a good target for the host cellular immune response to the virus[14,15].

The major natural routes of infection of HTLV-1 are from mother to infant and sexual contact[16]. Blood transfusion was once a route of HTLV-1 infection; however, the efficient screening of blood products has prevented new infection thus far[17]. Therefore, when we find a middle-aged person who tests positive for HTLV-1, he or she may have been infected from his/her mother while in infancy or from his/her spouse or blood products after reaching adulthood. Identifying the infection route is important because ATL appears to develop primarily in persons who acquire HTLV-1 perinatally, whereas HAM/TSP is associated with exposure to HTLV-I later in life[18].

Asymptomatic carriers are positive for HTLV-1 antibody. The peripheral blood mononuclear cells (PBMCs), primarily CD4 positive T-cells, harbor HTLV-1 provirus[19]. The level of proviral load is likely determined within a year of infection[20]. The development of a humoral and, probably, cellular immune response to virus occurs during this period. The nature of the host immune response may affect the burden of HTLV-1, which varies widely among asymptomatic carriers[21]. Most individuals infected with HTLV-1 develop into asymptomatic carriers with low proviral loads; however, some develop into carriers with high proviral loads[20]. Proviral load appears to remain stable, once a set point has been achieved[20,22]. The maintenance of the level of proviral load seems largely due to the relationship between the clonal expansion of HTLV-1-infected cells and the immune response to them[22-24].

Phenotypic change of T cells and immune dysfunction in HTLV-1 infection

The phenotypic changes of T-cells, which have been reported thus far, are described in Table 2. HTLV-1 infection has been shown to cause the expression of activation markers, such as interleukin-2 (IL-2) receptor, in T-cells[14]. HTLV-1 Tax has been reported to activate the promoter for IL-2 and IL-2 receptor[14]. Proliferation of unstimulated PBMCs in cell culture without interleukin 2 is observed in the HTLV-1 carriers (spontaneous proliferation)[25]. Spontaneous proliferation of PBMCs from HTLV-1 carriers is due to the polyclonal expansion of cells and correlates directly with the expression of viral genes such as Tax[26]. Activated T cells infected with HTLV-1 may subsequently activate resting T-cells through interaction among T cells.

Changes in circulating leukocytes, specifically an increase in the percentage of lymphocytes and a decrease in the percentage of eosinophils and basophils, have been reported in asymptomatic HTLV-1 carriers[27]. CD4+ and CD25+ cell levels were also shown to be increased among asymptomatic carriers[28]. The largest increase was seen in asymptomatic carriers whose peripheral blood smears were positive for atypical lymphocytes.

Recently, CD4+CD25+ cells were reported to be the surface phenotype of regulatory T-cells (Treg)[29]. The majority of CD4+ CD25+T cells were positive for Foxp3, a master gene for Treg, in normal individuals[30]. Increased expression of Foxp3 was reported in ATL cells[31]. However, not all CD4+CD25+T cells in the asymptomatic HTLV-1carriers were reported to be positive for the expression of Foxp3. In another words, CD4+ CD25+T cells in HTLV-1 carriers were inconsistently composed of the

Table 2 Abnormalities of immune system reported in HTLV-1 asymptomatic carriers

Continuous activation of T-cells
Clonal expansion of certain population of T-cells
Change of the level of regulatory T-cells
Decreased level of naïve T-cells
Spontaneous proliferation of peripheral blood mononuclear cells
Increased level of CD4+CD25+T-cells
Decreased reaction to recall antigen
Decreased level of serum IgE

two populations with and without Foxp3, suggesting that the former are Treg and that the latter are either activated T cells or aberrant Treg downregulating Foxp3 expression. Foxp3 expression in CD4+CD25+T cells from HAM/TSP patients was reported to be lower than that from HTLV-1 negative individuals[32]. Moreover, HTLV-1 Tax was shown to have a direct inhibitory effect on Foxp3 expression and the function of CD4+CD25+T cells. It was suggested that the imbalance of CD25 and Foxp3 expression in asymptomatic carriers is closely related to HTLV-1 infection. This modulation of Foxp3 in asymptomatic carriers may be causatively implicated in the autoimmune-like diseases in HTLV-1 infection.

In addition to HTLV-1 Tax, expression of HTLV-1 HBZ, which is transcribed from 3'LTR, has been shown to promote the proliferation of infected cells[33]. It has been postulated that clonal proliferation of HTLV-1-infected cells is likely responsible for maintaining proviral loads in carriers. Expansion of certain clones of HTLV-1-infected cells is frequently observed in asymptomatic carriers[34]. In a particular case, only one clone of HTLV-1 infected cells occupied almost 1% of the total PBMCs in the asymptomatic carrier[35]. This abnormal growth of certain T-cells due to HTLV-1 infection, especially in asymptomatic carriers with high proviral loads, may cause the immune system imbalance. In addition, the number of naïve T cells was reported to be low in the HTLV-1 carriers[36]. It was suggested that the low number of naïve T-cells was due to the suppressed production of T-cells in the thymus, which may account for the immunodeficiency observed in HTLV-1 infection.

HTLV-1 carriers have been shown to have a reduced response to recall antigens. For example, we previously showed that asymptomatic carriers had a significantly reduced delayed-type hypersensitivity response to purified protein derivative skin testing[37]. The reduced response to recall antigens was shown to be more evident in the HTLV-1 carriers who show a spontaneous proliferation of PBMCs[38]. HTLV-1 carriers were also shown to have lower levels of IgE and may have subclinical deficiencies in both type 1 and type 2 immunity[39]. Indeed, when the relationship between strongyloidiasis and HTLV-1 infection was evaluated in Okinawa, Japan, serum IgE levels and peripheral eosinophil counts were significantly lower in HTLV-1 co-infected patients compared with patients without HTLV-1 infection[40]. A low frequency of atopy among HTLV-1 carriers was also reported in Brazil[41].

The natural history of HTLV-1 infection and disease manifestations

A hypothesis for the natural history of HTLV-1 infection and its association of disease manifestations is shown in Figure 1. The major infection routes of HTLV-1 are breast feeding by the mother during the child's infancy and between spouses after reaching adulthood. The level of HTLV-1 proviral load is likely determined within a year of infection and varies widely among asymptomatic carriers. The balance between the proliferation of HTLV-1-infected cells and the host immune response to them determines the proviral load, which remains stable, once a set point has been achieved. HTLV-1 carriers with low proviral loads may remain in an asymptomatic state for their entire lives. In case of the carriers with high proviral loads, continuous activation and clonal expansion of T cells due to HTLV-1 infection may occur frequently. Escape from the immune system and accumulation of genetic abnormalities may lead the clonally expanded T cells infected with HTLV-1 to the initial stage of ATL. Even in HTLV-1 carriers who do not develop ATL, decreased levels of regulatory T cells and naïve T-cells are observed in the peripheral blood. Spontaneous proliferation of PBMCs and subclinical deficiencies in both type 1 and type 2 immunity are also observed. These immune system imbalances may result in an increased incidence of opportunistic infections, autoimmune diseases, chronic inflammatory diseases and virus-associated malignancies in HTLV-1 infection.

However, there has been no direct evidence to connect HTLV-1 infection and opportunistic infections, autoimmune diseases, chronic inflammatory diseases and virus-associated malignancies. T-cell dysfunction may not be the only factor involved in the process of the immune deficiencies seen in HTLV-1 infection. Indeed, impairment of antigen-presenting cells or inability of PBMC from HTLV-1-infected individuals to respond to interleukin-12 might also account for the immunodeficiency

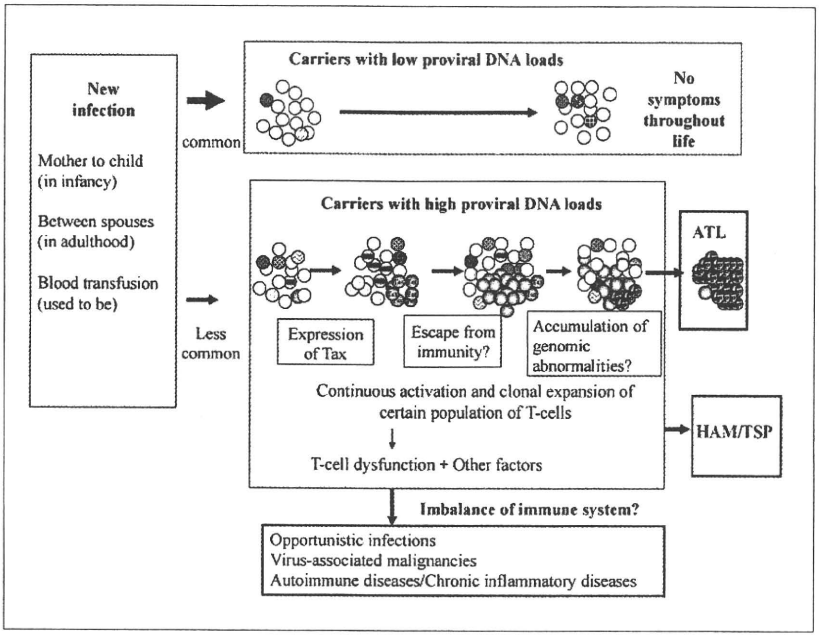


Fig.1 Natural history of human T-cell lymphotropic virus type 1 infection and possible association with the disease manifestations (hypothesis)

observed in HTLV-1 infection[42]. In addition, the natural history of HTLV-1 has been shown to differ markedly by geographic area, due in part to the host response to infection. For example, HTLV-1 infection was associated with diminished T-cell-mediated immunity in Japanese persons and with activated T-cell immunity in Jamaicans[43]. Markers of immune activation correlated more strongly with anti-HTLV-1 titers and provirus load in Jamaican than in Japanese individuals. Therefore, further studies are needed to clarify the mechanism of HTLV-1's contribution to the disease manifestations described above.

References

1) Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: Detection and isolation of type-C retrovirus particles from fresh and cultured lymphocytes of patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA, 7: 7415-7419, 1980.
2) Hinuma Y, Nagata K, Hanaoka M, et al: Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA, 78: 6476-6480, 1981.
3) Osame M, Usuku K, Izumo S, et al: HTLV-1 associated myelopathy, a new clinical entity. Lancet, 1: 1031-1032, 1986.
4) Mochizuki M, Yamaguchi K, Takatsuki K, Watanabe T, Mori S, Tajima K: HTLV-I and uveitis. Lancet, 339: 1110, 1992.
5) Ijichi, S, Matsuda T, Maruyama I, et al: Arthritis in a human T lymphotropic virus type I (HTLV-I) carrier. Ann Rheum Dis, 49: 718-721, 1990.
6) Nishioka K: HTLV-I arthropathy and Sjogren syndrome. J Acquir Immune Defic Syndr Hum Retrovirol, 13(Suppl 1): S57-S62, 1996.
7) Morgan OS, Rodgers-Johnson P, Mora C, Char G: HTLV-1 and polymyositis in Jamaica. Lancet, ii: 1184-1187, 1989.
8) Setoguchi, Y, Takahashi S, Nukiwa T, Kira S: Detection of human T-cell lymphotropic virus type I-related antibodies in patients with lymphocytic interstitial pneumonia. Am Rev Respir Dis, 144: 1361-1365, 1991.
9) LaGrenade L, Hanchard B, Fletcher V, Cranston B, Blattner W: Infective dermatitis of Jamaican children: a marker for HTLV-I infection. Lancet, 336: 1345-1347, 1990.
10) Robinson RD, Lindo JF, Neva FA, Gam AA, Vogel P, Terry SI, CooperES: Immunoepidemiologic studies of Strongyloides stercoralis and human T lymphotropic virus type I infections in Jamaica. J Infect Dis, 169: 692-696, 1994.
11) Okayama A, Maruyama T, Tachibana N, et al: Increased prevalence of HTLV-I infection in patients with hepatocellular carcinoma associated with hepatitis C virus. Jpn J Cancer Res, 86: 1-4, 1995.