Pseudotypes of human T-cell leukemia virus types 1 and 2: Neutralization by patients' sera

(adult T-cell leukemia-lymphoma/vesicular stomatitis virus)

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ABSTRACT   Pseudotypes of vesicular stomatitis virus (VSV) bearing envelope antigens of human T-cell leukemia virus (HTLV) types 1 and 2 were prepared by propagating VSV in cells lines productively infected with HTLV. Plaque assays of VSV(HTLV) pseudotypes were employed to determine the presence of (*i*) HTLV receptors on cells and (*ii*) neutralizing antibodies in the serum of patients with adult T-cell leukemia-lymphoma (ATLL). Cell surface receptors for HTLV-1 and HTLV-2 were found on nonlymphoid cells of human and mammalian origin. Neutralizing antibodies specific to VSV(HTLV-1) were found in sera of ATLL patients in titers varying from 1:50 to 1:30,000 and did not correlate closely with antibody titers for internal viral antigens. Sera from ATLL patients in the United Kingdom (Caribbean immigrants), United States, and Japan completely neutralized VSV(HTLV-1), indicating that the HTLV isolates from these distinct geographic regions represent a single envelope serotype. Neutralization of VSV(HTLV-1) was more specific and more sensitive than assays of syncytium inhibition. No cross-neutralization was observed between bovine leukosis virus and HTLV, and only limited cross-reaction was found for envelope antigens of HTLV-1 and HTLV-2. These studies show that VSV(HTLV) pseudotypes can be readily used to screen for neutralizing antibodies in patients' sera and to distinguish HTLV envelope serotypes.

Human T-cell leukemia virus type 1 (HTLV-1) is a C-type retrovirus associated with certain malignancies of mature T cells known as adult T-cell leukemia-lymphoma (ATLL) (1, 2). The first isolates of HTLV were made from tumor cells of American patients (3, 4). A virus formerly known as adult T-cell leukemia virus (ATLV) (5, 6), endemic in the regions of southwestern Japan where ATLL is prevalent (1, 7, 8), was found to be closely related or identical to HTLV (9–11). The Caribbean basin is another HTLV-endemic area (12) and the virus also appears to have a widespread distribution in tropical America and Africa (13). A related but distinct virus, HTLV type 2 (HTLV-2), has been isolated from an American patient with T-cell hairy cell leukemia (14).

Like many naturally occurring strains of animal retroviruses, a simple, quantitative bioassay of HTLV has proved difficult because the virus is not directly cytopathic or transforming for monolayer cells in culture. A syncytium assay of HTLV production (15–17) is not readily adaptable to a quantitative plaque assay because the *in vitro* infectivity of cell-free HTLV preparations is very low (18). An alternative approach is to use an indirect assay of pseudotypes rather than the virus itself. Pseudotypes are phenotypically mixed virions carrying the genome of one virus and the coat proteins of another. The most widely used pseudotype systems for retroviruses employ either related retrovirus genomes carrying oncogenes, which can be titrated by a focal cell transforma-

tion assay, or the unrelated rhabdovirus, vesicular stomatitis virus (VSV), which is titrated by plaque assay (19, 20). We have adopted the VSV system for HTLV studies.

When cells chronically infected with retroviruses are superinfected with VSV, a proportion of the progeny VSV particles bear the envelope glycoproteins of the retrovirus (20–22). Core antigens of the retrovirus are not assembled into VS virions. The VSV pseudotypes resist neutralization by anti-VSV antibodies but are sensitive to neutralizing antibodies specific to the retrovirus donating the envelope antigens. The host range for viral penetration of pseudotypes is restricted to cells bearing receptors for the retrovirus. Following penetration and uncoating, however, the VSV genome contained in the pseudotype particle replicates to produce nonpseudotype progeny. Thus, a cytopathic plaque assay of VSV pseudotypes can be used to determine receptor expression, receptor interference, and neutralizing antibodies specific to the retrovirus encoding the envelope glycoproteins.

VSV pseudotype particles have been employed to study the biological properties of the envelope glycoproteins of avian leukosis and reticuloendotheliosis viruses (21–24), murine leukemia viruses (21, 25, 26), murine mammary tumor virus (27, 28), bovine leukosis virus (BLV) (29), and primate retroviruses (30, 31). Here we report the production of VSV(HTLV) pseudotype particles and their neutralization by human sera.

MATERIALS AND METHODS

Viruses and Cells. *VSV.* VSV, Indiana serotype, a wild-type clone recently plaque-purified, was used for pseudotype production (24).

HTLV-1. HTLV-producing MT2 cells (5) were obtained from I. Miyoshi (Kochi, Japan). HUT78, HUT102, C10/MJ2, C91/PL, and MT1 cells (3, 32) were obtained from R. C. Gallo and M. Popovic (National Cancer Institute, Bethesda, MD, USA). HOS/PL and HOS/MT2 cells are human osteosarcoma cells infected with and producing HTLV derived from C91/PL cells and MT2 cells, respectively (18).

HTLV-2. C1218M cells are human umbilical cord T cells transformed by HTLV-2 from patient MO (14) and were obtained from R. C. Gallo and M. Popovic. Ton1 cells are human tonsil T cells transformed by HTLV-2 in this laboratory by cocultivation with x-irradiated C1218M cells.

BLV. Fetal lamb kidney (FLK) cells productively infected with BLV were obtained from A. Burny (Brussels, Belgium).

T cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum. Monolayers of human osteosarcoma (HOS), mink

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Abbreviations: HTLV, human T-cell leukemia virus; VSV, vesicular stomatitis virus; pfu, plaque-forming units; BLV, bovine leukosis virus; ATLL, adult T-cell leukemia-lymphoma.
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lung (CCL 64), feline (CCC S^+L^-), and bovine (MDBK) cells were used for VSV plaque assays. These cultures were maintained in Dulbecco modified Eagle's medium (DME medium) supplemented with 3–10% heat inactivated-fetal calf serum and antibiotics.

Antisera. Hyperimmune sheep antiserum against VSV was obtained from J. Zavada (Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia). Sera from West Indian ATLL patients in the United Kingdom were provided by D. Catovsky (Hammersmith Hospital), M. F. Greaves (Imperial Cancer Research Fund Laboratories), and D. Shanson (St. Stephen's Hospital, London). Sera of United States and Japanese patients were provided by R. C. Gallo and P. Sarin (National Cancer Institute, Bethesda). Neutralizing antiserum to BLV prepared from an infected animal was provided by J. Ghysdael (Brussels). All sera were heat-inactivated (56°C, 30 min).

Pseudotype Production and Assay. Retrovirus-producing cells were infected with VSV at a multiplicity of infection equivalent to 10 and were incubated at 37°C for 12–18 hr before harvesting progeny VSV, which was stored in 1-ml aliquots at −70°C. VSV was assayed by plaque titration on various monolayer cell types in 30-mm wells (24). Virus was incubated for 1 hr at 33°C with excess heat-inactivated sheep anti-VSV serum to titrate the pseudotype or without antiserum to titrate the total VSV. Human patients' sera or other retrovirus antisera were included at appropriate dilutions at this stage when required and serial dilutions were then plated on assay cell monolayers pretreated with 25 μg of DEAE-dextran per ml. After agar overlay, the assays were incubated at 33°C and plaques were counted after 2 or 3 days.

RIA. A competitive, solid-phase RIA for human sera used a C91/PL cell lysate as a source of HTLV internal antigens (unpublished data). Serial dilutions of sera were incubated with antigen followed by incubation with ^{125}I-labeled anti-HTLV IgG. The highest dilution displacing >50% ^{125}I binding was recorded as the serum titer.

Syncytial Assay. Syncytium induction and inhibition by antisera was conducted with HOS cells as described (15).

RESULTS

VSV(HTLV-1) Pseudotype Formation in HOS/PL Cells. As HOS/PL virions carry sufficient envelope antigens to confer cell-free infectivity (18), HOS/PL cells were chosen to prepare VSV(HTLV-1) stocks.

Fig. 1 shows the titration of VSV propagated in HOS and HOS/PL cells. VSV grown in control HOS cells was completely neutralized by anti-VSV but was unaffected by ATLL serum. With VSV grown in HOS/PL cells, the fraction resisting neutralization by anti-VSV serum represented about 10^{-5} of the total VSV yield. This surviving fraction $[10^{4.7}$ plaque-forming units (pfu)/ml] was completely neutralized by serum from an American patient with ATLL, previously shown to contain HTLV-1 antibodies to p24 and syncytium activity (15). These results show that the pfu surviving anti-VSV serum represent pseudotypes with HTLV-1 envelopes. When VSV(HTLV-1) pseudotype was plated on HTLV-producing HOS/PL cells, receptor interference was absolute, confirming the envelope specificity of the pseudotype.

Human, mink, and feline cells were equally sensitive to VSV(HTLV-1), whereas bovine MDBK cells were completely resistant (data not shown), indicating that cell surface receptors for HTLV-1 are widespread but not universal among mammalian cell types. A more detailed analysis of pseudotype host range will be reported elsewhere (33).

Propagation of Pseudotypes in Further HTLV-Producing Cells. Pseudotype formation was investigated with a number of HTLV isolates, including HTLV-2, and also with BLV.
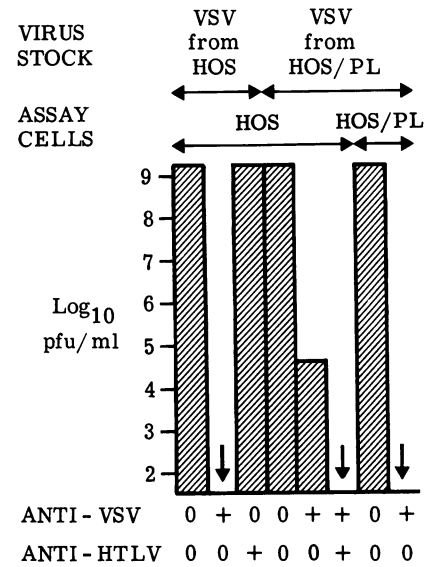

FIG. 1. Titration of VSV propagated in control HOS cells and in HTLV-producing HOS/PL cells. Virus stocks were treated with antisera as indicated before plating on assay cells. Anti-HTLV was serum from an American ATLL patient.

Table 1 shows that specific pseudotypes were obtained with VSV propagated in cells infected with the PL isolate of HTLV-1, whereas other HTLV-1 isolates did not unequivocally yield VSV pseudotypes. Some pfu surviving anti-VSV were obtained with MT2 and C10/MJ2 (cells that produce more HTLV than the other HTLV-1-infected cells), but they were not neutralized by high-titer sera of either American or Japanese ATLL patients. Therefore, the surviving pfu should not be strictly defined as pseudotypes, although their plating efficiency was reduced on MDBK cells lacking HTLV receptors (data not shown). However, specific pseudotypes were obtained with HTLV-2 (MO isolate in C1218M and Ton1 cells) and BLV. The VSV(HTLV-2) stocks had a higher plating efficiency on feline CCC cells than on HOS or mink cells, and CCC cells were therefore used for all comparative assays involving HTLV-2.

Table 1. Yield of total VSV and pseudotype propagated in various HTLV-producing cells

Retrovirus-producing cells	Titer, $\log_{10}$ pfu/ml		
		VSV surviving neutralization	
	Total VSV	Anti-VSV only	Anti-VSV and anti-retrovirus
No virus			
HUT78	8.3	<2.0	—
HOS	9.8	<2.0	—
HTLV-1			
C91/PL	8.7	3.9	<2.0
HOS/PL	9.4	4.6	<2.0
MT1	8.6	<2.0	<2.0
MT2	9.7	3.3	3.3
HOS/MT2	9.3	3.0	2.7
HUT102	8.4	<2.0	<2.0
C10/MJ2	8.9	3.4	3.4
HTLV-2			
C1218M	8.9	4.2	<2.0
Ton1	9.1	3.8	<2.0
BLV			
FLK	9.0	4.5	<2.0

VSV(HTLV) stocks were titrated on CCC feline cells and VSV(BLV) on mink cells.

Serology of VSV(HTLV) Pseudotypes. The titers of human sera neutralizing VSV(HTLV-1) are shown in Table 2. All sera from ATLL patients contained specific neutralizing activity with titers ranging from 1:50 to 1:30,000. Adsorption of sera with *Staphylococcus aureus* removed the neutralizing activity, indicating that it is mediated by immunoglobulin. Table 2 also shows that RIA titers of antibodies reacting mainly with HTLV-1 internal antigens do not correlate closely with the neutralization titers, suggesting that individual ATLL patients exhibit different humoral immune responses to the internal and envelope antigens of HTLV-1. Sera from patients in different geographic regions completely neutralized VSV(HTLV-1) prepared from the American PL isolate, demonstrating that HTLV-1 endemic in the United States, West Indies, and Japan represents a single envelope serotype.

Table 3 compares the pseudotype neutralizing properties and syncytium inhibitory activities of sera from individuals naturally infected with HTLV-1, HTLV-2, a simian virus closely related to HTLV, and BLV. Approximately 75% ATLL sera have antibodies specifically inhibiting HTLV-1 syncytia, whereas 25% sera show equivalent titers for inhibition of both HTLV-1 and HTLV-2 syncytia (17). Sera H0423 and F4093 represent the first category and sera F4387 and HS1 the second. Serum MO is from the patient with hairy cell leukemia from whom HTLV-2 was isolated (14). The vervet serum is from an African green monkey captured in Kenya. Captive vervets in Germany were found to have anti-HTLV antibodies (34) and our studies indicate that 40% vervets in Kenya have antibodies reacting with both HTLV-1 and HTLV-2 in syncytium inhibition tests (ref. 17; unpublished observations). The chimpanzee (bred in the United Kingdom) has serum with similar properties. These primates are presumed to be infected with HTLV-related viruses, as demonstrated for Japanese monkeys (35).

The data presented in Table 3 show that the envelope antigens of HTLV-1, HTLV-2, and BLV can be clearly differentiated serologically in pseudotype neutralization assays. No cross-reaction between BLV and either HTLV strain was observed with sera showing high titers for the envelope antigens of homologous viruses. The high-titer HTLV-1 sera had

Table 2. Neutralization of VSV(HTLV-1) pseudotype by sera from ATLL patients in United States, United Kingdom, and Japan: Comparison with RIA

Serum		Titer	
Country	Patient	Neutralizing	RIA
United States	F0781	50	30
	F0920	30,000	220
	F3634	30,000	2,100
	H0423	6,000	25
	Control	<10	<10
United Kingdom	HS1	1,250	400
	RM1	1,250	200
	T33.2	50	NT
	W23.5	625	30
	Control	<10	<10
Japan	S318	250	100
	S353	1,250	100
	T7236	1,250	10
	W0995	1,250	100

VSV(HTLV-1) prepared from HOS/PL cells as the fraction surviving excess anti-VSV serum was reacted at $10^{3.7}$ pfu/ml with serial 1:5 dilutions of human serum and plated on mink cells for plaque assay. The antibody titers are expressed as the reciprocal of the highest dilution causing >80% reduction of pfu (neutralization) or ≥50% displacement of ^{125}I (RIA); NT, not tested. The control sera were pooled from healthy adults; patient S318 is a clinically normal subject born in an endemic area.

Table 3. Cross-neutralization tests between HTLV-1, HTLV-2, and BLV envelope antigens

Antibody		Titer					
		Syncytium inhibition			Pseudotype neutralization		
Source	Serum	HTLV-1	HTLV-2	BLV	HTLV-1	HTLV-2	BLV
ATLL	H0423	300	10	<10	6,000	10	<10
	F4093	300	30	<10	30,000	50	<10
	F4387	300	300	<10	30,000	250	<10
	HS1	300	300	<10	1,250	250	<10
Hairy	MO	<10	300	<10	10	1,250	<10
Vervet	M106	30	30	<10	250	10	<10
Chimp	M52	30	30	<10	250	10	<10
Bovine	BB01	<10	<10	300	<10	<10	6,000

The serum titers are the reciprocal of the highest dilution causing >80% reduction of syncytia or pseudotype pfu.

much lower titers for VSV(HTLV-2), and conversely MO serum was specific for HTLV-2. The human, chimpanzee, and vervet sera, that showed equal cross-reaction with HTLV-1 and HTLV-2 in syncytium inhibition assays, all exhibited markedly stronger neutralization of VSV(HTLV-1) than VSV(HTLV-2).

DISCUSSION

We have demonstrated the use of VSV pseudotypes to investigate HTLV-1 and HTLV-2 envelope antigens, including titrations of human sera for neutralization of infectivity. VSV(HTLV) pseudotype formation was efficient in HTLV-1-producing HOS/PL and C91/PL and HTLV-2-producing C1218M and Ton1 cells but was inconclusive in other HTLV-1-producing T cells and in HOS/MT2 cells. Pseudotype yields from HOS/PL cells were equivalent to titers reported with other primate retroviruses (30) and BLV (29) but were 1/100th of the titers attainable with avian leukosis viruses (24).

Experimentation with mixed infections of VSV and HTLV may represent a potential biohazard greater than laboratory manipulation of HTLV alone. When rhabdoviruses and retroviruses are propagated in the same cells, reciprocal phenotypic mixing takes place (36, 37). Thus, HTLV(VSV) particles bearing stable VSV glycoproteins may be produced as well as VSV(HTLV) pseudotypes. These "reverse" pseudotypes are likely to be more infectious than HTLV itself, which may require cell-to-cell contact for transfer.

The ability of VSV(HTLV) to plaque on several non-lymphoid cell types demonstrates that the apparent T-cell tropism of HTLV is not due to the lack of receptors on other cell types, and at least one nonlymphoid line, HOS, is permissive for HTLV replication (18). Receptor interference, occurring when viral glycoproteins synthesized by infected cells bind to specific cellular receptor sites (19), was clearly shown by the resistance of HOS/PL cells to VSV(HTLV-1) infection. We have also observed cross-interference of receptors between HTLV-1 and HTLV-2 (33).

VSV(HTLV-1) was completely neutralized by sera from patients with ATLL. Sera from United States and Japanese patients and from British patients of West Indian origin all neutralized the VSV(HTLV-1) pseudotypes without a surviving fraction, though individual antibody titers varied considerably. These findings provide strong evidence that the envelope glycoproteins of HTLV-1 infecting ATLL patients in widely separated geographic areas are very closely related. No cross-reaction was found between neutralization of HTLV and BLV, although these viruses have structural similarities (38) and partial homology of p24 protein sequences (39). Furthermore, antiserum from the patient infected with

HTLV-2 (14) barely neutralized VSV(HTLV-1) at all and most sera of patients infected with HTLV-1 neutralized HTLV-2 only very weakly, indicating that the envelope glycoproteins of HTLV-1 and HTLV-2 are antigenically distinct. The chimpanzee and vervet sera, which cross-reacted with both HTLV-1 and HTLV-2 in syncytium assays, exhibited an apparent specificity to HTLV-1 by pseudotype neutralization. However, the amount of free envelope glycoprotein in pseudotype preparations of HTLV-1 and HTLV-2 is not known, and the absolute titers of neutralizing antibody may vary according to the quantity of competing antigen present.

Neutralization of VSV(HTLV) pseudotypes provides a convenient method for titrating serum antibodies specific for HTLV envelope antigens. It is more sensitive than the syncytium inhibition assay, with titers of some patients' sera as high as 1:30,000 and is as sensitive as other assays of antibodies to HTLV already in use. Our studies also indicate that, though all ATLL patients have serum antibodies to both core and envelope antigens of HTLV-1, the neutralizing titers are not closely correlated with RIA of antibodies reacting mainly with internal antigens. Further studies using purified antigens (40) will be needed to analyze differential immune responses to HTLV proteins and their antigenic epitopes. VSV(HTLV) pseudotypes will, therefore, be of use in clinical and epidemiological studies for monitoring neutralizing antibodies and for distinguishing different envelope serotypes presenting in T cell malignancies and other syndromes.

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