Virology **268,** 41–48 (2000)
doi:10.1006/viro.2000.0143, available online at http://www.idealibrary.com on IDEAL®

The HTLV Receptor Is a Widely Expressed Protein

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Received July 26, 1999; accepted December 14, 1999

The receptor for human T-cell leukemia virus type 1 (HTLV-1) was found to be expressed on a broad range of cell lines derived from multiple species. Receptor expression was assessed using human immunodeficiency virus type 1 particles, pseudotyped with the HTLV-1 envelope glycoprotein, and expressing luciferase under the control of an SV40 enhancer and promoter. Infection by pseudotyped virus was blocked with neutralizing antibodies to HTLV-1, and infection was dependent on the presence of the cleavage and fusogenic sequences in the envelope protein precursor. Trypsin treatment of susceptible target lymphocytes reduced entry. Entry was partially resistant to ammonium chloride.

Key Words: human T-cell leukemia virus; receptor; envelope; pseudotype.

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T cell leukemia/lymphoma and HTLV-associated myelopathy, occurring in 1–10% of infected individuals (Hollsberg and Hafler, 1996; Ratner and Poiesz, 1988). It is endemic in southern Japan, the Caribbean islands, and parts of South America and Africa and is frequently found in intravenous drug abusers in North America. The homologous virus, HTLV-2, although not clearly associated with a specific disease, is widespread among Native Americans, as well as intravenous drug abusers. Several simian T-cell leukemia viruses are found to be highly related to HTLV-1 or -2, present in a wide range of primates, and associated with lymphoma in primate centers.

The HTLV-1 envelope protein is synthesized as a 462-amino-acid, 62-kDa precursor glycoprotein that is cleaved, oligomerized, and glycosylated in the endoplasmic reticulum and Golgi (Paine *et al.,* 1994; Pique *et al.,* 1990). It is expressed on the surface of the infected cell and the virion as a complex of a 289-amino-acid, 46-kDa surface glycoprotein and a 176-amino-acid, 21-kDa transmembrane protein (TM). Analogous to other retroviruses, it is assumed that the surface glycoprotein binds to a cellular receptor, leading to conformational activation of the fusion sequence in the TM (Weiss *et al.,* 1985). The nature of the cellular receptor and cellular entry is poorly understood.

HTLV-1 and -2 and simian T-cell leukemia virus are thought to share a receptor, based on studies of mutual interference for infection (Sommerfelt and Weiss, 1990). Several candidate proteins have been proposed as the receptor for HTLV-1 or an adhesion molecule that promotes infection, including interleukin (IL)-2 receptor-α (Lando *et al.,* 1983), 71-kDa heat shock protein (Sagara *et al.,* 1998), vascular cell adhesion molecule-1 (Hildreth *et al.,* 1997), a 40- to 70-kDa glycoprotein designated C33 (Imai *et al.,* 1992), an 80-kDa glycoprotein analog of intercellular adhesion molecule-3 (Agadjanyan *et al.,* 1994), and a 30- to 31-kDa protein (Gavalchin *et al.,* 1995). In the current work, we used a highly sensitive pseudotype assay, first reported by Sutton and Littmann (1996), to demonstrate the pattern of expression of the HTLV-1 receptor, to examine several putative molecules as potential receptors, and to address the mode of viral entry.

RESULTS

Viral pseudotypes

A sensitive, quantitative single-cycle assay of HTLV-1 infection used viral pseudotypes of HIV particles with the HTLV envelope protein (Fig. 1). Negative and positive controls included nonpseudotyped particles and particles pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G), whose receptor is constitutively expressed on a wide array of cell types of various species (Friedmann and Yee, 1995). The HIV genome used in this study includes the luciferase gene under the regulation of the SV40 promoter in place of the HIV-1 *env* gene (HIVLuc+Env−, Fig. 1). Producer 293T cells were transfected with HIVLuc+Env− and Env expression plasmids, induced with butyrate, and after 60–72 h, pseudotyped viral particles were harvested from the culture supernatants, filtered through a 0.22-μm filter, and titered by an HIV-1 p24 antigen ELISA. Target cells were then infected with pseudotyped particles, and after 48 h, luciferase assays were performed to measure infection.

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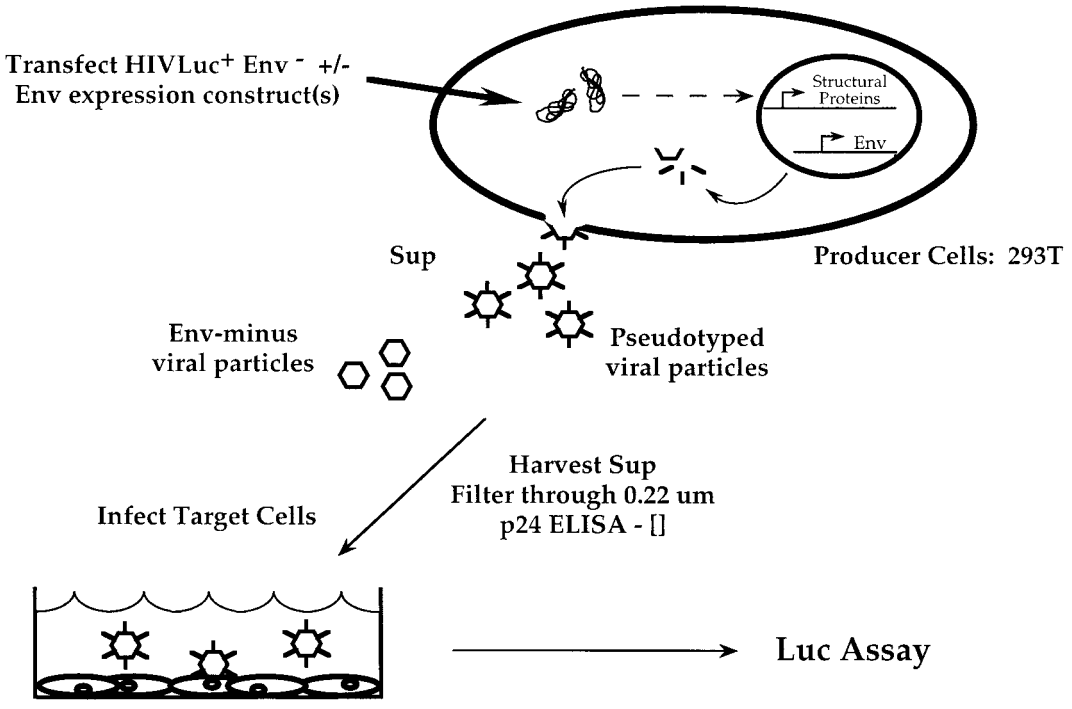

FIG. 1. Infection with viral pseudotypes. The schematic shows that viral pseudotypes were produced from 293T cells transfected with a HIVLuc+Env− plasmid with or without a plasmid expressing the HTLV-1 envelope protein or VSV-G glycoprotein. After 60–72 h, pseudotyped viral particles were harvested from the cell culture supernatant, purified through a 0.22-μm filter, and titered by a p24 antigen ELISA. The resultant particles were used to infect susceptible target cells for 3 h, and after an additional 48 h of incubation, luciferase assays were performed.

The quantitative characteristics of the assay were evaluated with increasing amounts of HTLV-1 envelope pseudotyped particles. Using 90, 460, 925, and 2700 ng of HTLV-1 envelope pseudotyped particles to infect HeLa cells, 500, 11,000, 20,000, and 52,000 light units, respectively, were measured (not shown). The luciferase activities reported are linear through this range. Using 8000 ng of nonpseudotyped particles, fewer than 100 light units was seen in these cells.

Expression of the HTLV-1 receptor

Pseudotyped virus particles were used to infect a wide variety of cell lines, including cell lines previously shown to be highly susceptible to HTLV-1 infection (human HOS, 293T, HeLa, and BJAB WH cells), as well as cell lines previously reported not to be susceptible to HTLV-1 infection (human BJAB CC84 and U87-MG cells, mouse C127/LT cells, pig MPK cells, hamster CHO cells, monkey CV-1 cells, rat NRK cells, bat Tb1lu cells, quail QM7 cells, and bovine MDBK cells; Fig. 2) (Copeland *et al.,* 1994; Li *et al.,* 1996; Sommerfelt *et al.,* 1988). Using particles pseudotyped with the HTLV-1 envelope, all cell lines, with the exception of MDBK cells, could be infected, although the levels of luciferase activity varied among cell lines between 6×10^3 and 3×10^7 light units (Fig. 2A). All cell lines could be infected with VSV-G pseudotyped particles (Fig. 2B). Ratios of activity of particles with HTLV-1 envelope versus those with VSV-G varied between 0.001 and 1.3 for the infectable cell lines, suggesting that there is significant variability in the level of HTLV-1 receptor on these cell lines. Limitations in assay sensitivity in previous studies may have been responsible for the failure to identify low but significant levels of HTLV-1 receptor activity.

The ratio of activity on MDBK cells of HTLV-1 envelope versus VSV-G pseudotyped particles was <0.0003. Although, a low level of HTLV-1 receptor expression on these cells cannot be ruled out, the level appears to be 4- to 4400-fold lower than that of the other cell lines tested.

Characterization of the HTLV-1 receptor

To examine the specificity of the interaction of HTLV-1 envelope pseudotyped particles with HOS cells, preincubation of particles with pooled anti-gp45 envelope neutralizing antibodies was examined (Fig. 3). A 97% decrease in luciferase activity was seen with the HTLV-1 envelope pseudotyped particles, but no effect was seen on infection by VSV-G pseudotyped particles (Fig. 3A).

Antibodies to heat shock protein 71 were also examined, in light of recent reports that this moiety may serve as the receptor for HTLV-1 (Sagara *et al.,* 1998). However, these antibodies had no effects on infection by HTLV-1 envelope or VSV-G pseudotyped particles (Fig. 3A). In this experiment, an antibody to platelet-derived growth

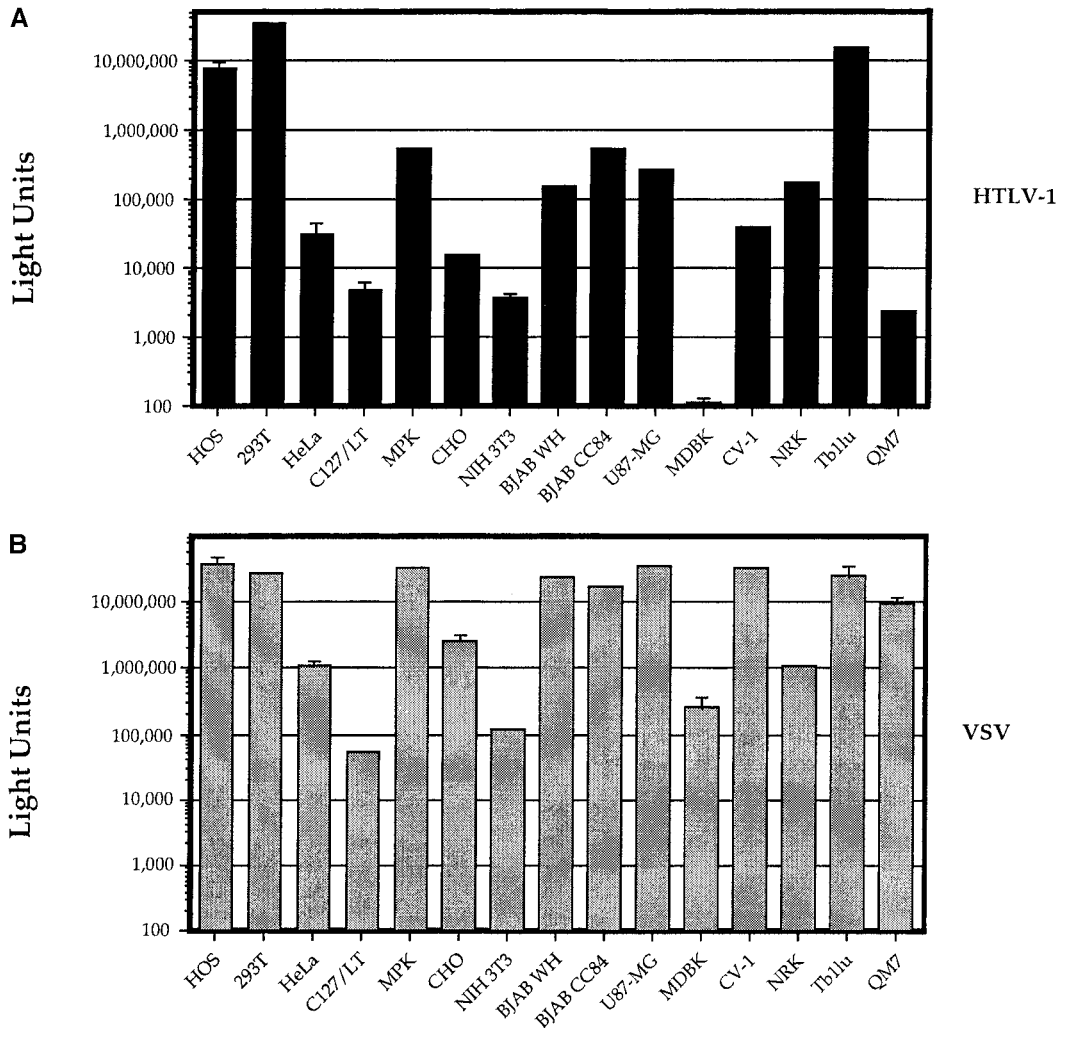

FIG. 2. The HTLV receptor is widely expressed on diverse cell types of different species. Infection titers, recorded as relative light units of luciferase activity, of a variety of cell lines incubated with virus particles pseudotyped with (A) the HTLV-1 envelope produced from pHTE-1 or (B) the VSV-G glycoprotein produced from pHCMV-G. Columns represent the mean levels of infection of duplicate assays, and the range of values is indicated.

factor-β receptor was used as an additional negative control, and no alteration in infection was seen (Fig. 3A).

An additional control to examine the specificity of HTLV-1 receptor activity used a mutant envelope precursor protein that is noncleavable and nonfusogenic. For this purpose, the RSRR cleavage site in the gp62 precursor protein was mutated to RSRT, and the N-terminal hydrophobic AVP residues of TM were converted to AED. Both of these alterations have been shown to disrupt fusion activity of the HIV-1 envelope precursor protein (Stein *et al.,* 1987). This plasmid was designated pHTE-1ΔSUTM, and the mutant precursor envelope protein was found by immunoblotting to be stably expressed in the transfected cells and the resultant pseudotyped virus particles (not shown). Nevertheless, the infectivity of particles pseudotyped with the mutant envelope precursor protein was 0.0015% of that of the properly processed form of the HTLV-1 envelope glycoprotein (Fig. 3B).

Because the HTLV-1 receptor is widely expressed, we also examined whether it was a cell-surface protein. For this purpose, phytohemagglutin-stimulated human peripheral blood mononuclear cells (PBMCs) were used, because their cell surface proteins were highly susceptible to trypsin treatment. Infection of trypsin-treated and nontreated PBMCs was examined with HTLV-1 envelope pseudotyped particles (Fig. 4). Positive and negative controls in this study included particles pseudotyped with the HIV-1 envelope, which uses a protein receptor and coreceptor at the cell surface, and VSV-G, which does not use a protein for binding (Dimitrov, 1997). Trypsin treatment of PBMCs reduced HTLV-1 receptor activity by 75% and HIV-1 receptor activity by 85%, whereas no effect was seen on VSV-G receptor activity. Although this study does not rule out the presence of a second trypsin-resistant HTLV-1 receptor on PBMCs, it indicates that the predominant activity is trypsin sensitive.

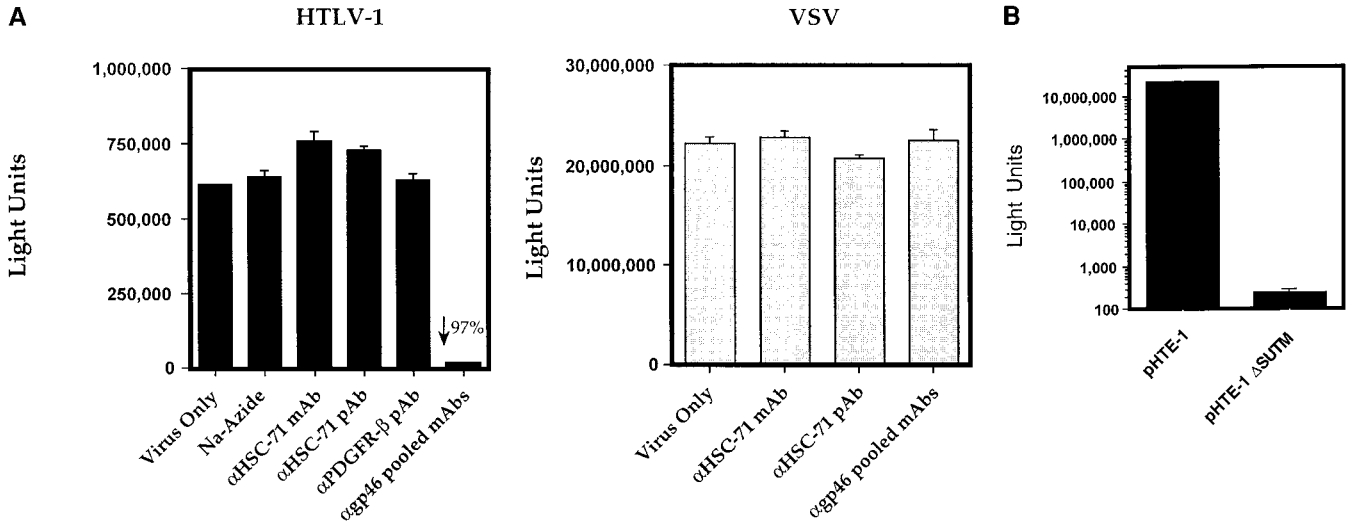

FIG. 3. Specificity of infection by pseudotyped viruses. (A) HOS cells were infected with HTLV-1 pseudotyped viruses produced with pHTE-1 (left) or VSV-G pseudotyped viruses produced with pHCMV-G (right). (B) HOS cells were infected with virus particles pseudotyped with the HTLV-1 envelope protein produced from pHTE-1 or a mutant envelope protein precursor derived from pHTE-1ΔSUTM, with alterations at the envelope precursor cleavage site and the fusion peptide of TM. Columns represent the mean levels of infection of duplicate assays, and the range of values is indicated.

Mode of HTLV-1 entry

Retroviruses have been found to enter susceptible target cells via pH-sensitive or pH-insensitive endocytic pathways or via pH-insensitive fusion of the viral lipid envelope with the plasma membrane (Kielian and Jungerwirth, 1990). In this experiment, a HeLa cell variant was used, designated Magi-CCR5 cells, to also test HIV-1 as a negative control, because HIV-1 enters susceptible target cells via a pH-insensitive fusion process (Fig. 5) (Stein *et al.,* 1987). These cells constitutively express HIV-1 receptor CD4 and coreceptors CXCR4 and CCR5 and carry the HTLV-1 long terminal repeat fused to the β-galactosidase gene. To examine the mode of entry by HTLV-1 envelope pseudotyped particles, the cells were pretreated with 30 mM ammonium chloride for 3.5 h preinfection with or without an additional 19 h of treatment postinfection (Fig. 5). A positive control used particles pseudotyped with VSV-G, because this virus is known to enter cells through a pH-sensitive endocytic

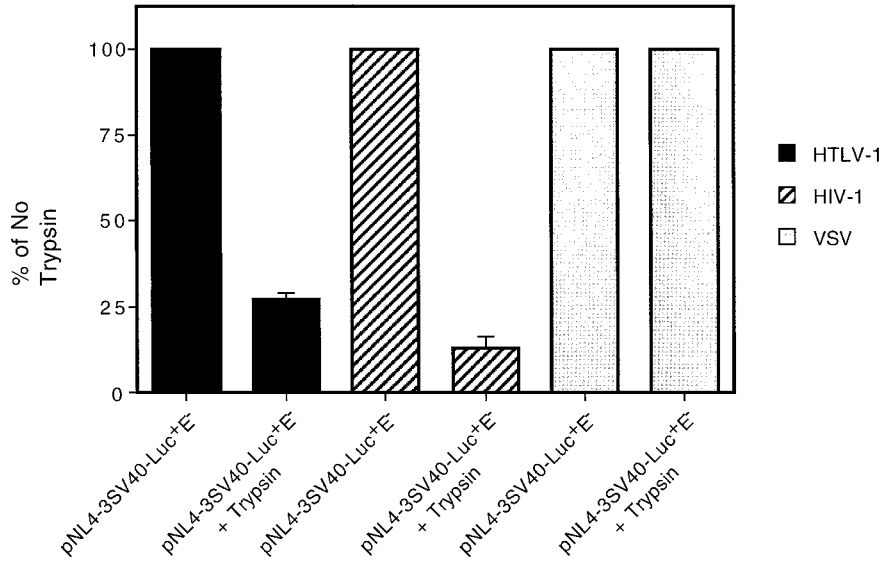

FIG. 4. The HTLV receptor is trypsin sensitive. PBMCs were pretreated with or without trypsin and then infected with viral particles pseudotyped with the HTLV-1 envelope protein (pHTE-1), the HIV envelope protein (pHXADA), or VSV-G (pHCMV-G). Luciferase activity in cell lysates was determined, and values from trypsin-treated cells were normalized to those of cell lysates from untreated cells. Columns represent the mean levels of infection of duplicate assays, and the range of values is indicated.

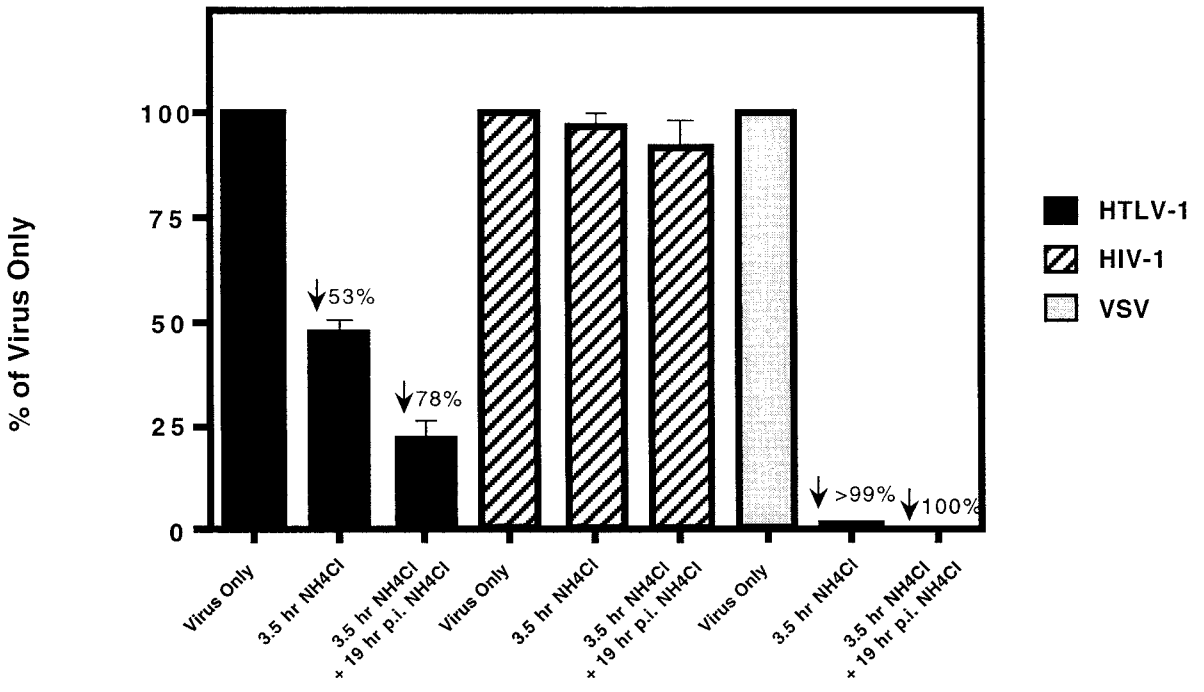

FIG. 5. HTLV entry is partially sensitive to ammonium chloride. Magi-CCR5 cells were untreated or pretreated for 3.5 h with ammonium chloride before infection with or without an additional 19 h of treatment with ammonium chloride post-infection (p.i.) with viral particles pseudotyped with the HTLV-1 envelope protein (pHTE-1), HIV envelope protein (pHXADA), or VSV-G (pHCMV-G). Luciferase activity in cell lysates was determined and values of ammonium chloride-treated cells were normalized to those of cell lysates from untreated cells. Columns represent the mean levels of infection of duplicate assays, and the range of values is indicated.

pathway (Kielian and Jungerwirth, 1990). Infection by HIV-1 was reduced <10% by the ammonium chloride treatments, which is consistent with previous observations (Stein *et al.,* 1987). In contrast, infection by VSV-G pseudotyped particles was reduced by >99% by ammonium chloride treatment. Intermediate results were obtained with HTLV-1 envelope pseudotyped particles, with reductions of 53–78%. Similar experiments with ammonium chloride and other endocytosis inhibitors could not be performed in PBMCs due to the toxicity of these agents.

DISCUSSION

In vitro infection experiments suggested that the HTLV-1 receptor has a wide species and cell type distribution (Clapham *et al.,* 1983; Hoxie *et al.,* 1984; Krichbaum-Stenger *et al.,* 1987; Nagy *et al.,* 1983; Watabe *et al.,* 1989; Yoshikura *et al.,* 1984). Similarly, cell fusion assays have also suggested that the HTLV-1 receptor is widely expressed (Li *et al.,* 1996; Okuma *et al.,* 1999). The current work extends the findings of Sutton and Littman (1996), using a sensitive pseudotype assay, to demonstrate that the distribution of HTLV-1 receptor is wider than appreciated in previous studies (Fig. 2). Thus most of the cell lines reported in previous studies as not infectable by HTLV-1 were in fact susceptible to infection by the HTLV-1 envelope pseudotyped virus. The discrepancy between these findings and fusion-based assays is most likely due to the higher sensitivity of the current assay. Low levels of entry through a non-fusion-based pathway may also be responsible for this finding.

The use of VSV-G pseudotyped particles provided an important control for infection by pseudotyped particles (Fig. 2B). Thus one cell line, MDBK, was identified as being poorly susceptible to HTLV-1 infection, if it is infectable at all. Although levels of infection of MDBK by VSV-G pseudotyped virus are depressed compared with 12 of the other cell lines tested, this activity level was >1000-fold above background levels, and higher than those of two other cell lines that were infectable by HTLV-1 envelope pseudotyped particles. This demonstrates that postentry events required for reverse transcription and expression of the SV40 promoter-luciferase indicator cassette are not compromised in these cells. MDBK cells were also not susceptible to HTLV-1 envelope-mediated fusion (data not shown and Li *et al.,* 1996). The lack of infection of MDBK cells does not reflect a general species restriction to HTLV-1 infection, because other bovine cell lines were susceptible to HTLV envelope-mediated fusion (Li *et al.,* 1996). The MDBK cells may lack either a critical component of the HTLV-1 receptor or a coreceptor required for entry. Alternatively, they may possess a specific inhibitor of HTLV-1 infection. Recent data suggesting that access to the HTLV-1 receptor can be restricted by other cell surface molecules

would be consistent with this idea (Hildreth, 1998). Experiments that examined somatic cell hybrids of MDBK cells and susceptible target cells could discriminate these possibilities. If MDBK cells are lacking a component specific for HTLV-1 infection, these cells could be suitable for genetic approaches aimed at determining whether a specific human cDNA clone can confer infection on these cells.

The current work also addressed several candidate molecules for the HTLV-1 receptor identified in previous studies. The current work is in accord with previous findings (Clapham *et al.,* 1983) that the IL-2 receptor is not the HTLV-1 receptor, because nonlymphoid cells (e.g., HOS, HeLa, and 293T cells; Fig. 2) are susceptible to infection. Furthermore, vascular cell adhesion molecule-1 is probably not the receptor, because cells other than endothelial cells can also be infected with HTLV-1 (Hildreth *et al.,* 1997). The 80-kDa protein expressed on BJAB-WH but not BJAB-CC84 cells (Agadjanyan *et al.,* 1994) is also not likely the HTLV-1 receptor, because both cell lines were infectable by HTLV-1 envelope pseudotyped viruses (Fig. 2). Furthermore, the 71-kDa heat shock protein (Sagara *et al.,* 1998) is also not likely the HTLV-1 receptor, because antibodies to this protein had no affect on pseudotype virus infection (Fig. 3). However, the receptor is likely to be a cell surface protein, because trypsin treatment of susceptible target cells markedly reduced infection by HTLV-1 envelope pseudotyped virus particles (Fig. 4).

The mechanism of entry by HTLV-1 after binding the receptor is also not understood. To determine whether a pH-sensitive endocytotic pathway is used, we examined inhibition of HOS cell infection by ammonium chloride, which prevents acidification of the endosome. Cellular entry by HIV-1 is not pH sensitive (Stein *et al.,* 1987), as confirmed in the current work (Fig. 5). In contrast, the entry of VSV-G pseudotyped particles was exquisively sensitive to ammonium chloride (Kielian and Jungerwirth, 1990; Marsh and Pelchen-Matthews, 1994). Studies with HTLV-1 envelope pseudotyped particles gave intermediate results, with only partial inhibition by ammonium chloride under these same conditions. The most likely explanation is that two distinct pathways of HTLV-1 entry exist, which may represent a high-affinity receptor that uses a pH-sensitive endocytotic pathway and a low-affinity receptor that uses a pH-insensitive endocytotic or fusion pathway. It is possible that only one or both of these pathways are present on cells of various tissues and species, accounting for the widespread expression of the receptor. Additional studies of the entry mechanism could use recently described transdominant dynamin mutants, to inhibit the formation of clathrin-coated vesicles (DeTulleo and Kirchhausen 1998).

In conclusion, the current study provides additional information about the characteristics of the HTLV-1 receptor that are critical for its definitive identification.

MATERIALS AND METHODS

Cell lines

HOS, 293T, HeLa, C127/LT, CHO, NIH 3T3, U87-MG, MDBK, CV-1, NRK, Tb11u, and QM7 cells were obtained from American Type Culture Collection (Rockville, MD). BJAB WH and BJAB CC84 were provided by Dr. M. Agadjanian (University of Pennsylvania). Tb1Lu cells were cultivated in modified Eagle's medium with Earle's basic salt supplement, 10% fetal calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. QM7 cells were cultivated in medium 199 with Earle's basic salt solution, 10% tryptose phosphate broth, 10% fetal calf serum, 1 mM sodium pyruvate, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All of the other cell lines were cultivated in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human PBMCs were purified by Ficoll gradient centrifugation from single donors and cultivated in RPMI 1640 medium, 10% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 U/ml IL-2 (provided by Chiron Corporation, Emeryville, CA). Magi-CCR5 cells were derived from stable transfection of a CCR5 expression clone into Magi cells, a HeLa cell line stably transfected with human CD4 and a plasmid containing the HIV-1 long terminal repeat fused to the β-galactosidase gene (Hung *et al.,* 1999).

Plasmid construction

The HIVLuc+Env− plasmid, pNL4-3SV40Luc+Env−, was constructed from pGB108, provided by Dr. A. Panganiban (University of Wisconsin). pGB108, a plasmid containing the HIV-1 provirus from strain NL4-3 (Freed *et al.,* 1992), was digested with *Nhe*I to remove the SV40 promoter-hygromycin cassette present within the HIV-1 *env* gene and to replace it with the *Nhe*I–*Xba*I fragment of pGL3 (Promega, Madison, WI), containing the SV40 promoter-luciferase gene.

pHTE-1, provided by Dr. M.-C. Dokehlar, includes the HTLV-1 CR strain *env* gene, flanked by two HTLV-1 long terminal repeats (Pique *et al.,* 1990). pHTE-1ΔSUTM was constructed by replacing the *Sal*I–NsiI fragment (nucleotides 493-1383) with a PCR amplified mutant fragment constructed by primer overlap extension with the following oligonucleotides: A, 5′-ACGCGTCGACGCTCCAGGATATGACCCCATC-3′; B, 5′-TGCATGCATGGTCCTGCAAGGATAACAAG-3′; C, 5′-CGTTCCCGCACAGCAGAAGACGTGGCGGTCTGGCTTG-3′; and D, 5′-CAAGCCAGACCGCCACGTCTTCTGCTGTGCGGGAACG-3′. The mutant fragment includes the mutations R312T, V314E, and P315D and abolished an *Age*I site and created a *Bbs*I site (nucleotide 1131). pHCMV-G, provided by Dr. J.-K. Yee (City of Hope National Medical Center), includes

the VSV-G gene under the regulation of a cytomegalovirus promoter (Chen *et al.,* 1996).

Infection with pseudotyped viruses

For production of pseudotyped viruses, 3×10^6 293 cells were plated overnight in a T75 flask and then transfected for 5 h with 50 μl of LipofectAMINE, 750 μl of OptiMEM, and 10 μg of pNL4-3SV40Luc+Env− with or without 10 μg of pHTE-1, pHTE-1ΔSUTM, or pHCMV-G. The cells were washed with OptiMEM and incubated for 12–16 h at 37°C and 5% CO_2 with 8 ml of fresh medium and 20 mM sodium butyrate and then for 60–72 h in 10 ml of fresh medium. The culture supernatant was removed, filtered through a 0.22-μm filter, and titered by an HIV-1 p24 antigen ELISA (Abbott Laboratories, Chicago, IL). The typical yield of virus particles was 1 μg/ml.

Freshly prepared virus was used to infect 3×10^6 target cells plated onto a 100-mm plate for 3 h at 37°C and 5% CO_2. The cells were washed and incubated with fresh medium for 48 h at 37°C and 5% CO_2. The cells were washed, and cell lysates were prepared in 100 μl of luciferase assay buffer containing 0.1 M potassium phosphate, pH 7.8, 0.2% Triton X-100, and 1 mM fresh DTT. Luciferase assays were performed on Optocomp I (MGM Instruments).

For antibody inhibition assays, HOS cells were incubated with 0.1% sodium azide, 10 μg/ml antibody to the 71-kDa heat shock protein (SC7298 or SC1049; Santa Cruz Biotechnology, Santa Cruz, CA), 10 μg/ml antibody to the platelet-derived growth factor-β receptor (SC432; Santa Cruz Biotechnology), or 10 μg/ml concentration of each of neutralizing anti-gp46 HTLV-1 antibodies PRH7A and PRH11A (provided by Dr. Steve Foung, Stanford University; Hadlock *et al.,* 1999). These antibodies blocked 50% of gp45 binding at 0.4 μg/ml and completely blocked binding at 4 μg/ml (Hadlock *et al.,* 1999). For trypsin inhibition experiments, PBMCs were stimulated with PHA-P (Difco) for 3 days and then preincubated with 500 μg/ml trypsin at 37°C and 5% CO_2, washed, and infected with viral pseudotypes. For ammonium chloride inhibition experiments, Magi-CCR5 was preincubated with 30 mM ammonium chloride with or without an additional 19 h of treatment postinfection.

ACKNOWLEDGMENTS

We thank Michael Robek and Nancy Vander Heyden for critical discussions. This work was supported by U.S. Public Health Service grant CA 63417.

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