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Cell-free Human T-cell Leukemia Virus Type 1 Binds to, and Efficiently Enters Mouse Cells

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Human T-cell leukemia virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia/lymphoma and other HTLV-1-associated diseases. However, the interaction between HTLV-1 and T cells in the pathogenesis of these diseases is poorly understood. Mouse cells have been reported to be resistant to cell-free HTLV-1 infection. However, we recently reported that HTLV-1 DNA could be observed 24 h after cell-free HTLV-1 infection of mouse cell lines. To understand HTLV-1 replication in these cells in detail, we concentrated the virus produced from c77 feline kidney cell line and established an efficient infection system. The amounts of adsorption of HTLV-1 are larger in mouse T cell lines, EL4 and RLm1, than those in human T cell lines, Molt4 and HUT78, and are similar to that in human kidney cell line, 293T. Unexpectedly, however, the amounts of entry of HTLV-1 are about 10-fold larger in the two mouse cell lines than those in the three human cell lines employed. Moreover, viral DNA was detectable from 1 h in EL4 and RLm1 cells, but only from 2–3 h in 293T, Molt4 and HUT78 cells. However, the amount of viral DNA in EL4 cells became smaller than that in Molt4 cells. HTLV-1 expression could be detected until day 1–2 in RLm1 and EL4 cells, and until day 4 in Molt4 cells. Our results suggest that mouse cell experiments would give useful information to dissect the early steps of cell-free HTLV-1 infection.

Key words: Cell-free HTLV-1 — Entry — Expression — Mouse cells

Human T-cell leukemia virus type 1 (HTLV-1) is believed to be a causative agent of adult T-cell leukemia/lymphoma (ATL),[1, 2] HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP),[3–5] and other HTLV-1-associated diseases.[6, 7] Among infected individuals, only 2 to 3% develop ATL.[8] In addition, there is a latency of 40 to 50 years before manifestation of ATL. A small animal model should be useful to study the pathogenesis of HTLV-1-associated diseases, which is not understood. Although rat models of HTLV-1 infection[9] and HAM/TSP[10–13] have been reported, a mouse model would be desirable due to the accumulated information on the genetic background of mice and the advances in transgenic technology.

Recently, we reported HTLV-1 transmission to newborn mice[14–16] by inoculating them with an HTLV-1-producing human T cell line, MT-2.[17] In addition, although it was reported that mouse cell lines are very resistant to cell-free HTLV-1 infection,[18] we have recently found that cell-free HTLV-1 can enter many mouse cell lines.[19]

The kinetics of HTLV-1 replication is not well understood, mainly due to the unavailability of efficient cell-free HTLV-1 infection. Although Fan *et al.* developed a sensitive HTLV-1 infection assay with cell-free HTLV-1 produced from MT-2 cells, only low levels of viral DNA have been detected.[20] Haraguchi *et al.* reported that the cell-free HTLV-1 produced from a feline kidney cell line, c77, is highly transmissible to human cells. The transmission of cell-free HTLV-1 produced from c77 cells was estimated to be more than 3000 times higher than that of HTLV-1 derived from MT-2, C91/PL or HOS/PL cells by PCR assay. The transmission of HTLV-1 from c77 is inhibited by treatment of the virus with neutralizing antibodies.[21] This cell-free infection system is expected to be useful for analyzing the early stages of viral infection. Recently, we used this system to demonstrate that cell-free HTLV-1 can enter mouse cells.[19]

In this study, we established a more efficient cell-free HTLV-1 infection system by concentrating the cell-free HTLV-1 produced from c77 cells. With this system, we analyzed the adsorption and entry of the virus, and the kinetics of viral DNA synthesis and expression as compared to those in human cells. Our results showed that HTLV-1 enters mouse cells more efficiently than it enters the human cells employed. Viral expression was detected in both mouse and human cells.

MATERIALS AND METHODS

Cells and cell culture The HTLV-1-producing cell line, c77 feline kidney cell line,[22–24] was used as a source of

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cell-free HTLV-1. Mouse T cell lines, EL4 (CD4+ cell) and RLm1 (CD4+, CD8+ cell), and human T cell lines, Molt4 (CD3+, CD4+ cell) and HUT78 (CD4+, CD8−) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Human kidney cell line, 293T, was maintained in Dulbecco's minimal essential medium. c77 cells were maintained in Eagle's minimum essential medium supplemented with 10% FCS. EL4 was a gift of Dr. T. Honjo (Kyoto University). RLm1 and 293T were gifts of Dr. H. Nakauchi and Dr. M. Onodera (University of Tsukuba).

Cell-free virus preparation and assays Cell-free HTLV-1 was prepared as described,[21, 25] with a slight modification. Four million c77 cells in 8 ml of medium were seeded in each 10-cm plastic dish and cultured for 2 days. The medium was replaced by fresh medium and cultured for 24 h. One thousand milliliters of culture medium was harvested and centrifuged at low speed to remove cells and debris. The supernatant was centrifuged with a JS4.2 rotor at 5000*g* for 20 h at 4°C or centrifuged on a cushion of 20% sucrose with a SW28 rotor at 100 000*g* for 3 h at 4°C. The pellet was suspended with 1 ml of RPMI medium as a concentrated virus fraction. The concentrated and unconcentrated virus fractions were treated with RNase-free DNase 1(80 u/ml, Promega, Madison, WI) for 1 h at 37°C, and passed through 0.22-μm-pore-size cellulose acetate membrane filters. To determine the infectivity of cell-free HTLV-1 preparation, the concentrated and unconcentrated virus fractions were serially diluted with culture medium and inoculated immediately into Molt4 cells. After 24 h of incubation, HTLV-1 DNA in Molt4 cell lysate was detected by PCR. The virus concentration was determined by an HTLV-1 core protein p19 enzyme-linked immunosorbent assay (ELISA) (RETRO-TEK, ZeptoMetrix Corp., Buffalo, NY).

Virus adsorption and entry assays In general, retroviruses bind efficiently to target cells at 4°C.[26] For analysis of the virus adsorption and entry into mouse and human cells, we followed the procedure used by Sun *et al.* for human immunodeficiency virus (HIV)-1.[27] We prepared the concentrated cell-free HTLV-1 fraction by centrifugation on a cushion of 20% sucrose with an SW28 rotor at 100 000*g* for 3 h at 4°C after Derse *et al.*[28] with slight modifications. Then we immediately inoculated the concentrated virus into 10^6 mouse EL4 and RLm1 cells, and human Molt4, HUT78 and 293T cells. The cells were incubated for 30 min by rotating the tubes continuously at 4°C, then washed 5 times with ice-cold phosphate-buffered saline (PBS) to remove unbound virus. Then the cells were lysed in 200 μl of disruption buffer (2.5% Triton X-100 in PBS). The cell lysate was stored at −20°C until assayed for viral p19 content by ELISA. For entry assay, we inoculated another lot of the concentrated virus into the same number of the same cells and incubated them in petri dishes for 30 min at 37°C. The unpenetrated viruses remaining on the cell surface were eliminated by treatment with ice-cold RPMI 1640 (without FCS) containing 0.1 mg/ml of pronase (Roche Diagnostics, Basel, Switzerland) for 5 min at 4°C. Then the cells were washed 4 times with ice-cold PBS, lysed and assayed for intracellular p19 by ELISA.

Experiments for analysis of viral DNA synthesis and viral expression EL4, RLm1, Molt4, HUT78 and 293T cells, at a concentration of 2×10^6 cells per ml, were incubated with the concentrated DNA-free virus at 37°C. One million cells were harvested at 0, 1, 2, 3, 6, 9 and 12 h later and washed with ice-cold PBS. The cells were suspended in lysis buffer (10 m*M* Tris-HCl, pH 8.3, 1 m*M* EDTA, 0.45% NP-40, 0.45% Tween 20 and 300 μg/ml proteinase K) and incubated for at least 5 h at 55°C. The HTLV-1 *pX* sequence in lysates corresponding to 1×10^5 cells was detected by PCR and subsequent Southern hybridization.

For quantitation of viral DNA and detection of virus expression, EL4 and Molt4 cells, at a concentration of 2×10^6 cells per ml, were cultured with the DNA-free virus preparation for 3 h at 37°C. The cells were washed with ice-cold PBS and then treated with 1 ml of ice-cold RPMI 1640 (without FCS) containing 0.1 mg/ml pronase for 5 min at 4°C. Cells were washed immediately with 2 ml of ice-cold RPMI containing 10% FCS. The inoculated cells were maintained for up to 30 days in continuous culture. Half of the culture was harvested every 24 h, and analyzed for HTLV-1 DNA by semi-quantitative PCR, for HTLV-1 expression by RT-PCR and for virus production by ELISA.

PCR for HTLV-1 DNA Cellular DNAs from the infected and uninfected cells were prepared by sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction.[9] As a template for semi-quantitative PCR, the DNA or lysate of the infected cells was serially diluted with that of uninfected cells. One microgram of DNA or the lysate corresponding to 1×10^5 cells was used as a template for each PCR. The HTLV-1 *pX* sequence was detected by PCR (95°C for 10 min, then 50 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1.5 min, and finally at 72°C for 7 min) with *Taq* Gold polymerase and Southern blot analysis, as described previously.[9] The probe for the *pX* region was labeled by digoxigenin (DIG oligonucleotide Tailing Kit, Roche Diagnostics). The mouse c-*myc* sequence or human β-*globin* sequence, which served as an internal control to assure the quality of the template, was amplified in the same reaction tube used for multiplex PCR as described previously.[19]

RT-PCR for HTLV-1 expression RNA was extracted from the infected and uninfected cells with an ISOGEN (Nippon Gene, Osaka). RNA from the c77 cells was used as a positive control. One microgram of RNA was used

for reverse transcriptase-PCR (RT-PCR) analysis, as described by Kinoshita *et al.*[29] Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase. Fifty cycles of amplification were performed with RPX3 and RPX4 primers,[29] and with *Taq* Gold polymerase (Applied Biosystems, Foster City, CA). The PCR products were detected with the Dig-labeled probe RPXPR1.[29] Amplification of a cDNA fragment of the glyceraldehyde-3-phosphate dehydrogenase gene was performed as an internal control.

RESULTS

Concentration of cell-free HTLV-1 The concentrated and unconcentrated HTLV-1 fractions were serially diluted with culture medium and inoculated immediately into Molt4 cells. After 24 h of incubation, the *pX* DNA fragments in Molt4 cell lysates were detected by PCR. The *pX* DNA fragments were detectable in Molt4 cells inoculated with the concentrated virus fraction till 3125-fold dilution (Fig. 1A), and with the unconcentrated virus fraction till 25-fold dilution (Fig. 1B). Thus, the infectivity of cell-free HTLV-1 was increased about 100 times by centrifugation of the culture supernatant of HTLV-1-producing c77 cells.

The amounts of adsorption and entry of virus in the cell-free HTLV-1-inoculated cells For virus adsorption assay, one million EL4, Rlm1, Molt4, HUT78 and 293T cells were incubated with the concentrated cell-free HTLV-1 fraction for 30 min at 4°C. For virus entry assay, we incubated concentrated virus with these cell lines for 30 min at 37°C. The amounts of adsorption and entry of HTLV-1 was measured in terms of p19 viral protein in the cell lysates by ELISA. The amounts of adsorbed virus were much lower in human Hut78 cells than those in mouse EL4 and RLm1 cells, but those in 293T and Molt4 cells were similar to those in mouse EL4 and RLm1 cells (Fig. 2A). The amounts of entered virus in EL4 and RLm1 cells were about 10 times those in 293T, Molt4 and HUT78 cells (Fig. 2B). Thus, cell-free HTLV-1 enters more efficiently into mouse cells than it does into the human cells used in this work.

Detection of viral DNA in cell-free HTLV-1-inoculated human and mouse cells We inoculated the mouse and human cell lines with the concentrated HTLV-1, and detected HTLV-1 *pX* DNA fragments in the inoculated cells at 0, 1, 2, 3, 6, 9 and 12 h later by PCR assay. *pX* DNA fragments were detected in EL4 and RLm1 cells

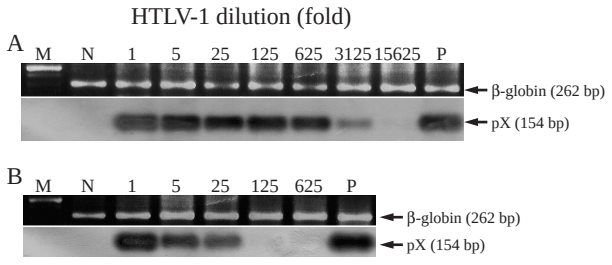

Fig. 1. Concentration of infectivity of cell-free HTLV-1. Concentrated virus (A) and unconcentrated virus (B) were 5-fold serially diluted with medium, and inoculated into Molt4 cells. HTLV-1 *pX* (Southern blot) and human *β-globin* (ethidium bromide staining) sequences in the lysates of cells harvested 24 h after inoculation were analyzed by PCR. Uninfected Molt4 cell lysates were used as negative controls (N). HTLV-1-positive MT-2 cell lysates, diluted 100-fold with uninfected Molt4 cell lysate, were used as positive controls (P). M indicates DNA size markers.

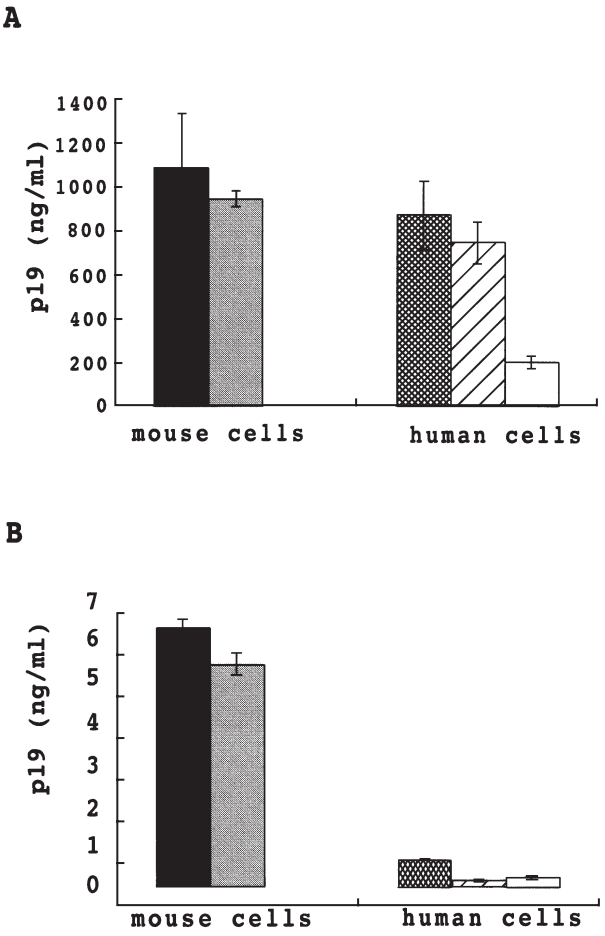

Fig. 2. Comparison of cell-free HTLV-1 adsorption and entry between mouse cells and human cells. For entry assay, one million EL4 (■), RLm1 (▩), 293T (▨), Molt4 (▨) and HUT78 (□) cells were incubated with virus preparation for 30 min at 4°C (A). For entry assay, those cells were incubated with virus preparation for 30 min at 37°C (B). The titer of p19 antigen in the lysates was determined by ELISA. All the experiments were performed in triplicate.

from 1 h, in Molt4 and HUT78 cells from 3 h and in 293T cells from 2 h after virus inoculation (Fig. 3). Viral DNA appears earlier in mouse cell lines than in the human cell lines used in this work.

Kinetic analysis of HTLV-1 provirus in the cell-free HTLV-1-inoculated human and mouse cells To analyze the kinetics of viral DNA synthesis, we infected Molt4 and EL4 cells with concentrated virus and maintained the cells for up to 30 days in continuous culture. We harvested half of the cells every day and extracted the cellular DNA. We detected the HTLV-1 *pX* fragments in cellular DNA serially 5-fold diluted with uninfected cellular DNA by PCR assay. About 3000 copies of HTLV-1 *pX* sequence was detected in 10^5 infected Molt4 cells 1 day after viral inoculation. The copy number decreased to about 25 copies from day 2 and remained at a similar level until day 30. In contrast, about 100 copies of the HTLV-1 *pX* sequence were detected in 10^5 infected EL4 cells 1 day after viral inoculation. The viral DNA decreased to about one copy in 10^5 EL4 cells on day 3, and from day 20, it was undetectable (Fig. 4). The amount of viral DNA was smaller in EL4 cells than in Molt4 cells during the observation period. In Molt4 cells, the viral DNA persisted for at least 1 month of the observation period.

Detection of tax/rex mRNA in the cell-free HTLV-1-inoculated human and mouse cells We also extracted mRNA of the infected cells at the indicated time points (Fig. 5), and detected HTLV-1 tax/rex mRNA by RT-PCR. Tax/rex mRNA was detectable from 1 h to 48 h after virus inoculation in EL4 cells and from 1 h to 24 h in

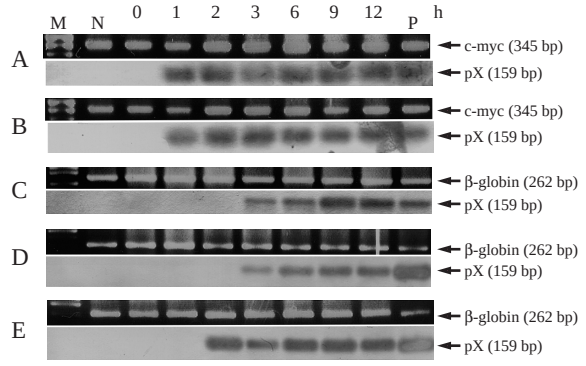

Fig. 3. Viral DNA was detected earlier in the mouse cell lines than in the human cell lines after viral inoculation. Concentrated virus fraction was inoculated into EL4 (A), RLm1 (B), Molt4 (C), HUT78 (D) and 293T (E) cells. The cells were harvested at 0, 1, 2, 3, 6, 9 and 12 h after viral inoculation. Mouse c-*myc*, human *β-globin* (ethidium bromide staining) and HTLV-1 *pX* (Southern blot) sequences in the harvested cell lysates were analyzed by PCR. Lysates of uninfected target cells were used as negative controls (N). HTLV-1-positive human MT-2 cell lysates, diluted 100-fold with uninfected target cell lysates, were used as positive controls (P). Mouse c-*myc* and human *β-globin* bands served as internal controls. M indicates DNA size markers. For EL4, RLm1 and Molt4 cells, a representative result from 3 independent experiments is shown.

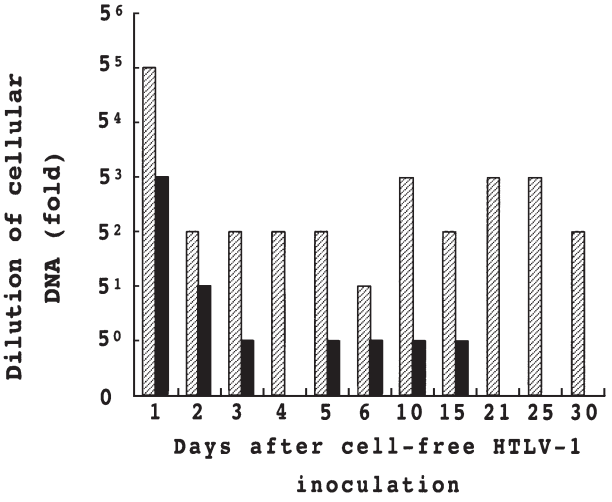

Fig. 4. Presence of viral DNA in EL4 and Molt4 cells after viral inoculation. DNA was extracted from harvested EL4 cells (■) and Molt4 cells (▨) inoculated with the concentrated viral fraction at the time points shown, and serially diluted with uninfected Molt4 or EL4 cell DNAs. The HTLV-1 *pX* and human *β-globin* sequences in the diluted cell DNA were analyzed by PCR in duplicate. Mouse c-*myc* and human *β-globin* were used as internal controls.

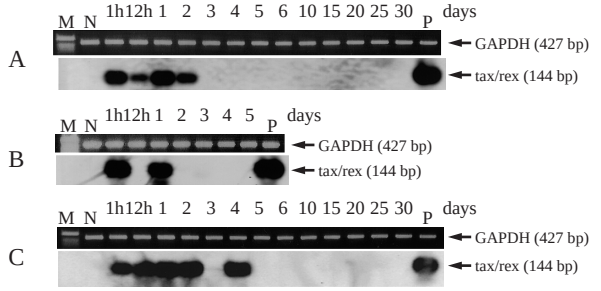

Fig. 5. HTLV-1 tax/rex mRNA was detected in cell-free HTLV-1-inoculated EL4, RLm1 and Molt4 cells by RT-PCR. Total cellular RNA was extracted from EL4 (A), RLm1 (B) and Molt4 (C) cells infected with cell-free HTLV-1 at the time points shown. HTLV-1 tax/rex (Southern blot) and GAPDH (ethidium bromide staining) mRNA sequences in 1 μg of RNA were analyzed. Uninfected human Molt4 cell and EL4 cell RNA were used as negative controls (N). HTLV-1 positive human MT-2 cell RNA was used as positive controls (P). M indicates DNA size markers. For EL4 and Molt4 cells, a representative result from 2 independent experiments is shown.

RLm1 cells. In Molt4 cells, the mRNA was detected from 1 h to day 4 after viral inoculation. The mRNA became undetectable thereafter (Fig. 5). There was no detectable viral expression at 12 h after infection in RLm1 cells or at 3 days in Molt4 cells. p19 antigen in the supernatants of cell-free HTLV-1-infected human and mouse cells was below the level of detectability during culture for 30 days.

DISCUSSION

The infection of cells with cell-free HTLV-1 has been reported to be quite inefficient, compared with other retroviruses.[20, 28, 30] Fan *et al.* reported that they could infect Molt4 cells with cell-free HTLV-1 isolated from MT-2 cells, and the copy number of HTLV-1 provirus was calculated as 168 per 2×10^6 cells 4 h after infection.[20] We estimated that the transmission of cell-free HTLV-1 produced from c77 cells was more than 3000 times higher than that of the cell-free HTLV-1 derived from MT-2, C91/PL or HOS/PL cells, and was completely inhibited by HTLV-1 antibody-positive human sera.[21] Thus, it is not clear at present why HTLV-1 derived from c77 is easily transmissible. In the present work, we improved the infectivity of culture supernatant of c77 about 100 times by centrifuging the culture supernatant (Fig. 1). We also found that at least 3125 copies of viral DNA per 10^5 cells were present 1 day after infection (Fig. 4). Our data suggested that our system is suitable for analysis of the early stage of HTLV-1 infection in cultured cells. Using this system, we analyzed the early stage of cell-free HTLV-1 infection of mouse cells and human cells.

Our adsorption assay showed a comparable binding of cell-free HTLV-1 on mouse cells and on human cells. In accordance with our findings, Jassal *et al.* recently showed that recombinant HTLV-1 surface glycoprotein gp46 binds significantly to murine cells as well as to human cells.[31] Our assay should be useful in future studies on the interaction between HTLV-1 envelope proteins and cell surface receptors.

Interestingly, our entry assay showed that the amount of entry of the virus into mouse cell lines is 10-fold more than that into the human cell lines employed. After viral entry into the target cells, the viral DNA is synthesized by reverse transcriptase. The viral DNA was detected from 1 h in mouse cells, but from 2 or 3 h in human cells. The HIV DNA synthesis was reported to be detectable 4 h after cell-free infection of human cells.[32] The early step of cell-free HTLV-1 infection seems more efficient in mouse cells than in human cells.

Previously we could not detect the tax/rex mRNA, presumably due to the low titer of cell-free HTLV-1.[19] In the present work, the tax/rex mRNA of HTLV-1 was detectable in mouse and human cell lines infected with concentrated cell-free HTLV-1. The tax/rex expression was undetectable in RLm1 cells and Molt4 cells at 12 h and 3 days, respectively, after infection. Further work is needed to elucidate the mechanism of this transient down-regulation of viral mRNA.

Although the viral DNA persisted in human cells during the 30-day observation period, it became undetectable in mouse cells 21 days after infection. One possible explanation is that the HTLV-1-infected mouse cell population in this system might not have had a growth advantage and was lost during daily passage of the cell culture. Another possibility is that most HTLV-1-infected cells proceeded to cell death. Apoptosis of infected cells has been reported with other retroviruses.[33–36]

Further analysis of cell-free HTLV-1 infection of mouse cells would give information about viral adsorption at the receptor, entry of cell-free HTLV-1, and host factors involved in HTLV-1 replication, and should be helpful in establishing a useful mouse model of HTLV-1 infection and HTLV-1-associated diseases.

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