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VIRAL TRANSFER TECHNOLOGY ***RESEARCH ARTICLE***

Gene transfer into stimulated and unstimulated T lymphocytes by HIV-1-derived lentiviral vectors

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Genetic modification of T lymphocytes holds great potential for treatments of cancer, T cell disorders and AIDS. While in the past recombinant murine retroviruses were the vectors of choice for gene delivery to T cells, vectors based on lentiviruses can provide additional benefits. Here, we show that VSV-G pseudotyped HIV 1 vector particles delivering the enhanced green fluorescent protein (EGFP) efficiently transduce human T lymphocytes. Transduction efficiency was optimal when infection included centrifugation of cells with concentrated vector supernatant in the presence of Polybrene. In contrast to previous reports describing murine retrovirus-mediated gene transfer to T lymphocytes, fibronectin did not improve the transduction efficiency of the VSVG-pseudotyped HIV-1 particles. Similar gene transfer efficiencies were observed following stimulation of cells with PHA/IL-2 or anti-CD3i/CD28i antibodies, although greater transgene expression was observed in the latter case. Interestingly, production of vectors in the absence of the accessory proteins Vif, Vpr, Vpu and Nef was accompanied by a 50% decrease in transduction efficiency in activated T cells. Transduction of T cells that were not stimulated before infection was achieved. No transduction of non-prestimulated cells was observed with a GALV-pseudotyped murine retroviral vector. The requirement for accessory proteins in non-prestimulated cells was more pronounced. Our results have implications for lentiviral vector targeting of other cells of the hematopoietic system including stem cells. Gene Therapy (2000) **7,** 596–604.

Keywords: *gene transfer; gene therapy; lentiviral vector; T lymphocyte; EGFP; fibronectin*

Introduction

Genetic modification of T lymphocytes offers potential correction or treatment of diseases, including certain inherited disorders, eg adenosine deaminase deficiency; and acquired diseases, eg acquired immune deficiency syndrome (AIDS) and cancer. To date the most widely investigated method for gene transfer to human T cells has involved murine retroviral vectors.[1–3] These vectors are appealing as tools for gene therapy because they have a relatively large cloning capacity, do not transfer viral genes and the transferred therapeutic gene is stably integrated into the chromosome of the host cell. However, efficient infection depends on expression by the target cell, of the receptor for the amphotropic retroviral envelope. This potential obstacle can be overcome by the use of alternative viral envelopes that target distinct receptors on the target cell, eg the gibbon ape leukemia virus (GALV) envelope or the G protein of vesicular stomatitis virus (VSV). An additional drawback of murine retroviral vectors is the inability to transduce cells which do not undergo division following infection. Retroviral vectors based on lentiviruses do not have this limitation. These vectors may be a useful alternative for gene therapy of human T cells.

Retroviruses belong to a family of RNA viruses whose replication involves the production of a DNA intermediate. The simpler retroviral genomes have three genes, *gag*, *pol* and *env*. The more complex lentiviruses, as well as encoding Gag, Pol and Env, also encode several regulatory and accessory proteins. HIV-1, for example, encodes the regulatory proteins Rev and Tat and the accessory proteins Vif, Vpr, Vpu and Nef. Vectors based on HIV-1 have been described.[4] Most are produced by co-transfection of three plasmid constructs: (1) a packaging construct containing all the HIV-1 genes except env; (2) an expression plasmid for the G protein of VSV; and (3) a transfer vector containing a promoter-driven gene of interest. These vectors have been shown to infect a wide range of different cell types *in vitro* and *in vivo*. Notably, transduction of non-dividing cells has been demonstrated, including growth arrested fibroblasts and differentiated macrophages in culture and neuronal cells, hepatocytes, and myocytes *in vivo*.[5–8]

A primary concern over the use of lentiviral vectors is their biosafety. Several modifications to the producer system described above have been made to avoid toxicity and reduce the possibility of producing replication-competent HIV-1. Viral accessory genes, which are associated with virulence, have been eliminated from the vector genome,[9,10] genes on the packaging plasmid have been further separated on to two plasmids[11] and production of self-inactivating vectors has been described.[12] Rev independent vector systems have also been proposed.[8,13] More recently, a vector system which packages an HIV-1 transfer cassette into a simian immunodeficiency virus particle has been developed.[14]

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Vectors based on HIV may be ideally suited to deliver anti-HIV genes. The HIV long terminal repeat (LTR) and the Rev responsive element (RRE) present on the transfer vector RNA genome may compete with those of a HIV-1 viral genome, effectively acting as decoys for Tat and Rev activity, thus impairing viral replication.[15] In addition, vector RNA may compete with genomic RNA for packaging and thus be mobilised to other target cells. Human $CD4^+$ T lymphocytes are a natural host for infection by HIV-1. Non-pseudotyped HIV-1-derived vectors have been shown to transduce $CD4^+$ T cell lines.[16–18] Matsuoka *et al*[19] demonstrated transduction of human primary T cells with non-pseudotyped HIV-1-based vectors but the apparent efficiency of transduction was extremely low. In this study, we show that (1) VSVG-pseudotyped lentiviral vectors efficiently transduce primary T lymphocytes, and we describe adaptations to the transduction protocol that resulted in high levels of gene transfer. (2) The lentiviral vectors are able to transduce freshly isolated T cells in the absence of any prestimulation, whereas a murine retroviral vector was unable to do so under the same conditions. (3) HIV accessory proteins are required for maximal transduction of T cells, particularly when the cells were unstimulated before infection.

Results

Optimisation of VSVG-pseudotyped lentiviral vector-mediated gene transfer to T-lymphocytes

Modifications, in recent years, to protocols for retrovirus-mediated gene delivery to T lymphocytes have resulted in improved gene transfer efficiencies. Such modifications include incubation of vector supernatant and target cells in the presence of polycations such as Polybrene, co-centrifugation of vector and cells[20] and co-localisation of retrovirus and target cells on the extracellular matrix molecule fibronectin.[21–23] We first examined whether using VSVG-pseudotyped lentiviral vectors, we could accomplish the same levels of gene transfer that are currently achieved with optimised protocols for murine retrovirus-mediated gene transfer to T cells. To generate infectious HIV-1 based vectors containing all accessory proteins, a packaging plasmid PCWT (see Materials and methods, Figure 1a), a transfer vector containing the EGFP gene under the control of the murine pgk promoter (see Materials and methods, Figure 1b), and a plasmid expressing the VSVG protein from a CMV promoter (pMD.G[5], Figure 1c) were co-transfected into 293T cells. Following concentration, vectors with a titer (on HeLa cells) in excess of 1×10^7 were obtained. T cells were activated, following isolation of PBMCs, for 48 h with PHA/IL-2. This treatment led to a cell population, 90% of which were T cells, as assessed by anti-CD3 staining and FACS analysis (data not shown). The cells were transduced at a nominal (based on HeLa cells) MOI of 0.6. This resulted in transduction of approximately 4% of the cells (Figure 2a), indicating that the effective MOI of T cells, calculated using the Poisson distribution, was approximately 0.04. Incubation of the vector supernatant and cells in the presence of 5 μg/ml Polybrene (higher concentrations of Polybrene were toxic to T cells, data not shown) resulted in an approximate two-fold increase in the percentage of cells transduced. Centrifugation of vector supernatant and cells during infection was accompanied by a five-fold increase in transduction efficiency. The combination of both Polybrene and centrifugation resulted in a slightly higher percentage of cells transduced (six-fold increase over control). Thus, both Polybrene and centrifugation enhanced transduction efficiency although the fold-enhancement seen with centrifugation was higher than that measured with Polybrene.

Several reports have shown that transduction of T lymphocytes on human fibronectin improved efficiency of retrovirus gene transfer.[21,23] PBMCs were stimulated with immobilized antibodies to CD3 and CD28 (CD3i/CD28i), a treatment which results in the selective growth of T cells. We measured the percentage of EGFP positive cells following transduction on plates coated with BSA or recombinant fibronectin fragments (Figure 2b). Only a modest increase in transduction was found when fibronectin was used. This small increase (approximately 1.1-fold) in transduction efficiency with fibronectin was consistently observed in multiple experiments ($n = 7$) and depended neither on the MOI nor on the mode of stimulation. In contrast, fibronectin-mediated transfer was significantly enhanced using a GALV-pseudotyped murine retroviral vector (data not shown), demonstrating that the effect of fibronectin depends upon the nature of the pseudotyped envelope.

Nevertheless, upon optimisation of the lentivirus protocol by including a centrifugation step with Polybrene, and a (nominal) MOI in excess of two, transduction efficiencies of 30% or greater were obtained. Figure 2c shows the result of lentiviral vector infection of stimulated T cells from three independent donors. Transduction efficiencies ranged from 35 to 46%, with a mean of 40%. It should be noted that in general, the levels of EGFP positive cells observed on day 3 after transduction were 1.5- to 1.9-fold higher than those observed on day 5 after transduction. From day 5 onwards, the percentage of EGFP+ cells remained relatively stable for 14 days after transduction. Thus, the initial high level of transduction measured, likely reflects pseudo-transduction or transient expression from non-integrated vector DNA.[24]

To determine whether both $CD4^+$ and $CD8^+$ T lymphocytes were transduced, EGFP fluorescence and CD4 or CD8 expression were monitored concurrently. Equivalent percentages of $CD4^+$ and $CD8^+$ cells were found to be positive for EGFP (Figure 2d) indicating that both populations are equal targets for transduction by the lentiviral vector.

Effect of T cell activation on VSVG-pseudotyped lentivirus-mediated gene transfer

Human peripheral blood lymphocytes are predominantly in a resting state but can be activated *in vitro* by a variety of methods. Protocols for murine retrovirus-mediated gene transfer included a T cell activation step to induce cells to divide. The manner in which T lymphocytes are activated has been shown to affect efficiency of transduction by retroviral vectors.[21,23,25] We compared the levels of transduction observed with lentiviral vectors following stimulation of PBMCs for 48 h with CD3i/CD28i or with PHA and IL-2. On day 5 after transduction a similar percentage of EGFP positive cells was observed with both methods of stimulation (Figure 3a), although we consistently found that the efficiency of transduction was donor-dependent. Interestingly, while the percentage of cells

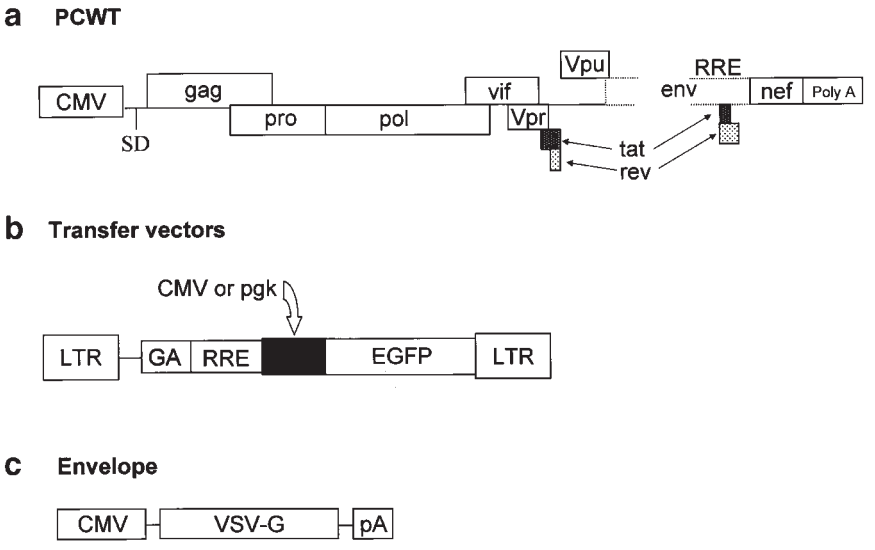


Figure 1 *Schematic representation of (a) the packaging construct, (b) transfer vectors and (c) the envelope expression vector.*[5]

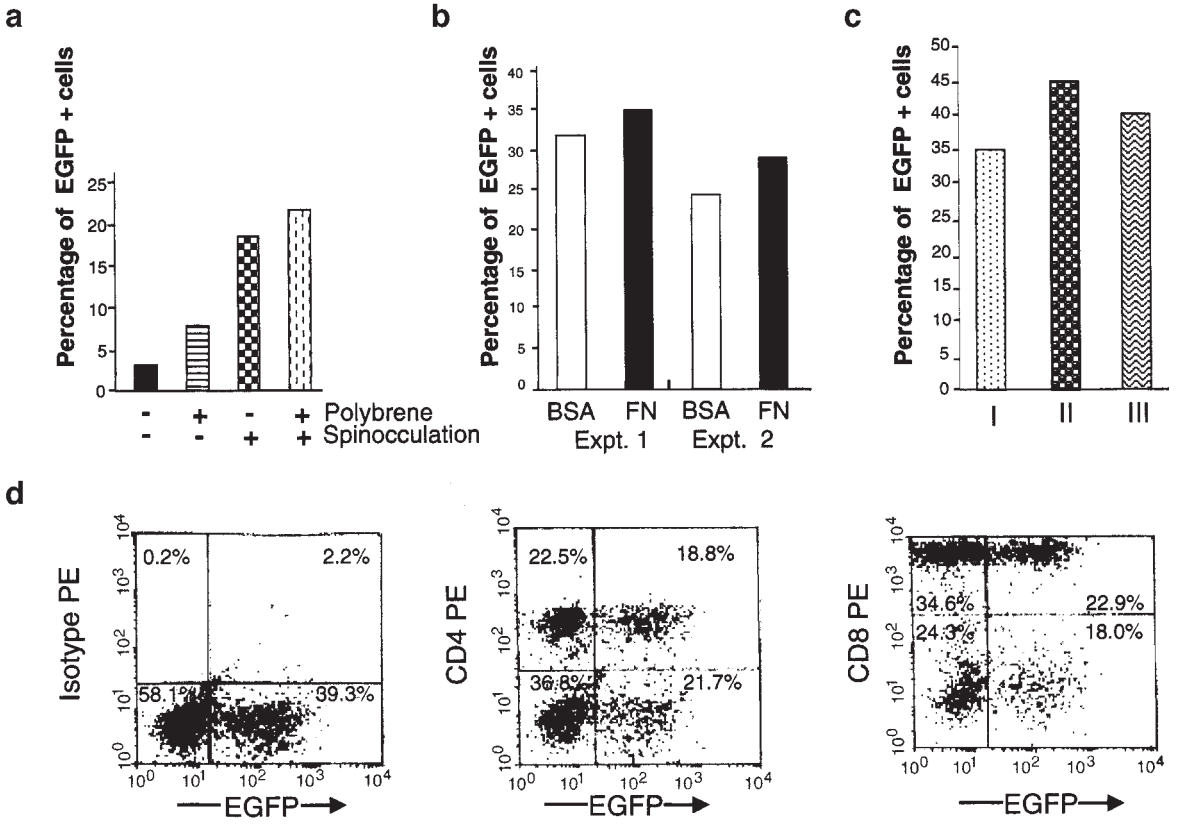


Figure 2 *Optimisation of VSVG-pseudotyped lentiviral vector-mediated transduction of T lymphocytes. (a) T lymphocytes, stimulated for 48 h with PHA/IL-2 were transduced with the PCWT vector (MOI = 0.6) for 3 h in tubes in the absence/presence of Polybrene (5 μg/ml) or with/without centrifugation as indicated. Following transduction, cells were processed as described in Materials and methods and EGFP expression measured 5 days later. (b) PBMCs were stimulated for 48 h with CD3i/28i and subsequently transduced by incubation for 4 h in 100 μl of PCWT supernatant on BSA-coated plates or on CH-296 (fibronectin)-coated plates. Following transduction, cells were washed, expanded in medium containing IL-2 and analysed for EGFP expression 5 days later. The results are representative of seven independent experiments. (c) PBMCs from three independent donors (I to III) were stimulated for 48 h with PHA/IL-2 and subsequently transduced by centrifugation of 1×10^6 cells with 50 μl of a PCWT supernatant (MOI = 2.5) in the presence of Polybrene as above. Following transduction, cells were washed, expanded as before and analysed for EGFP expression 5 days later. (d) The phenotype of the transduced cells was assessed by monitoring both EGFP fluorescence and CD4 or CD8 expression. Percentages of cells are indicated in each quadrant. Similar results were obtained in three independent experiments.*

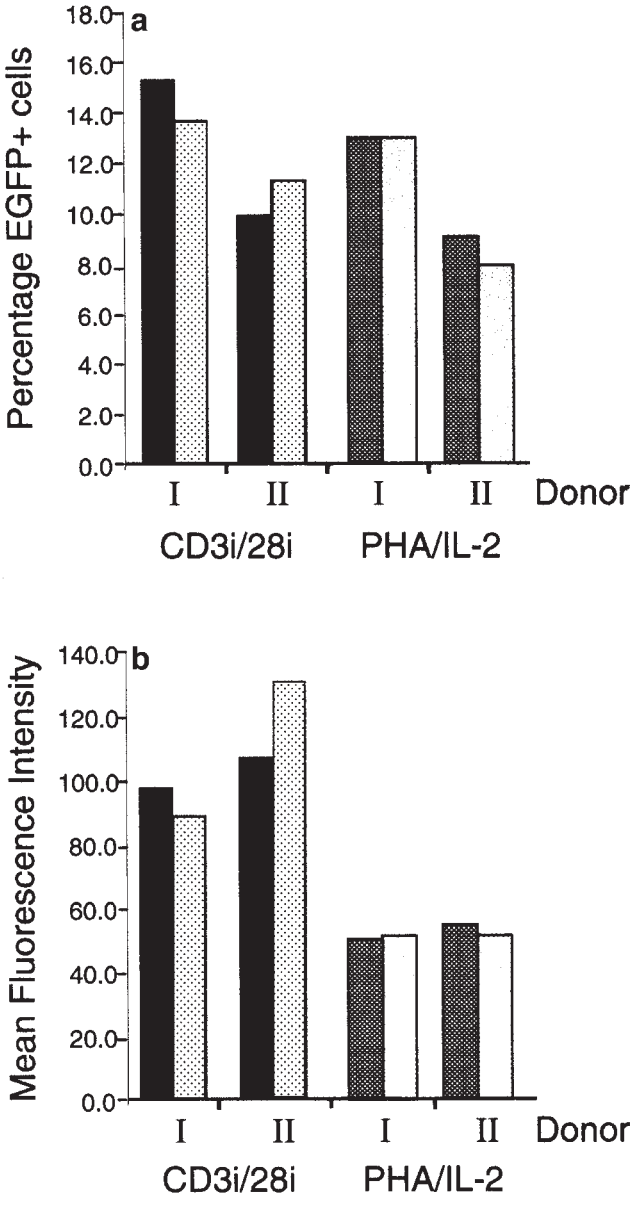


Figure 3 *Effect of T cells stimulation on the efficiency of lentivirus transduction. PBMCs from two donors (I and II) were stimulated for 48 h with CD3i/CD28i or with PHA/IL-2 and subsequently transduced, in duplicate, as follows: 5×10^6 cells were centrifuged at 22°C, 1500 g for 3 h with 50 μl of PCWT vector supernatant (MOI = 1.6) in the presence of Polybrene. Following transduction, cells were washed and expanded in medium containing IL-2 (50 U/ml). EGFP expression was assessed 5 days later. The percentage of EGFP expressing cells for each duplicate is shown (a). The mean fluorescence intensity (MFI) of each duplicate transduction is plotted (b).*

transduced was similar following either method of stimulation, the level of EGFP expression, as assessed by mean fluorescence intensity (MFI), was two-fold greater in CD3i/CD28i stimulated cells (Figure 3b). However, in the absence of continuous CD3i/CD28i stimulation this difference was not maintained. At 11 and 14 days after transduction, EGFP expression in CD3i/CD28i-stimulated cells decreased to that observed in PHA/IL-2-stimulated cells (MFI approximately 50).

Higher transduction efficiencies were obtained by vectors produced in the presence of the accessory proteins of HIV-1

The biosafety of lentiviral vectors derived from HIV-1 is a legitimate concern. Although the risk of insertional mutagenesis is the same as for retroviral vectors, the proteins encoded by HIV accessory genes may have toxic or otherwise harmful effects in the target cells. However, they may also play a positive role in the transduction process. The requirement for lentiviral accessory proteins to establish efficient transduction is cell type dependent.[7,8,10] We examined whether vectors produced in the absence of Vif and Vpr, Vpu and Nef would transduce activated T lymphocytes as efficiently as those produced in the presence of these accessory proteins. The vectors used for these experiments were produced by co-transfection of the pgk-EGFP transfer vector and the VSVG expression plasmid (pMD.G) into 293T cells together with either pCMVΔR8.2 or pCMVΔR8.9 packaging plasmids. pCMVΔR8.2 encodes all HIV-1 proteins except Env while pCMVΔR8.9 does not encode Env, Vif, Vpr Vpu or Nef.[10] Viral supernatants with equivalent MOIs, as determined on Hela cells, were then used to infect T lymphocytes which had been pre-stimulated for 48 h. Transduction efficiencies ranging from 30% to 36% (mean 32.7%) were observed in three donors with vector produced from the packaging construct containing all the HIV-1 accessory genes (Table 1). Interestingly, infection with vector produced in the absence of the HIV-1 accessory genes resulted in a two-fold decrease in transduction efficiency: the percentage of transduced cells ranged from 14.9 to 17.1, with a mean of 16.2. Thus, although HIV accessory proteins are not strictly required for T lymphocyte transduction, their presence enhances this process.

Transduction of non-stimulated T cells

One reason for developing lentiviral vectors is the ability of lentiviruses to infect non-cycling cells, in contrast to murine retroviruses, which need host-cell-DNA replication for infection. We therefore attempted to transduce

Table 1 Higher transduction efficiencies are obtained by vectors produced in the presence of the accessory proteins of HIV-1

Donor	*Vector*	*Percentage (%) of EGFP+ cells*	
		Experiment 1	*Experiment 2*
I (MOI = 2.1)	pCMVΔR8.2	34.9	31.3
I (MOI = 2.1)	pCMVΔR8.9	16.8	16.2
I	MLV (GALV env)	16.6	19.9
II (MOI = 3.7)	pCMVΔR8.2	36.0	31.9
II (MOI = 3.8)	pCMVΔR8.9	16.4	17.1
III (MOI = 3.1)	pCMVΔR8.2	29.5	ND
III (MOI = 3.3)	pCMVΔR8.9	14.9	ND

Activated T cells were transduced by centrifugation with Polybrene using vectors prepared in the presence (pCMVΔR8.2) or in the absence of the HIV-1 accessory proteins Vif, Vpr, Vpu and Nef (pCMVΔR8.9) or with a GALV pseudotyped MLV vector. Following transduction, cells were processed as described in Materials and methods and EGFP expression measured 5 days later. Three independent donors were used and the MOI (as determined on HeLa cells) in each lentivirus experiment is indicated.
ND = not determined.

freshly isolated T cells, of which greater than 99% are in the G_0/G_1 phase of the cell cycle. PBMCs were infected either immediately following isolation (and therefore contain a mix of cells, including T cells, B cells and monocytes; donor IV and V, Table 2) or following enrichment for $CD4^+$ T cells by immunomagneting sorting (donor III, Table 2; 80% of these cells were $CD3^+$ at the time of transduction, as assessed by anti-human CD3 antibody labelling and FACS analysis, not shown). The efficiency of transduction was measured 5 days after infection. Measurement at 5 days after infection, and not before, was carried out to avoid the appearance of false positives (pseudo-transduction or transient expression from non-integrated vector DNA). To maintain T cell viability during this time, they were stimulated. Thus, following transduction, cells were stimulated for 48 h with PHA/IL-2 and maintained in IL-2 containing medium until the time of harvest. This process also serves to greatly enrich for T cells (Materials and methods), hence, analysis was carried out on T cells and not on a mixed population. Significant levels of transduction were achieved with lentiviral vectors. A GALV-pseudotyped MLV-based vector, as expected, was unable to transduce these non-pre-stimulated T cells (Table 2, compare pCMVΔR8.2 with MLV (GALV env)). An equivalent innoculum of this MLV-based vector led to transduction of a significant portion of prestimulated T cells (Table 1). Failure to detect EGFP expressing cells following infection with the GALV-pseudotyped MLV vector could be explained if EGFP was poorly expressed from the Mo-MuLV LTR compared with the pgk promoter in the lentiviral vector system. To examine this possibility, we compared the level of EGFP expression (MFI) following infection of pre-stimulated T cells with lentiviral vectors and MLV-based vectors (Figure 4). The MFI following infection of two independent donors with the lentiviral vector was between 40 and 55 (Figure 4a). This value corresponds with the MFI values measured in other experiments using this vector. Infection with the GALV envelope pseudotyped murine retrovirus gave rise to a wider range of EGFP expression. The MFI was approximately 20 times higher (Figure 4b) than that measured with the pgk-EGFP vectors. It is thus likely that if transduction of resting T cells with the MLV vector had occurred, we would have detected it.

Table 2 Transduction of non-stimulated T cells

Donor	*Vector*	*Percentage of EGFP+ cells*
III (MOI = 3.1)	pCMVΔR8.2	18.3
III (MOI = 3.3)	pCMVΔR8.9	5.2
III	MLV (GALV env)	0.0
IV (MOI = 1.5)	pCMVΔR8.2	8.0
IV (MOI = 1.6)	pCMVΔR8.9	2.3
IV	MLV (GALV env)	0.0
V (MOI = 1.8)	pCMVΔR8.2	11.0
V (MOI = 1.8)	pCMVΔR8.9	6.6
V	MLV (GALV env)	0.0

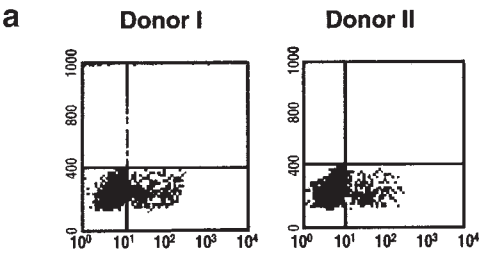
Freshly isolated PBMCs were transduced by centrifugation with Polybrene using vectors prepared in the presence (pCMVΔR8.2) or in the absence of the accessory proteins HIV-1 Vif, Vpr, Vpu and Nef (pCMVΔR8.9) or with a GALV pseudotyped MLV vector. Immediately following transduction, cells were plated in medium containing PHA/IL-2 for 48 h and subsequently in medium containing IL-2 only. EGFP expression was measured at day 5 after transduction. Three independent donors were used and the MOI (as determined on HeLa cells) in each lentivirus experiment is indicated.

Transduction of non-prestimulated T cells with the pCMVΔR8.9 vector showed an increased dependence on HIV accessory proteins compared with prestimulated cells. Whereas vectors devoid of accessory proteins were two-fold less efficient in transducing stimulated cells, their ability to transduce non-stimulated cells was decreased by 3.5-fold in some experiments. These data demonstrate the advantage of lentiviral vectors for the infection of non-cycling T cells and suggest a role for HIV accessory genes in the packaging component.

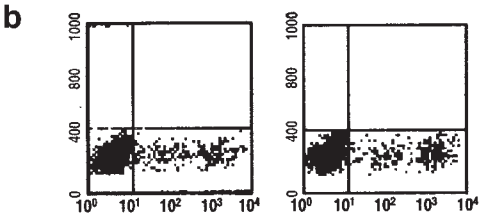
Discussion

We show that VSV-G pseudotyped retroviral vectors based on HIV-1 can efficiently mediate gene transfer to human T lymphocytes. In the past, attention has focused on using onco-retroviral vectors to transduce T cells. Recent improvements to transduction protocols have increased the efficiency of gene transfer. We show here that some, but not all, of these developments can be adapted to VSVG-pseudotyped lentivirus-mediated gene transfer to T lymphocytes. A two-fold enhancement in T cell transduction efficiency was observed when Polybrene was incubated with T cells and lentiviral vector at the time of infection. Use of the less toxic polycation, protamine sulphate, may allow higher concentrations to be used and in turn may result in higher transduction efficiencies. Of greater significance was the five-fold enhancement observed following centrifugation of cells and virus. The combination of centrifugation and Polybrene resulted in a slightly higher overall enhancement of transduction efficiency. Consequently, transduction was achieved with low titres of vector (all of the experiments were carried out with MOIs, as determined on HeLa cells, less than or equal to 3.3). These modifications to the transduction protocol may have applications for gene delivery to other hematopoietic cell types including stem cells. Miyoshi *et al*[26] detected GFP expression in approximately 35 and 54% of $CD34^+$ cells following transduction with a VSVG-pseudotyped lentiviral vector using MOIs of 60 and 300, respectively. Case *et al*[24] using similarly high MOIs detected GFP expression in approximately 24% of $CD34^+$ cells. Inclusion of a centrifugation step in these transduction protocols may result in higher transduction efficiencies or, equally importantly, may allow the use of less transducing virus.

Pollok *et al*[21] reported that gene transfer to T-lymphocytes was three- to nine-fold more efficient when supernatant infection was carried out on a recombinant fragment (CH-296) of fibronectin, compared with BSA with Polybrene. Dardalhon *et al*[23] also showed efficient murine retrovirus-mediated gene transfer to human T cells when infection was carried out on fibronectin. Interestingly, we found no significant enhancement in VSVG-pseudotyped lentiviral vector-mediated gene transfer to T lymphocytes in the presence of fibronectin. This likely reflects a lack of binding of the VSV glycoprotein to fibronectin and a consequent lack of co-localisation of retroviral particles and cells. This finding highlights an important distinction



pgk	Donor I	Donor II
% cells EGFP +	18.2	16.9
MFI	54.4	40.8



MuLV	Donor I	Donor II
% cells EGFP +	17.7	21.4
MFI	1042.6	1088.2

Figure 4 *PBMCs from two donors (I and II) were stimulated for 48 h with PHA/IL-2 and subsequently transduced, by centrifugation at 22°C, 1500 g for 3 h in the presence of Polybrene, with lentiviral vector supernatant (a) or with supernatant containing GALV pseudotyped MLV vector (b). EGFP fluorescence was monitored by FACS and the percentage of EGFP+ cells and the MFI values for each transduction are shown.*

between the commonly used murine retroviruses and VSVG-pseudotyped lentiviral vectors and should be taken into consideration when these vectors are being examined as gene transfer tools to other hematopoietic cells. Many of the protocols describing efficient murine retrovirus-mediated gene transfer to T cells involve transduction of cells on 2 to 3 consecutive days.[21,23,27] Our protocol achieves efficient transduction with a single infection cycle, providing an advantage for clinical applications. Recently, Douglas *et al*[28] demonstrated efficient transduction of activated T lymphocytes using a Tat-dependent lentivirus-based vector. Thus, the transfer vector contained a HIV LTR-promoter driven *tat* gene as well as a GFP reporter gene. The expression of Tat in target cells curtails the use of this vector for clinical trials of human gene therapy.

Several reports using murine retroviral vectors have indicated that the way in which T cells are activated influences transduction efficiency. Pollok *et al*[21] activated T cells in several ways including exposure to interleukin-2 (IL-2), soluble anti-CD3 monoclonal antibody (CD3s), immobilised anti-CD3 monoclonal antibody (CD3i), or co-immobilised CD3 and CD28 monoclonal antibodies (CD3i/CD28i). They observed highest fibronectin-mediated transduction efficiencies following activation with CD3i/CD28i. Similarly, Yang *et al*[25] found that activation of T lymphocytes by bead-conjugated anti-CD3 and anti-CD28 antibodies resulted in higher levels of transduction by MLV based vectors than activation by other means, eg CD3i and recombinant IL-2 or phytohemagglutinin (PHA) and IL-2. Dardalhon *et al*[23] reported that peripheral blood lymphocytes, stimulated for 2 days with either PHA or immobilised antibodies (anti-CD3 and anti-CD28) did not significantly alter the level of fibronectin-mediated murine retroviral gene transfer (transduction efficiencies ranged from 15 to 45%). We observed that similar percentages of cells were targeted with VSVG-pseudotyped lentiviral vectors when cells were pre-stimulated with CD3i/CD28i or PHA/IL-2, however the level of gene expression was approximately two-fold higher in the CD3i/CD28i stimulated cells. This may reflect an overall higher activation status achieved with CD3i/CD28i than with PHA/IL-2. Quinn *et al*[27] studied the relationship between T cell activation and proviral gene expression. Under conditions where cells were most highly activated and producing highest levels of cytokines (IFN-γ, TNF-α, IL-4), proviral protein expression was also highest. Pollok *et al*[21] observed that fibronectin-facilitated murine retroviral gene transfer to T cells was more successful following activation of cells with CD3i/CD28i than with CD3i, CD3s or IL-2 alone. In our experiments, following transduction, cells were maintained in IL-2 containing medium. The initial difference in EGFP expression between CD3i/CD28i and PHA/IL-2 stimulated cells was not sustained following 2 weeks of culture. This apparent down-regulation of proviral gene expression in the absence of T cell signalling has been reported by Quinn *et al*[27] and by Pollok *et al*.[21] In both these studies, reactivation of transduced T cells was accompanied by re-initiation of proviral gene expression.

HIV-based vectors devoid of one or more accessory genes/proteins have been developed, with the aim of improving their biosafety. Such vectors retained the ability to transduce non-dividing 293T cells,[9,10] growth arrested HeLa cells,[8] differentiated macrophages and contact-inhibited primary human skin fibroblasts *in vitro*,[8,10] as well as neuronal cells, hepatocytes and myocytes *in vivo*.[7,10] In some instances, eg transduction of rat neurons *in vivo*, no reduction in gene transfer was observed with vectors produced in the absence of accessory proteins. However, in other cases, the efficiency of transduction was diminished. Vector particles produced in the absence of Vpr showed a 50% decreased transduction efficiency in differentiated macrophages in culture.[10] Vif/Vpr deleted vectors displayed diminished transduction efficiency in rodent liver *in vivo*,[7] while Gasmi *et al*[8] described a three-fold reduction in infectivity of accessory protein (Vif, Vpr, Vpu and Nef)-deficient vector in contact-inhibited human primary skin fibroblasts. We

report here a two-fold reduction in gene transfer to activated T lymphocytes with vectors produced in the absence of Vif, Vpr, Vpu and Nef compared with transduction with vectors produced in the presence of these proteins. Deleted vectors were even less efficient at transducing cells that had not previously been activated. In these cells we observed a difference in efficiency of up to 3.5-fold. The reasons why accessory proteins are dispensable for efficient transduction of some cell types and not others are not entirely known. Human T lymphocytes are a natural host for HIV and it is likely that the virus has become adapted for replication in these cells. It is, therefore, not surprising that the presence of accessory proteins increases transduction efficiency. Vif, a late HIV-1 gene product, has been reported to be essential for the establishment of productive HIV-1 infection in peripheral blood T lymphocytes.[29] While productive infection is not a feature of lentiviral vectors, it is possible that a necessary viral function associated with gene delivery, eg reverse transcription, is less efficient following infection with virions produced in the absence of Vif. This accessory protein has been shown to increase the infectivity of progeny virions in a producer cell-dependent manner.[30] Vif-deficient virions are defective with respect to the capacity of the HIV-1 reverse transcription complex to elongate viral DNA efficiently. The result is an inability to produce full-length viral DNA genomes.[31]

The largest of the accessory proteins, Nef, is associated with enhanced infectivity in primary T cells. The difference in infectivity between Nef+ and Nef– virions is more pronounced following infection of freshly isolated T cells which are stimulated after infection than it is following infection of T cells which are activated before infection.[32] Consistent with this, we observed a greater decrease in transduction efficiency with deleted vectors in freshly isolated T cells compared with cells that were pre-stimulated. Hence, the absence of Nef may play a role in the decreased transduction efficiency we observed with deleted vectors in T lymphocytes. Vpr, a late HIV-1 gene product, with a role in mediating the nuclear import of the HIV-1 pre-integration complex has a modest effect on HIV-1 replication in primary T cells, although its effect in growth arrested cells such as differentiated macrophages is far greater.[33] While it is unlikely that the two-fold decrease in gene transfer to activated T lymphocytes is due to the lack of Vpr, it may account, in part, for the larger decrease observed in unstimulated cells. On the whole, gene transfer with particles produced from packaging constructs harbouring genes for accessory proteins may be achieved at lower MOIs compensating perhaps for the increased toxicity. Nevertheless, transduction of T lymphocytes whether prestimulated or not was observed with deleted vectors. Given correct conditions, eg transduction with more concentrated vector, efficiencies of gene transfer compatible with clinical application may well be achieved.

Quiescent T cells become infected by HIV-1 but the reverse transcription process does not go to completion, preventing successful infection. However, application of a mitogenic signal shortly after infection will allow the completion of reverse transcription.[34] We observed transduction of freshly isolated T cells which had not been stimulated before infection, and underwent activation only starting immediately after infection. In contrast to the results obtained with lentiviral vectors was the lack of EGFP positive cells following infection of these cells with a GALV pseudotyped MLV-based vector. For successful gene delivery with murine retroviral vectors, cells must undergo division. However, as was the case for the lentiviral vectors, PBMCs incubated with the MLV vector were also stimulated in our experiments immediately after transduction. Thus, it appears that the lentiviral vectors have an advantage over onco-retroviral vectors for the transduction of freshly isolated, non-prestimulated T lymphocytes. The data presented here demonstrate that VSVG-pseudotyped lentiviral vectors can effectively mediate gene transfer to T lymphocytes. Thus, they have enormous potential both as research tools and in a clinical setting to study and treat T cell-associated diseases.

Materials and methods

Plasmid construction

A packaging construct PCWT (Figure 1a), expressing all the HIV-1 genes except *env* under the control of the human cytomegalovirus (CMV) promoter was prepared by a series of steps as follows: a 0.7-kb *Bss*HII–*Sph*I fragment of pNL43 (from positions 711 to 1443) was subcloned into pSL1180 (Pharmacia-Biotech, UK) generating pSLga. A 33-bp deletion in the putative packaging signal, between the major splice donor and the start site of the *gag* gene, was then performed by site directed mutagenesis with the oligonucleotide 5′GACGCTCTCGC ACCCATCTCTCACCAGTCGCCGCCCCTC3′. Following verification by sequencing, a further 4.3-kb fragment *Sph*I–*Sal*I of pNL43 (from positions 1443 to 5785) was cloned into pSLga generating pSLgagpol. An *env* gene containing fragment of pNL43, *Eco*RI–*Bam*HI (from positions 5743 to 8465) was cloned into pCi-neo (Promega, Wallisellen, Switzerland) and a subsequent *Bgl*II flanked fragment (0.5 kb) removed, creating pΔenv. A fragment from the *Bam*HI site in pNL43 (position 8465) to the end of the *nef* coding sequence was amplified by PCR and cloned 3′ of the deleted *env* gene in pΔenv creating pΔenvnef. Finally a single fragment encompassing the sequence from *Bss*HII to *Eco*RI of pSLgagpol was cloned upstream of the deleted env in pΔenvnef generating PCWT (Figure 1a). The packaging constructs pCMVΔR8.9 which does not encode Env, Vif, Vpr Vpu or Nef and as control, the plasmid from which it was derived pCMVΔR8.2 (which like PCWT, encodes all proteins of HIV-1 except Env) were described previously.[10] pMD.G (schematically represented in Figure 1c) which encodes the heterologous VSV envelope from the immediate–early enhancer/promoter of CMV was described previously.[5] Transfer vectors (Figure 1b) were made as follows: pHR CMV EGFP was produced by removing the *LacZ* gene (*Xba*I–*Bam*HI fragment) from pHR CMV *LacZ*,[5] and replacing it with the gene encoding the enhanced green fluorescent protein (EGFP) (*Xho*I–*Nhe*I fragment) from PEGFP-C1 (CLONTECH Laboratories Basel, Switzerland). PHR pgk EGFP was produced by replacing the CMV promoter in pHR CMV EGFP with the murine phosphoglycerate kinase (pgk) promoter.[35]

Production and assays of vectors

Human embryonic kidney 293T cells[36] were maintained in Dulbecco modified Eagle's medium supplemented with 10% foetal calf serum (FCS). For vector production,

cells were plated at 70% to 80% confluence on 15-cm plates and transfected by calcium phosphate DNA precipitation[37] with 26 μg of PCWT, 10 μg of pMD.G and 40 μg of transfer vector. The following morning, cells were washed and medium replaced, this was repeated 24 h later and 64 h after transfection conditioned medium was harvested. Preparations of VSV-pseudotyped vectors were filtered through 0.45 μm filters and concentrated by ultracentrifugation (50 000 *g* for 90 min). Titres of vector preparations were determined by plating HeLa cells at a density of 1×10^5 cells per 5-cm plate, transducing the cells with serial dilutions of vector supernatants, followed by fluorescence-activated cell sorting (FACS) analysis 5 days later. Titres ranged from $0.5–5 \times 10^7$ IU/ml. Vector supernatants were tested for the presence of replication competent HIV by innoculation of HTLV II transformed cells MT-2. Cells were maintained in culture for 3 weeks to 1 month, samples of culture supernatant were taken twice weekly and stored at –20°C. The p24 level in the supernatant was measured by ELISA (Abbott). No replication-competent virus was detected. Supernatant containing GALV pseudotyped MLV, described in Dardalhon *et al*[23] was a kind gift of V Dardalhon and N Taylor (Institute of Molecular Genetics, Montpellier, France). Infections, using 500 μl of this supernatant were carried out as for lentiviral vectors.

Isolation and transduction of primary T lymphocytes

Peripheral blood mononuclear (PBMCs) cells from healthy donors were isolated by centrifugation on Ficoll–Hypaque (density, 1.077 g/ml, Pharmacia) for 30 min (800 *g*) at 25°C and washed twice with PBS. The resulting primary lymphocyte population was grown in RPMI 1640 supplemented with 20% FCS and 1% penicillin-streptomycin (GIBCO-BRL, Life Technologies, Paisley, UK). T lymphocytes were activated for 48 h by phytohemagglutinin A (5 μg/ml) and interleukin-2 (50 U/ml) or immobilised monoclonal antibodies, anti-CD3 (OKT3) and anti-CD28 (clone-CD28.2; PharMingen; CD3i/CD28i). For the immobilisation of antibodies, 24-well non-tissue culture-treated plates (Costar) were coated with antibody (1 μg/μl in PBS) at 0.5 ml/well for 2 to 4 h at 37°C. The coated plates were then blocked with 1% BSA in PBS for 20 min at 37°C, washed once with PBS and then used for activation. At day 2 after activation the cells were harvested and counted. Flow cytometric analysis indicated that these populations were routinely up to 90% positive for CD3 and hence highly enriched for T lymphocytes.

To deplete PBMCs of B cells, monocytes, and $CD8^+$ T cells, freshly isolated PBMCs were incubated for 1 h at 4°C with Dyna beads (M-450 CD19, M-450 CD14 and M-450 CD8 respectively: Dynal, Chester, UK). Following removal of the beads, the remaining cells (80% of which were $CD3^+$, as assessed by anti-human CD3 antibody (PE, PharMingen, Becton Dickinson, Oxford, UK) labelling and FACS analysis) were immediately infected.

Cell transduction and analysis

T cells were transduced with vector as follows unless otherwise stated: 1×10^6 cells were collected in 15 ml polypropylene Falcon tubes, resuspended in a given volume of vector supernatant (25–100 μl) in the presence or absence of Polybrene (5 μg/ml). Tubes were centrifuged at 1500 *g* for 1.5–3 h at room temperature or incubated at room temperature without centrifugation. For transduction on fibronectin, non-tissue culture treated 24-well plates (Costar, High Wycombe, UK) were coated with (20 $\mu g/cm^3$) or 4 ml polystyrene tubes (Falcon, Fred Baker, Runcorn, UK) with 400 μl (32 μg/ml) recombinant fibronectin (fragment CH-296, Takara Schuzo, Otsu, Shiga, Japan) or with bovine serum albumin (BSA) overnight at 4°C or for 2 h at room temperature. Plates or tubes were subsequently blocked with 1% BSA (20 min, 37°C) and washed once with PBS before use.

Following transduction, cells were washed once with PBS and resuspended in medium supplemented with 50 U/ml IL-2. Cells were split or medium changed every 3 days when 50% of the medium was replaced with fresh medium containing IL-2. On indicated days after transduction, cells were harvested, washed and fixed before flow cytometric analysis. Fluorescence activated cell sorting (FACS) was carried out using a FACScan flow cytometer (Beckton Dickinson) and Lysis II software. Transduction of cells was detected by the presence of GFP (488 nm) laser. CD4 and CD8 expression was assessed by staining with anti-human antibodies labelled with phycoerythrin (PE, PharMingen, Becton Dickinson).

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