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Infection of Peripheral Blood Mononuclear Cells and Cell Lines by Cell-Free Human T-Cell Lymphoma/Leukemia Virus Type I

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Previous studies of in vitro infection by human T-cell lymphoma/leukemia virus type I (HTLV-I) have required cocultivation of target cells with HTLV-I cell lines or vesicular stomatitis virus pseudotypes containing HTLV-I envelope proteins. We report here the development of a cell-free infection assay for HTLV-I. Target cells were incubated with purified, DNase-treated HTLV-I virions for 4 h at 37°C. Target cell DNA was then analyzed for the presence of newly synthesized HTLV-I proviral DNA by the highly sensitive polymerase chain reaction. Using this assay system, we have been able to consistently detect in vitro infection of a variety of cellular targets by different HTLV-I isolates. Optimal infection required the presence of 10 μg of DEAE-dextran per ml. The assay was dose dependent with respect to virus input. In general, the amount of proviral DNA detected correlated with the level of HTLV-I receptors present on the surface of the target cells, as measured by fluorochrome-labelled HTLV-I binding. Finally, the specificity of the assay was confirmed by demonstrating that the cell line, L1q, a somatic cell hybrid containing human chromosome 17q, to which the gene for the HTLV-I receptor has been mapped, was susceptible to infection by HTLV-I, while the parental mouse cell line from which it was derived, LMTK−, which lacks human chromosome 17q, was not.

Human T-cell lymphoma/leukemia virus type I (HTLV-I) is believed to be the etiologic agent for adult T-cell leukemia and HTLV-I-associated myelopathy (1, 18; see reference 20 for a review). The virus demonstrates a general T-lymphocyte tropism in vivo, with $CD4^+$ lymphocytes appearing to be a primary target for infection (5, 18, 19). However, HTLV-I-infected B cells (13) and $CD8^+$ T cells (5) have been identified in vivo.

It has been somewhat difficult to demonstrate in vitro infection with HTLV-I compared with doing so with other retroviruses. This difficulty has been overcome by several groups utilizing cocultivation systems which combine lethally treated HTLV-I-infected donor cells with target cells (7, 14, 15). Successful infection in this assay is determined by the induction of syncytia or the detection of viral proteins, nucleic acids, or both in infected target cells (25). With this type of assay system, a limited number of cell types have been examined for their susceptibility to infection by HTLV-I. Thus far, $CD4^+$ and $CD8^+$ T cells (7, 14, 15), B cells (26), fibroblasts (3), endothelial cells (10), and glial cells (21) have been successfully infected with the virus. Rare infection of target cells and live animals by cell-free virus has been reported (3, 4, 23).

The use of cocultivation assays for HTLV-I infection has several important limitations. Such assays have proven to be very labor intensive. Further, cocultivation assays are not very quantitative, although a variation on this format, whereby infected targets are plated on semisolid media and grown as infected colonies for 2 weeks, has been demonstrated to be a somewhat cumbersome solution to this problem (7). Most importantly, cocultivation assay systems, because they already contain viral protein and nucleic acids from the virus donor cells, are not suitable for the analysis of the kinetics of HTLV-I infection.

The classical methods to detect cell-free retrovirus infection have required a high efficiency of infection. Only a very small percentage of target cells have been shown to be infected with HTLV-I after exposure to the virus (4, 7); thus, the amount of HTLV-I proteins and nucleic acids is below the limitation of standard assays. The polymerase chain reaction (PCR) allows for the facile detection of human retroviral DNA (17). We and others have recently shown that PCR can be utilized to detect low-level HIV-1 infection in a cell-free system (17, 28). Employing the same technology, we have developed the quantitative cell-free HTLV-I infection assay described below.

MATERIALS AND METHODS

Cells. Normal adult peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized blood on a Ficoll-Hypaque density gradient. Activated PBMC were generated by culturing the above cells in RPMI 1640 medium, 10% fetal calf serum, 3 μg of phytohemagglutinin (PHA) per ml (Burroughs Wellcome, Research Triangle Park, N.C.), and 10% delectinated, partially purified human interleukin 2 (IL-2) (Cellular Products, Buffalo, N.Y.) for 72 h at 37°C in 5% CO_2. The cell lines used in the cell-free infection assay were obtained from the American Type Culture Collection (ATCC) and include Molt-4 (CRL 1582), an immature human T-leukemia-cell line; Hut 78 (TIB 161), a mature human T-leukemia-cell line; CEM (CCL 119), a human T-lymphoblastoid-cell line; HSB-2 (CCL 120.1), a human T-lymphoblastic-leukemia-cell line; Jurkat (CRL 8163), a human T-lymphoma-cell line; Daudi (CCL 213) and Raji (CCL86), human Burkitt lymphoblast cell lines; RPMI 8226 (CCL 155), a human B-myeloma-cell line; HL-60 (CCL 240), a human promyelocytic cell line; U937 (CRL 1593), a human premonocyte cell line; K562 (CCL 243), a human

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myelogenous cell line; SK-N-MC (HTB 10), a human neuroblastoma cell line; U-87 MG (HTB 14) and U-373 MG (HTB 17), two human glioblastoma cell lines; HOS (CRL 1453), a human osteosarcoma cell line; Vero (CCL 81), an African green monkey kidney cell line; and LMTK− (CCL 1.3), a mouse fibroblast line. The cell lines were maintained according to ATCC protocols. RPMI 8402 (11), a human intrathymic pre-T-cell line, was the gift of A. Silverstone. The human megakaryocytic cell line, Dami (8), was the kind gift of Simon Karpatkin. L1, a hybrid of LMTK− containing human chromosome 17, was a kind gift of E. Stubblefield (22). L1q is a subclone of L1 which contains only the q arm of human chromosome 17 (12a).

Virus stocks and assays. Purified HTLV-I virions were prepared from the growth media of the virus-producing cell line MT-2, containing an HTLV-I isolate from an adult T-cell leukemia patient (15). After the cells and debris were removed by low-speed centrifugation, the virus was purified by centrifugation through a 20 to 60% sucrose gradient at 150,000 × *g* for 18 h or by pelleting of virions from HTLV-I-containing media at 19,000 × *g* for 60 min. Before its use in the cell-free infection assay, the virus preparation was treated with DNase (80 U/ml) (Promega, Madison, Wis.) for 1 h at 37°C and filtered through a 0.45-μm-pore-size filter. The virus concentration was determined by an HTLV-I core protein p19 enzyme-linked immunosorbent assay (16) with an HTLV-I virus standard (Cellular Products) and by an HTLV-I RNA-directed reverse transcriptase PCR assay with a synthetic HTLV-I RNA standard (2). One microgram of HTLV-I contains approximately 1.5×10^7 RNA molecules.

Additional HTLV-I-producing cell lines were utilized to produce cell-free virus. These included UMC-CTL 20, 21, and 22, derived from the peripheral-blood samples and cerebrospinal fluid of two HTLV-I-associated myelopathy patients (1), and HUT 102 B2, derived from an adult T-cell leukemia patient (18).

HTLV-I cell surface binding. The procedure for examining the ability of HTLV-I to bind to target cells is as previously reported (12). Sucrose-purified HTLV-I was labelled with either Rhodamine 18 (R-18) or Bodipy [9-(1,3,5,7-tetra methyl Bodipy)-4 nonanoic acid] (Molecular Probes, Inc., Eugene, Oreg.). Five hundred thousand cells were incubated with 10 μg of Bodipy–HTLV-I or R-18–HTLV-I virions in medium containing various concentrations of Polybrene or DEAE-dextran for 45 min on ice. Following adsorption of the virus, the cells were pelleted, washed, and fixed with 1.0% paraformaldehyde and subjected to flow cytometric analysis.

Cell-free infection of target cells with HTLV-I. Sucrose-gradient-purified or pelleted HTLV-I virions from infected cell culture supernatants were treated with DNase (80 U/ml) and filtered. The DNA-free virus preparation was then resuspended at the required concentration in culture media containing 10 μg of DEAE-dextran per ml. Activated PBMC, at a concentration of 2×10^6 cells per ml, were incubated with various concentrations of virus preparations at 37°C for 4 h. The unadsorbed virus was then removed by centrifugation of the cells and aspiration of the supernatant. The cells were washed five times, and then they were analyzed for HTLV-I proviral DNA by PCR. Some target cells were maintained in continuous culture after infection for up to 30 days. Sequential aliquots were removed and analyzed for HTLV-I DNA by PCR and HTLV-I protein expression, including reverse transcriptase (18), p19 Gag (16), and gp46 Env (15a).

TABLE 1. Effects of DEAE-dextran and Polybrene on R-18–HTLV-I binding to PBMC[a]

Treatment of PBMC (μg/ml)[b]	R-18–HTLV-I[c] added	Mean channel fluorescence	Increase in binding over virus alone (%)[d]
None	No	137	None
None	Yes	597	None
DEAE-dex (40)	Yes	701	23
DEAE-dex (20)	Yes	677	17
DEAE-dex (10)	Yes	654	12
DEAE-dex (5)	Yes	607	2
PB (40)	Yes	581	None
PB (20)	Yes	611	3
PB (10)	Yes	605	2
PB (5)	Yes	585	None

[a] PBMC were activated with PHA and IL-2. DEAE-dex, DEAE-dextran; PB, Polybrene.

[b] A total of 5×10^5 PBMC at day 3 following activation with PHA (10 μg/ml) and delectinized IL-2 (20%, vol/vol). Virus was incubated with target cells for 30 min at room temperature in the presence of DEAE-dextran, Polybrene, or buffer alone.

[c] A total of 10 μg of HTLV-I protein normalized to p19; equal to 50% of saturating dose of R-18–HTLV-I on PBMC.

[d] Calculated by dividing the net mean channel of the sample minus the autofluorescence of the negative control by the net mean channel of the virus alone sample minus the autofluorescence of the negative control.

PCR for detection of HTLV-I DNA. As previously described (17), total cellular DNA was isolated by sodium dodecyl sulfate-proteinase K digestion of the infected cells. Isolation was followed by phenol and chloroform extraction. Amplification of the HTLV-I proviral sequence was accomplished by the use of PCR with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) and a heat-stable DNA polymerase isolated from *Thermus aquaticus* (a kind gift of Cetus Corporation). The HTLV-I *pol* primer pair HTIP (4757-4778)$^+$/HTIP (4942-4850)$^-$ and detector probe HTIP (4825-4850)$^+$d were used. Primers and probe to detect HTLV-I strong stop DNA were HTIL (490-515)$^+$/HTIL (655-630)$^-$ and HTIL (564-596)$^+$d, respectively. Four micrograms of whole cellular DNA was added to a reaction cocktail and subjected to 30 cycles of amplification. Thereafter, aliquots of amplified DNA were mixed with ^{32}P-labelled probe and allowed to hybridize. The hybridized products were loaded directly onto 8% polyacrylamide gels and electrophoresed, and then gels were autoradiographed. The copy number of proviral DNA was determined by densitometry with an HTLV-I proviral DNA standard curve from UMC-CTCL-11b (5, 17), which contains two copies of HTLV-I per cell.

RESULTS

Effects of DEAE-dextran and Polybrene on the binding of fluorochrome-labelled HTLV-I to target cells. Several different groups of investigators have shown that the addition of polycationic or polyanionic compounds to virus preparations during infection of target cells leads to enhanced infection of the target cells (12, 25), presumably by stabilization of the interaction of the surface viral envelope protein and the cell surface receptor. In previous studies examining HTLV-I binding to target cells (12), we used Polybrene at a concentration of 1 μg/ml. In order to optimize the conditions for binding of the virus to its target cells, we tested several concentrations of DEAE-dextran and Polybrene for the ability to enhance the binding of HTLV-I to targets (Table

1). Ten micrograms of rhodamine-labelled virus was incubated with activated PBMC targets in the presence of twofold dilutions of either DEAE-dextran or Polybrene starting at 40 μg/ml, and the amount of bound virus was quantitated by flow cytometry. An increase in binding of HTLV-I to the target cells ranging from 2 to 23% was observed when the virus was added to the cells in the presence of DEAE-dextran compared with the binding of the virus in media alone. Little or no increase in virus binding over control was observed when HTLV-I was added to the target in the presence of Polybrene at any concentration. Toxic effects on the target cells were observed at the higher (20 and 40 μg/ml) concentrations of DEAE-dextran, so a concentration of 10 μg/ml, which still gave some enhanced binding with no toxic effects, was chosen for use in subsequent assays. This concentration of DEAE-dextran was also optimal in the cell-free infection experiments performed as described below (data not shown).

Titration of HTLV-I input for cell-free infection assay. Two million PHA- and IL-2-activated PBMC (day 3) were incubated with 250, 50, 10, or 0 μg of DNase-treated HTLV-I normalized to p19 in the presence of 10 μg of DEAE-dextran per ml for 4 h at 37°C. The cells were then pelleted and vigorously washed, and the DNA was extracted. HTLV-I proviral DNA content was analyzed by PCR with the *pol* and strong stop primer-detector systems. The copy number of HTLV-I proviral DNA was determined from a standard curve of DNA from UMC-CTC-11b, which contains two copies of HTLV-I per cell (Fig. 1).

As can be seen in Fig. 1, no DNA signal was detectable after DNase treatment of the HTLV-I virions, indicating that all subsequent DNA detected was the product of reverse transcription of viral RNA following infection of the target cells. When PHA- and IL-2-activated PBMC were incubated with increasing amounts of purified HTLV-I, reproducible copies of either HTLV-I strong stop or *pol* DNA were subsequently detected (Fig. 1 and 2). While the assay was linear with up to 250 μg of input HTLV-I, we have found that increasing the input virus much beyond this amount resulted in a strong toxic syncytium formation among target cells. Interestingly, the HTLV-I copy number detected with the strong stop primer-detector system was approximately 1.5 times that of the *pol* primer-detector system at all input concentrations of virus tested (Fig. 2). These data would imply that there were more incomplete than complete proviral DNA copies synthesized, because the strong stop system recognizes both species of DNA, while the *pol* system detects only complete proviral DNA copies.

All of the HTLV-I isolates, regardless of their source of origin, were able to infect activated target PBMC as identified by PCR (data not shown).

Negative-stained electron microscopic evaluation of HTLV-I virions purified from MT-2 cell culture-conditioned media indicates that 250 μg of HTLV-I approximates 10^9 virus particles (unpublished observation). The 4 μg of target cell DNA analyzed in each PCR represents approximately 30% of the total DNA extracted from the 2×10^6 target cells used in each infection. Hence, the total mean number of HTLV-I proviral DNA copies in 2×10^6 target cells exposed to 250 μg of HTLV-I input virus (Fig. 1) would be 1.3×10^3, indicating that there is roughly one infectious particle per 10^6 virions or 3.25×10^6 copies of HTLV-I RNA.

HTLV-I binding and susceptibility to infection of different cell lines. The susceptibility of various cell lines to infection by HTLV-I was evaluated by two methods. First, the binding of Bodipy-labelled virus to target cells was quantitated. Second, each cell line was incubated with 250 μg of DNase-treated HTLV-I virions for 4 h and the copy number of HTLV-I proviral DNA after 4 h was determined by PCR. The results of this assay are shown in Table 2. A total of seven T-cell lines, three B-cell lines, and one monocytelike cell line were studied. Other cell lines examined for their susceptibility to HTLV-I infection included two glioblastoma cell lines, a human fibroblast cell line, a neuroblastoma cell line, a megakaryocytic cell line, an erythroleukemic cell line, and African green monkey kidney cells. In general, the

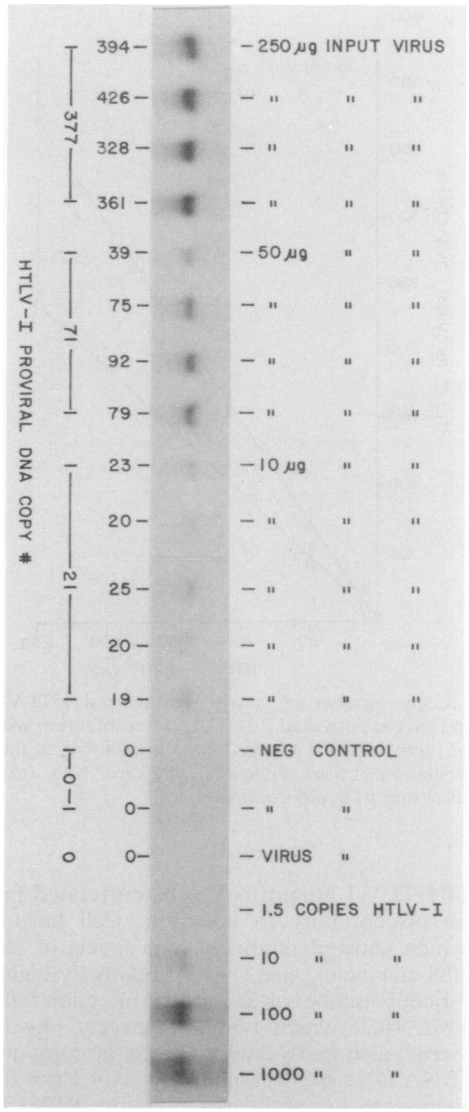

FIG. 1. Autoradiograph of liquid hybridization of DNA amplified with HTLV-I strong stop primer pairs and ^{32}P-end-labelled strong stop detector oligonucleotide. Replicate samples were analyzed after infection of activated PBMC with various inputs of DNase-treated infectious HTLV-I. Appropriate negative controls, including 250 μg of DNase-treated virions alone, were also analyzed, as was a reference dilution series of known HTLV-I copy number. The autoradiograph was analyzed on a densitometer, and a standard curve was developed with the positive controls. Copy number of each individual sample was then determined by comparing its signal intensity with the standard curve. The individual HTLV-I DNA copy number is shown for each sample, as is the mean DNA copy number for a given input of virus (⊢—⊣).

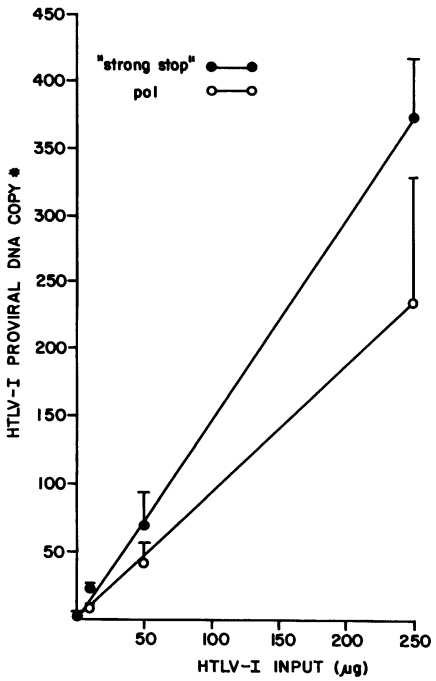

FIG. 2. Copy number of newly synthesized HTLV-I *pol* or strong stop DNA in activated PBMC 4 h after infection with various amounts of input HTLV-I virions. Data are shown as the mean ± standard deviation of four replicates. The error bars are shown in only one direction to avoid confusion.

TABLE 2. Correlation of binding of Bodipy–HTLV-I with cell-free infection

Cell and cell line	Level of Bodipy–HTLV-I binding (channels)[a]	Level of HTLV-I infection[b]
T		
PHA-, IL-2-activated PBMC[c]	77	377
Molt-4	88	168
Jurkat	74	75
HUT 78	65	57
HSB-2	63	38
CEM	52	44
RPMI 8402	27	12
B		
RPMI 8226	89	57
Daudi	77	46
Raji	27	0
Other		
U-937	282	56
U-373	143	76
U-87	117	46
SK-N-MC	112	0
K562	49	35
Malme-3	46	20
Vero	22	125
Dami	15	0

[a] Net mean channel fluorescence after 5×10^5 cells reacted with 10 μg of Bodipy–HTLV-I, listed in decreasing order of levels of virus binding.
[b] Copy number of proviral HTLV-I DNA per 2×10^6 cells 4 h after infection with 250 μg of HTLV-I.

amount of HTLV-I bound to T cells correlated fairly well with their susceptibility to infection. Cell lines such as Molt-4, which showed relatively high levels of binding of HTLV-I (88 channels), also showed relatively higher levels of HTLV-I copy number after cell-free infection followed by PCR. RPMI 8402, which bound relatively low levels of HTLV-I virus, also had very low levels of copy number of HTLV-I DNA after cell-free infection. Not all of the B-cell lines studied were susceptible to infection. RPMI 8226 and Daudi bound HTLV-I at very similar levels and also demonstrated similar levels of copy number of HTLV-I proviral DNA following cell-free infection. Proviral DNA could not be detected in Raji cells after infection. A very low level of HTLV-I binding to target cells was also observed. Whether the binding of HTLV-I to Raji cells is specific is yet to be determined. Less correlation of HTLV-I binding with susceptibility to infection, as determined by PCR, was observed with the non-T-, non-B-cell lines studied. The two glioma cell lines bound high levels of the virus and were susceptible to infection by HTLV-I, while the neuroblastoma cell line, SK-N-MC, although binding high levels of the virus, was not susceptible to HTLV-I infection as determined by PCR. In contrast, Vero cells bound relatively few HTLV-I virions but gave a relatively high level of HTLV-I proviral DNA copy number after PCR analysis.

Cell-free infection of LMTK− and L1q cells with HTLV-I. Work by Sommerfelt et al. (24) with vesicular stomatitis virus pseudotypes and syncytium formation to measure susceptibility to HTLV-I infection suggested that the gene encoding the HTLV-I receptor mapped to human chromosome 17q. We therefore attempted to demonstrate specific infection of L1q cells, which contain the entire q arm of human chromosome 17. As seen in Fig. 3, the L1q hybrid cell line could be infected by HTLV-I, while the parental cell line, LMTK−, could not, in general agreement with the results of Sommerfelt et al. Two different purified preparations of virus from MT-2 cells and pelleted virus from MT-2 culture supernatant were all able to infect the L1q cell line.

Kinetics of HTLV-I proviral DNA synthesis and analyses for viral protein expression. During the first 24 h postinfection, there is a gradual increase in detectable HTLV-I proviral DNA, which peaks at about 4 to 6 h and then decreases. Proviral DNA levels then remain constant or disappear over the next several days but eventually are detectable again by day 30, albeit still in low levels (data not shown). During 30

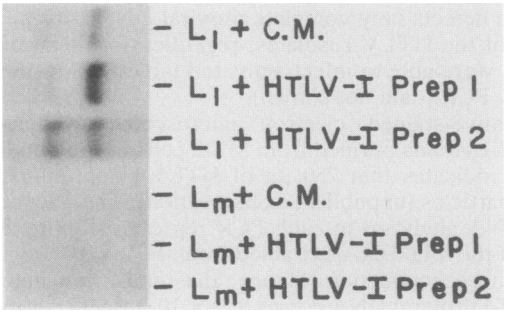

FIG. 3. Autoradiograph of liquid hybridization of DNA amplified by using the HTLV-I *pol* primer pair and the ^{32}P-end-labelled oligonucleotide *pol* detector. Target cells are either the somatic cell hybrid L1q (L_1), which contains human chromosome 17q, or the rat fibroblast parental cell line LMTK− (L_m), which does not. DNA was extracted and amplified 4 h after exposure to 250 μg of any of three HTLV-I virus samples (pelleted particles [C.M.] or two different sucrose-gradient-purified virus samples [Prep 1 and Prep 2]).

days of culture, there was no detectable HTLV-I reverse transcriptase released into the cell culture-conditioned media. Likewise, after an initial washout period of several days, there was no detectable HTLV-I p19 or gp46 protein in the cultures (data not shown).

DISCUSSION

HTLV-I is a clinically relevant human retrovirus associated with several neoplastic and nonneoplastic diseases (20). The virus has been detected worldwide and has been found to be endemic in certain parts of the world, including Japan, sub-Saharan Africa, the Caribbean, and Melanesia (20, 27). Throughout the world, intravenous-drug abusers have particularly high prevalence rates of HTLV-I infection (6).

Studies on the biology of HTLV-I and the efficacy of antiviral agents have been hampered by the lack of a reproducible assay for cell-free HTLV-I transmission. Using the capability of PCR to amplify rare DNA sequences, we have been able to develop a sensitive and quantitative assay to detect in vitro HTLV-I infection by measuring nascent proviral DNA synthesis. The primer pairs and detector oligonucleotides used here have been able to detect infection by all of the HTLV-I isolates studied. The number of proviral DNA copies detected in target cells 4 h after exposure to DNase-treated virions indicates a low multiplicity of infection consistent with previous in vitro and clinical observations. Preliminary data suggest that, as with human immunodeficiency virus type 1 (HIV-1) (28), more strong stop DNA than full-length proviral DNA may be transcribed and, hence, the former may be a more sensitive indication of virus transmission.

The validity of the cell-free transmission assay is supported by the fact that the somatic-cell hybrid cell line L1q, containing human chromosome 17q as its only human genetic material, could be successfully infected with HTLV-I, while the parental mouse fibroblast cell line, LMTK−, could not.

The ability to infect different target cells with HTLV-I, as measured by detection of newly synthesized proviral DNA copies, correlated in general with the amount of fluorochrome-labelled HTLV-I which bound to those same target cells. This correlation was particularly true for the T-cell lines studied. Several cell lines, most notably the human neuroblastoma cell line SK-N-MC and the African green monkey kidney endothelial cell line Vero, were exceptions to the rule, with the former demonstrating high levels of virus binding but no detectable proviral DNA synthesis and the latter showing minimal levels of virus binding but relatively efficient viral DNA synthesis. The reason(s) for these discrepancies is unclear, but it is well known that proviral DNA synthesis is the end product of several steps in addition to retrovirus binding (for a review, see reference 25). For example, the SK-N-MC cells might lack accessory molecules which could facilitate HTLV-I entry into the cell, similar to the role of LFA-1 in HIV-1 infection (9). Alternatively, impaired viral uncoating and/or reverse transcription of HTLV-I RNA may account for the results observed for these cells. Future studies using cationic liposomes preincubated with HTLV-I might resolve whether the block to HTLV-I infection in SK-N-MC cells is at the level of the cell membrane.

Given the observations above, it is evident that this assay will prove useful in future studies on the interaction between HTLV-I envelope proteins and cell surface receptors and those on antiviral agents that impact on the early events of the HTLV-I life cycle. The low multiplicity of infection and apparent low levels of subsequent virus protein expression evidenced in this and previous studies still preclude the facile dissection of the later events involved in HTLV-I replication. However, the use of reverse transcriptase-directed PCR should ultimately allow for careful analyses of viral RNA transcription following cell-free infection of target cells by HTLV-I.

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