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Human Immunodeficiency Virus Type 1 Vectors with Alphavirus Envelope Glycoproteins Produced from Stable Packaging Cells

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Alphavirus glycoproteins have broad host ranges. Human immunodeficiency virus type 1 (HIV-1) vectors pseudotyped with their glycoproteins could extend the range of tissues that can be transduced in both humans and animal models. Here, we established stable producer cell lines for HIV vectors pseudotyped with alphavirus Ross River virus (RRV) and Semliki Forest virus (SFV) glycoproteins E2E1. RRV E2E1-stable clones could routinely produce high-titer pseudotyped vectors for at least 5 months. SFV E2E1-stable clones, however, produced relatively low titers. We examined the properties of RRV E2E1-pseudotyped vectors [HIV-1(RRV)] and compared them with amphotropic murine leukemia virus Env- and vesicular stomatitis virus glycoprotein G-pseudotyped vectors. HIV-1(RRV) displayed a number of characteristics which would be advantageous in ex vivo and in vivo experiments, including resistance to inactivation by heat-labile components in fresh human sera and thermostability at 37°C. Upon single-step concentration by ultracentrifugation of HIV-1(RRV), we could achieve vector stocks with titers up to 6×10^7 IU/ml. HIV-1(RRV) efficiently transduced cells from several different species, including murine primary dendritic cells, but failed to transduce human and murine T cells as well as human hematopoietic stem cells (HSC). These results indicate that HIV-1(RRV) could be used in a number of applications including animal model experiments and suggest that expression of RRV cellular receptors is limited or absent in certain cell types such as T cells and human HSC.

Vectors based on the lentivirus human immunodeficiency virus type 1 (HIV-1) can deliver therapeutic transgenes into both dividing and nondividing cells (23, 33). One important factor for vector performance is the viral envelope glycoproteins (Env) with which the vector is pseudotyped, as Env dictates not only vector host range and tissue tropism but also vector stability. We have recently described a novel, stable HIV-1 vector packaging system, STAR, which continuously produces high-titer HIV-1 vectors bearing gammaretrovirus Env (13). With the advent of stable pseudotyped HIV-1 producer cell lines, the ability of an Env to be incorporated into such a cell line is a significant consideration. It is notable that the commonly used vesicular stomatitis virus G glycoprotein (VSV-G) is highly fusogenic and thus cannot be used in constitutive stable producer cells (1, 5). Moreover, the VSV-G-pseudotyped HIV-1 vectors are particularly susceptible to inactivation in human sera, whereas vectors pseudotyped with gammaretrovirus Env are not (9, 31).

Experiments utilizing animal models are useful and often required in preclinical gene therapy research. It would, therefore, be ideal if the same gene transfer vector can be used in both humans and the model animals. Recently, we reported the high-titer stable producer cell lines for the HIV-1 vectors with amphotropic murine leukemia virus (MLV-A), feline endogenous RD114, and gibbon ape leukemia virus (GALV) Env (13, 31). The RD114- and GALV-pseudotyped vectors, however, cannot be used in murine models, as there is no functional cellular receptor for RD114 and GALV Env in mouse cells (24). Although the HIV-1 vectors pseudotyped with MLV-A Env [HIV-1(MLV-A)] can transduce both human and mouse cells through its cellular receptor, Pit-2 (24), their transduction efficiency may depend on the expression levels of its receptors on the target cells (16). Therefore, an HIV vector pseudotyped with an alternative broad-host-range Env, which can utilize the other cellular factor(s) as a receptor(s), would be necessary when the target cells poorly express Pit-2.

Alphaviruses belong to the family *Togaviridae* and are found in insects, reptiles, birds, and mammals. Although it remains unknown what molecules act as cellular receptors for these viruses, it has been suggested that several receptors or several receptor-coreceptor combinations are involved in virus entry (12, 32). Alphaviruses exhibit a wide host range and cellular tropism, most of which are important gene therapy targets including antigen-presenting cells, neurons, and muscle cells (12, 32). Recently, pseudotyping of HIV-1 vectors has been shown with glycoproteins from members of the alphaviruses, namely, Ross River virus (RRV), Semliki Forest virus (SFV), and Sindbis virus (14, 21). As RRV glycoproteins E2E1 could be stably expressed as part of an MLV producer cell line (29), we considered the use of alphavirus glycoproteins in our stable HIV-1 packaging cell line STAR (13).

In this study, we first examined the efficiency of pseudotype production when alphavirus Env was incorporated into the STAR producer cell line. Next, parameters that would affect

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alphavirus pseudotyped HIV-1 use in vivo and ex vivo, such as resistance to inactivation in human sera and thermostability, were investigated. This investigation was performed in comparison to HIV-1 virions pseudotyped with VSV-G or MLV-A Env. VSV-G is most commonly used in vitro and ex vivo, while MLV-A Env has been used in the transduction of several clinically relevant cell types in clinical trials (3, 4, 6). Finally, we assessed the host cell ranges of the alphavirus Env-pseudotyped HIV-1 vectors and how efficiently the vectors would transduce a number of cell lines from different species as well as human and murine primary cell cultures.

MATERIALS AND METHODS

Cell lines. Human BC3 and Jurkat and mouse EL4 and P815 cells were maintained at 37°C, 5% CO_2, in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). All the other cell lines were maintained at 37°C, 10% CO_2, in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Transient virus production. Virus was harvested from 10-cm-diameter plates. A total of 4×10^6 293T cells were plated 24 h before transfection. Cells were transiently transfected with plasmid at a 3:2:1 weight ratio of vector plasmid (pHR′SINcPPT-SE [7]) to packaging plasmid (pCMVΔR8.91 [33]) to Env plasmid by using Lipofectamine (Gibco BRL) according to the manufacturer's instructions. The Env plasmids pMD-G (VSV-G) (23), phCMV-A (MLV-A) (2), pRRV-E2E1A (RRV) (29), and pSFV-E2E1A (SFV) (14) were used. After washing with OptiMEM, virus was harvested for 48 h at 37°C in 8 ml of OptiMEM (Gibco BRL). Viral supernatant was then passed through a 0.45-μm-pore-size filter.

Generation of STAR cells producing alphavirus pseudotypes. The HIV-1 vector-harboring STAR cell clone (STAR-HV#12) was transfected with pRRV-E2E1A or pSFV-E2E1A and then selected in the presence of 100 μg of zeocin/ml for 2 weeks.

Stable virus production. HIV-1(RRV), HIV-1(SFV), and HIV-1(MLV-A) vectors were harvested from STAR cell clones STAR-HV12-RRV#B, STAR-HV12-SFV#A, and STAR-Ampho-HV#2, respectively. A total of 5×10^6 cells were plated onto 10-cm-diameter plates with Dulbecco's modified Eagle medium with 10% FCS. The cells were maintained at 37°C for 36 h, and the supernatants were replaced with the FCS-free medium OptiMEM (Gibco BRL). The cells were cultured at 37°C for 24 h and further maintained at 32°C for 18 h. The culture supernatants were harvested as RRV, SFV, and MLV-A Env pseudotypes. STAR-HV#12 was transfected with pMD-G in OptiMEM, and the FCS-free culture supernatants were used as HIV-1(VSV-G). For human hematopoietic stem cell (HSC) experiments, we harvested HIV-1(RRV) and HIV-1(MLV-A) in FCS-free medium (X-vivo15; Biowhittaker Cell Biology) instead of OptiMEM. The supernatants from STAR cells constantly contained 450 to 650 ng of p24 of HIV-1 Gag/ml, while HIV-1 vectors produced by conventional three-plasmid transfection had about 100 ng of p24/ml (13, 31).

Viral titer determination. A total of 2×10^5 cells were inoculated with serial dilutions of viral supernatant. Forty-eight hours after infection, enhanced green fluorescent protein (eGFP) titers (international units per milliliter) were determined by using a fluorescence-activated cell scanner (FACS). Where indicated, titrations were carried out in the presence of 8 μg of Polybrene (hexadimethrine bromide; Sigma, Poole, United Kingdom)/ml or with centrifugal inoculation, or "spinoculation" (1,200 × *g*, 2 h, 25°C), or both. Unless otherwise stated, the minimum level of detection by FACS in our experiments was 2×10^3 IU/ml.

Concentration. All pseudotypes were harvested in OptiMEM. Ten milliliters of each supernatant was concentrated at $10^5 \times g$ (SW41 rotor at 35,000 rpm, 1.5 h, 4°C) by using a Beckman L7 ultracentrifuge. The pellet was resuspended in 500 μl of OptiMEM. Thirty milliliters of supernatant was concentrated at 3,000 × *g* with a Heraeus (Sepatech) Megafuge 2.0R bench top centrifuge. Here, the pellet was resuspended in 1.5 ml of OptiMEM. Where indicated, virus was diluted with OptiMEM.

Preparation of human sera. Human peripheral blood was incubated on ice overnight. Serum from the clot was aliquoted and frozen at −80°C (fresh serum preparation) until required or heat inactivated at 56°C for 45 min and then frozen at −80°C (heat-inactivated serum preparation).

Isolation of murine DC. Murine dendritic cells (DC) from bone marrow were prepared by using a standard protocol (25). Briefly, bone marrow cells were grown in Iscove's modified Dulbecco's medium (Gibco BRL) supplemented with 10% FCS, 5 μg of apotransferrin (Sigma)/ml, 5×10^{-5} M 2-mercaptoethanol (Gibco BRL), 100 U of penicillin/ml, 100 μg of streptomycin (Gibco BRL)/ml, and 50 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF)/ml.

Isolation of human monocyte-derived DC. Peripheral blood mononuclear cells were isolated on Ficoll gradients. Monocytes were allowed to adhere for 2 h on non-tissue-culture-treated plates and washed extensively. Monocytes were then removed in EDTA, washed, and replated at the desired density on tissue culture plates and grown in RPMI medium plus 10% FCS, antibiotics, interleukin-4 (IL-4), and GM-CSF (1,000 U/ml) as described previously (27). Four days later, DC cultures were depleted of CD2-, CD3-, and CD19-expressing cells by negative immunomagnetic selection and replated at the desired density. After infection studies, DC were identified by expression of CD1a by FACScan.

Isolation of human lymphocytes. T lymphocytes were isolated on the basis of nonadherence to tissue culture after isolation of peripheral blood mononuclear cells on Ficoll gradients. T lymphocytes were then cultured in RPMI medium plus 10% FCS supplemented with 100 U of IL-2 (Peprotech)/ml and 1 μg of concanavalin A (Sigma)/ml for 48 h. Cells were then washed, replated, and cultured for a further 3 days in RPMI medium plus 10% FCS supplemented with IL-2.

Transduction of dendritic and lymphocyte cell cultures. After 5 days of culture, DC were washed and exposed to virus at the required multiplicity of infection (MOI) on 293T cells, as standardized on 293T cells, overnight and then washed thoroughly and cultured for a further 7 days in culture medium. The percent cell transduction was then determined by FACScan. Virus used in these experiments was titrated onto 293T cells in the presence of 8 μg of Polybrene/ml in parallel.

Isolation and infection of human CD34$^+$ cells. Isolation and infection of human CD34$^+$ cells were carried out as previously described (26).

RESULTS

Transient and stable production of alphavirus pseudotypes. First, we investigated the pseudotyping efficiency of HIV-1 vectors with alphavirus glycoproteins by transient three-plasmid transfection (23, 34). The vector titers were determined on 293T cells in the presence of Polybrene. RRV E2E1-pseudotyped HIV vector [HIV-1(RRV)] had a titer of 4×10^5 IU/ml, while SFV E2E1-pseudotyped vector [HIV-1(SFV)] had a titer of 10^5 IU/ml. These titers compare to 4×10^6 IU/ml with VSV-G and 1×10^6 IU/ml with MLV-A Env used to produce HIV-1 vectors by transient three-plasmid transfection.

Next, we examined if HIV-1 vectors with alphavirus glycoproteins could be generated from stable HIV-1 producer cells. The HIV-1 vector-harboring STAR cells were transfected with the RRV, SFV, and MLV-A Env expression plasmids and then selected in phleomycin for 3 weeks. We selected 48 clones of each plasmid and screened them for vector production. Although a significant number of STAR cell clones producing HIV-1(RRV) or HIV-1(SFV) could be obtained after the first screening, many clones lost their titers during prolonged culture (up to 5 months) (Table 1). MLV-A Env expression, however, appeared to be more stable than the alphavirus Env, as most of the positive clones remained positive for up to 5 months. When screened after 20 weeks in culture, vector titers from the five RRV-stable clones were 1×10^5 to 4×10^5 IU/ml, while the three SFV clones could produce virus with titers of 5×10^3 to 2×10^4 IU/ml on 293T cells. Therefore, unlike MLV-A Env, introduction of RRV and SFV Envs into STAR cells did not increase viral titers above those seen in transient virus production experiments. Due to the relatively low titer of HIV-1(SFV), the properties of HIV-1(RRV) were examined in subsequent experiments.

Stability of RRV pseudotypes. The ability of a pseudotyped virus to resist inactivation in human serum is a useful characteristic for in vivo use. Each pseudotype was therefore mixed at

TABLE 1. Screening of STAR cells expressing RRV and SFV Env[a]

Screening (time point)	No. of vector-producing clones/total clones (eGFP titer [IU/ml]) for		
	MLV-A	RRV	SFV
1 (5 weeks)	22/48	16/48	11/48
2 (11 weeks)	19/22 ($5 \times 10^5 \sim 2 \times 10^7$)	6/16 ($10^5 \sim 10^6$)	3/11 ($5 \times 10^3 \sim 2 \times 10^4$)
3 (20 weeks)	19/19 ($5 \times 10^5 \sim 2 \times 10^7$)	5/6 ($10^5 \sim 4 \times 10^5$)	3/3 ($5 \times 10^3 \sim 2 \times 10^4$)

[a] Infectious vector production from phleomycin-resistant clones was monitored for up to 5 months. Vector production was examined by inoculating the culture supernatants of the clones on 293T cells in the presence of 8 μg of Polybrene/ml at the indicated time points.

a ratio of 1:1 with fresh or heat-inactivated human serum and incubated at 37°C for 1 h. After incubation, each virus was titered on 293T cells in the presence of Polybrene. The results of this experiment, expressed as a percentage of virus titer upon incubation with heat-inactivated human sera, are shown in Fig. 1. Consistent with previous observations, HIV-1 pseudotyped with the VSV-G Env shows poor stability in fresh human sera, while HIV-1(MLV-A) virions are quite stable (9, 31). The stability of HIV-1(RRV) in fresh human sera was similar to that of HIV-1(MLV-A). Another characteristic that is advantageous for the ex vivo and in vivo use of HIV vectors is good thermostability, i.e., the ability to resist inactivation both at 37°C and during freeze-thaw cycling. HIV-1(RRV), HIV-1(MLV-A), and HIV-1(VSV-G) were incubated as previously described (31) at 37°C for 1, 3, or 6 h or subjected to three rounds of freeze-thaw cycling between −80 and 37°C. HIV-1(RRV) also showed good stability at 37°C (Fig. 2A), and was resistant to freeze-thaw, compared to HIV-1(MLV-A) and HIV-1(VSV-G) (Fig. 2B).

The effect of spinoculation on RRV Env pseudotype titer. We next considered the use of centrifugal inoculation, or spinoculation, of HIV-1(RRV) onto cells to enhance ex vivo cell transduction, as this may be a useful technique to employ in conjunction with concentration. To examine the effect of spinoculation on HIV-1(RRV) infection of tissue culture cell lines, the titration of virus on 293T cells was carried out with or without spinoculation and in the presence and absence of Polybrene. The results from this experiment are shown in Fig. 3. The presence of Polybrene enhanced HIV-1(RRV) infection on 293T cells up to sevenfold, whereas spinoculation had no effect on HIV-1(RRV) titer. Consistent with our previous observations (31), spinoculation had little effect on HIV-1(VSV-G) titer, while the effects of Polybrene and spinoculation on HIV-1(MLV-A) titer appeared to be additive.

Enhancement of HIV-1(RRV) titer by concentration. It is often necessary to concentrate viral stocks, either to enhance viral titer for ex vivo transduction or to allow the injection of high-titer stocks into animal models. We therefore investigated how efficiently HIV-1(RRV) produced from STAR cells might be concentrated in comparison to HIV-1(VSV-G). HIV-

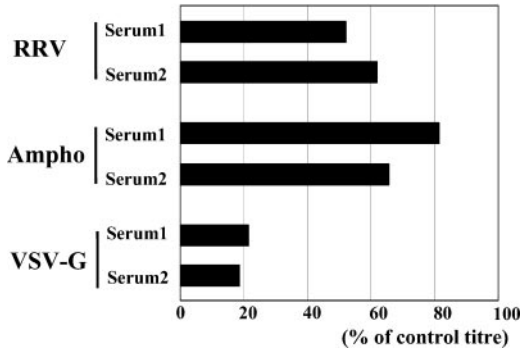

FIG. 1. Alphavirus pseudotype inactivation in fresh human sera. MLV-A (Ampho) and RRV pseudotypes were harvested from STAR cells. HIV(VSV-G) was produced by transient transfection of 293T cells. Each virus was incubated (37°C for 1 h) 1:1 with heat-inactivated FCS and fresh or heat-inactivated human sera. Titrations were carried out on 293T cells in the presence of 8 μg of Polybrene/ml. eGFP titer was determined by FACS. Titer after incubation with fresh or heat-inactivated human sera is represented as a percentage of the viral titer after incubation with heat-inactivated FCS. Serum 1 and Serum 2 refer to different sera from different donors.

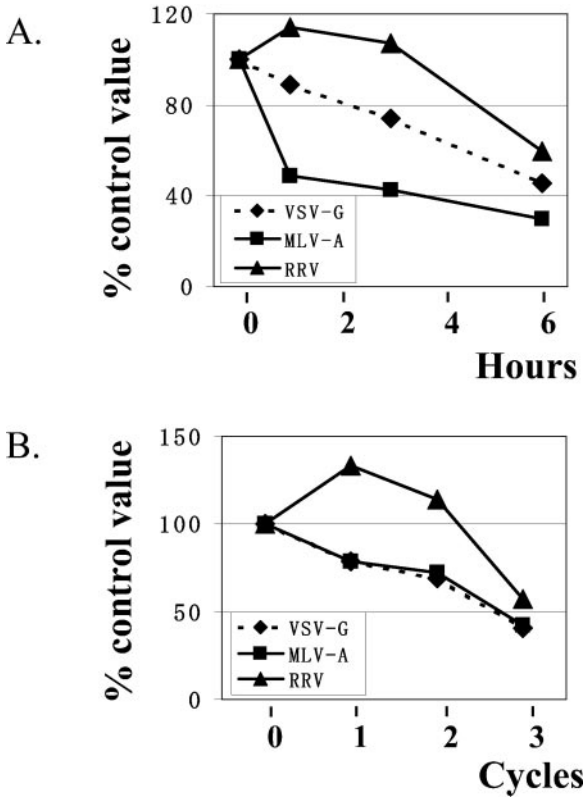

FIG. 2. Stability of alphavirus pseudotypes. Virus was titrated onto 293T cells in the presence of 8 μg of Polybrene/ml after incubation at 37°C for certain time points (A) or after a number of cycles between 37 and −80°C (B). eGFP titer was determined by FACS. Control titer is virus titer before treatment. This result is representative of two independent experiments.

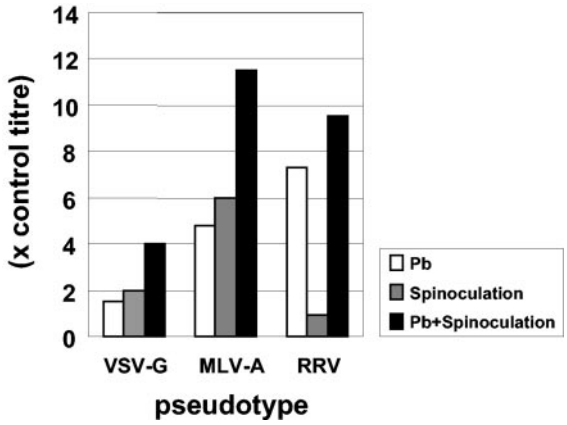

FIG. 3. Effect of Polybrene and spinoculation on HIV-1(RRV) infection. Each pseudotype was titrated onto 293T cells and spun down at 1,200 × *g* for 2 h at 25°C or titrated onto 293T cells in the presence of Polybrene (Pb) (8 μg/ml), or both. eGFP titer was determined by FACS. Values of relative titer compared to control titer in the absence of Polybrene and without spinoculation are shown. Data for HIV-1(RRV) are representative of at least two independent experiments.

1(VSV-G) was generated by transient transfection of the VSV-G expression plasmid into the HIV vector-harboring STAR cells. Supernatants were titrated onto 293T cells before and after concentration by ultracentrifugation (10^5 × *g* for 1.5 h) and centrifugation (3,000 × *g* for 8 h). Both ultracentrifugation and low-speed centrifugation methods could efficiently increase the HIV-1(RRV) titers. However, HIV-1(RRV) virus recovery after concentration was poor, especially by low-peed concentration, compared to that of HIV-1(VSV-G) (Table 2).

Recently, we showed that soluble gammaretrovirus Env concentrated with HIV-1 particles could compete with pseudotypes for their cellular receptor, thereby inhibiting infection at a high dose (31). Thus, we examined if this was the case with HIV-1(RRV) by attempting to achieve more than 95% infection at a high dose by using the concentrated viral stocks described above. We used human 293T and mouse NIH 3T3 cell lines as target cells. As expected, the infectivity of HIV-1(VSV-G) was dose dependent, and we could achieve 100% infection with a high multiplicity of infection (Table 3). Transduction efficiency of HIV-1(RRV) infection, however, did not reach more than 85% even at an MOI of 25 (Table 3).

TABLE 2. Concentration of alphavirus pseudotypes

Envelope	eGFP titer (% recovery after concn)[b]		
	Unconcentrated	After centrifugation at[a]	
		10^5 × *g*/1.5 h	3000 × *g*/8 h
VSV-G	59	220 (93.2)	180 (76.3)
RRV	7.6	82 (53.9)	41 (27.0)

[a] Alphavirus pseudotypes were concentrated 20-fold and HIV-1(VSV-G) was concentrated 4-fold by centrifugation at 10,000 or 3,000 × *g* Virus was titrated on to 293T cells with 8 μg of Polybrene/ml, and eGFP expression was assayed by FACS.

[b] Titer is expressed as 10^5 IU/ml. Percent recovery [(100 × titer after concentration)/(concentration factor × unconcentrated titer)] is shown in parentheses.

TABLE 3. Correlation of virus dose and infection[a]

Target cells	Envelope	% Infection at MOI of			
		0.2	2	8	25
293T	VSV-G	18	74	97	100
	RRV	19	57	78	80
	MLV-A	22	78	96	66
NIH3T3	VSV-G	16	66	90	100
	RRV	20	55	76	82
	MLV-A	18	77	98	74

[a] The indicated cell lines were inoculated with the indicated virus dose. Percent eGFP expression was determined by FACS.

Unlike amphotropic MLV pseudotype, we did not observe any decrease in infectivity of HIV-1(RRV) at high dose.

Cell specificity of RRV Env-pseudotyped vector. To examine the range of cells that could be infected by HIV-1(RRV), we first infected a range of cell lines from different origins (Fig. 4). HIV-1(SFV) was also tested in order to determine if tropism was common to the two alphavirus pseudotypes. Although HIV-1(RRV) transduced human HeLa, 293T, NP2, TE671, HT1080, and BC3 cell lines, the Rhesus monkey FrhK-4 cell line, the murine NIH 3T3 cell line, the cat CRFK cell line, and the quail QT6 cell line efficiently, we could not detect an eGFP-positive population in the HIV-1(RRV)-infected human and mouse T-cell lines (Jurkat and EL4, respectively) as well as a mouse mast cell-like line, P815. HIV-1(SFV) also transduced the cells from each species, although the infectivity of HIV-1(SFV) was in general about 20 times lower than that of HIV-1(RRV) in the tested cell lines. The titers of HIV-1(SFV) were under detectable levels (<500 IU/ml) in mouse EL4 and P815 and rhesus monkey FrhK-4 cells, while the SFV Env pseudotypes could transduce the human T-cell line Jurkat (4,000 IU/ml). There was, therefore, no strict similarity in tropism between the two alphavirus pseudotypes. In contrast, VSV-G-pseudotyped vectors could efficiently transduce all cell lines tested.

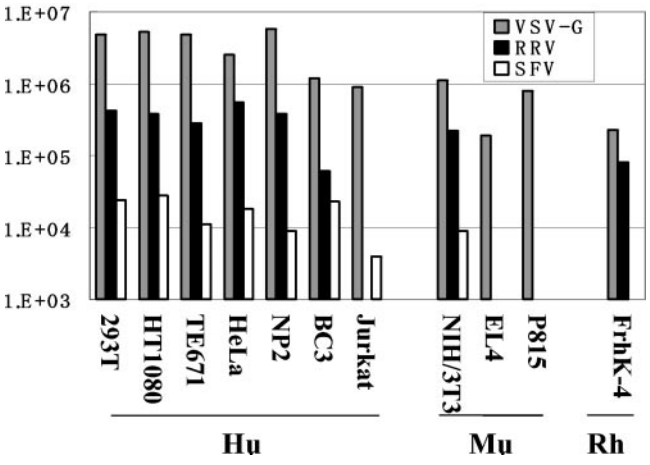

FIG. 4. Infection of cell lines derived from several species. Titrations were carried out on the cell lines indicated in the presence of 8 μg of Polybrene/ml. eGFP titer was determined by FACS. Averages of two independent experiments are shown. Minimum level of detection by FACS in these experiments was 5 × 10^3 IU/ml. Hu, human; Mu, murine; Rh, rhesus monkey.

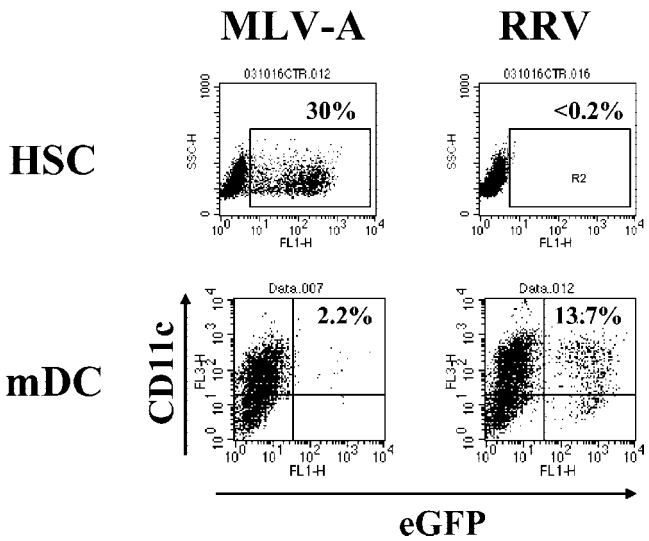

FIG. 5. Transduction of human HSC and C57BL/6-derived mouse dendritic cells (mDC). Vector transduction was examined by FACS. Mouse DC were also stained by anti-CD11c DC monoclonal antibody conjugated with Cy5.

Infection of human hematopoietic progenitor cells. Next, we examined the ability of HIV-1(RRV) to transduce human HSC, as permanent gene transfer to HSC has the potential to provide a cure for monogenic disorders of the hematopoietic system. Thus, we tested the ability of HIV-1(RRV) virions to transduce human cord blood-derived $CD34^+$ hematopoietic progenitor cells. $CD34^+$ cells were cultured for 24 h in serum-free medium with the cytokines SCF, Flt-3 ligand, and TPO and transduced on Retronectin preloaded with HIV-1(RRV) or HIV-1(MLV-A). Transduction efficiency was determined by flow cytometry to detect expression of eGFP 48 h after transduction. Although HIV-1(MLV-A) could transduce 30 to 60% of the HSC, we could not detect eGFP-positive cells when HSC were challenged with HIV-1(RRV) (Fig. 5).

Infection of human and murine dendritic cells. We further tested how efficiently HIV-1(RRV) pseudotypes transduce primary cell cultures of gene therapy targets. DC are necessary for the initiation of most T-cell-mediated immune responses. Antigen presentation to lymphocytes by DC can lead to the generation of antigen-specific effector T cells. Recently, we and others reported the application of lentivirally modified DC for tumor immunotherapy (11, 20, 25).

Bone marrow cells from BALB/c or C57BL/6 mice were cultured with GM-CSF for 5 days. These immature mouse DC were then challenged with HIV-1(RRV) and HIV-1(MLV-A) produced from STAR cells as well as transiently produced HIV-1(VSV-G). Transduction efficiency was examined 7 days after infection. As depicted in Table 4, HIV-1(RRV) efficiently transduced murine DC. The relative efficiency of HIV-1(RRV) infection is evident when the input viral titers of HIV-1(RRV), HIV-1(VSV-G), and HIV-1(MLV-A) were adjusted to the same MOI (MOI of 3). As reported previously (11, 20, 25), HIV-1(VSV-G) efficiently transduced murine DC, particularly at a high dose. HIV-1(MLV-A), meanwhile, poorly transduced mouse DC.

We also tested the ability of HIV-1(RRV) to transduce human DC. Human DC were prepared from the peripheral blood of healthy human donors and challenged with the same pseudotypes. Phytohemagglutinin-activated peripheral blood lymphocytes (PBL) harvested during preparation of DC were also tested. Although HIV-1(VSV-G) efficiently transduced human DC, transduction of human DC by HIV-1(RRV) and HIV-1(MLV-A) was inefficient (Table 4). For transduction of human activated PBL, HIV-1(MLV-A) was most efficient. Consistent with the HIV-1(RRV) transduction result with a human T-cell line, Jurkat (Fig. 4), no infection of human PBL could be observed with HIV-1(RRV) infection.

TABLE 4. Infection of murine and human primary cell cultures[a]

Envelope	MOI	% Infected cells (±SD)			
		mDC (B6)	mDC (B/c)	hDC	hPBL
RRV	3	14.3 ± 4.2	13.4 ± 1.4	1.9 ± 1.1	<0.1
MLV-A	32	2.0 ± 0.3	1.6 ± 0.6	1.9 ± 0.1	26.5 ± 6.4
MLV-A	3	0.8 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	6.8 ± 2.8
VSV-G	24	53.9 ± 12.7	38.5 ± 2.4	18.7 ± 11.2	7.1 ± 0.3
VSV-G	3	8.4 ± 2.1	5.9 ± 0.6	6.3 ± 0.8	3.5 ± 1.0

[a] Primary cell cultures of murine DC (mDC) from BALB/c (B/c) and C57BL/6 (B6) mice, human DC (hDC), and human peripheral blood lymphocytes (hPBL) were infected with the indicated pseudotypes. Shown in the table is the percentage of cells infected. Vector inputs were adjusted to the same MOI (MOI, 3) or to the same volume [MOI of 32 for HIV-1(MLV-A) and MOI of 24 for HIV-1 (VSV-G)]. MOI was determined by titration of pseudotypes onto 293T cells in the presence of 8 μg of Polybrene/ml in parallel with primary cell culture infection.

DISCUSSION

Alphavirus glycoproteins possess characteristics attractive for use in pseudotyping HIV-1 vectors. First, they have extremely broad host range, including insects, reptiles, birds, and mammals, most likely due to their use of multiple cellular factors or ubiquitous proteins as receptors (12, 32). Thus, HIV-1 vectors pseudotyped with their glycoproteins could extend the range of tissues that can be transduced in both humans and animal models. Second, since the alphaviruses are transmitted by arthropods, they should be stable in the bloodstream (12, 32), suggesting that alphavirus glycoprotein-pseudotyped vectors would be useful in systemic delivery. Third, unlike VSV-G, alphavirus glycoproteins can be stably expressed (29).

Here, we examined whether alphavirus glycoproteins E2E1 could be used in our stable HIV vector packaging cell line STAR (13). Indeed, we could obtain several STAR cell clones stably expressing RRV or SFV E2E1 (Table 1). Two RRV clones constantly produced 2×10^5 to 4×10^5 IU of HIV-1(RRV)/ml for more than 5 months. During screening of our clones, however, we noticed that many RRV or SFV E2E1-positive clones ceased producing infectious vectors after 5 to 11 weeks in culture (Table 1). This observation suggests that expression of RRV or SFV E2E1 was in general not as stable as MLV-A Env expression in STAR cells. We speculate that the expression of RRV and SFV proteins might be slightly cytotoxic in the context of STAR cells, which already express HIV-1 proteins (Gag, Pol, Rev, and Tat). It is also possible that HIV-1(RRV), like VSV-G pseudotypes (1), superinfects its own producers, causing genotoxic effects. Therefore, the cells

gradually lose the Env expression, making virions eventually noninfectious.

Another possible explanation for loss of infectious vector production in RRV and SFV but not MLV-A Env-stable clones may be the difference in expression systems between the alphavirus and MLV-A Env constructs. The MLV-A Env is expressed by the MLV long terminal repeat, a construct that has been successfully used in long-term, high-titer retroviral-lentiviral vector production (8, 13). In contrast, alphavirus Env expression is controlled by the cytomegalovirus immediate-early promoter, which has been reported to be silenced by methylation during prolonged culture (18, 22). If this is the case, we may be able to improve the stability of RRV and SFV Env expression by cloning the alphavirus Env genes into the MLV long terminal repeat construct. It is, however, noted that a level of vector production similar to the transient system was maintained long term in several HIV-1(RRV) producer lines.

HIV-1(RRV) pseudotypes displayed a number of characteristics which would be advantageous in ex vivo and in vivo experiments. HIV-1(RRV) pseudotypes are resistant to inactivation by heat-labile components in fresh human sera (Fig. 1) and exhibit good thermostability at 37°C (Fig. 2A), which should be advantageous in systemic gene delivery. We also demonstrated that the HIV-1(RRV) can be concentrated by ultracentrifugation, although the vector recovery after concentration was not as good as that for HIV-1(VSV-G) (Table 2). Upon single-step concentration of RRV pseudotypes by ultracentrifugation (reduction in volume of 200-fold), we could achieve titers of up to 6×10^7 IU of HIV-1(RRV) stocks/ml (data not shown). Our concentration results are comparable to those obtained with RRV Env-pseudotyped feline immunodeficiency virus (FIV)-based vector (15). In that study, Kang and coworkers achieved titers of 5×10^4 to 5×10^5 IU/ml prior to concentration and 2×10^7 to 1.5×10^8 IU/ml after concentration (reduction in volume of 250-fold).

The ratio of cells transduced with VSV-G-pseudotyped MLV or HIV-1 vectors generally corresponds closely to $1-e^{(-MOI)}$, as predicted from a Poisson distribution of transduction of the entire cellular population. At high MOI, VSV-G-pseudotyped vectors transduce more than 95% of the culture population and can achieve multiple transduction of more than 10 copies per cell (1, 31). Such transduction characteristics are believed to be due to high-level expression of its cellular receptor on the target cells. It is thought that phosphatidylserine is a receptor for VSV or, at least, acts as part of the cellular receptor complex for VSV-G (19, 28). Given the extremely broad host or cell range of the wild-type RRV, we expected that the transduction kinetics of HIV-1(RRV) would be similar to those of HIV-1(VSV-G). Unexpectedly, the transduction kinetics of HIV-1(RRV) were not dose dependent and reached a plateau at high MOI on human 293T and NP2 as well as mouse NIH 3T3 cell lines (Table 3). Unlike HIV-1(MLV-A), however, we did not observe any inhibitory effects at MOI 25 with the RRV pseudotypes. Since particle-free, soluble MLV-A Env in the HIV-1(MLV-A) preparation plays a major role as an inhibitor of HIV-1(MLV-A) infection (31), we speculate that the RRV E2E1-stable STAR cells do not shed soluble RRV glycoproteins into the culture supernatant or that glycoproteins shed into the supernatant cannot interact with RRV cellular receptors. Indeed, given HIV-1(MLV-A) titer, but not HIV-1(RRV) titer, can be improved by spinoculation (Fig. 3), it would appear that the Envs on these two pseudotypes interact with their cellular receptor(s) by different mechanisms.

HIV-1(RRV) could efficiently transduce cell lines from different species including human, rhesus monkey, mouse, cat, and quail but failed to transduce human and mouse T-cell lines, human primary T cells, and human HSC (Table 4 and Fig. 4). These results indicate that RRV can utilize the cellular receptors from different host species but that their expression is limited or absent on certain cell types such as T cells or HSC. We believe failure to transduce the cells mentioned above is most likely due to the lack or absence of cellular receptor expression on those cells, not a postentry block to infection, given the efficient transduction of HIV-1(VSV-G) on all cell lines. Previously, Kang and coworkers (15) demonstrated that in vivo administration of FIV(RRV) resulted in efficient transduction of hepatocytes and neuroglial cells, while transduction of neurons, skeletal muscle, or airway epithelial cells was inefficient. Again, we speculate that the inability to transduce these cell types may be due to limited receptor expression or the absence of receptor(s).

DC are potent antigen-presenting cells that play a critical role in the initiation of host immune responses. Genetically modified DC, which express certain antigens, have been used to elicit antigen-specific, major histocompatibility complex-restricted cytotoxic-T-lymphocyte responses (30). Recently, we and others demonstrated that lentiviral transduction of murine DC in vitro and in vivo is a powerful immunotherapeutic strategy (10, 25). Although infectivity of RRV in DC has not been characterized, in vitro studies demonstrate that RRV can infect murine macrophage (17). We therefore reasoned that HIV-1(RRV) might efficiently transduce mouse DC. This appeared to be the case (Table 4). The RRV-pseudotyped vectors could efficiently transduce bone marrow-derived premature DC from BALB/c and C57BL/6 mice. Since FIV(RRV) causes less cytotoxicity in comparison to FIV(VSV-G) after systemic delivery (15), in vivo administration of HIV-1(RRV) may be a useful tool in immunotherapeutic strategies.

In conclusion, HIV-1 virions can be pseudotyped with RRV and SFV glycoproteins, and stable HIV packaging lines producing HIV-1(RRV) and HIV-1(SFV), equivalent in titer to transient production, were established. HIV-1(RRV) virions derived from STAR cells display characteristics, such as resistance to inactivation in fresh human sera, good stability at 37°C, and efficient concentration by ultracentrifugation, that would be advantageous in ex vivo and in vivo experiments. Furthermore, HIV-1(RRV) can be used to transduce cells from many species, including murine DC. Our data, however, indicate that the cellular receptor(s) for RRV is not as ubiquitous as that of VSV-G. Identification of the cellular receptor(s) for RRV will be helpful for a better understanding of how HIV-1(RRV) can be applied in the future.

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