using an ABI Prism7000 under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles (95°C for 15 s and 60°C for 1 min). The oligos and TaqMan probe for *TBP-2* were purchased from Applied Biosystems. 18S ribosomal RNA was used as an internal control.

Sodium bisulfite sequencing of genomic DNA
The genomic DNA was prepared from HTLV-I-infected IL-2-dependent or -independent cells using a Puregene DNA isolation kit (Gentra). Sodium bisulfite treatment was performed as reported previously (Nosaka *et al.*, 2000; Yasunaga *et al.*, 2004). For the sequencing of the *TBP-2* promoter region, 100 ng of genomic DNA was amplified by PCR using as primers, 5′-GGAGAAGACATCGGTCCT-3′ (forward) and 5′-CATGATGGAACTGAGTTGGT-3′ (reverse). For bisulfite-sequencing analyses, 100 ng of sodium bisulfite-treated genomic DNA was amplified by PCR using two pairs of methylation-specific primers. For the TATA-box region, a first round of amplification was performed with 5′-GGTTTTAGGGTTAGTGGGA-3′ (forward) and 5′-AAAAACCTTCTTTCCCCCAA-3′ (reverse), followed by a second round of nested PCR with 5′-TTTATTGGATTTGG GAGAA-3′ (forward) and 5′-ATCCAATCTCCACAAACA CTCC-3′ (reverse) primers. For the exon-1 region, a first round of amplification was performed with 5′-GGAAAGAA GGTTTTTTTTTTGA-3′ (forward) and 5′-CCACTTACCT ATTAACTAATCT-3′ (reverse), followed by a second round of nested PCR with 5′-TGATTTTGTTTAGTGTAATTAG-3′ (forward) and 5′-CCTATTAACTAATCTTCCAA-3′ (reverse). Amplification was carried out under the following conditions: 30 cycles (denaturing at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 2 min), followed by a final extension at 72°C for 8 min. The PCR products were purified using a QIAEX II Gel extraction kit (Qiagen) and ligated into the vector pCR2.1 TOPO (Invitrogen). Inserts from five positive clones of each product were sequenced using M13 primers. Sequencing was performed using Big Dye Terminator reagent (Applied Biosystems) with an ABI PRISM310 Genetic Analyzer.

Chromatin immunoprecipitation (Chip) assay
For analyzing the histone acetylation status in TBP-2-restored cells, a Chip assay was performed as described previously (Spencer *et al.*, 2003). In brief, a chromatin solution was immunoprecipitated overnight at 4°C, using antiacetylated H3 and H4 antibodies (Upstate Biotechnology). Precipitated DNA was analysed by PCR amplification, using a KlenTaq LA DNA Polymerase Mix (Sigma). The following promoter-specific primers were used: *TBP-2* promoter region (207 bp); 5′-TCCAGAGCGCAACAACCAT-3′ (forward) and 5′-AAG CAGGAGGCGGAAACGT-3′ (reverse) or, *β-globin* promoter region (237 bp); 5′-GGCAAGGTGAACGTGGATG AAGTTGGTG-3′ (forward) and 5′-GGAGTGGACAGA TCCCCAAAGGACTCAAAG-3′ (reverse). PCR was carried out under the following conditions: 30 cycles for *TBP-2* (denaturing at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 2 min) or, 28 cycles for *β-globin* (denaturing at 95°C for 30 s, annealing at 66°C for 1 min, and extension at 72°C for 2 min). The PCR products were visualized by electrophoresis in 3% NuSieve GTG agarose gel.

Western blot
Cell lysates were prepared with lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl (pH 7.5), 2.5 mM sodium pyrophosphate, 1 mM *β*-glycerophosphate, 1 mM sodium orthovanadate, 1 mM NaF and protease inhibitor cocktail (Roche)). Western blotting was performed as described previously (Nishinaka *et al.*, 2004a). The membrane was blocked with 10% (w/v) skim milk in Tris-buffered saline containing 0.05% Tween-20, then incubated with monoclonal anti-TBP-2 or anti-*β*-actin (Sigma, St MO) antibodies, followed by peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Tokyo, Japan). An ECL Western blot detection kit (Amersham, Tokyo, Japan) was used to visualize the epitopes.

Knockdown of TBP-2 expression by RNA interference (RNAi)
Double stranded oligonucleotides (rACAGACUUCGGA GUACCUGdTT) for selective silencing of *TBP-2* or control oligonucleotides (rUUCUCCGAACGUGUCACGUdTT) (Qiagen) were transfected into cells using a human T cell nucleofector kit (Amaxa biosystems, Tokyo, Japan). Eight hours after transfection, IL-2 was added to the culture.

Cell proliferation assay
To analyse the cellular growth, cell proliferation was assayed based on [^{3}H]-thymidine incorporation or with the MTT assay using WST-1 reagent (TaKaRa). Cells ($2–5 \times 10^3$/well) were seeded in 96-well flat-bottomed microtiter culture plates. Cells were cultured for 3 days in the presence or absence of IL-2 (7.5–15 ng/ml) and cell proliferation was assessed at different time points of the culture (day 0–3). For the [^{3}H]thymidine incorporation assay, cells were labeled with 1 μCi of [^{3}H]methyl-thymidine (Amersham) for the last 6 h and radioactivity was measured. For the MTT assay, cells were incubated with WST-1 reagent for the last 2 h and analysed with a Thermo-Max microplate reader.

Acknowledgements

We thank Dr Koichi Ikuta for discussions, and Ms Yoko Kanekiyo and Ms Satoko Maeji for secretarial assistance. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Research and Development Program for New Bio-Industry Initiatives.

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Donor-Derived T-Cell Leukemia after Bone Marrow Transplantation

TO THE EDITOR: Asymptomatic carriers of human T-cell lymphotropic virus type I (HTLV-I) are considered acceptable as donors in allogeneic stem-cell transplantation for patients with adult T-cell leukemia–lymphoma (ATL).[1] However, the infusion of HTLV-I–infected cells from HTLV-I–seropositive donors could lead to the development of donor-derived ATL under immunosuppressive conditions after stem-cell transplantation. Here we describe a patient in whom ATL derived from donor cells developed four months after transplantation of stem cells from a sibling with HTLV-I.

A 44-year-old Japanese man with lymphoma-type ATL underwent transplantation of bone marrow from his HLA-identical brother in February 2004. The conditioning regimen included intravenous cyclophosphamide (120 mg per kilogram of body weight) and total-body irradiation (12 Gy). Cyclosporine and a short course of methotrexate were given as prophylaxis against graft-versus-host disease (GVHD). On day 26, low-dose prednisone was instituted because of GVHD-associated fever. With stable hematopoietic engraftment, complete donor chimerism was confirmed in a T-cell fraction on day 20 (Fig. 1). On day 133, the patient's white-cell count increased to 49.1×10^9 per liter with 89 percent ATL cells, although the original tumor had completely disappeared. Southern blot analysis revealed monoclonal integration of HTLV-I provirus in the ATL cells. Although we discontinued immunosuppressive therapy and administered chemotherapeutic agents, the patient died of the tumor in August 2004 (day 177).

A test for the status of donor–recipient chimerism in a T-cell–enriched fraction at the onset of ATL after transplantation showed a donor pattern (Fig. 1). In September 2004, hematologic and blood chemical values of the donor were almost normal, although the white-cell count included 1 percent atypical lymphocytes. Southern blot analysis showed no monoclonal integration of the HTLV-I provirus in the peripheral-blood mononuclear cells of the donor. These findings suggest that the donor was still an asymptomatic carrier without substantial clonal proliferation of HTLV-I–infected cells.

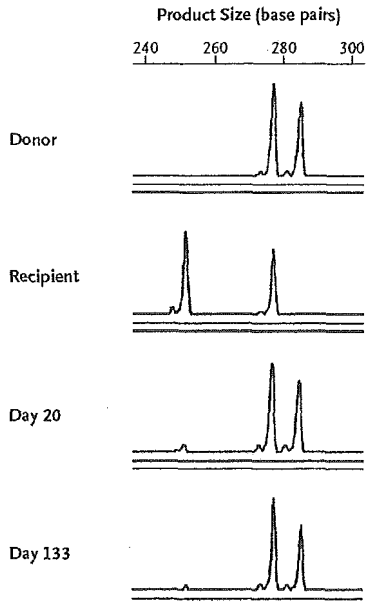

Figure 1. Analysis of T Cells at Engraftment (Day 20) and at the Onset of ATL (Day 133).

Genomic DNA from peripheral blood was amplified by polymerase-chain-reaction (PCR) assays with the use of fluorescent primers flanking an informative microsatellite region. Fluorescent peak controls for the donor and the recipient before stem-cell transplantation were obtained from whole peripheral-blood cells. At the onset of ATL after stem-cell transplantation, enriched CD3-positive cells, which were obtained with a purity of more than 95 percent, showed typical morphologic features of ATL cells on blood smears, and most of them expressed both CD4 and CD25, as assessed by flow cytometry. Fluorescent PCR products and GeneScan ROX 500 Size Standard were analyzed in a genetic analyzer with the use of Genescan software (Version 3.1.2) (ABI-310, Applied Biosystems).

Suppression of the host immune system increases the occurrence of virus-associated lymphoid cancers.[2] ATL develops in approximately 5 percent of HTLV-I carriers after an incubation period of several decades.[3] However, ATL has been reported to develop at a younger age in renal-transplant recipients with HTLV-I infection during immunosuppressive therapy[4] and sooner after the transmission of HTLV-I infection through blood transfusion in patients under immunosuppressive conditions.[5] Thus, the immunosuppressive

status in recipients of stem-cell transplants also potentially contributes to the development of ATL in donor-derived T cells that are infected with HTLV-I.

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A Case of Platydeoxia?

TO THE EDITOR: The Clinical Problem-Solving article by Hegland et al. (Dec. 1 issue)[1] concerned a case of platypnea–orthodeoxia, in which a positionally dependent intracardiac right-to-left shunt led to arterial desaturation when the patient was in the upright position but not the supine. We report a case in which the converse occurred.

An 81-year-old woman was brought to the emergency room by her neighbors, who found her lying on the floor of her apartment, markedly confused. On examination, the patient was cooperative but delirious. She was hemodynamically stable, her respiratory rate was 13 breaths per minute, and her initial oxygen saturation was 95 percent while breathing room air. Repeated oximetry several hours later revealed an oxygen saturation of 80 percent and blood gas measurement yielded a partial pressure of arterial oxygen (PaO_2) of 34 mm Hg that did not improve despite the delivery of high-flow oxygen by aerosol mask. The patient was intubated and transferred to the intensive care unit. A spiral computed tomographic scan was negative for pulmonary embolism but revealed a very large aneurysm in the ascending aorta (8.2 cm by 8.0 cm) that was compressing the right main pulmonary artery.

In the intensive care unit, the patient was placed in a sitting position and the PaO_2 increased to 486 mm Hg. Her PaO_2 remained elevated despite the reduction of the fraction of inspired oxygen (F_IO_2) to 35 percent, and she was extubated and transferred back to the ward. On the ward, her oxygen saturation dropped to 60 percent and rose only marginally while she was breathing supplemental oxygen. It was then noted that the oxygen saturation rapidly corrected to 100 percent when the patient was placed lying on her side or sitting upright. A transthoracic echocardiogram with bubble study was performed; it revealed a patent

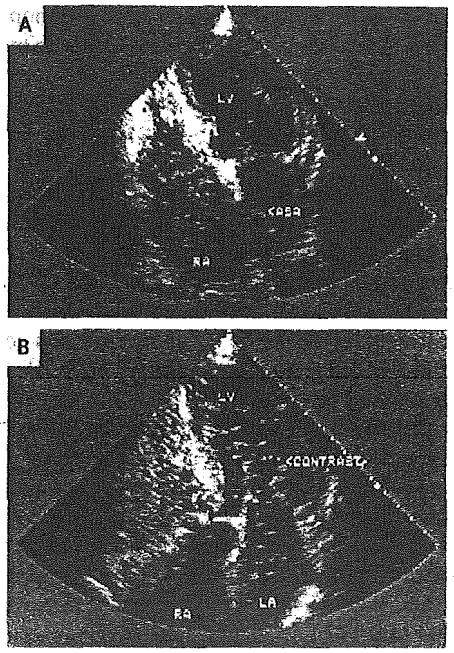
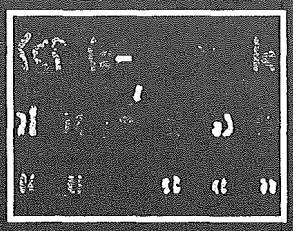
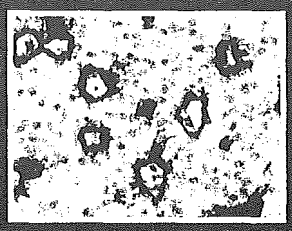
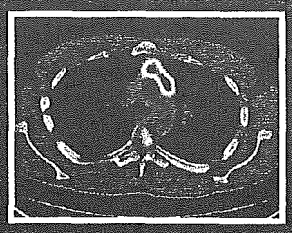


Figure 1. Transthoracic Echocardiography with Microbubble Contrast.
Panel A shows the echocardiogram that was obtained with the patient in the left lateral decubitus position and shows abundant microbubbles in the right atrium (RA) and only a few in the left ventricle (LV). ASA denotes atrial septal aneurysm. The echocardiogram in Panel B was obtained while the patient was in the supine position and shows the increased presence of microbubbles in the left atrium (LA) and left ventricle (LV).

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29

Human T-Cell Leukemia Virus Type I

Masao Matsuoka, M.D., Ph.D.

Human T-cell leukemia virus type I (HTLV-I) is the first human retrovirus shown to be linked with human disease. In 1977, adult T-cell leukemia/lymphoma (ATL) was proposed as a distinct clinical entity from its unique geographic distribution and clinical features.[1] Then, its causative retrovirus, HTLV-I, was identified in the human T-cell line,[2] which finally clarified that HTLV-I was the causative virus of ATL.[3] The discovery of HTLV-I leads to further characterization of ATL and HTLV-I infection, and discloses the virologic mechanism of transformation.

VIROLOGY OF HTLV-I

HTLV-I belongs to the delta-type retroviruses, which also include bovine leukemia virus (BLV), human T-cell leukemia virus type II (HTLV-II), and simian T-cell leukemia virus (STLV).[4] BLV and STLV have also been associated with neoplastic diseases as well as HTLV-I. The structure of HTLV-I provirus is similar to other retroviruses that contain *gag*, *pol*, and *env* genes flanked by long terminal repeat (LTR) sequences at both ends.[5] A unique structure was found between *env* and the 3'-LTR, denoted the pX region, which encodes the regulatory proteins, $p40^{tax}$ (Tax), $p27^{rex}$ (Rex), p12, p13, p30, p21, and HBZ (Fig. 29–1). The presence of accessory genes is the characteristics of delta-type retroviruses as well as foamy virus and lentivirus including human immunodeficiency virus type 1 (HIV-1).

Function of Tax

Among these accessory proteins encoded by HTLV-I, Tax protein plays a central role in the proliferation of infected cells and the leukemogenesis because of its pleiotropic actions (Fig. 29–2).[6] Tax potently increases the expression of viral genes through the viral LTR and stimulates the transcription of cellular genes through cellular signaling pathways of NF-κB, CREB, SRF, and AP-1. Tax does not bind to promoter or enhancer sequences by itself, but it interacts with cellular proteins that are transcriptional factors or modulators of cellular functions.

Transcriptional Activation

Tax can activate the NF-κB pathway by interacting with IKKγ. IKKα, β, and γ form a 700-kDa complex in which IKKγ functionally adapts Tax into this large complex.[7] The activated complex phosphorylates IκB, which detach NF-κB, result in activation of NF-κB. Among the various functions of the Tax protein, activation of NF-κB has been shown to be essential to transformation by HTLV-I.[8] Activation of the NF-κB pathway induces transcription of various cytokines and their receptor genes as well as numerous genes associated with apoptosis and the cell cycle. For example, Tax can activate the transcription of interleukin (IL)-2Rα and IL-2 genes through the NF-κB pathway. In addition, the transcription of IL-6, IL-15, and GM-CSF genes can be activated by the Tax protein via NF-κB. Such activation of genes associated with cell proliferation seems to be involved in the growth of HTLV-I–infected cells both in vitro and in vivo. Tax can also induce expression of Bcl-xL via activation of NF-κB, which renders ATL cells apoptosis-resistant.[9] As well as HTLV-I–transformed cell lines, increased expression of Bcl-xL was observed in fresh ATL cells,[10] which may account for the resistance of ATL cells to chemotherapy.

For activation of the viral LTR, Tax requires at least two 21-bp enhancers containing an imperfect cAMP-responsive element bound to a cyclicAMP response element binding protein (CREB).[11] Tax can bind to both CREB- and CREB-binding protein (CBP), the latter of which is a transcriptional co-activator.[12] Under physiologic conditions, only phosphorylated CREB induced by stimulation can bind to CBP. Tax shunts this pathway, resulting in stimulation-independent activation of the CREB pathway. CBP acetylates histone and opens the nucleosome structure around the transcriptional site.

Transcriptional Repression

Conversely, Tax can trans-repress transcription of certain genes, such as DNA polymerase β, *lck*, *p18*, and *p53* genes. For trans-repression of *p18* gene transcription, the E-box, which binds to transcriptional factor E47, is critical. Tax protein itself could not bind to E-box or E47, but interferes with binding of E47 to the transcriptional co-activator, p300, resulting in repression of transcription.[13] p53-dependent transcription is also repressed by Tax protein. Similar to trans-repression of p18, Tax does not bind to the p53- or p53-binding site, but rather inhibits the recruitment of CBP to p53 on the p53-binding sites.[13] This mechanism of trans-repression contrasts with that of trans-activation of the CREB pathway by Tax protein. Although both mechanisms depend on binding the Tax protein to the transcriptional activator, CBP/p300, their effect on transcription is quite different.[6]

Functional Inhibition

Apart from transcriptional regulation, Tax can influence the function of cellular factors. Tax protein can interact with a negative inhibitor of cyclin-dependent kinase (CDK) 4, $p16^{INK4a}$, via its ankyrin motif, and impair its function.[14] Since $p16^{INK4a}$ is an inhibitor of CDK4, its functional inactivation leads to activation of CDK4/6, phosphorylation of Rb, and finally G1/S transition.

Transforming growth factor β (TGF-β) is an inhibitory cytokine that plays important roles in development, the

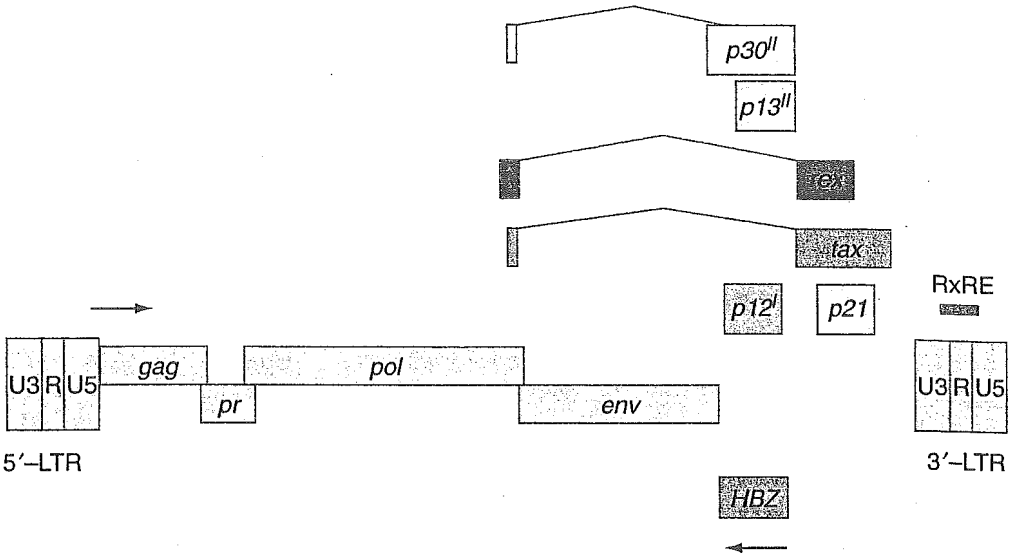


Figure 29–1. Structure of HTLV-I provirus and its encoding genes. The structure of HTLV-I provirus genome, and its encoded accessory gene have been shown. *Arrows* indicate orientation of viral gene transcription. Pr, protease.

immune system, and oncogenesis. Since TGF-β generally suppresses the growth of tumor cells, most tumor cells acquire escape mechanisms to inhibit signaling from TGF-β, which include mutation of its receptor and mutation of Smad molecules that transduce the signal from the receptor. Tax is also reported to inhibit the signal of TGF-β by its binding to Smad2, 3, and 4, or CBP/p300.[15,16] Inhibition of TGF-β signaling enables HTLV-I–infected cells to escape TGF-β–mediated growth inhibition.

ATL cells are well known to show remarkable chromosomal abnormalities, which are thought to reflect chromosomal instability. Tax has been reported to interact with the checkpoint protein, MAD1, which forms a complex with MAD2 and controls the mitotic checkpoint. The functional hindrance of MAD1 by Tax protein causes chromosomal instability, suggesting the involvement of this mechanism in oncogenesis.[17]

Rex

Rex acts at the post-transcriptional level to regulate viral gene expression, which enhances the expression of the unspliced *gag/pol* and singly spliced *env* transcripts, and decreases *tax/rex* mRNAs.[6] Therefore, Rex is a negative regulator for viral transcription by inhibiting the expression of the *tax* gene. Rex binds to the Rex-responsive elements (RxRE), which is located in the 3'-LTR (R/U3 region) (Fig. 29–1), which mediates the nuclear transport of unspliced viral mRNA, and also regulates RNA processing.

p30

p30 interacts with the transcriptional co-activator, CBP/p300, which competes with Tax at 5'-LTR, resulting in reduced viral gene expression. In addition, p30 specifically binds to *tax/rex* mRMA, and retains it in the nucleus. Thus,

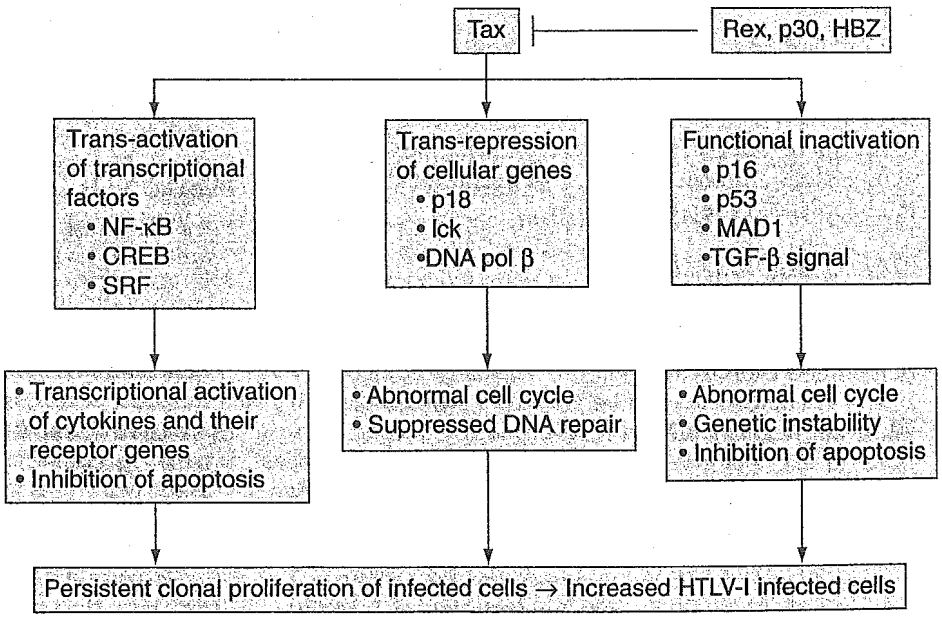


Figure 29–2. Pleiotropic actions of Tax. Pleiotropic actions of Tax proteins are summarized. Expression of Tax is also down-regulated by Rex, p30, and HBZ.

p30 reduces the production of Tax protein by a post-transcriptional mechanism, leading to suppression of viral gene transcription.[18]

HBZ

HBZ, which is encoded by the complementary strand of HTLV-I (Fig. 29–1), binds to c-Jun and JunB, and decreases the DNA-binding activity of these transcriptional factors,[19] which in turn reduces the viral transcription from LTR. This finding shows that HBZ is another negative regulator for virus gene expression.

p12

Open reading frame (ORF) 1 of the pX region encodes p12 (Fig. 29–1), which is present in the endoplastimic reticulum and Golgi apparatus. p12 has been shown to play an important role in establishment of HTLV-I infection and optimal viral infectivity in vivo and quiescent primary lymphocytes.[20,21] As a mechanism, p12 has been shown to interact with calreticulin and calnexin, and increase cytoplasmic calcium, leading to NFAT activation in T lymphocytes.[22] With this action, p12 facilitates host cell activation and establishment of persistent infection. Although the expression of p12 protein in vivo has remained obscure, cytotoxic T lymphocytes (CTLs) against p12 have been demonstrated in individuals infected with HTLV-I, indicating that p12 protein is expressed in vivo.[23]

p13

p13 is gene product encoded by ORFII of pX region, and selectively targeted to mitochondria, which is inserted in the inner mitochondrial membrane.[24] The presence of cytotoxic T cells against p13 in the carriers shows its expression in vivo.[23] Expression of p13 suppresses the growth of tumor cells and also increases the sensitivity to Ca2+-mediated stimuli.[25] The function of p13 in viral replication and cell proliferation needs further study.

Thus, HTLV-I has redundant strategies to increase infected cells through its encoded proteins, Tax and p12, which promote cell proliferation, inhibit apoptosis, and increase genetic and chromosomal instability (Fig. 29–2). With these strategies, HTLV-I increases its copies in vivo by promoting the proliferation and survival of infected cells, and causes ATL as a consequence of the strategies. On the other hand, Tax is also a major target recognized by cytotoxic T lymphocytes (CTL) in vivo. To suppress the *tax* gene expression, HTLV-I also has redundant mechanisms to suppress the expression and function of Tax by Rex, p30, and HBZ as described above, which reduce viral gene expression and virus production. Such mechanism enables HTLV-I–infected cells to suppress viral gene expression and escape from the host immune system.

EPIDEMIOLOGY OF HTLV-I

On a global basis, 10 million to 20 million people are estimated to be infected with HTLV-I. HTLV-I is endemic in southwest Japan, the Caribbean Islands, countries surrounding the Caribbean Basin, parts of Central Africa, and South America. In addition, epidemiologic studies of HTLV-I revealed high seroprevalence rates in Melanesia, Papua New Guinea, and the Solomon Islands, and among Australian aborigines.[26] In Japan, approximately 1.2 million individuals were estimated to be infected by HTLV-I, and more than 800 cases of ATL are diagnosed each year.[27] The cumulative risk of ATL among HTLV-I carriers in Japan was estimated at about 6.6% for men and 2.1% for women, indicating that most of HTLV-I carriers are asymptomatic throughout their lives.[28]

TRANSMISSION OF HTLV-I

HTLV-I is transmitted by three routes: (1) mother to infant (via breast milk); (2) horizontal (sexual); and (3) parenteral (blood transfusion or intravenous drug use). Infected cells are essential for transmission of HTLV-I via any of these routes, which has been demonstrated by the absence of seroconvertors among recipients of fresh frozen plasma transfusions.[29] The transmission efficiency of free virion is estimated to be 1 in 10^5 to 10^6 virion, whereas cell-to-cell transmission is much more efficient.[30] Thus, transmission of HTLV-I requires cell-to-cell contact. Such contact induces polarizations of the cytoskeleton of an infected cell to the cell-to-cell junction (virologic synapse), and then Gag protein complexes containing the virus genome accumulate at this junction, finally leading to the transfer of these complexes to uninfected cells.[31] This mechanism can explain why cell-to-cell contact is essential to transmission of HTLV-I.

HTLV-I can infect various cell types, including B lymphocytes, dendritic cells, fibroblast, rat cells, and mouse cells, indicating that its receptor is ubiquitously expressed on cell surfaces. Glucose transporter, GLUT-1, has been identified as a receptor of HTLV-I,[32] which also proved that the receptor for HTLV-I is ubiquitously expressed. However, after transmission in vivo, HTLV-I provirus was predominantly found in the CD4-positive T lymphocytes.[33] This suggests that HTLV-I could increase the number of infected CD4-positive T lymphocytes in vivo after infection, coinciding with the finding that HTLV-I can transform only T lymphocytes in vitro, most of which were CD4-positive. Even a retrovirus vector expressing only Tax could transform CD4-positive T lymphocytes in vitro.[34] Taken together, these findings suggest that Tax promotes proliferation, and inhibits apoptosis, of HTLV-I–infected CD4-positive T lymphocytes in vivo.

IMMUNOLOGICAL CONTROLS OF HTLV-I INFECTION

Although HTLV-I can promote the proliferation of infected cells by viral gene products such as Tax, it also induces the immune response to HTLD-1 to eliminate the infected cells (Fig. 29–3).

Immune Response Against Tax Protein

Among viral proteins, Tax has been shown to be a major target of CTLs in vivo.[35] Therefore, Tax-expressing cells are considered to be eliminated in vivo. Indeed, depletion of CD8-positive T lymphocytes from the peripheral blood

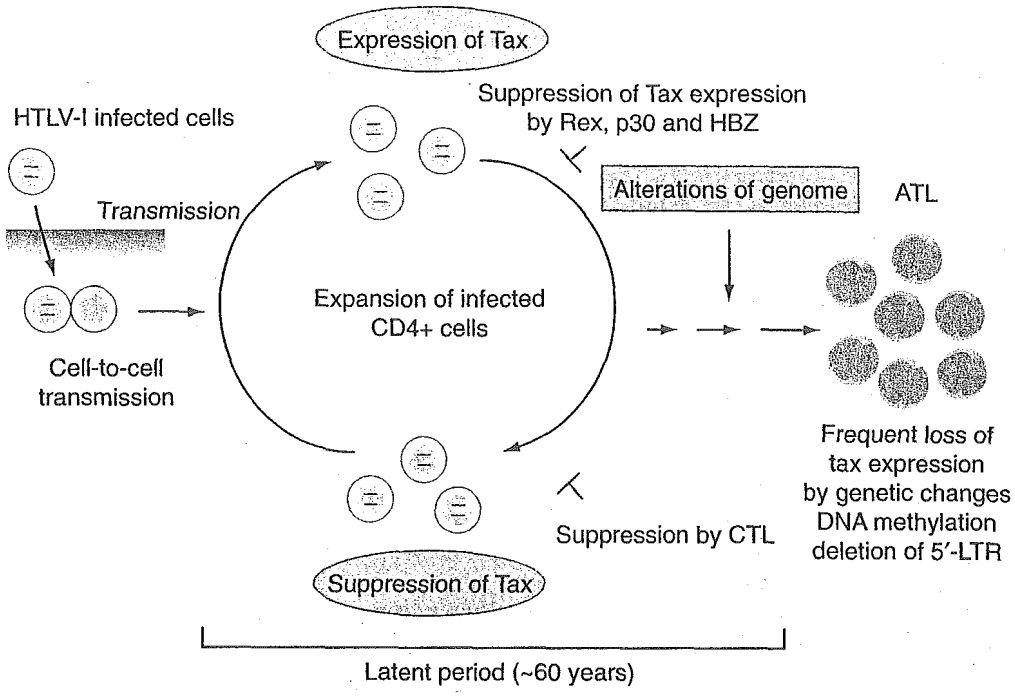


Figure 29–3. Natural course from the infection of HTLV-I to onset of ATL. HTLV-I is transmitted via three routes, and infected cells are necessary in all three. After infection, HTLV-I promotes clonal proliferation of infected cells by pleiotropic actions of Tax. Proliferation of HTLV-I–infected cells is controlled by cytotoxic T cells in vivo. After a long latent period, ATL develops in about 5% of asymptomatic carriers. The expression of Tax is inactivated by several mechanisms, suggesting that Tax is not necessary in this stage. Alternatively, alternations in the host genome accumulate during the latent period, finally leading to onset of ATL.

mononuclear cells of HTLV-I–infected individuals in vitro promoted Tax expression in the CD4-positive subpopulation, indicating that CD8-positive CTLs suppressed Tax expression in vivo.[36] Thus, the survival of HTLV-I–infected cells depends on the balance of proliferative actions of Tax and the host immune system. These findings suggest that in asymptomatic carriers, CTLs against HTLV-I can control the growth of cells carrying the HTLV-I provirus, resulting in preventing the development of ATL.

Immunodeficiency and Development of ATL

Opportunistic infections such as fungal, protozoal, and viral infections are common in patients with ATL due to the inevitable impairment of T-cell function. To a lesser extent, impaired cell-mediated immunity has also been demonstrated in HTLV-I carriers. Such immunodeficiency in the carrier state might be associated with leukemogenesis of ATL by allowing proliferation of HTLV-I–infected cells. A prospective study of HTLV-I–infected individuals identified carriers who later developed ATL and showed that the anti-Tax antibody level was low in all ATL cases for up to 10 years preceding their diagnosis. This finding indicates that HTLV-I carriers with a higher anti–HTLV-I titer, which is roughly correlated with HTLV-I provirus load, and a lower anti-Tax reactivity may be at greatest risk of ATL.[37] The levels of anti–HTLV-I antibody and soluble IL-2 receptor (sIL-2R) have been shown to be correlated with HTLV-I provirus load,[38] and high antibody titers, and high sIL-2R were risk factors for developing ATL among carriers.[39] Taken together, these findings suggest that a higher proliferation of HTLV-I–infected cells, and a low immune response against Tax, might be associated with the onset of ATL. Given this finding, potentiation of CTLs against Tax by a vaccine strategy might be useful in preventing the onset of ATL.[40]

EBV-associated lymphomas frequently develop in individuals with an immunodeficient state associated with transplantation or AIDS. Does such an immunodeficient state, which abrogates the immune function suppressing HTLV-I–infected cells, affect the onset of ATL? Among 24 patients with post-transplantation lymphoproliferative disorders (PT-LPD) after renal transplantation in Japan, five cases of ATL have been reported. Considering that most of PT-LPD is of B-cell origin in Western countries, this frequency of ATL was quite high. Although high seroprevalence of HTLV-I is due to blood transfusion during hemodialysis, the immunodeficient state during renal transplantation apparently promotes the onset of ATL.[41]

Impaired cell-mediated immunity, such as suppressed T-cell response to EBV, and seronegativity against purified protein derivative, has been reported in HTLV-I carriers, also indicating relationship to immunodeficiency. One mechanism of immunodeficiency is that HTLV-I infects CD8-positive T lymphocytes, which might impair the function of CD8-positive T lymphocytes.[33] Indeed, the immune response against Tax, via HTLV-I–infected CD8-positive T cells, renders these cells susceptible to fratricide mediated by autologous HTLV-I–specific CD8-positive T lymphocytes.[42] Fratricide among virus-specific CTLs could impair the immune control of HTLV-I. Another mechanism of immunodeficiency is based on the observation that the number of naive T cells decreased in individuals infected with HTLV-I via decreased thymopoiesis.[33]

HTLV-I–INFECTED CELLS IN VIVO

HTLV-I provirus load, which is correlated with the number of HTLV-I–infected cells, varied by more than 100-fold among HTLV-I carriers. When the sequential DNA samples from peripheral blood mononuclear cells of the same HTLV-I carriers who were followed in a cohort study were analyzed, provirus loads fluctuated only 2- to 4-fold in most

carriers, showing that provirus loads were relatively constant over time for up to 7 years in individual carriers.[38] Analyses of HTLV-I provirus load in seroconverters showed that the same virus sequences were identified in the married couples (infected persons transmitted virus to partners); however, their provirus loads were quite different, suggesting that provirus load was determined not by HTLV-I itself, but rather by host factors.[43] One of the candidates for such host factors is the immune response, especially CTLs against HTLV-I, which control the number of HTLV-I–infected cells (Fig. 29–3).[44]

Clonal Expansion of HTLV-I–Infected Cells

Since HTLV-I provirus is randomly integrated in the host genome,[45] the integration site is specific to each HTLV-I–infected cell. When integration sites of HTLV-I provirus were identified in infected cells by inverse PCR, HTLV-I–infected cells clonally proliferated in carriers, some clones were shown to persist for a long time in the same individuals.[46,47] Most of these persistent clones were CD4-positive lymphocytes, which is consistent with the finding that HTLV-I predominantly immortalizes CD4-positive T lymphocytes in vitro.

The HTLV-I provirus is genetically stable, especially compared with the other major human retrovirus, human immunodeficiency virus (HIV). It has been postulated that increased HTLV-I load is achieved not by virus replication, but by clonal proliferation of infected cells. Since reverse transcriptase is an error-prone DNA polymerase, a higher replication rate generates the vast diversity in the virus genome. In HIV, a higher rate of mutation generated in the viral replication results in acquisition of drug resistance, and escape from the host immune system. On the other hand, HTLV-I increases its copies mainly by proliferation of HTLV-I–infected cells. In such situation, HTLV-I provirus in the host cells is replicated by cellular DNA polymerase with proofreading activity. Therefore, HTLV-I provirus is genetically stable in striking contrast to HIV-I. Such clonal expansion of HTLV-I–infected cells is directly associated with the onset of ATL (Fig. 29–3).

However, the reverse transcriptase (RT) inhibitor inhibits the replication of HTLV-I in vitro,[48] and in addition, in vivo administration of RT inhibitor also suppresses the provirus load.[49] This finding shows the possibility that HTLV-I replicates in vivo, although it remains unknown how much such replication can account for provirus load in vivo.

GENETIC FACTORS AFFECTING PROVIRUS LOAD AND SUSCEPTIBILITY TO HTLV-I–ASSOCIATED DISEASES

Cellular immunity, including MHC molecules, influences the provirus load in HTLV-I–infected individuals. In addition, other polymorphisms of genes might influence the provirus load. For example, although the higher provirus load is associated with the risk of HAM/TSP, polymorphism of the TNF gene (*TNF-863A*) increases susceptibility to the disease, whereas SDF-1 (*SDF-1 +801A* 3'UTR), and IL-15 (*IL-15 191C*) gene polymorphism are protective at onset.[50] Among these polymorphisms, *IL-15 191C* has been implicated in reducing the provirus load. Familial clustering of ATL patients has been reported, suggesting predisposing genetic factors. Polymorphism of tumor necrosis factor α (TNF-α) has been shown to be associated with ATL in comparison with asymptomatic carriers, suggesting that genetic polymorphism, which increases the production of TNF-α, is associated with susceptibility to ATL.[51] Such genetic analysis utilizing rapidly accumulating knowledge and developing technology will clarify the genetic basis of the familial clustering of ATL patients.

ADULT T-CELL LEUKEMIA/LYMPHOMA

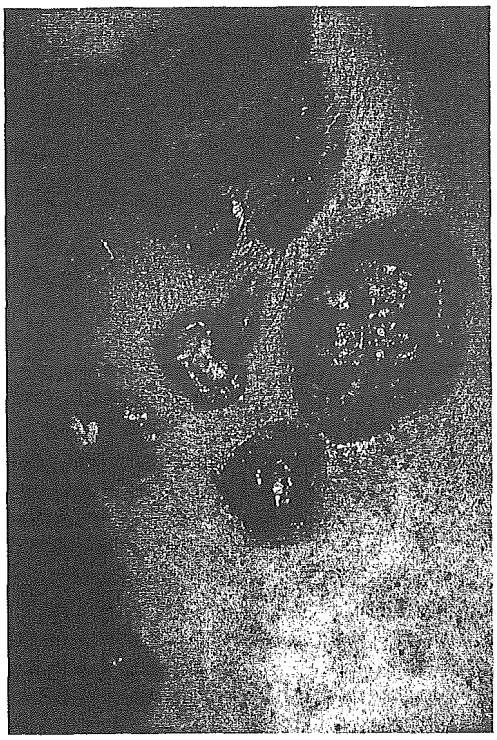
Clinical Features of ATL

The onset of ATL is slightly more common in males (male-to-female ratio is 1.16:1). However, since female carriers are predominant in HTLV-I infection, the risk for developing ATL is three times higher for males. Average age at the onset of ATL is 60 years in Japan. On the other hand, the average age of Caribbean and African ATL patients is about 43 years, suggesting that the onset is influenced by genetic or environmental factors.[52]

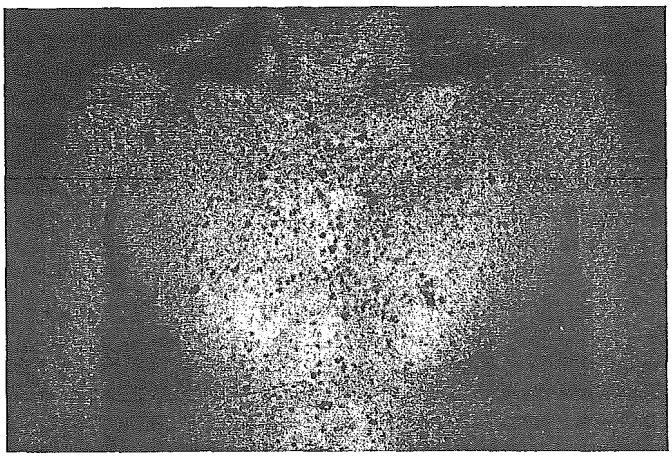
The findings at onset are abdominal pain, diarrhea, pleural effusion, ascites, cough, sputum, and an abnormal shadow on chest x-ray films. Predominant physical findings are peripheral lymph node enlargement (72%), hepatomegaly (47%), splenomegaly (25%), and skin lesions (53%).[52] ATL cells tend to infiltrate into various organs/tissues, including skin, liver, lung, gastrointestinal tract, central nervous system, and bone. Various skin lesions, such as papules, erythema, and nodules are frequently observed in ATL patients (Fig. 29–4). In the skin, ATL cells densely infiltrate the dermis and epidermis, forming Pautrier's microabscesses in the epidermis (Fig. 29–5). In the bone, infiltration of ATL cells causes punched-out lesions (Fig. 29–6). Pulmonary complication is also frequently observed in patients with ATL, which includes leukemic infiltration (Fig. 29–7) and pulmonary infections. The high frequency of hypercalcemia is the most striking feature of ATL; about 70% of ATL patients have high serum Ca2+ levels during the clinical course of the disease, particularly during the aggressive stage of ATL. In the bone of ATL patients with hypercalcemia, the number of activated osteoclasts increase (Fig. 29–8).

White blood cell (WBC) count ranges from normal to 500 × 10^9/L. Blood involvement is frequently observed in patients with ATL, and leukemic cells in peripheral blood resemble Sézary cells, having indented or lobulated nuclei (Fig. 29–9). Since infiltration of ATL cells into bone marrow is usually not so dense, anemia and thrombocytopenia are rare. Eosinophilia is frequently observed in ATL patients, as well as other T-cell malignancies.

Serum lactate dehydrogenase (LDH) is elevated in most ATL patients, and higher LDH levels indicate an advanced or aggressive disease state. Thus, serum calcium and LDH levels reflect the extent of disease, and are useful for monitoring the remaining tumor or disease activity. Hyper-



A



B

Figure 29–4. Skin lesions in ATL patients. Skin lesions observed in ATL patients are variable. Tumor formation is common among skin involvement in ATL patients (A). Papule is also observed in a patient with acute ATL (B). (See color insert.)

bilirubinemia, observed when ATL cells infiltrate the liver, indicates a poor prognosis. Hypergammaglobulinemia is very rare in ATL, which is consistent with the fact that ATL cells have suppressor-inducer activity to immunoglobulin synthesis in vitro. ATL cells are known to express interleukin 2 (IL-2) receptor alpha chain on their surfaces, and also secrete its soluble forms. Therefore, levels of SIL-2R are elevated in the sera of patients with ATL, the levels of SIL-2R being correlated with the tumor mass and clinical course.

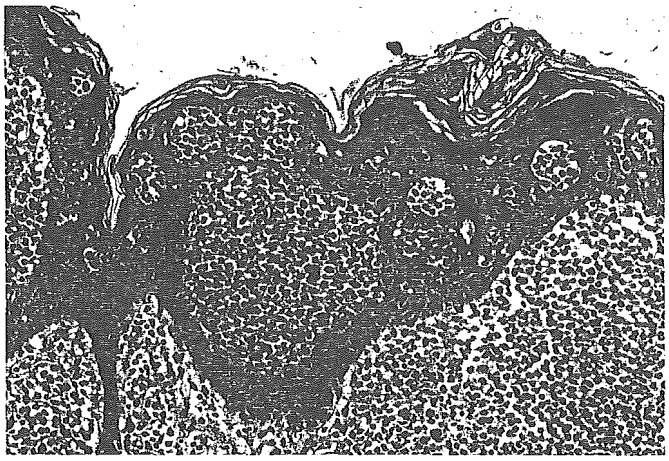


Figure 29–5. Skin involvement of ATL. ATL cells infiltrate into epidermis and form Pautrier's microabscesses. (See color insert.)

Pathogenesis of ATL

ATL is a neoplasm of activated helper T lymphocytes, which elaborate various cytokines, and express the immunoregulatory molecules on the surface. Such cytokines produced by ATL cells influence the pathophysiology of ATL. Eosinophilia is frequently observed in patients with ATL, which is caused by elevated IL-5. In addition, elaborated parathyroid hormone–related protein (PTH-rP) from ATL cells activates osteoclasts and promotes bone resorption, which is implicated in hypercalcemia. ATL cells produce

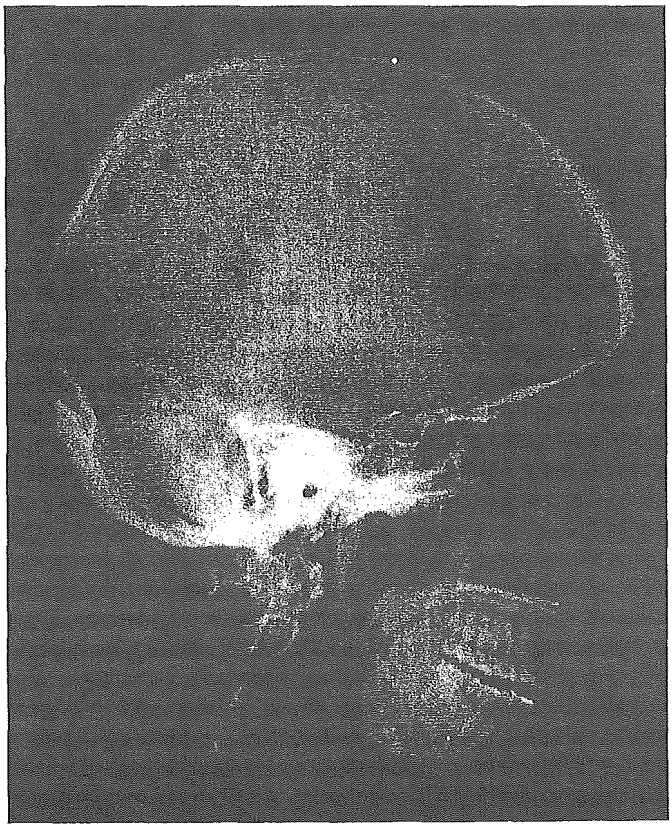


Figure 29–6. Punched-out lesions in the skull of an acute ATL patient.

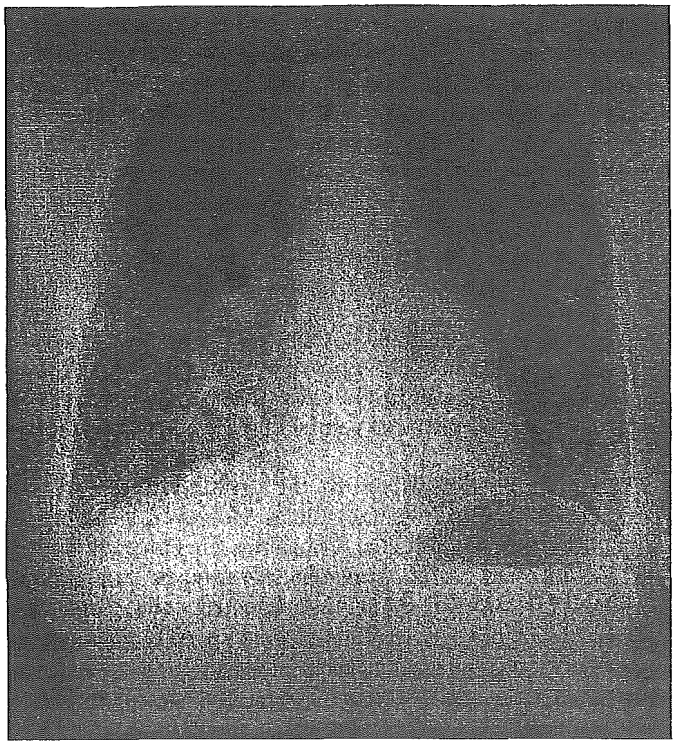


Figure 29–7. Pulmonary infiltration of ATL cells.

other cytokines, including TGF-β, IL-10, IL-8, and M-CSF, which modify the pathogenesis of ATL.

Hypercalcemia is more severe in patients with ATL than those with other hematologic malignancies. Several pathologic studies of ATL patients with hypercalcemia have indicated that high serum Ca2+ levels are due to an increased number of osteoclasts and accelerated bone resorption (Fig. 29–8). Bone is constitutively remodeled by osteoblasts (matrix synthesis) and osteoclasts (bone resorption). Osteoclasts are derived from hematopoietic precursor cells and belong to the monocyte macrophage lineage. During differentiation of osteoclasts, precursor cells sequentially express c-Fms (receptor of M-CSF) followed by receptor activator nuclear factor κB (RANK).[53] M-CSF and RANK

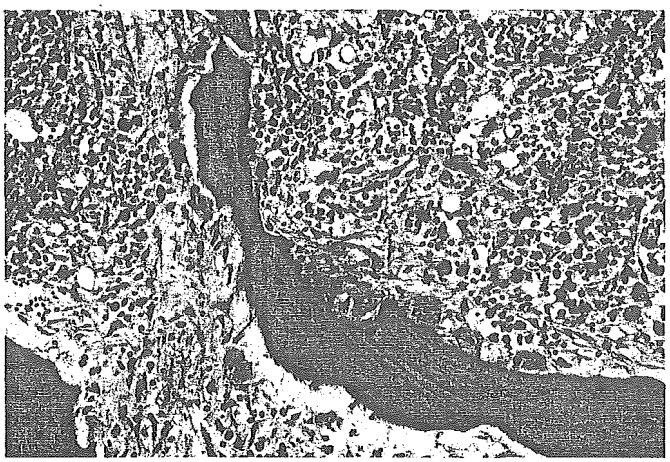


Figure 29–8. Increased osteoclasts in a hypercalcemic ATL patient. In a hypercalcemic patient, the number of osteoclast increased in the bone, which accelerated bone resorption. (See color insert.)

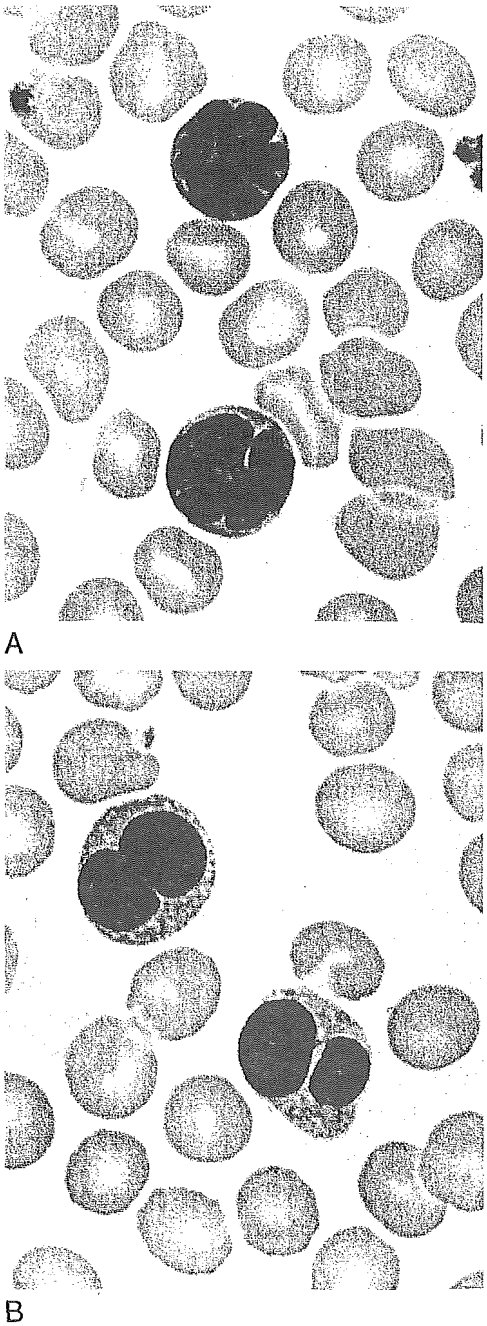


Figure 29–9. Cell morphology of ATL cells. ATL cells from an acute ATL (A) and a chronic ATL (B) are shown. (See color insert.)

ligand (RANKL) have been shown to be critical factors for the differentiation of osteoclasts, which are physiologically produced by stromal cells and osteoblasts. ATL cells from patients with hypercalcemia, which highly expressed the transcripts of the RANKL gene, induced the differentiation of hematopoietic precursor cells into osteoclasts in vitro in the presence of M-CSF.[54] It showed that RANKL expressed on ATL cells induced the differentiation into osteoclasts, resulting in increased number of osteoclasts and hypercalcemia in cooperation with PTH-rP. Thus, the immunoregulatory molecules on the ATL cells modify the pathogenesis.

Chemokines and their receptors have been implicated in the migration and tissue localization of lymphocytes. ATL

cells are well known to infiltrate into various organs or tissues, frequently invading skin or lymphoid tissues, which suggests that the differential expression of chemokine receptors might determine the migration of ATL cells. Analysis of chemokine receptor expression revealed that CCR4 was frequently expressed on HTLV-I–transformed cell lines and fresh ATL cells.[55] CCR4-positive T lymphocytes contain skin-seeking memory T cells, suggesting that expression of CCR4 accounts for frequent infiltration of ATL cells into skin. On the other hand, expression of CCR7 was reported to be associated with involvement of lymphoid tissues and lymph node enlargement.[56]

Diagnosis of ATL

The diagnostic criteria for ATL have been defined as follows: (1) histologically and/or cytologically proven lymphoid malignancy with T-cell surface antigens; and (2) abnormal T lymphocytes present in the peripheral blood except for the lymphoma-type. These abnormal T lymphocytes include not only typical ATL cells, the so-called flower cells, but also the small and mature T lymphocytes with incised or lobulated nuclei that are characteristic of the chronic or smoldering type. Additional criteria follow: (3) antibody to HTLV-I present in the sera at diagnosis; and (4) demonstration of monoclonal integration of HTLV-I provirus by the Southern blot method.

Morphology of ATL Cells

Most ATL cells characteristically exhibit lobular division of their nuclei; most are bi- or multi-foliate, and are separated by deep indentations. Cells with such a nuclear configuration are designated as "flower cells" (Fig. 29–9A). Cells from chronic ATL are relatively uniform in size and nuclear configuration, and smaller than those seen in either acute or smoldering ATL (Fig. 29–9B). Cells in this type also exhibit lobular division of their nuclei. However, most of the lobulated nuclei are bi- or tri-foliate. Cells from the smoldering type are relatively large and lack cytoplasmic granules or vacuoles. The nuclear chromatin is in coarse strands and is deeply stained.

Serology of HTLV-I

Anti-HTLV-I antibodies are positive in almost all patients with ATL, although seronegative ATL cases have been rarely reported.[52] The presence of serum antibodies against HTLV-I can be demonstrated by enzyme-linked immunosorbence, gelatin particle hemagglutination, indirect immunofluorescence, and the Western blot method.

Immunologic Characterization of ATL Cells

Surface phenotype of typical ATL cells is positive for CD2, 3, 4, 25, and HLA-DR, and is negative for CD7 and 8, indicating that ATL cells are derived from activated helper T lymphocytes. A characteristic feature of ATL cells is the decreased expression of T-cell receptor (TCR)/CD3 complex on their surfaces. There are several reports of ATL cases with different leukemic cell phenotypes, such as CD4+ and CD8+, CD4– and CD8–, and CD4– and CD8+.[29] Most of these variant forms are observed in patients with acute ATL and indicate poor prognosis. CD95 (Fas/APO-1) antigen is highly expressed on the leukemic cells in most patients with ATL, and such ATL cells are highly susceptible to antibody against Fas antigen. On the other hand, Fas-negative ATL cases were also reported and underlying mutations of the Fas gene were identified.[52]

HTLV-I Provirus Genome in Leukemic Cells of ATL

Definitive diagnosis of ATL requires detection of monoclonal integration of HTLV-I provirus in genomic DNA from tumor cells, especially in endemic areas. Monoclonal integration of HTLV-I provirus can be detected by the Southern blot method.

Tax plays a critical role in the proliferation of HTLV-I–infected cells and leukemogenesis. However, it is a major target of cytotoxic T cells. Thus, the presence of Tax in HTLV-I–infected cells provides advantages and disadvantages for survival of HTLV-I–infected cells. Since Tax expression is not frequently detected in ATL cells,[58] they presumably acquire the ability to proliferate independent of Tax function during the leukemogenesis. ATL cells frequently lose Tax expression by several mechanisms. Although the 5′-LTR is a viral promoter for transcription of viral genes, it was reported that 5′-LTR of HTLV-I provirus was deleted in 39% of cases examined, indicating that the viral gene transcription was impaired in ATL cells with such a provirus.[59] The second mechanism is the nonsense or missense mutation of the *tax* gene in fresh ATL cells. It is noteworthy that ATL cells in some cases had mutations in the Class I MHC recognition site of the Tax protein, resulting in escape from immune recognition.[60] The third mechanism is epigenetic change of the 5′-LTR: the 5′-LTR was selectively methylated, which silenced the transcription of viral genes.[58,61] With these mechanisms, ATL cells lost Tax expression, and could escape from the host immune surveillance system. It is speculated that Tax plays an important role in persistent proliferation of HTLV-I–infected cells during the carrier state, and then genetic and epigenetic changes accumulate in the host genome mediated by mutator phenotype of Tax,[62] which finally leads to Tax-independent proliferation and escape from the host immune system by inactivation of the *tax* gene.[4]

Chromosomal Abnormalities of ATL Cells

Karyotype analyses of 107 patients with ATL revealed several chromosomal abnormalities[63]: trisomies of chromosome 3 (21%), 7 (10%), and 21 (9%); monosomy of the X chromosome (38%) in females; loss of a Y chromosome (17%) in males; structural abnormalities, including translocations involving 14q32 (28%) or 14q11 (14%), and deletion of 6q (23%). Although there is no specific chromosomal abnormality for ATL, chromosomal abnormalities accumulate during disease progression. Comparative genomic hybridization (CGH) analyses of ATL revealed gains at chromosome 14q, 7q, and 3p. Genomic alterations increase in aggressive ATL compared with indolent forms (chronic and smoldering), which indicate the marked chromosomal instability in ATL cells.[64]

Classification of ATL

Clinical features of ATL vary among patients. Therefore, ATL patients can be classified into four clinical subtypes according to the clinical features: acute, chronic, smoldering, and lymphoma type. The Lymphoma Study Group (1984–1987) in Japan proposed the following diagnostic criteria for classifying ATL into the following four subtypes[65]:

1. *Smoldering type.* Five percent or more abnormal lymphocytes of T-cell nature in peripheral blood (PB); normal lymphocyte level (<4×10⁹/L); no hypercalcemia; LDH value of up to 1.5 times the normal upper limit; no lymphadenopathy; no involvement of liver, spleen, central nervous system (CNS), bone, or gastrointestinal tract; and neither ascites nor pleural effusion. Skin and pulmonary lesion(s) may be present. In patients with less than 5% abnormal T lymphocytes in PB, at least one histologically proven skin or pulmonary lesion should be present.
2. *Chronic type.* Absolute lymphocytosis of more than 3.5×10⁹/L; LDH value up to twice the normal upper limit; no hypercalcemia; no involvement of CNS, bone, or gastrointestinal tract; and neither ascites nor pleural effusion. There may be histologically proven lymphadenopathy with or without extranodal lesions, and there may be involvement of liver, spleen, skin, and lung, and 5% or more abnormal lymphocytes.
3. *Lymphoma type.* No lymphocytosis, 1% or less abnormal lymphocytes; histologically proven lymphadenopathy with or without extranodal lesions.
4. *Acute type.* The most common subtype, highly aggressive malignancy that shows lymphadenopathy, hepatosplenomegaly, and skin lesions, but does not meet the criteria of the other types.

Treatment and Prevention of ATL

Aggressive forms of ATL (acute and lymphoma-type) are generally treated with combination chemotherapy, although long-term success has been very limited. The acute form, with hypercalcemia, high LDH levels, and an elevated white blood cell count, carries a particularly poor prognosis. Sequential trials in Japan have resulted in the complete remission rate being increased from 16% with a four-drug combination to 43% with eight drugs.[66] In the recent study by Lymphoma Study Group in Japan, the improved survival of ATL patients has been reported by a granulocyte colony–stimulating factor–supported multiagent chemotherapy protocol. Although the median survival remains 13 months in this study, the estimated 2-year survival was 31.3%.[67] In contrast, smoldering ATL, or some cases of chronic ATL, may have a more protracted natural course, which may be compromised by aggressive chemotherapy. Alternative strategies for both acute and chronic forms are clearly needed.

The major obstacles in the treatment of patients with ATL include drug resistance and development of opportunistic infections caused by various organisms, such as *Pneumocystis jiroveci*, cytomegalovirus, *Strongyloidiasis*, and a variety of fungi, indicating that cell-mediated immunity is severely impaired in these patients.[52] The prophylactic measures should be taken for patients with ATL. The mechanism of drug resistance in ATL cells has been studied. One mechanism is that ATL cells show elevated NF-κB activity, which induces antiapoptotic genes such as *bcl-xL* and *inhibitor of apoptosis protein*.[9] To overwhelm the drug resistance mediated by NF-κB, the proteasome inhibitor, PS-341, is a candidate for treatment.[68,69]

Although the frequency of opportunistic infections is much higher in patients with ATL than in those with other hematologic malignancies, the underlying mechanism(s) remained unsolved. Opportunistic malignancies, Kaposi's sarcoma,[70] and Epstein–Barr virus (EBV)–associated lymphoma[71] have also been reported in patients with ATL, which also indicates a state of immunodeficiency in these patients.

α-Interferon (α-IFN) combined with azidothymidine (AZT) was administered to 19 patients with ATL, and major responses (complete plus partial remissions) were achieved in 58% of the patients (11 of 19), including complete remission in 26% (5 of 19).[72] The mechanism of this combination therapy remains unknown since these drugs have no effect in vitro. In primary effusion lymphoma (PEL), α-IFN induced the expression of tumor necrosis factor–related apoptosis-inducing ligand and AZT suppressed NF-κB activity.[73] Both effects are considered to synergistically induce the apoptosis of PEL cells. Humanized monoclonal antibody against IL-2 receptor was also used for patients with ATL,[74] although its effect was limited.

Allogeneic stem cell transplantation (allo-SCT) for patients with ATL has been reported.[75] Median leukemia-free survival after allo-SCT was more than 17.5 months. Since autologous transplantation has been shown to be ineffective for ATL, graft versus leukemia should play an important role in anti-ATL effect. In this regard, it is noteworthy that two patients without graft-versus-host disease relapsed with ATL. The immune response by cytotoxic T lymphocytes against Tax protein has been augmented after BMT, indicating that such anti-Tax cytotoxic T cells exert an antitumor effect on ATL cells.[76]

It is obvious that the reduced transmission of HTLV-I directly leads to prevention of ATL. Since the breast feeding is thought to be major route of vertical transmission, bottle feeding is recommended for seropositive mothers instead of breastfeeding in Japan. About 18% of infants have been seroconverted by breast feeding from seropositive mothers. Refraining from breastfeeding could reduce the seroconversion rate to about 3%.[77] The transmission route of HTLV in seroconverted children in spite of complete bottle feeding remains unknown, although intrapartum transmission is suspected.

For HTLV-I transmission by blood transfusion, all donated blood at blood centers were subjected to HTLV-I antibody testing beginning in November 1986 in Japan. After this, none of the recipients, even patients with hematologic disorders who received multiple transfusions, were seroconverted. In Japan, absolute decline of the carrier rate among the young generation was observed, possibly due to complete achieving of blood donor screening, and success in preventing most maternal transmission by refraining from breastfeeding.

In HIV-1, the prophylactic administration of antiviral drugs to individuals who had accidental exposure to

infected blood can reduce the transmission. Since nucleoside reverse transcriptase inhibitors such as azidothymidine can block the replication of HTLV-I,[48] prophylactic administration should be considered after accidental exposure to blood of HTLV-I–infected individuals.

HTLV-I–RELATED DISORDERS

HTLV-I is the causative virus of not only ATL, but also other inflammatory diseases, such as HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP), chronic lung diseases, infective dermatitis, arthropathy, and uveitis. In patients with HAM/TSP and HTLV-I uveitis, an increased number of HTLV-I–infected cells has been reported, suggesting that inflammatory reactions, which include excessive production of cytokines and increased expression of adhesion molecules in HTLV-I–infected cells, and host immune response against infected cells, play a critical roles in the pathogenesis of these diseases.

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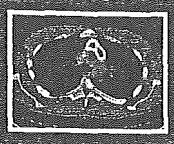

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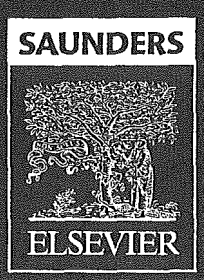

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Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms

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Abstract

Background: Human T-cell leukemia virus type I (HTLV-I) causes adult T-cell leukemia (ATL) after a long latent period. Among accessory genes encoded by HTLV-I, the *tax* gene is thought to play a central role in oncogenesis. However, Tax expression is disrupted by several mechanims including genetic changes of the *tax* gene, deletion/hypermethylation of 5'-LTR. To clarify the role of epigenetic changes, we analyzed DNA methylation and histone modification in the whole HTLV-I provirus genome.

Results: The *gag*, *pol* and *env* genes of HTLV-I provirus were more methylated than pX region, whereas methylation of 5'-LTR was variable and 3'-LTR was not methylated at all. In ATL cell lines, complete DNA methylation of 5'-LTR was associated with transcriptional silencing of viral genes. HTLV-I provirus was more methylated in primary ATL cells than in carrier state, indicating the association with disease progression. In seroconvertors, DNA methylation was already observed in internal sequences of provirus just after seroconversion. Taken together, it is speculated that DNA methylation first occurs in the *gag*, *pol* and *env* regions and then extends in the 5' and 3' directions *in vivo*, and when 5'-LTR becomes methylated, viral transcription is silenced. Analysis of histone modification in the HTLV-I provirus showed that the methylated provirus was associated with hypoacetylation. However, the *tax* gene transcript could not be detected in fresh ATL cells regardless of hyperacetylated histone H3 in 5'-LTR. The transcription rapidly recovered after *in vitro* culture in such ATL cells.

Conclusion: These results showed that epigenetic changes of provirus facilitated ATL cells to evade host immune system by suppressing viral gene transcription. In addition, this study shows the presence of another reversible mechanism that suppresses the *tax* gene transcription without DNA methylation and hypoacetylated histone.

Background

Human T-cell leukemia virus type I (HTLV-I) is associated with a neoplastic disease, adult T-cell leukemia (ATL), and inflammatory diseases, such as HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) and HTLV-I-associated uveitis [1,2]. Among HTLV-I carriers, a part of infected individuals develop ATL after a long latent period. During the leukemogenesis by HTLV-I, Tax protein is considered to play a critical role through its pleiotropic actions, which include transactivation of NF-κB, CREB and SRF pathways, transrepression of *lck*, *p18* and DNA polymerase β gene transcriptions, and functional inactivation of p53 and MAD1 [3-6]. Through these actions, Tax induces the proliferation of HTLV-I infected cells and inhibits their apoptosis, resulting in an increase in the number of infected cells. However, since Tax protein is the major target of cytotoxic T-lymphocytes (CTLs) *in vivo*, the expression also has a negative effect on the survival of ATL cells [7-9]. In some ATL cells, *tax* gene expression is inactivated by genetic and epigenetic changes, which include deletion, insertion or mutation of the *tax* gene, and DNA methylation or deletion of 5'-LTR [10-13]. Such inactivation of Tax expression is considered to allow ATL cells to escape from the host immune system.

DNA methylation of retroviruses is regarded as a host defense mechanism for inactivating retrovirus expression [14]. However, it is also recognized as a mechanism for virus-infected cells to escape from the host immune system and establish the latent state. In contrast, human immunodeficiency virus (HIV) is resistant to silencing *in vivo*. It is because HIV is frequently integrated into active transcriptional unit *in vivo* [15]. These findings coincide with the fact that HIV vigorously replicates *in vivo*. On the other hand, DNA methylation accumulated in HTLV-I 5'-LTR has been shown to silence viral gene transcription in leukemic cells [12,13]. In addition, the frequency of integration of HTLV-I provirus into transcriptional units was equivalent to that calculated based on random integration [16], which also increased the silencing. It remains unclear where and when DNA methylation occurs within the HTLV-I provirus genome.

In this study, we analyzed DNA methylation and histone modification in the whole HTLV-I provirus, and observed the progressive accumulation of DNA methylation. In addition, another reversible mechanism silenced viral gene transcription regardless of hyperacetylated promoter region.

Results

Analyses of DNA methylation of HTLV-I provirus

To reveal DNA methylation status within the HTLV-I provirus, we analyzed the DNA methylation by sodium bisulfite sequencing and combined bisulfite restriction analysis (COBRA). Initially, DNA methylation in 5'-LTR, gag, pol, env, pX and 3'-LTR was identified by sodium bisulfite sequencing. In an ATL case (Fig. 1A), the internal regions of the HTLV-I provirus, including gag, pol and env, were heavily methylated. On the other hand, 5'-LTR and pX were partially methylated, and 3'-LTR was not methylated at all. In an ATL cell line, ATL-48T (Fig. 1A), the internal sequences of the HTLV-I provirus were partially methylated, whereas both LTRs were not methylated. Since the analyses by sodium bisulfite sequencing were time-consuming, we established the COBRA method to detect and analyze DNA methylation in a large number of samples, and then compared the results obtained with the two methods. After amplification of sodium bisulfite treated DNAs with each primer sets, the products were digested with TaqI or AccII, which contain one (TaqI) or two (AccII) CpG site(s) within the recognition sequences. When CpG site is methylated, the products retain CpG site, resulting in digestion by these enzymes. On the other hand, CpG is converted to UG when it is unmethylated. Therefore, PCR products are resistant to restriction enzymes (Fig. 1B). With the COBRA method, the extent of DNA methylation was quantified in eight CpG sites throughout the HTLV-I provirus: 5'-LTR (620 according to the numbering by Seiki et al. [17]), gag (1753), pol (2988, 4187 and 5151), env (6113), pX (7258) and 3'-LTR (8342) (Fig. 1C). The extent of DNA methylation detected by the COBRA method was well correlated with that obtained by sodium bisulfite sequencing in both cases studied, as shown in Fig. 1A and 1C.

DNA methylation throughout the HTLV-I provirus in HTLV-I-transformed and ATL cell lines

Using the COBRA method, we analyzed the DNA methylation throughout the whole HTLV-I provirus of the cell lines (Fig. 2B and 2C). In addition, we also analyzed the tax gene transcription by RT-PCR (Fig. 2A) and the number of integrated HTLV-I proviruses in each cell lines by Southern blot method. Among the tax gene-expressing cell lines (ATL-35T, MT-2, Sez627, MT-4, ATL-55T, MT-1 ATL-48T and ATL-2) (Fig. 2A), internal sequences from gag to pX were variably methylated. However, 5'-LTR was not methylated or partially methylated, while 3'-LTR was not methylated in all cell lines (Fig. 2B). In ATL-43T and TL-Oml, which did not show tax gene transcription (Fig. 2A), 5'-LTR and the internal sequences were heavily methylated (Fig. 2C), indicating the close correlation between the extents of DNA methylation of the provirus, particularly 5'-LTR, and tax gene transcription. As previously reported, the treatment by 5-aza-deoxy-cytidine can recover the tax gene expression of these cell lines, indicating that the latent state by DNA methylation of 5'-LTR is reversible [13].