

RESEARCH ARTICLE

Characterization of HIV-1 vectors with gammaretrovirus envelope glycoproteins produced from stable packaging cells

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We have recently described a novel, stable human immunodeficiency virus type 1 (HIV-1) vector packaging system, STAR. High-titre HIV-1 vectors bearing gammaretrovirus envelopes (Env) are continuously produced from STAR cells. Here we compare the properties of such vectors, with the amphotropic murine leukaemia virus (MLV-A) Env, a modified gibbon ape leukaemia virus (GALV) Env and two modified versions of the cat endogenous retrovirus RD114 Env, produced from STAR cells, to transiently produced HIV-1 vectors with vesicular stomatitis virus G protein (VSV-G). Our results indicate that gammaretrovirus pseudotypes from STAR cells are relatively stable at 37°C and are resistant to

inactivation by freeze/thaw cycling or incubation with human sera. HIV-1(VSV-G) was, however, sensitive to freeze/thaw when harvested in serum-free media and was readily inactivated in human sera. Furthermore, the titre of 'gammaretrovirus' pseudotypes, but not HIV-1(VSV-G), could be increased by the use of a combination of polybrene and spinoculation. All pseudotypes could be efficiently concentrated, but soluble gammaretrovirus Env could act as an inhibitor of infection.

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Introduction

Vectors based on human immunodeficiency virus type 1 (HIV-1) can deliver therapeutic transgenes to a variety of cell types, both dividing and nondividing. HIV-1-based vectors have previously been produced either transiently¹ or using packaging cell lines in which vector production is induced.^{2–4} Until recently, no stable packaging cells to produce continuously high-titre HIV-1 vectors were available, because it has been difficult to stably express large amounts of HIV-1 Gag-Pol. Furthermore, it has also proven to be difficult to produce continuously the rhabdovirus vesicular stomatitis virus G protein (VSV-G), which is most commonly used to replace the HIV-1 envelope proteins and 'pseudotype' HIV-1 vector particles.^{1,5–7}

Recently, we have developed a stable, constitutive HIV-1 packaging system, STAR, based on 293T cells.⁸ This system rapidly and reproducibly generates large batches of high-titre vectors. High-level expression of HIV-1 Gag-Pol was achieved by transducing 293T cells with a murine leukaemia virus (MLV) vector encoding a codon-optimized *gag-pol* gene.⁶ Subsequently, the HIV-1 regulatory proteins Tat and Rev, for efficient vector genome production, gammaretrovirus envelopes (Env)

and HIV-1 vector genomes were introduced, resulting in continuous high-titre recombinant vector production.⁸ The Env were from three gammaretrovirus strains, amphotropic MLV (MLV-A), gibbon ape leukaemia virus (GALV)⁹ and the feline endogenous virus RD114,¹⁰ which unlike VSV-G have been successfully used in stable packaging cell lines for MLV vectors. These Env use distinct cellular receptors, all of which are found on a wide variety of human cell types.¹¹ MLV vectors bearing these Env have been used for the transduction of primary cells such as lymphocytes and CD34⁺ progenitor cells in both preclinical^{12–14} and clinical^{15–18} applications.

The mature gammaretrovirus Env consists of a trimer of heterodimers. Each dimer is composed of the extracellular surface subunit (SU) and the membrane spanning subunit (TM). In the mature protein, SU and TM are connected via non-covalent interactions and a labile disulphide bond.^{19,20} Given the relative weakness of these interactions, thermostability and the ability of infectious particles to be concentrated are dictated by Env, as dissociation of the surface and transmembrane envelope subunits inactivates the virus. Resistance of vectors to inactivation by human complement, which is partly determined by Env, is an important characteristic for *in vivo* applications.²¹

High-titre HIV-1 vectors produced from STAR cells and pseudotyped with gammaretroviral Env may be advantageous for several reasons. Firstly, the time and costs of vector production for research or clinical

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applications would be reduced. Secondly, large batches of HIV-1-based vector could be reproducibly generated, and a single set of exhaustive safety tests could then be carried out. Furthermore, virus producer cells could be co-cultivated long term with target cells, such as haematopoietic stem cells, or injected *in vivo* without any decrease in vector production. Therefore, information on the basic properties of these vectors is important in order to assess their utility. In this study, HIV vectors bearing the MLV-A Env, a modified GALV Env and two modified versions of the RD114 Env were produced from STAR cells and their characteristics were examined in comparison to transiently produced HIV-1(VSV-G).

Results and discussion

Generation of STAR cells producing gammaretrovirus pseudotypes

HIV-1 vectors pseudotyped with the Env derived from MLV-A, RD114 and GALV have been produced in transient systems.^{14,22,23} Previous studies have shown that the titre of lentivirus vectors pseudotyped with the Env from GALV and RD114 can be improved with the substitution of their cytoplasmic tail by that of the MLV envelope.^{22–24} Therefore, constructs with these substitutions, GALV +²⁵ and RD +,²⁴ were used. It is possible that these modifications facilitate gammaretrovirus Env function by enhancing reaction between HIV-1 protease (PR) and Env.^{22,24} Therefore, as an alternative strategy to enhance PR–Env interaction, the RD114 Env was also modified by replacing the R peptide cleavage site sequence with that of a matrix-capsid cleavage site in HIV-1 Gag to create RDpro.⁸

The envelope expression plasmids for MLV-A, GALV +, RD + and RDpro were transfected into STAR cells and clonal cell lines for envelope expression were obtained. These clones were infected with HIV-1(VSV-G) carrying the vector HV, encoding eGFP.⁸ The envelope clones producing the highest titre virus were selected. The vector rescue was dependent on gammaretrovirus Env and not due to carryover of VSV-G because HV was not rescued from STAR cells without Env in a parallel experiment. Culture supernatant was harvested after 4–16 weeks cell cultivation and analysed for Env incorporation and infectivity on several cell lines.

Figure 1 shows immunoblotting of pellets from STAR cell supernatants concentrated by ultracentrifugation. The presence of vector particles was demonstrated for all STAR cell lines, but not for parental 293T cells, by the presence of HIV-1 p24. Antibodies raised against the RD114 SU recognized the RD114 gp70 in the supernatant from STAR cells expressing RD + or RDpro envelopes (Figure 1, lanes 5 and 6). The same membrane was probed with polyclonal antibodies raised against an MLV envelope SU. It has been shown that these antibodies strongly recognize the MLV-A SU but show only weak crossreaction with the GALV SU.²⁶ Using this serum, the MLV-A but not GALV SU was detected (Figure 1, lanes 3 and 4). A monoclonal antibody (42/411) that recognizes an epitope in the ectodomain of TM from both MLV-A and GALV²² did not react with the two RD114 constructs, but detected both immature (p15) and mature (p12) forms of the MLV-A and GALV + envelope TMs (Figure 1, lanes 3 and 4).

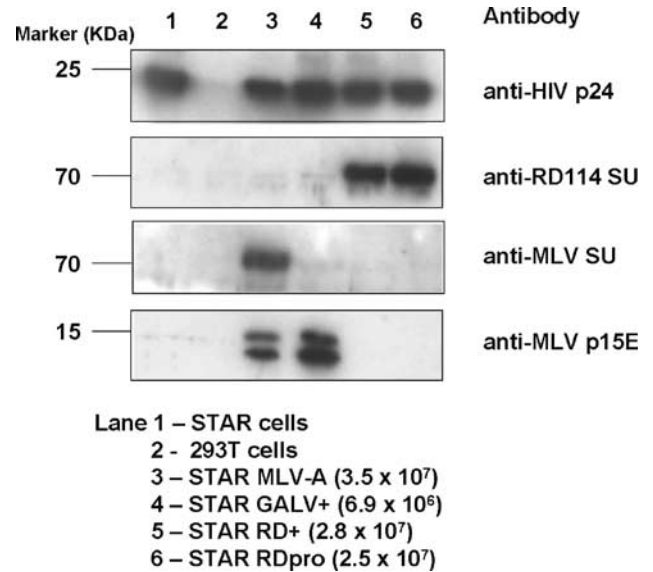


Figure 1 Incorporation of gammaretrovirus envelopes in HIV-1 vectors. In all, 8 ml of the supernatant was concentrated by ultracentrifugation and the pellet resuspended in 30 μ l of loading buffer. A volume of 15 μ l of each sample was run on 10% (three upper panels) or 14% (bottom panel) polyacrylamide gels. After transfer, membranes were probed with a 1:1 mixture of the murine monoclonal antibodies ADP365 and ADP366 raised against HIV CA (anti-HIV p24), goat polyclonal serum raised against RD114 SU (anti-RD114 SU), goat polyclonal serum raised against Rauscher MLV SU (anti-MLV SU) or rat monoclonal antibodies 42/411 raised against MLV TM (anti-MLV p15E). The position of protein markers (in kDa) are shown. The eGFP titre of each virus supernatant on 293T cells in the presence of polybrene is also shown.

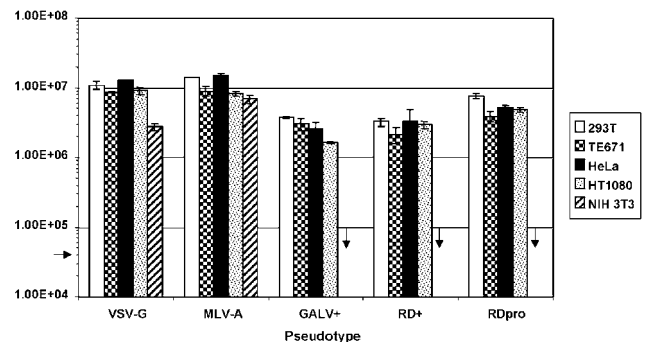


Figure 2 Titration of HIV-1 vectors on a range of cell lines. HIV-1 vectors pseudotyped with gammaretrovirus envelopes (MLV-A, GALV +, RD + and RDpro) were harvested in OptiMEM from STAR cells or, in the case of HIV-1(VSV-G), harvested in OptiMEM during transient virus production. Each pseudotype was titrated onto the human cell lines 293T, TE671, HeLa and HT1080 or the murine cell line NIH 3T3 in the presence of polybrene (8 μ g/ml). Titre was assayed by FACS 48 h post infection. Detection limit of 4×10^4 IU/ml is indicated by arrows. The titre of parallel harvest from STAR cells with the eGFP vector but without envelope was less than the detection limit.

eGFP titre was compared to transiently produced HIV-1(VSV-G) (Figure 2). HIV-1 vectors bearing gammaretrovirus Env had titres in the range of 10^6 – 10^7 IU/ml on the human cell lines tested, with MLV-A the highest and GALV + lowest. These titres were similar to or slightly lower than that of HIV-1(VSV-G). It should be noted that the supernatants of STAR cell lines had p24 in the range

of 450–650 ng/ml (data not shown). This compares to about 100 ng/ml for transiently produced HIV-1(VSV-G), indicating less infectivity per vector particle for stably produced gammaretroviral pseudotypes than for transiently produced HIV-1(VSV-G).⁸ As expected vectors with GALV+, RD+ and RDpro did not infect murine NIH3T3 cells, because mice do not have functional receptors. Correct receptor usage was further demonstrated by receptor interference: HIV-1(MLV-A) did not infect TE671 cells chronically infected with replication-competent MLV-A, HIV-1(GALV+) did not infect TE671 cells chronically infected with GALV, and HIV-1(RD+) and HIV-1(RDpro) did not infect TE671 cells chronically infected with RD114 (data not shown). No crossinterference was observed.

Stability at 37°C

Stability of pseudotyped HIV-1 vectors during storage and transduction is an important practical consideration. We examined HIV-1 vectors with gammaretrovirus Env and HIV-1(VSV-G) for stability at 37°C, sensitivity to freeze/thaw cycles and inactivation by human sera. In these experiments, vectors were harvested in either OptiMEM or DMEM + 10% FCS, as the absence of FCS in the vector preparation is desirable in many gene therapy applications.

To assess vector stability, decay of infectivity was measured after incubation at 37°C. Aliquots of each virus in OptiMEM or DMEM + 10% FCS were incubated at 37°C and titrated on 293T cells 2 and 6 h after the start of incubation (Figure 3). No substantial difference in stability at 37°C between virus harvested and incubated in OptiMEM *versus* that in DMEM + 10% FCS was observed. HIV-1(MLV-A) and HIV-1(RDpro) appeared to be least stable with a half-life less than 2 h, while HIV-1 bearing either the GALV+ or RD+ appeared to be slightly more stable over 2 h incubation (half-life between 2 and 6 h). The HIV-1(VSV-G) virus, meanwhile, had lost only up to 40% of its original titre after 6 h incubation at 37°C. Virus titration after 24 h incubation at 37°C showed that HIV-1(VSV-G) titre had fallen by 90% (data not shown). The half-life of this virus at 37°C was therefore shorter than 24 h.

Resistance to freeze/thaw cycling

Vector stability during freeze/thaw cycles was then examined by titrating virus on 293T cells, after cycling

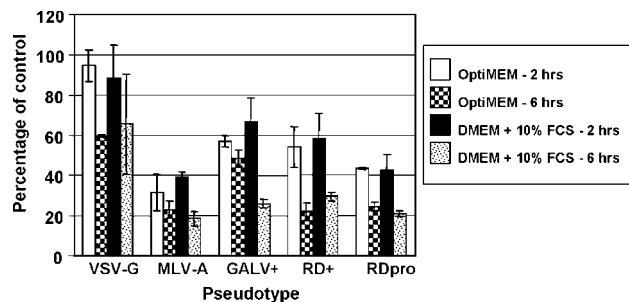


Figure 3 Stability of HIV-1 vectors at 37°C. Vectors were harvested in either OptiMEM or DMEM + 10% FCS. Vector stocks were incubated at 37°C and titrated at different time points. eGFP titre on 293T cells in the presence of 8 µg/ml polybrene is presented as a percentage of the viral titre before incubation. Values shown are the mean of two experiments and the error bars show the actual data points.

between –80 and 37°C. The titre is presented as a percentage of titre before commencing the first cycle (Figure 4). All gammaretrovirus pseudotypes showed some resistance to freeze/thaw. After one cycle, the MLV-A pseudotyped vector lost less than 5% of its original titre in either media. All other retroviral pseudotypes appeared to be more sensitive to freeze/thaw, although after three cycles no retroviral pseudotype lost greater than 50% of its original titre. There was no substantial difference in stability between vectors harvested in OptiMEM and DMEM + 10% FCS. This, however, was not the case for the HIV-1(VSV-G) vector. After one cycle the virus harvested in OptiMEM lost up to 65% of its original titre, while virus titre in DMEM + 10% FCS appeared to be stable. Moreover, after three cycles HIV-1(VSV-G) vector in OptiMEM lost 90% of its original titre while that in DMEM + 10% FCS lost less than 10%. While the reason for this difference is unclear, conditions for freezing HIV-1(VSV-G) may need careful optimization.

Resistance to inactivation in fresh human sera

Each virus, in either OptiMEM or DMEM + 10% FCS, was incubated at 37°C for 1 h with an equal volume of fresh or heat-inactivated human serum and then titrated on 293T cells. A percentage of the viral titre after incubation with fresh frozen serum in relation to incubation with the corresponding heat-inactivated serum is shown (Figure 5). All gammaretroviral pseudotypes in both media exhibited good stability when

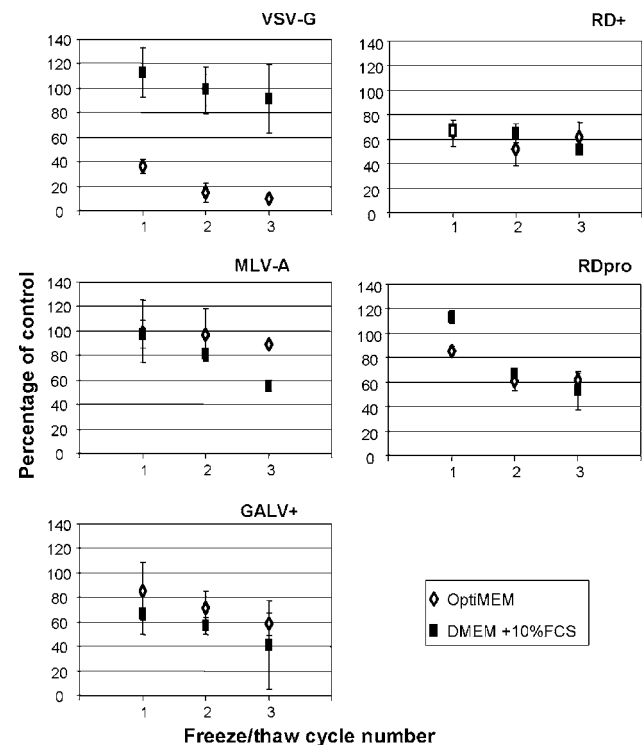


Figure 4 Resistance of HIV-1 vectors to freeze/thaw cycling. Pseudotyped HIV vectors were frozen and thawed at –80 and 37°C, respectively, up to three times. Relative eGFP titre to the control titre before initiating the first cycle on 293T in the presence of 8 µg/ml polybrene is presented in percentages. Values shown are the mean of two experiments and the error bars indicate the actual data points.

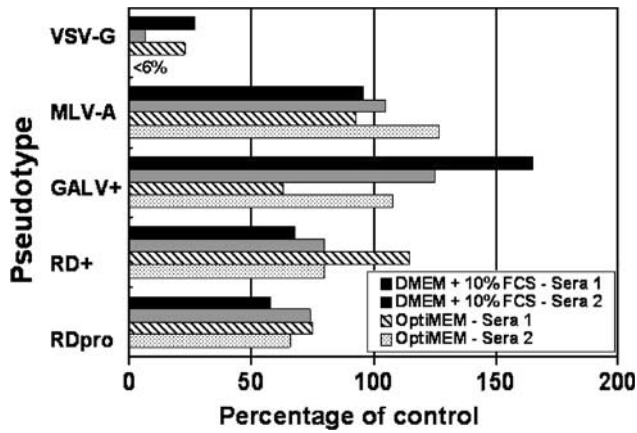


Figure 5 Resistance of HIV-1 vectors to human sera. Pseudotyped HIV vectors harvested in either OptiMEM or DMEM + 10% FCS were exposed to human sera. Titre on 293T cells in the presence of 8 µg/ml polybrene is represented as a percentage of the viral titre after incubation with fresh frozen serum in relation to incubation with the corresponding heat-inactivated serum (100% corresponds to the titre after treatment with heat-inactivated serum). Sera harvested from two different healthy donors were used (Sera 1 and 2). Values shown are the mean of two experiments. In the two experiments, viral titre did not vary by greater than two-fold. Values below 6% of the control titre could not be detected by FACS.

exposed to human sera. HIV-1(MLV-A) lost at most 8% of titre while those bearing the GALV + envelope showed a loss of titre (27%) in one instance. The highest reduction in retroviral pseudotype titre of HIV-1(RDpro) harvested in DMEM + 10% FCS exposed to Serum 1 is no greater than 40%. HIV-1(VSV-G) virus, in contrast, appeared to be sensitive to inactivation by fresh human sera. There was no substantial difference in serum sensitivity between vectors with different gammaretrovirus Env in this study. In contrast, we previously reported that replication-competent MLV-A was more sensitive than RD114 or GALV when produced by galactosyl(α1-3)galactosyl (αGal)-negative human cells.²⁷ The reason for this difference is unclear.

HIV-1(VSV-G) was more sensitive to inactivation in fresh human serum than HIV-1 vectors pseudotyped with gammaretroviral Env. Exposure to Serum 1 causes a loss of up to 80% of titre, whereas incubation with Serum 2 caused a drop in titre of this virus to below the detection threshold. These results are consistent with data by DePolo *et al*²⁸ indicating that a VSV-G pseudotyped HIV vector is readily inactivated in human serum by complement. Furthermore, our results and those of DePolo *et al* are consistent with previous observations that wild-type VSV replicating in human cells, devoid of the xenogeneic αGal antigen, were sensitive to inactivation in fresh human serum, although less so than VSV bearing αGal antigens.^{29,30}

Enhancement of vector titre using polybrene and spinoculation

We investigated vector titre enhancement by the use of polybrene and centrifugal inoculation (spinoculation). Pseudotype vectors were harvested in OptiMEM. Each pseudotype was titrated onto 293T cells in the presence of polybrene, or with spinoculation, or both. The results from these experiments are shown in Figure 6. Polybrene

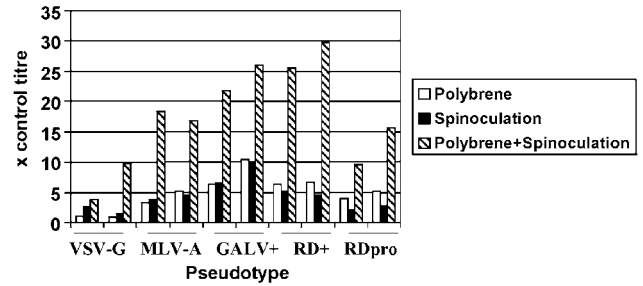


Figure 6 Effect of polybrene and spinoculation on HIV-1 vector infection. HIV-1 vectors were harvested in OptiMEM. Each pseudotype was titrated on 293T cells and spun down at 1200 g for 2 h at 25°C or titrated on 293T in the presence of polybrene (8 µg/ml) or both. Values of relative titre to control titre in the absence of polybrene and without spinoculation are shown for two independent experiments.

enhanced infection of HIV-1 vectors with gammaretrovirus Env on average 5–6 ×, whereas spinoculation enhanced infection on average 4 ×. The presence of polybrene did not substantially enhance HIV-1(VSV-G) titre. When polybrene and spinoculation were used together, their effect in all cases was additive.

The enhancement effect of polybrene on different cell lines (the human cell lines 293T, TE671, HeLa and HT1080 and the murine cell line NIH 3T3) was also investigated (data not shown). Polybrene did not raise HIV-1(VSV-G) titre by greater than 2 × on any cell line tested. The effect of polybrene on gammaretrovirus pseudotype titre was most notable on 293T or TE671 cells (8–16 ×), while its effect is less dramatic on HeLa or HT1080 cells (2–4 ×). The infection enhancement of polybrene on NIH 3T3 cells was negligible.

Concentration of STAR cell derived pseudotypes

Concentration of vector stocks is often necessary. Ultracentrifugation, low-speed centrifugation and centrifugal filtration (or ultrafiltration) have been used to concentrate HIV-1(VSV-G) vectors.^{1,31,32} Among gammaretrovirus pseudotypes, only HIV-1(MLV-A) has been examined for its susceptibility to concentration by ultracentrifugation and centrifugal filtration. We therefore carried out a comparative study, concentrating the four different pseudotypes by four different methods. Vectors bearing MLV-A, GALV + and RDpro Env as well as VSV-G were harvested in OptiMEM and concentrated 40-fold in volume by centrifugal filtration using a filter with a cutoff of 100 kDa, or centrifugation at three different speeds: 100 000 g for 1.5 h; 10 000 g for 1.5 h; 3000 g for 7 h. Before and after concentration, the percentage of eGFP-transduced cells was measured in two-fold serial dilutions on 293T cells in the presence of polybrene. Figure 7 shows examples of the titration curves for HIV-1(MLV-A) and HIV-1(VSV-G). eGFP titre and % recovery of each vector preparation after the concentration procedure were estimated using % transduction data for dilutions in linear range of titration and are shown in Table 1.

Limited damage to vectors by concentration was observed, as vector recovery was more than 50% in most experiments (Table 1). In some cases, recovery was more than 100%, which may be due to either removal of factors

inhibiting infection or concentration of factors increasing stability and/or infectivity of the vector. Centrifugation at 10 000 or 3000 g generally resulted in higher recovery for gammaretroviral pseudotypes. HIV-1(VSV-G) may be more stable than HIV-1 vectors with gammaretrovirus Env when concentrated at higher ultracentrifugation

speeds or by centrifugal filtration. Also, in an extension of this study, it was noted that greater concentration, up to 100-fold, could be achieved with high yield by one-step centrifugation at either 10 000 or 3000 g, resulting in a titre of 2.0×10^8 – 1.5×10^9 IU/ml (data not shown).

While HIV-1(VSV-G) achieved 100% infection at high doses after concentration in all conditions (Figure 7a), gammaretroviral pseudotypes could only achieve 100% infection on 293T cells when viral supernatant was subjected to milder conditions of centrifugation (Figure 7b for HIV-1(MLV-A), data not shown for HIV-1(GALV+) and HIV-1(RDpro)). At high doses, infection of gammaretrovirus pseudotypes was inhibited when virus particles were concentrated by either ultracentrifugation at a speed of 100 000 g or centrifugal filtration.

It has been previously reported³³ that supernatant from an MLV vector packaging cell line, FLYA13,¹⁰ contains large amounts of free MLV-A Env. Excess soluble Env appeared to inhibit vector transduction, competing with vector particles for their cellular receptor.^{34,35} To determine if soluble MLV-A Env was concentrated with vector particles during centrifugal filtration and inhibits infection of HIV(MLV-A) from STAR cells, the concentrated preparation was fractionated by Sepharose gel filtration. Gel filtration fractions were analysed for infectivity and for the presence of HIV-1 capsid (CA) and MLV-A Env (Figure 8a and b). eGFP infectivity and HIV-1 p24 were detected in the flow-through fractions, 3–5, which were capable of efficient transduction (95–100% transduction using 20 μ l) compared to 5% transduction using 20 μ l of the column load (Figure 8c). This indicates that gel filtration separated an inhibitor(s) from the vector particles. An immunoblot probed with anti-MLV SU revealed that majority of MLV-A Env SU appeared in fractions 10–13 (Figure 8b). In order to test for inhibition of infection, fractions 9–22 were mixed with HIV-1(MLV-A) or HIV-1(RDpro) and titrated onto 293T cells (Figure 8c). Substantial reduction of eGFP transduction by HIV-1(MLV-A), but not HIV-1(RDpro), was observed for fractions 10–13, which contain most MLV-A Env. This suggests that the non-virion-associated envelope present in fractions 10–13 is the most probable inhibitor of HIV-1(MLV-A) infection, presumably by competing for cellular receptors.

Our results show inhibition of infection when STAR cell supernatants are concentrated by centrifugal filtration using a filter with a cutoff of 100 kDa, or centrifugation at 100 000 g for 1.5 h. There does not,

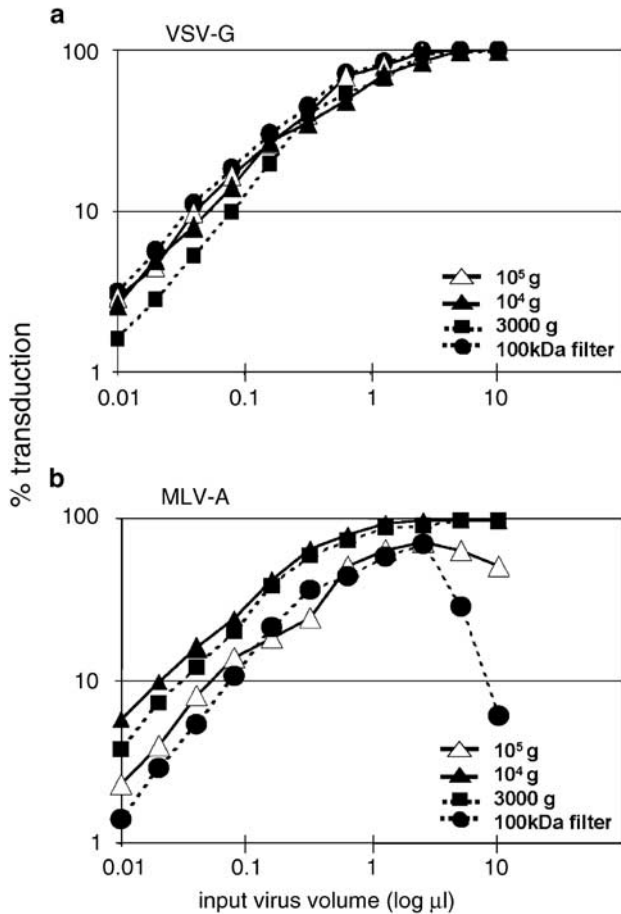


Figure 7 Titration of concentrated HIV-1 vectors. HIV-1(VSV-G) (a) and HIV-1(MLV-A) (b) harvested in OptiMEM were concentrated 40-fold by a range of methods: ultracentrifugation at 100 000 and 10 000 g, low-speed centrifugation at 3000 g and centrifugal filtration using Centricon-20 filters (100 kDa filter). Each concentrated stock was diluted in a two-fold series and titrated onto 293T cells in the presence of 8 μ g/ml polybrene. eGFP-transduced cells were counted by FACS.

Table 1 Concentration of HIV-1 vectors

Envelope	Unconcentrated	Centrifugation			
		10 ⁵ g	10 ⁴ g	3000 g	100 kDa filter ^a
VSV-G	1.5 ^b	56 (92) ^c	52 (85)	28 (47)	36 (60)
MLV-A	2.1	40 (60)	100 (117)	72 (180)	28 (32)
GALV+	0.5	11 (55)	18 (90)	9 (45)	10 (50)
RDpro	1.5	45 (75)	136 (226)	59 (98)	13 (21)

^aThe volume of virus was reduced 40-fold by centrifugation at 100 000, 10 000 or 3000 g and centrifugal filtration using a 100 kDa cutoff filter. Virus was titrated on 293T cells with 8 μ g/ml polybrene and eGFP expression assayed by FACS.

^bTitre is expressed as 10⁷ IU/ml.

^c% recovery (100 \times titre after concentration)/(40 \times unconcentrated titre) is shown in parentheses.

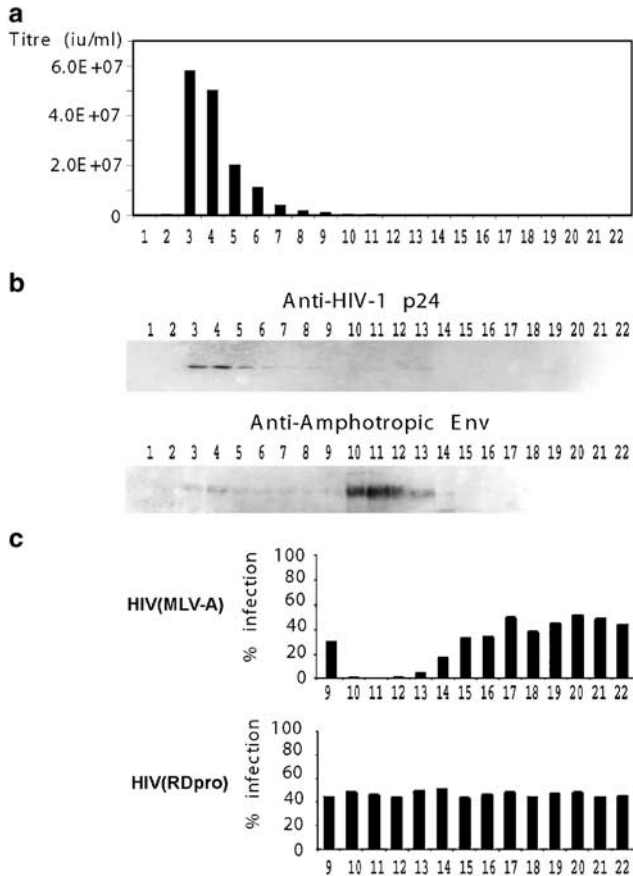


Figure 8 Gel filtration analysis of HIV(MLV-A) preparation by centrifugal filtration. HIV-1(MLV-A) pseudotypes were harvested in OptiMEM and subjected to concentration by centrifugal filtration. The resulting concentrated stock was fractionated by Sepharose gel filtration. Aliquots of each fraction were analysed for eGFP transduction (a) and the presence of HIV-1 CA and MLV-A Env by Western blot using antibodies against CA and Env gp70 (b). Aliquots from fractions 9–22 were mixed with fixed doses of either HIV(MLV-A) or HIV(RDpro) and then plated onto 293T cells in the presence of 8 µg/ml polybrene. Per cent eGFP transduction was measured by FACS (c).

however, appear to be inhibition of infection at high dose when virus particles are concentrated at lower speeds. Centrifugation at speeds between 3000 and 10 000 g could, therefore, be used in applications that require the preparation of concentrated virus stocks. Furthermore, low-speed centrifugation, albeit taking longer time, may be more suitable for concentrating large batches of vector stocks.

In summary, HIV vectors produced from STAR cells and pseudotyped with gammaretroviral Env can infect human cells as efficiently as VSV-G pseudotyped HIV-1 vectors. They are more stable in fresh human serum and can be more effectively used with polybrene and spinoculation in *ex vivo* applications than HIV-1(VSV-G). They are resistant to freeze/thaw, but their decay rate at 37°C is faster than that of HIV-1(VSV-G). This may be a disadvantage in systemic gene delivery, but potentially advantageous where local gene transfer without unwanted vector spread is required. Soluble Env in vector preparation may be problematic when concentration and/or high-dose vector input are required. Vector centrifugation at lower speed, however, circumvents this

problem. These results, along with the ease of quality control in stable, continuous vector production compared to that in transient or inducible systems, support further consideration of preclinical and clinical application of HIV-1 vectors from STAR cells pseudotyped with gammaretroviral Env.

Materials and methods

Cell lines

All cells were maintained at 37°C, 10% CO₂, in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml), with the exception of NIH 3T3 cells, which were maintained in DMEM supplemented with 10% donor calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). RD + Env sequence from pRD + plasmid²⁴ was introduced into RDL plasmid,¹⁰ resulting in RD + L plasmid. STAR RD + cells were generated by transfecting RD + L plasmid into STAR cells and used to produce an eGFP-encoding HIV-1 vector, HIV-1(RD+), as previously described.⁸ STAR Ampho, STAR RDpro and STAR GALV + cells producing eGFP-encoding HIV-1 vectors, HIV-1(MLV-A), HIV-1(RDpro) and HIV-1(GALV+) respectively, were previously described.⁸

Viral vector preparation

Viruses were harvested from 10 cm plates. For virus harvest from STAR cells, 4 × 10⁶ cells were plated 24 h before the start of virus harvest. Virus was harvested in 8 ml of either DMEM + 10% FCS, plus penicillin and streptomycin or OptiMEM (GibcoBRL) for 48 h at 37°C. Viral supernatant was then passed through a 0.45-µm-pore-size filter. HIV-1(VSV-G) virus was generated by transient transfection of 293T cells with a weight ratio of 3:2:1 of vector (pHV⁸) to packaging (pCMVΔR8.91⁷) to envelope (pMD-G¹) plasmids using Lipofectamine (GibcoBRL) as per the manufacturer's instructions. After washing with OptiMEM, virus was harvested for 48 h at 37°C in 8 ml of either DMEM + 10% FCS, plus penicillin and streptomycin or OptiMEM. Viral supernatant was then passed through a 0.45-µm-pore-size filter.

Viral titre determination

In all, 2 × 10⁵ cells were inoculated with serial dilutions of viral supernatant. At 48 h after infection, eGFP titres (IU/ml) were determined by using a fluorescence-activated cell scanner (FACS). The effect of passive transfer of the eGFP protein on the titre determination by FACS analysis has been excluded by the following observations: (1) eGFP-positive cell population remained fluorescent when typical transduced cell cultures were maintained for longer cultivation up to 2 weeks; (2) our titre determination was based on the experiments where eGFP-positive (gene-transduced) and -negative (with possible protein transfer) populations were well separated as shown previously.⁸ Where indicated, titrations were carried out in the presence of 8 µg/ml polybrene (hexadimethrine bromide (Sigma)) or with spinoculation (1200 g, 2 h, 25°C) or both.

Western blotting

Virus in 8 ml of the supernatant was pelleted for analysis by ultracentrifugation in an SW41 Beckman Rotor (30 000 rpm, 1 h, 4°C). Pellets were resuspended in 30 µl of 6 × loading buffer diluted in OptiMEM. Samples were boiled for 5 min and frozen at -20°C until further analysis. Samples were run on 10 or 14% polyacrylamide (SDS) gels. Protein was then transferred onto Hybond ECL nitrocellulose filters (Amersham) using semi-dry transfer apparatus and transfer buffer (39 mM glycine, 48 mM Tris base, 20% methanol).

The TM subunit of MLV-A and GALV envelopes was detected using undiluted supernatant from the rat hybridoma 42/114.³⁶ MLV-A SU was detected with goat polyclonal anti-Rauscher leukaemia virus gp70 (Quality Biotech Inc., Camden, NJ, USA), diluted 1/1000. RD114 SU was detected with goat polyclonal anti-RD114 gp70 (Quality Biotech Inc., Camden, NJ, USA), diluted 1/5000. HIV-1 CA was detected with a 1:1 mixture of the murine monoclonal antibodies ADP365 and ADP366 (MRCARD) both diluted 1/200. MLV capsid (CA) was detected with goat polyclonal anti-Rauscher leukaemia virus gp70 (Quality Biotech Inc., Camden, NJ, USA), diluted 1/1000. Blots were developed with horseradish peroxidase-conjugated anti-immunoglobulin (DAKO), diluted 1/1000, and an enhanced chemiluminescence (ECL) kit (Amersham Life Science).

Preparation of human sera

Human peripheral blood was clotted on ice overnight at 4°C. Serum was separated from the clot and 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).

Vector concentration

HIV-1(VSV-G) and gammaretrovirus pseudotypes were harvested in OptiMEM. In all, 10 ml of each supernatant was concentrated at 10⁵ g using a Beckman L7 ultracentrifuge (SW41 rotor, 35 000 rpm, 1.5 h, 4°C) and the pellet resuspended in 250 µl of OptiMEM. A Beckman L7 ultracentrifuge was also used to concentrate virus at 10⁴ g (SW41 rotor, 15 000 rpm, 3 h, 4°C). Again the pellet was resuspended in 250 µl of OptiMEM. A volume of 30 ml of the supernatant was concentrated at 3000 g (4000 rpm, 8 h, 4°C) using a Heraeus Megafuge 2.0R bench top centrifuge (Sepatech). The pellet was resuspended in 750 µl of OptiMEM. Virus was concentrated by centrifugal filtration using Centricon Plus-20 (Amicom) filters as per the manufacturer's instructions. Then, 16 ml of the supernatant was added to each filter and the virus recovered in 400 µl.

Gel filtration

In all, 100 µl of concentrated viral supernatant was loaded on a Sepharose G PC 3.2/30 column (Amersham) previously equilibrated in OptiMEM. The fractionation was performed using an LKB:µSeparation unit (Amersham) controlled using Smart Manager software. The flow rate of the column was maintained at 40 µl/min and 22 fractions of 100 µl were collected. A measure of 25 µl of each fraction of the gel filtration was mixed with 6 × loading buffer, boiled and then separated on 10%

polyacrylamide (SDS) gels and probed as described above.

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