

Cell-free Entry of Human T-Cell Leukemia Virus Type 1 to Mouse Cells

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Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent for adult T-cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis. Recently we infected newborn mice by inoculating HTLV-1-producing human cells, and found that T-cells, B-cells and granulocytes were infected *in vivo*. To understand the mechanism of viral-cell interaction and the pathogenesis of HTLV-1 using the mouse model, it is important to clarify the cellular tropism using a cell-free HTLV-1 transmission system. We employed a highly transmissible cell-free HTLV-1 produced by a feline kidney cell line, c77, and studied the susceptibility of 9 kinds of mouse cell lines, EL4, RLM1, CTLL-2, J774.1, DA-1, Ba/F3, WEHI-3, NIH3T3 and B1, and two kinds of human cell lines, Molt-4 and Hut78. HTLV-1 proviral sequence was found by PCR in all 9 mouse cell lines as well as in 2 human cell lines and viral entry was blocked with sera from an HTLV-1 carrier and an adult T-cell leukemia patient. Unexpectedly, mouse cell lines EL4 and RLM1 and human cell lines Molt-4 and Hut78 showed similar efficiency for viral entry. These results suggest a wide distribution of HTLV-1 receptor in mouse cells.

Key words: Entry — Human T-cell leukemia virus type 1 — Cell-free transmission — Mouse cell lines — Receptor

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL),¹⁾ HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)²⁾ and some other HTLV-1-associated diseases. The latency from HTLV-1 infection to the manifestation of ATL, a typical HTLV-1-associated disease, is 50 years or more, with only about 3% of HTLV-1 carriers developing the disease.³⁾ It would be useful to establish a small animal model to study the pathogenesis of HTLV-1-associated diseases. Although there are some HTLV-1-infected animal models,^{4–6)} a mouse model is desirable because of the accumulated knowledge of the genetic background and the advanced transgenic technology for production of transgenic or knockout mice. However, it was reported that mouse fibroblasts were very resistant to infection by vesicular stomatitis virus (VSV) pseudotype, VSV (HTLV-1), that has envelope antigens of HTLV-1.⁷⁾

We have recently succeeded in inducing HTLV-1 infection in C3H/HeJ mice by inoculating them with an HTLV-1-producing human T cell line, MT-2 cells.^{8–10)} However, the mechanism of entry of HTLV-1 into mouse cells is unclear. There are two possible routes for HTLV-1 entry into mouse cells: (I), cell-to-cell route: direct contact and subsequent fusion of HTLV-1 producing cells (MT-2 cells) with the target cells (mouse cells) and (II), the cell-free route: interaction of virus particles from MT-2 cells

with the appropriate receptor and/or coreceptor on the mouse cell surface.

The analysis of cellular tropism of HTLV-1 is important for understanding the viral-cell interaction and pathogenesis of HTLV-1. It is necessary to use a cell-free HTLV-1 transmission system in human cells and in experimental animal (e.g., mouse) cells. However, an efficient cell-free transmission system is not available thus far. Haraguchi *et al.* reported initially that cell-free HTLV-1 virus produced from a feline kidney cell line, c77, can efficiently transmit to human cells.¹¹⁾ We employed a highly transmissible cell-free HTLV-1 produced by c77 and examined whether the cell-free HTLV-1 could also transmit to mouse cells by inoculating 9 mouse cell lines. PCR and subsequent Southern hybridization were used to search for the HTLV-1 provirus sequence in the infected cell lysates. In this report we demonstrate that cell-free HTLV-1 could enter all the mouse cell lines and that mouse T cell lines are just as susceptible as human T cell lines to cell-free HTLV-1.

MATERIALS AND METHODS

Cells and cell culture The HTLV-1-producing cell line used in this experiment was c77 feline kidney cell line.¹²⁾ The mouse cell lines used as target cells were EL4 (CD4+ T-cell),¹³⁾ RLM1 (CD4+, CD8+ T-cell),¹⁴⁾ CTLL-2 (CD8+ T-cell),¹⁵⁾ J774.1 (macrophage),¹⁶⁾ Ba/F3 (Pro-B-cell),¹⁷⁾ DA-1 (myeloid cell),¹⁸⁾ WEHI-3 (myeloma cell),¹⁹⁾ NIH3T3 (fibroblast cell)²⁰⁾ and B1(Leydig cell).²¹⁾ Human

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T-cell lines, Hut78 (CD4+, CD8-)²² and Molt-4 (CD3+, CD4+),²³ were also used as target cells. Hut78, Molt4, EL4, RLm1, CTLL-2, J774.1, WEHI-3, Ba/F3 and DA-1 were maintained in RPMI1640 supplemented with 10% fetal calf serum. To this 50 U/ml recombinant human interleukin-2 (Shionogi, Saitama) was added for CTLL-2, and 10% of WEHI-3 cell-conditioned medium as a source of interleukin-3 for Ba/F3 and DA-1. c77 was maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum. NIH3T3 was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. B1 was maintained in EMEM supplemented with 10% fetal calf serum and 10^{-8} M β -estradiol and 0.1 μ g/ml insulin. EL4 and RLm1 were the gifts of Drs. T. Honjo (Kyoto University) and H. Nakauchi (University of Tsukuba), respectively. B1 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka). CTLL-2, J774.1, WEHI-3, Ba/F3 and DA-1 were obtained from RIKEN Cell Bank (Tsukuba).

Virus preparation Cell-free HTLV-1 had been prepared as described¹¹ with a slight modification. c77 cells were seeded at 5×10^5 per ml in culture medium, and incubated for 2 days. The medium was replaced by fresh medium which was harvested after incubation for 24 h. Cells and debris were removed from the culture supernatant by centrifugation for 15 min at 800g, then filtered through a 0.45- μ m-pore-size filter. In some experiments, the filtrate was further incubated with 80 U/ml of RNase-free DNase I (Promega, Madison, WI) for 1 h at 37°C. The filtrates were used immediately. The HTLV-1 p19 antigen in the virus preparation was measured.

Inoculation of target cells with cell-free HTLV-1 and detection of provirus and p19 antigen Mouse cell lines, at a concentration of $5-10 \times 10^5$ cells per ml, were incubated with the virus preparation for 24 h at 37°C. The cells were harvested, washed twice with phosphate-buffered saline (PBS) and divided into two aliquots. One of the aliquots was lysed with lysis buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 0.45% NP-40, 0.45% Tween 20 and 30 μ g/ml proteinase K) and incubated for 5 h at 55°C. The HTLV-1 *pX* sequence in the lysates was determined by PCR and subsequent Southern hybridization. The other aliquot of cells was maintained in the culture medium for a further 24 h. The cells were then harvested as above for analysis of HTLV-1 provirus by PCR. The culture supernatants were collected to measure the p19 antigen of HTLV-1 by using an HTLV-1 p19 ELISA kit (Cellular Products Inc., New York, NY) according to the manufacturer's instructions. The lysate of 5×10^4 cells was used for each PCR. HTLV-1 *pX* sequence was detected by PCR amplification (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, ended by 72°C for 10 min) with *Taq* polymerase and Southern hybridization, as described previously.¹⁰ The mouse *c-myc*

sequence or human β -globin sequence which served as the internal control to assure the quality of the template was amplified in the same reaction tube as multiplex PCR.¹⁰ The primers for the *pX* region were 5'-CGGATACCAGTCTACGTGT-3' (nt 7336-7355) and 5'-GAGCCGATAACGCGTCCATC-3' (nt 7475-7494), those for mouse *c-myc* were 5'-ACAACGTCTTGGAAACGTCAG-3' (nt 4069-4088) and 5'-GGTTAGGCTTTGAGCATGCA-3' (nt 4394-4413) and those for human β -globin were 5'-GGTTGGCCAATCTACTCCCAGG-3' (nt 85-106) and 5'-TGGTCTCCTTAAACCTGTCTTG-3' (nt 325-346). The probe for the *pX* region was 5'-CTGTGTACAAGGC-GACTGGTG-3' (nt 7363-7383). To exclude the possibility of contamination of the *pX*-containing DNA fragments from c77 cells, the filtrate of the culture supernatant of c77 cells was subjected to PCR. Namely, a mixture of 12.5 μ l of the cell-free virus preparation and 1 μ g of DNA of HTLV-1-negative Hut78 cell was used as a template for PCR to detect *pX* sequence.

Neutralization of HTLV-1 entry To analyze the neutralizing activity of cell-free HTLV-1 entry, the virus preparation was incubated with various dilutions of serum at 37°C for 60 min, and then used to inoculate the target cells. Sera from a healthy donor, an HTLV-1 carrier and an ATL patient were used. The titers of anti-HTLV-1 antibodies in the sera were assayed with a particle agglutination kit (Serodia HTLV-1; Fujirebio, Tokyo). The titer of anti-HTLV-1 antibodies in the serum from a non-infected healthy donor was less than 1:16, that from an HTLV-1 carrier was 1:4096, and that in the serum from an ATL patient was 1:2048. Sera were heated at 56°C for 30 min and filtered through a 0.45- μ m-pore-size filter (Code, 03CP045AS, ADVANTEC, Tokyo) for sterilization before use. Antibody neutralization was performed by preincubating the virus preparation with serum for 1 h at 37°C before use for inoculation to the target cells.

Susceptibility of human and mouse cells to cell-free HTLV-1 In order to check the susceptibility of human and mouse cells to cell-free HTLV-1, the virus preparation was serially diluted with culture medium and then added to the target cells (Molt-4, Hut78, EL4 and RLm1). After 24 h of incubation the cells were washed with PBS and maintained in the culture medium. After further incubation for 24 h, the cells were washed with PBS twice and lysed with lysis buffer. The lysates were used as templates for PCR.

RESULTS

Validity of cell-free preparation as a source of HTLV-1 virion Virus preparation from HTLV-1-producing c77 cells was filtered through a 0.45 μ m-pore-size filter, treated with 80 U/ml of RNase-free DNase I for 1 h at 37°C, and used to inoculate Hut78 cells, an HTLV-1 sus-

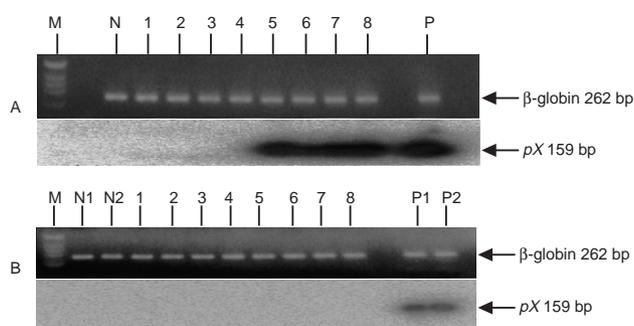


Fig. 1. A. Entry of cell-free HTLV-1 into Hut78 cells. Hut78 cells were harvested at 0 and 24 h after inoculation with cell-free HTLV-1 treated with RNase-free DNase I for 1 h at 37°C. The harvested cells were heated at 95°C for 10 min to inactivate DNase I. Quadruplicate assays for HTLV-1 *pX* and human β -globin sequences from cells harvested at 0 h (lanes 1, 2, 3 and 4) and 24 h (lanes 5, 6, 7 and 8) were analyzed by PCR. The set of ethidium bromide staining patterns (upper panel) and Southern hybridization patterns (lower panel) are presented. Human β -globin band, which serves as an internal control, was seen by ethidium bromide staining. Uninfected Hut78 cell DNA was used as a negative control (N). HTLV-1-immortalized human ATL-1K cell DNA mixed with 1 μ g of Hut78 cell DNA was used as a positive control (P). M indicates DNA size markers. B. The filtrate of cell-free HTLV-1 inoculum is free of proviral DNA. The virus preparation was mixed with 1 μ g of carrier DNA, Hut78 cell DNA, as described in "Materials and Methods." HTLV-1 *pX* and human β -globin sequences were analyzed by PCR. Sets of ethidium bromide staining patterns (upper panel) and Southern hybridization patterns (lower panel) are presented. HTLV-1-immortalized human ATL-1K cell DNA mixed with 1 μ g of uninfected Hut78 cell DNA served as positive controls (P1 and P2). Uninfected Hut78 cell DNA served as negative controls (N1 and N2). M indicates DNA size marker. Lanes 1, 2, 3 and 4 were treated with RNase-free DNase I, and lanes 5, 6, 7 and 8 were not treated with RNase-free DNase I.

ceptible human T-cell line. After 0 and 24 h of cultivation, Hut78 cells were harvested, heated for 10 min at 95°C to inactivate any remaining DNase I and stored at -20°C until PCR. As shown in Fig. 1A, HTLV-1 *pX* sequence was detected from the DNA of Hut78 cells incubated for 24 h after inoculation, but not at 0 h.

The filtered virus preparation was assayed by PCR for any possible contaminating DNA fragments of c77 cells which contain the *pX* sequence. As shown in Fig. 1B, no amplified HTLV-1 *pX* sequence was found in the virus preparation treated or untreated with 80 U/ml RNase-free DNase I for 1 h at 37°C, indicating that there is no detectable amount of DNA fragments with *pX* sequence, but there are infectious particles of HTLV-1, in the viral preparation. These data strongly suggest that the cell-free filtrate of c77 cells contains infectious particles of HTLV-1,

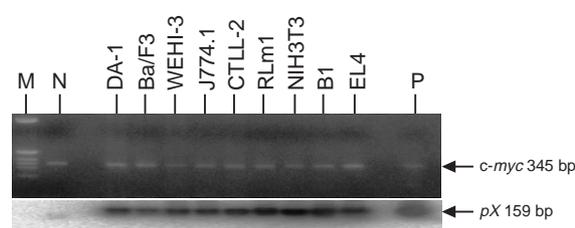


Fig. 2. Entry of cell-free HTLV-1 into mouse cell lines. Lysates of 5×10^4 cell equivalents from individual mouse cell lines inoculated with cell-free HTLV-1 were analyzed by PCR for the presence of HTLV-1 *pX* and mouse *c-myc* sequences, as previously described.¹⁰⁾ Uninfected mouse J774.1 cell lysate and HTLV-1-immortalized human ATL-1K cell lysate diluted with uninfected mouse J774.1 cell lysate were analyzed in parallel as a negative control (N) and a positive control (P), respectively. M indicates DNA size marker. The name of the mouse cell line is indicated above each lane.

but not significant DNA fragments from c77 cells containing HTLV-1 provirus.

Entry of cell-free HTLV-1 into mouse cell lines Nine mouse cell lines were inoculated with cell-free HTLV-1 for 24 and 48 h at 37°C. HTLV-1 *pX* sequence was detectable by PCR at 24 h (data not shown) and 48 h after inoculation from the lysates of all 9 mouse cell lines as shown in Fig. 2.

Blocking of HTLV-1 entry by anti-HTLV-1 sera Virus preparations were incubated with RNase-free DNase I and with serially diluted normal human serum or serum from an HTLV-1 carrier. Then they were used to inoculate either EL-4 or WEHI-3 cells. After 48 h of incubation, the cell lysates of EL-4 or WEHI-3 were analyzed by PCR for *pX* sequence. As shown in Fig. 3, *pX* sequence was detected from both EL-4 and WEHI-3 cells treated with normal human serum-treated virus preparation, but not by HTLV-1 carrier serum-treated virus preparation, even at 100-fold serum dilution. These results suggest that entry of HTLV-1 is mediated by a specific interaction between HTLV-1 and the cell membrane, presumably an HTLV-1 receptor.

Quantification of viral DNA molecules in target cells As shown in Table I, while *pX* sequence of HTLV-1 was positive until 10-fold dilution of the lysates, it was negative at 100-fold dilution in the case of medium alone or normal human serum. This indicates that the number of viral DNA molecules in the cells as determined by semi-quantitative PCR²⁴⁾ is more than 10 but less than 100 per 5×10^4 target cells. There was no significant difference among the 4 mouse cell lines. Again, the entry of HTLV-1 was blocked by anti-HTLV-1 antibody, as was clear at 10-fold dilution of the lysates in the presence of the serum from an ATL patient.

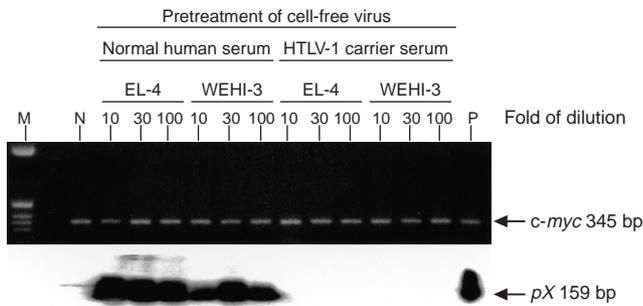


Fig. 3. Blocking of entry of cell-free HTLV-1 into mouse cells by serum from an HTLV-1 carrier. The filtrate of *c77* culture supernatant was incubated with 80 U/ml of RNase-free DNase I for 1 h at 37°C and then virus preparations were treated for 1 h at 37°C with HTLV-1 uninfected human serum or serum from an HTLV-1 carrier and used to inoculate target cells. HTLV-1 *pX* and mouse *c-myc* sequences in the harvested cell lysates were analyzed by PCR as described in the legend to Fig. 2. The name of the target cells and the serum dilution are written on top.

Similar susceptibility of human and mouse cell lines to entry of cell-free HTLV-1 Cell-free HTLV-1 preparation, the culture supernatant of *c77* cells, was serially diluted and used to inoculate human T cell lines Molt-4 and Hut78, and mouse T cell lines EL4 and RLm1. As shown in Fig. 4, HTLV-1 *pX* sequence was positive at viral dilutions of 1/1 and 1/5 in Molt-4 and EL4 cell lysates, and at the viral dilution of 1/1 in Hut78 and RLm1 cell lysates. These results indicate that human T cell lines and mouse T cell lines are similarly susceptible to cell-free HTLV-1.

Analyses for viral protein expression The HTLV-1 p19 antigen concentration in the culture supernatants of *c77* cells and the target cells 48 h after inoculation was assayed by using an HTLV-1 p19 ELISA kit. There was 28.3 ng/ml p19 antigen in the cell-free filtrate of *c77* cells. The concentration of p19 antigen in the supernatants of all

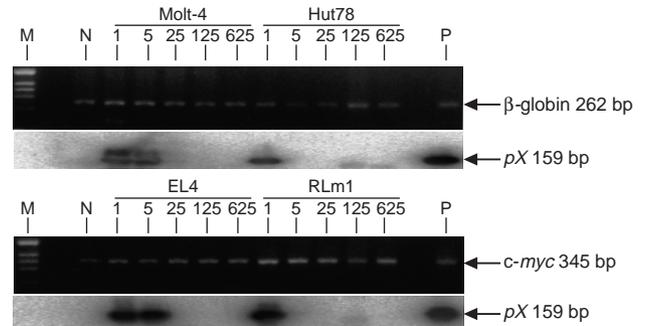


Fig. 4. Similar susceptibility of human and mouse cell lines to entry of cell-free HTLV-1. HTLV-1 *pX* sequence in the lysates of Molt-4, Hut78, EL4 and RLm1 cells inoculated with serially diluted cell-free HTLV-1 was detected by PCR. Human β -globin and mouse *c-myc* sequence were amplified with *pX* sequence in the same incubation mixture as multiplex PCR for human cells and mouse cells, respectively. The ethidium bromide-stained gel patterns are shown above the results of Southern hybridization. The name of the target cells and the virus dilution are written on top. Uninfected human Hut78 cell or mouse J774.1 cell lysates were used as negative controls (N). HTLV-1 positive human ATL-1K cell lysate, diluted with uninfected mouse J774.1 cell or human Hut78 cell lysate, were used as positive controls (P). M indicates DNA size markers.

9 mouse cell lines at 48 h after infection with cell-free HTLV-1 was below the level of detectability of 25 pg/ml.

DISCUSSION

We demonstrated that cell-free HTLV-1 particles produced by a feline kidney cell line can enter mouse cells as efficiently as human cells. This transmission is inhibited by anti-HTLV-1 antibody. These data suggest that mouse cells have HTLV-1 receptor.

To establish an efficient strategy to approach the problem of viral-cell interaction and the pathogenesis of

Table I. Detection of HTLV-1 *pX* Sequence in the Lysates of Mouse Cell Lines Inoculated with Cell-free HTLV-1 Pretreated with or without Normal Human Serum or Serum from an ATL Patient

Pretreatment of cell-free virus ^{a)}	DA-1			NIH3T3			EL4			RLm1		
	$\times 1$ ^{b)}	$\times 10$	$\times 100$	$\times 1$	$\times 10$	$\times 100$	$\times 1$	$\times 10$	$\times 100$	$\times 1$	$\times 10$	$\times 100$
None	+	+	-	+	+	-	+	+	-	+	+	-
Normal human serum	+	+	-	+	+	-	+	+	-	+	+	-
Serum from an ATL patient	+	-	-	+	-	-	+	-	-	+	-	-

a) Half milliliter of the virus preparation was preincubated with 10 μ l of sera or without serum for 1 h at 37°C and added to 0.5 ml culture medium. The incubated cells were washed after 24 h and harvested 48 h after the start of culture.

b) Cell-free HTLV-1-infected cell lysates were diluted 1-, 10- and 100-fold with the lysate of HTLV-1 uninfected mouse cell line, J774.1.

HTLV-1 using a mouse model, it is important to study HTLV-1 tropism using an HTLV-1 transmission system. It was reported that mouse fibroblast cell lines, including LMTK- and NIH3T3 TK- cells, were very resistant to HTLV-1 infection compared to human cell lines by monitoring plaque-forming units after VSV (HTLV-1) pseudotype infection and by syncytia formation assay after cocultivation with the HTLV-1 producing human cell line C91/PL.⁷⁾ Furthermore, the somatic-cell hybrid cell line L1q, which contains human chromosome 17q, could be infected with HTLV-1, but the parental mouse fibroblast cell line, LMTK-, could not.²⁵⁾ Thus, the receptor gene for HTLV-1 has been assigned to human chromosome 17q.^{7, 26, 27)} However, Sutton and Littman reported that HIV (HTLV-1) pseudotype virus could infect mouse fibroblast LMTK- cells at 4.5% efficiency and four other LMTK- cell lines which contained at least human chromosome 17q at 0.015–2.0% efficiency as compared to a human osteosarcoma cell line, HOS cells.²⁸⁾

The reason for these discrepancies is unclear. The possibilities include differences in the virus preparations, the target mouse cell lines and/or the sensitivity and specificity of the assay systems. The cell-free HTLV-1 used in earlier studies was mostly from MT-2 cells,^{25, 29, 30)} C91/PL cells³⁰⁾ or HOS/PL cells.³¹⁾ The filtered virus in the culture fluids of C91/PL cells could infect human lymphocytes from cord blood and peripheral blood, and that of MT-2 cells could infect human lymphocytes from cord blood only, while that of C10/MJ could infect neither of them.³⁰⁾ Since the native infectious particles of HTLV-1 were difficult to obtain, various kinds of pseudotype viruses have also been used for cell-free infection.^{7, 28, 32)} We took advantage of the cell-free HTLV-1 produced by c77 cells, a feline kidney cell line, which was reported to infect human cells about 3000 times as efficiently as those from MT-2, C91/PL and HOS/PL cells.^{11, 33, 34)} The endogenous feline xenotropic CCC retrovirus (CCC virus) was produced from c77 cells.³⁵⁾ However, the present work using mouse cell lines, EL-4 and WEHI-3 cells, showed complete neutralization of cell-free HTLV-1 transmission by HTLV-1 antibody-positive human sera, and we have reported that there was little or no HTLV-1(CCC) pseudotype in the c77 culture supernatant.¹¹⁾ It was also reported that the supernatant of irradiated MT-2 cells contained about 250 pg/ml of HTLV-1 p19 antigen.³⁶⁾ The virus preparation from c77 cells used in this study contained about 100 times more p19 antigen than the reported MT-2 cell culture supernatant. Mouse cell lines used previously are mostly fibroblasts, LMTK- and LMTK- derived mouse/human hybrid cells.^{7, 25)} Cell adhesion molecules were considered to be co-receptors for HTLV-1 transmis-

sion, based on syncytium formation assay.³⁷⁾ We used various kinds of cells of mouse origin to show that the receptor or co-receptor of HTLV-1 is widely distributed in mouse cells. Our data are consistent with reports that the receptor for HTLV-2, which shares a common receptor with HTLV-1,³⁸⁾ is widely distributed among species and cell types including murine cells, based on a highly sensitive fusion assay,³⁹⁾ and that the receptor for HTLV-1 was expressed on a broad range of cell lines derived from multiple species, including NIH3T3 mouse cell line, based on a highly sensitive pseudotype assay.³²⁾ The broad cell type specificities in mouse cell lines are also consistent with those in human cell lines, including U251-MG, human glioma-derived, HOS, human osteosarcoma-derived and HeLa cells.¹¹⁾

HTLV-1 p19 core antigen expression in the HTLV-1-infected mouse cells could not be detected in the culture fluid. The following possibilities can be considered. The number of cells infected and amount of viral antigen expressed are too small to be detectable by the present ELISA kit, or infected cells might not express viral antigen. This possibility could be tested by examining the infectivity of HTLV-1 virion produced in the supernatant of the susceptible cells, when we obtain efficient methods of concentration of biologically active HTLV-1 particles. Our previous result that HTLV-1-infected mouse splenocytes did not express HTLV-1 Tax mRNA is consistent with the present findings.¹⁰⁾ Human CD4/CCR5 transgenic mouse could be infected with HIV-1, but tat/rev mRNA could not be detected from spleen and lymph node RNA by RT-PCR.⁴⁰⁾ It is known that overexpression of human cyclin T enhances Tat transactivation in rodent cell lines.⁴¹⁾ It is possible that HTLV-1-infected mouse cells lack cellular factors required for *HTLV-1* gene expression.

In conclusion, efficient viral entry into various mouse cell lines was demonstrated with the cell-free HTLV-1 preparation produced by c77 cell line. These findings are relevant to further analysis of virus-cell interaction and may help to clarify the factors necessary for HTLV-1 infection.

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