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Lentiviral Vectors Pseudotyped with Glycoproteins from Ross River and Vesicular Stomatitis Viruses: Variable Transduction Related to Cell Type and Culture Conditions

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HIV-1-derived lentiviral vectors have been pseudotyped with various envelope glycoproteins to alter their host range. Previously, we found that envelope glycoproteins derived from the alphavirus Ross River virus (RRV) can pseudotype lentiviral vectors and mediate efficient transduction of a variety of epithelial and fibroblast-derived cell lines. In this study, we have investigated transduction of hematopoietic cells using RRV-pseudotyped vectors encoding the enhanced green fluorescent protein (EGFP). RRV-mediated transduction of human CD34$^+$ cord blood cells and progenitors was very inefficient, even at multiplicities of infection of 100 (0.4% EGFP-positive progenitor colonies). Inefficient transduction was also observed in a variety of hematopoietic cell lines. However, two erythroleukemia-derived cell lines and monocytic cells that were driven to macrophage-like differentiation were moderately transduced. Transduction of hematopoietic cells with a control VSV-G-pseudotyped lentiviral vector was generally efficient, but unexpectedly decreased up to threefold upon stimulation of lymphocytic cell lines or primary murine bone marrow cells. Also, the tested hematopoietic cell lines were essentially nonpermissive for adeno-associated type 2 (AAV) vectors, and this was not affected by lineage, activity, or differentiation. Treatment of permissive 293 cells with proteases revealed that transduction with both the RRV- and the VSV-G-pseudotyped vectors in part depends on the presence of cell surface proteins. These results show a severely restricted ability of RRV glycoproteins to mediate transduction in hematopoietic cells that is likely due to specific receptor requirements that differ from those of VSV-G and AAV. Conversely, transduction with the VSV glycoprotein is affected by cellular activation more than widely believed. Our findings suggest that the envelope glycoproteins and culture conditions employed need to be carefully evaluated for each application. Furthermore, the uniquely restricted host range of RRV-pseudotyped vectors may aid in the design of novel cell-selective transduction strategies.

Key Words: lentiviral vectors, Ross River virus, VSV-G, pseudotyping

INTRODUCTION

Genetically engineered lentiviruses derived from the human immunodeficiency virus (HIV-1) are promising tools for gene transfer. One of their main advantages is their apparent ability to integrate into nondividing cells, such as hematopoietic stem and progenitor cells [1–6]. As wild-type HIV envelope glycoprotein restricts infection to cells expressing CD4, the incorporation of foreign glycoproteins such as the vesicular stomatitis virus glycoprotein (VSV-G) into the lentiviral envelope has been shown to expand considerably the naturally restricted host range of the HIV-1 lentivirus [1,7–10]. The VSV glycoprotein in particular allows transduction of a very wide range of cell types from a variety of organisms [11]. Similarly, alphaviruses can infect a variety of organisms, ranging from insects and birds to mammals [12].

Our laboratory was the first to show that the envelope glycoproteins from Ross River virus (RRV) can be incorporated into HIV-1 lentiviral vectors, forming infectious

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pseudotypes that are capable of efficiently infecting a range of adherent cell types [13]. Alphaviruses contain an RNA genome of positive polarity that is surrounded by an icosahedral capsid. A host-derived envelope comprising a lipid bilayer protects the capsid structure. Embedded in the envelope are glycoprotein spikes that are arranged as 80 trimers on the viral surface [14]. They are composed mainly of the virally encoded E1 and E2 glycoproteins: a trimer of E1–E2 heterodimers for each spike [15]. E2 is thought to be responsible for viral binding to host cell receptors, whereas E1 mediates fusion [12]. The cellular receptors for RRV are still unknown, but protein receptors, specifically integrins, have been suggested [16]. Two recent studies have demonstrated that RRV glycoproteins can be efficiently incorporated into murine leukemia virus (MuLV) and feline immunodeficiency virus vectors. The resulting pseudotyped viruses can effectively transduce cells in cell culture and *in vivo* [17,18]. Furthermore, the RRV E1/E2-pseudotyped MuLV vectors were stable during concentration by ultracentrifugation and could be stably expressed in a vector packaging cell line, without apparent toxicity [18].

In this study, we have analyzed the ability of the RRV glycoproteins to mediate transduction into primary hematopoietic $CD34^+$ cells and cell lines representing different hematopoietic lineages. Our results reveal that transduction of hematopoietic cells by RRV-pseudotyped lentiviral vector is significantly impaired. While the receptor for RRV remains to be identified, our preliminary findings indicate that the receptor/coreceptor differs from that of adeno-associated virus (AAV), a virus that also demonstrated restricted infection of hematopoietic cells. An unexpected finding was also observed. Specifically, cell stimulation and differentiation decreased transduction with VSV-G- but not RRV-pseudotyped lentiviral vectors. These findings point out the importance of pseudotype and transduction conditions when attempting to maximize lentiviral transduction of hematopoietic cells.

RESULTS

Limited Transduction of $CD34^+$ Cord Blood Cells and Hematopoietic Cell Lines by RRV Glycoprotein-Pseudotyped Lentiviral Vector

Previously, we have demonstrated that envelope glycoproteins from the alphaviruses RRV and Semliki Forest virus (SFV) can be incorporated into HIV-1-derived lentiviral vectors, forming infectious pseudotypes that can be produced and concentrated to high titer [13]. As RRV-pseudotyped lentiviral vectors were able to transduce a variety of adherent cell lines, we sought to investigate their potential for transduction of primary hematopoietic cells. We produced viral vectors by transient transfection and concentrated them by ultracentrifugation as described underMaterials and Methods. The transfer vector utilized for these experiments encodes the enhanced green fluorescent protein (EGFP), allowing assessment of transduction by flow cytometric analysis. We used lentiviral vector pseudotyped with the VSV-G as a control when assessing gene transfer by vectors pseudotyped with the RRV or SFV envelope [19].

We determined titers of the EGFP-expressing vectors pseudotyped with RRV, SFV, and VSV-G envelope glycoproteins on 293 cells. We then diluted the vectors to the same multiplicity of infection (m.o.i.) and used them to transduce human $CD34^+$ cord blood cells. Cells were prestimulated for 3 days with granulocyte-colony stimulating factor (G-CSF), stem cell factor (SCF), and megakaryocyte growth and development factor (MGDF) (100 ng/ml of each) and transduced using a single overnight exposure of vector in the presence of Polybrene and the fibronectin fragment CH-296. We have previously shown that the combination of Polybrene and CH-296 provides improved gene transfer over either agent alone when transducing 293 and HT-1080 cells with RRV- and VSV-G-pseudotyped lentiviral vectors [13]. Also, this transduction protocol did not alter cell viability of cord blood cells, as assessed by trypan blue exclusion (data not shown).

As shown in Table 1, 24–28% of transduced cord blood cells maintained in bulk culture expressed EGFP 3 days after exposure to VSV-G-pseudotyped vectors (m.o.i. 100). Similar findings were noted when the transduced cells were plated in methylcellulose and assayed for progenitor-derived colonies, with 20–26% of colonies expressing EGFP 2 weeks after plating. In sharp contrast, RRV-pseudotyped vector showed little EGFP expression in bulk cultures and only 5 of 1199 (0.4%) total colonies analyzed showed evidence of EGFP expression (Table 1). SFV, the other alphavirus envelope studied, also failed to show significant transduction of human $CD34^+$ cord blood cells (Table 1).

As the results in our first experiment with primary cord blood cells were not predicted from our work with adherent cell lines, our second experiment included cord blood cells and a variety of hematopoietic cell lines (Table 1, experiment 2, and Fig. 1A). Consistent with the findings in primary cells, hematopoietic cell lines of various lineages were efficiently transduced by VSV-G-pseudotyped vector, but not by vector containing the RRV glycoproteins. In agreement with our previous findings [13], nonhematopoietic adherent cell lines were transduced to virtually 100% by both pseudotyped vectors at an m.o.i. of 40 (Fig. 1A). To show that the VSV-G and RRV vector preparations were similar in potency, we prepared serial dilutions of the vector and tested them on 293T cells and the MDA-231 breast cancer cell line. As shown in Fig. 1B, the RRV preparation used in these experiments transduced permissive cell lines at similar or higher efficiencies compared to the VSV-G pseudotype. The restricted transduction of hematopoietic

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TABLE 1: Transduction of CD34$^+$ cord blood cells with VSV, RRV, and SFV glycoprotein-pseudotyped lentiviral vectors

	TE FACS (%)[a]		Progenitor-derived colonies	
Pseudotype	m.o.i. 100	m.o.i. 20	GFP$^+$ CFU–GM/total CFU–GM	GFP$^+$ CFU–GM (%)[b]
Experiment 1				
VSV-G	28.2 (±1.6)	24.2 (±0.7)	27/103 (m.o.i. 100)	26.2 (±3.0)
RRV	1.8 (±0.3)	1.7 (±0.6)	0/94 (m.o.i. 100)	0 (±0)
SFV	ND[c]	0 (±0)	0/109 (m.o.i. 20)	0 (±0)
Experiment 2				
VSV-G	24.1 (±1.7)	16.1 (±1.5)	226/1120 (m.o.i. 100)	20.0 (±3.1)
RRV	3.8 (±0.7)	1.3 (±0.5)	5/1105 (m.o.i. 100)	0.4 (±0.4)
SFV	ND[c]	1.27[d]	0/413 (m.o.i. 20)	0

[a] TE indicates transduction efficiency assessed by flow cytometry 3 days after transduction and is given as the mean and standard deviation from transductions performed in triplicate, except transduction with SFV pseudotype in experiment 2, which was performed only once.
[b] Progenitors were plated in triplicate in methylcellulose. Results are given as means and standard deviations. CFU–GM, colony-forming unit–granulocyte, macrophage.
[c] ND, not determined.
[d] Fluorescence intensities for GFP were close to background levels.

cells by RRV-pseudotyped vector was confirmed by quantitative PCR analysis. As shown in Fig. 2, low quantities of vector DNA were detected in hematopoietic cells transduced with the RRV-pseudotyped vector, while DNA was readily detected in hematopoietic cells transduced with VSV-G-pseudotyped vector. In contrast, adherent cancer cell lines transduced with either VSV-G- or RRV-pseudotyped vector showed similarly high levels of the vector sequences. The data also support prior observations that the number of vector integrations exceeds that predicted by GFP expression, due either to multiple integrations in a single cell or to expression of GFP below the level detected by FACS analysis [20].

We explored further the ability to transduce preferentially cancer cells that contaminate hematopoietic stem cell sources by mixing G-CSF-mobilized peripheral blood stem cells from a normal donor with the breast cancer cell line MDA-MB-231/neo. The breast cancer cell line contains the neomycin phosphotransferase gene introduced by retroviral transduction followed by G418 selection. After 2 days of prestimulation in cytokines, we transduced a mixture of 95% CD34$^+$ cells and 5% MDA-MB-231/neo cells with lentiviral vectors pseudotyped with VSV-G or RRV envelope. Transduction was performed overnight in the presence of the fibronectin fragment CH-296. We harvested the cells and incubated them in fresh plates for an additional 24 h, after which time the we maintained the nonadherent cells in liquid culture and also placed them in methylcellulose. We cultured adherent cells (i.e., MDA-MB-231/neo) in G418 to eliminate nontumor cells. We analyzed the adherent G418-selected cells by FACS analysis 6 days after transduction. Colony-forming units were analyzed at day 12 for GFP expression to determine gene transfer into hematopoietic progenitors. As shown in Table 2, the VSV-G- and RRV-pseudotyped lentiviral vectors transduced greater than 90% of the MDA-MB-231-neo cells as assessed by flow cytometry. In contrast, 9 ± 4% of the progenitor colonies were transduced with VSV-G-pseudotyped vector, while there were no fluorescent colonies detected in the RRV-pseudotyped transduction. In addition to hematopoietic cells, a small number of MDA-MB-231/neo colonies were also observed in the methylcellulose. These colonies were fluorescent regardless of pseudotype, again confirming the high gene transfer into the cancer cell population. These data confirm the findings provided above and suggest that further exploration of RRV-pseudotyped vectors for hematopoietic cell purging is warranted.

Cell Adhesion Properties Influence Transduction by RRV-Pseudotyped Vector

The finding that RRV glycoproteins did not mediate efficient transduction of hematopoietic cells was intriguing. Both VSV-G- and RRV-pseudotyped vectors share the same underlying lentiviral core proteins that mediate the steps that lead up to viral integration. Furthermore, VSV and RRV glycoproteins have been shown to utilize the endocytotic pathway for entry into cells and are thought to fuse in acidified endosomes [18,21]. Thus, we favored the possibility that infection by the RRV pseudotype is inhibited at the cell surface. As all the cells that had limited transduction by RRV-pseudotyped vectors were nonadherent, we speculated that the presence of cellular adhesion molecules may be necessary to mediate infection by RRV glycoproteins. As an initial test, we compared transduction of 293 and 293T-NA cells. 293 cells are adherent cells that are known to be permissive for high-efficiency RRV-mediated gene transfer. The 293T-NA cells were selected for their ability to grow in suspension, thereby mimicking the culture condition of hematopoietic cell lines. We performed transduction of adherent 293 cells in tissue culture dishes, whereas we transduced 293T-NA cells in suspension using petri dishes to prevent adherence. As shown in Fig. 3, there was only a slight decrease in transduction efficiency of

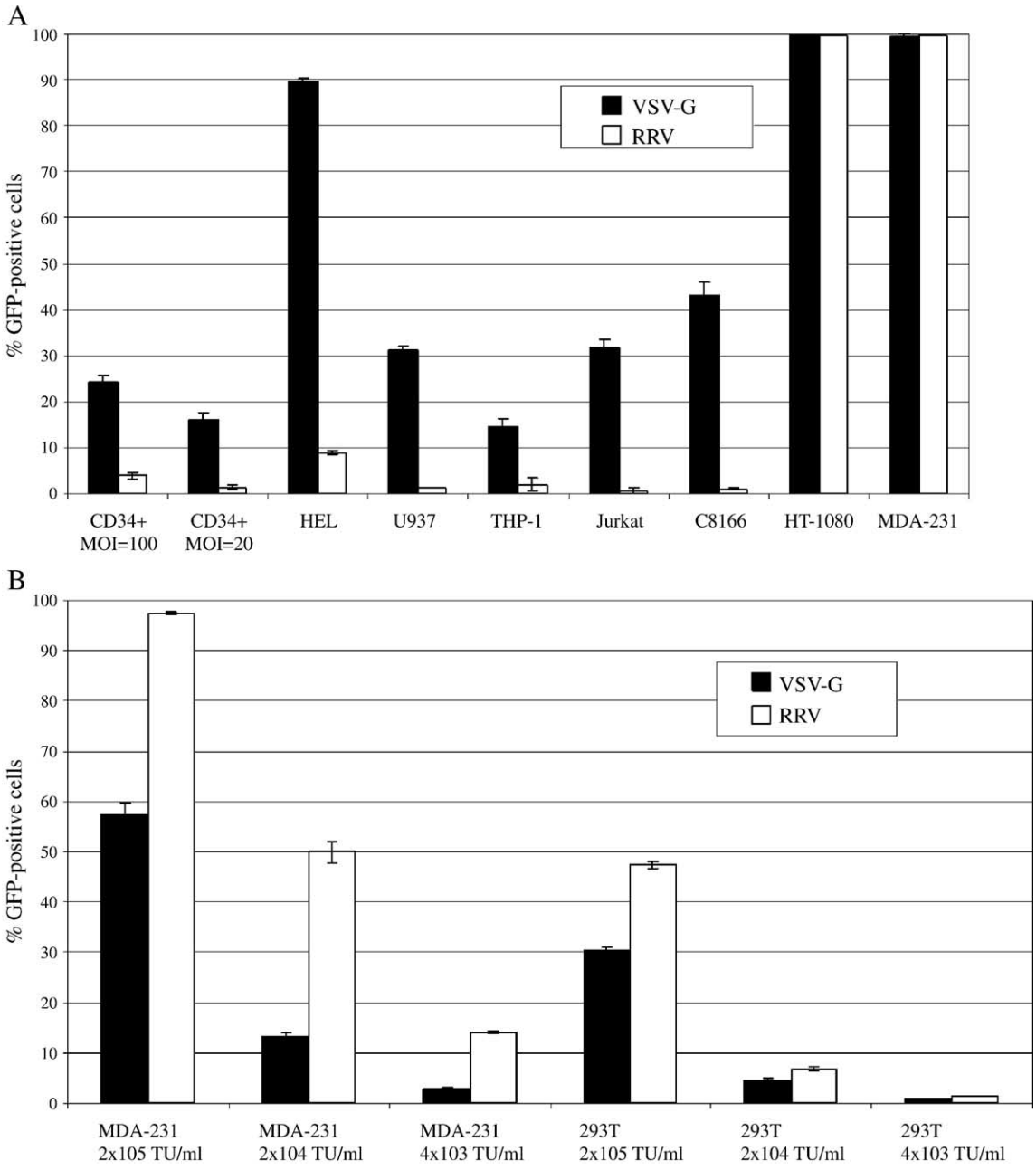

FIG. 1. Transduction of nonadherent hematopoietic cells and adherent cancer cell lines by VSV-G- and RRV-pseudotyped lentiviral vectors. (A) $CD34^+$ cord blood cells and hematopoietic cell lines were transduced with VSV-G- and RRV-pseudotyped lentiviral vectors overnight. The m.o.i. for $CD34^+$ cells are indicated (100 and 20). The m.o.i. for both nonadherent and adherent cell lines was 40. Cells were expanded for another 72 h, and the number of EGFP-positive cells was evaluated by flow cytometry. Results present the means and standard deviations of transductions performed in triplicate. (B) Control transductions were performed on adherent cancer cell lines to demonstrate equal concentrations of infectious viral vector for both VSV-G and RRV pseudotypes. Vector concentrations used for transduction are indicated below the bars.

293T-NA cells with the RRV-pseudotyped vector. The data suggest that cellular adhesion per se is not the major factor responsible for the limited transduction of hematopoietic cell lines observed with RRV-pseudotyped vectors.

Transduction Patterns Differ Between RRV-Pseudotyped Lentiviral Vectors and Adeno-associated Virus Type 2 Vectors

AAV vectors have been shown to have limited transduction of hematopoietic cells [22,23], a factor that was

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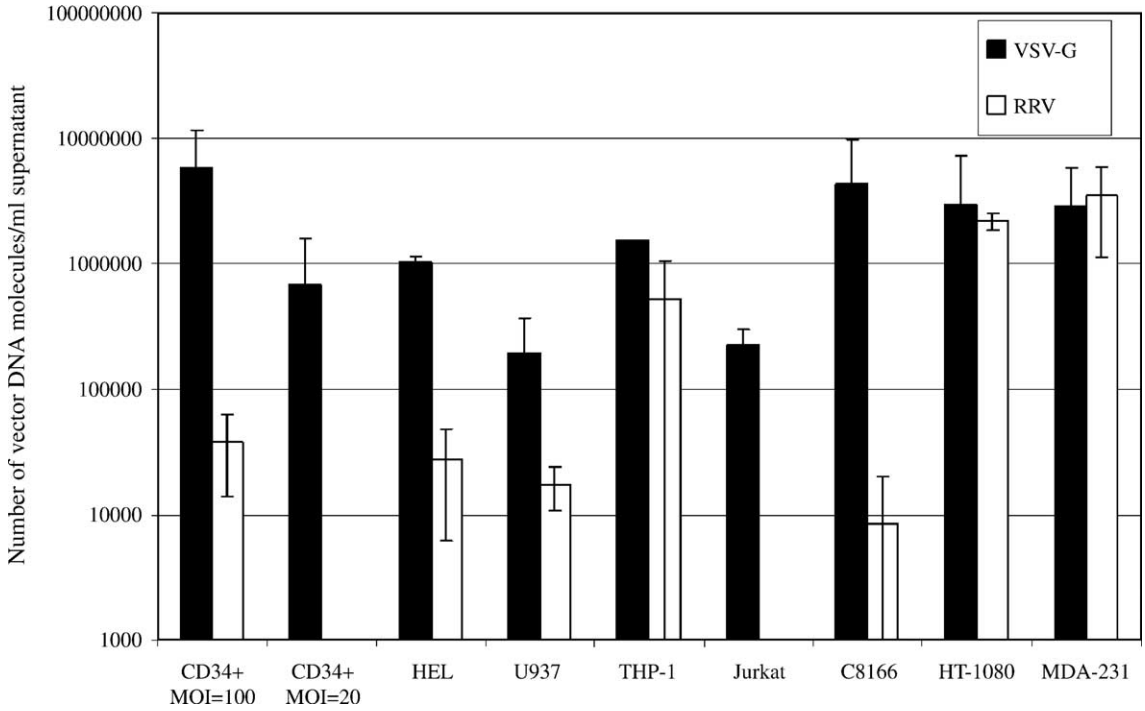

FIG. 2. DNA titers of nonadherent hematopoietic cells and adherent cancer cell lines transduced by VSV-G- and RRV-pseudotyped lentiviral vectors. Genomic DNA was extracted from transduced cells and cells exposed to medium only (mock-transduced cells) and subjected in duplicate to quantitative PCR to detect integrated EGFP transfer vector. Quantitation of vector copy number was performed by comparing the signal obtained from the genomic DNA to a serially diluted plasmid standard and then subtracting the background signal from mock-transduced controls.

later exploited in identifying receptor and coreceptors for AAV viral entry [24–27]. To explore if AAV and RRV might share similar pathways for viral entry, we compared the patterns of transduction of RRV- and VSV-G-pseudotyped lentiviral vectors with that of an AAV vector expressing EGFP. As shown in Fig. 4, all hematopoietic cell lines were resistant to AAV transduction, while all were permissive for VSV-G lentiviral transduction. In contrast, AAV and VSV-G-pseudotyped lentiviral vectors had similar transduction efficiencies in the permissive 293 cell line (Fig. 5A). Interestingly, RRV-pseudotyped lentiviral vector and AAV showed similar patterns of transduction for most hematopoietic cell lines but with two marked differences. Specifically, HEL and K562 cells were moderately permissive to transduction with RRV-pseudotyped lentiviral vectors but were not transduced by AAV (Fig. 4). This agrees with the moderate transduction rates in HEL cells previously observed (Fig. 1A), and a repeat experiment with K562 cells also confirmed significant transduction with RRV-pseudotyped vector (data not shown). While RRV-pseudotyped vector was superior to AAV for transduction of HEL and K562 cells, RRV was still inferior to VSV-G-pseudotyped lentiviral vectors.

These findings suggest that AAV and RRV-pseudotyped lentiviral vectors may differ in their viral entry pathways. While the receptor(s) required for RRV entry remains to be determined, it is thought to involve proteins [12,16]. As

TABLE 2: Transduction of mobilized CD34$^+$ peripheral blood progenitor cells mixed with MDA-MB-231/neo cells

		Day 6 % EGFP[a] (mean ± SD)		Day 12 progenitor assay (mean ± SD)	
Pseudotype	m.o.i.	Nonadherent	Adherent, G418 selected	Hematopoietic colonies[b]	Nonhematopoietic[c]
VSVG	100	11.5 ± .8	92.3 ± 3.0	33/371 (9 ± 4%)	35/35 (100%)
RRV	100	3.3 ± .3	93.3 ± 0.7	0/252 (0 ± 0%)	14/14 (100%)
Mock	0	0 ± 0	0 ± 0	0/265	0

[a] Transduction efficiency was assessed by flow cytometry 6 days after transduction and is given as the mean and standard deviation from transductions performed in triplicate.
[b] Progenitors were plated in triplicate in methylcellulose. Results are given as means and standard deviations. Hematopoietic colonies were the number of fluorescent colonies divided by the total number of colony-forming unit–granulocyte, macrophage and burst-forming unit erythroid colonies.
[c] A small number of MDA-MB-231/neo cell colonies were identified in the methylcellulose cultures and were assessed for fluorescence.

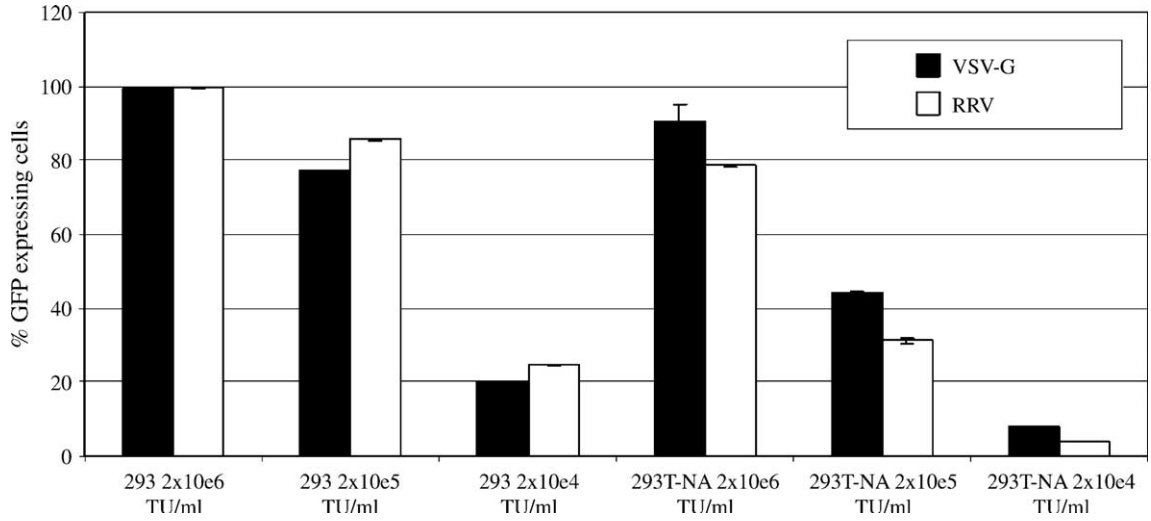

FIG. 3. Transduction of adherent 293 cells and 293T cells selected for growth in suspension by VSV-G- and RRV-pseudotyped lentiviral vectors. 293 cells and 293T-NA cells were transduced in triplicate by different dilutions of VSV-G- and RRV-pseudotyped lentiviral vectors. 293 cells were transduced in tissue-culture-treated six-well plates, whereas transduction of 293T-NA cells was performed in untreated petri dishes to prevent adhesion of cells to the plastic. The vector concentrations are indicated below the bars. 72 h posttransduction, the number of EGFP-positive cells was evaluated by flow cytometry.

shown in Figs. 5A and 5B, trypsin and protease XIV pretreatment of 293 cells decreased transduction by AAV and the RRV-pseudotyped lentiviral vector by up to 60%, while transduction with VSV-G decreased by less than 30%. Heparin completely inhibited AAV transduction at concentrations of 1 μg/ml and markedly limited lentiviral transduction at 100 μg/ml (Fig. 5A). The minimal effect of heparin at 1 μg/ml likely relates to the presence of Polybrene in the transduction medium of lentiviral but not AAV vectors. Heparin interferes with AAV viral

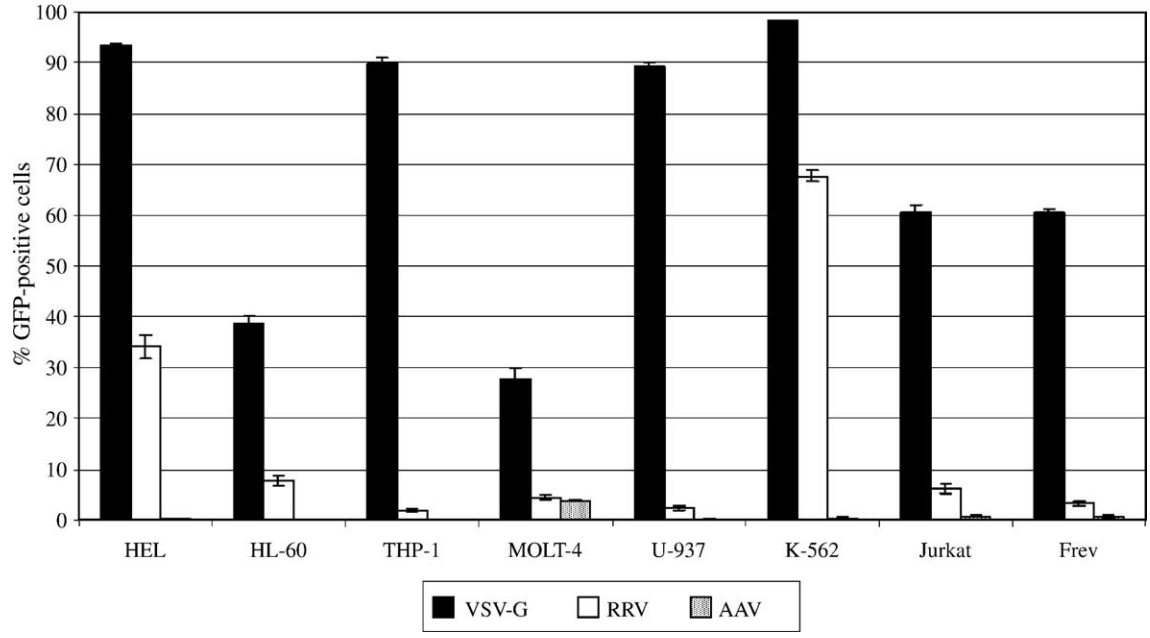

FIG. 4. Transduction of hematopoietic cells by VSV-G- and RRV-pseudotyped lentiviral vectors and AAV vector. Various hematopoietic cell lines were transduced in triplicate by lentiviral vectors or AAV vector at an m.o.i. of 100 overnight. Cells were then expanded for another 72 h and evaluated for EGFP expression by flow cytometry.

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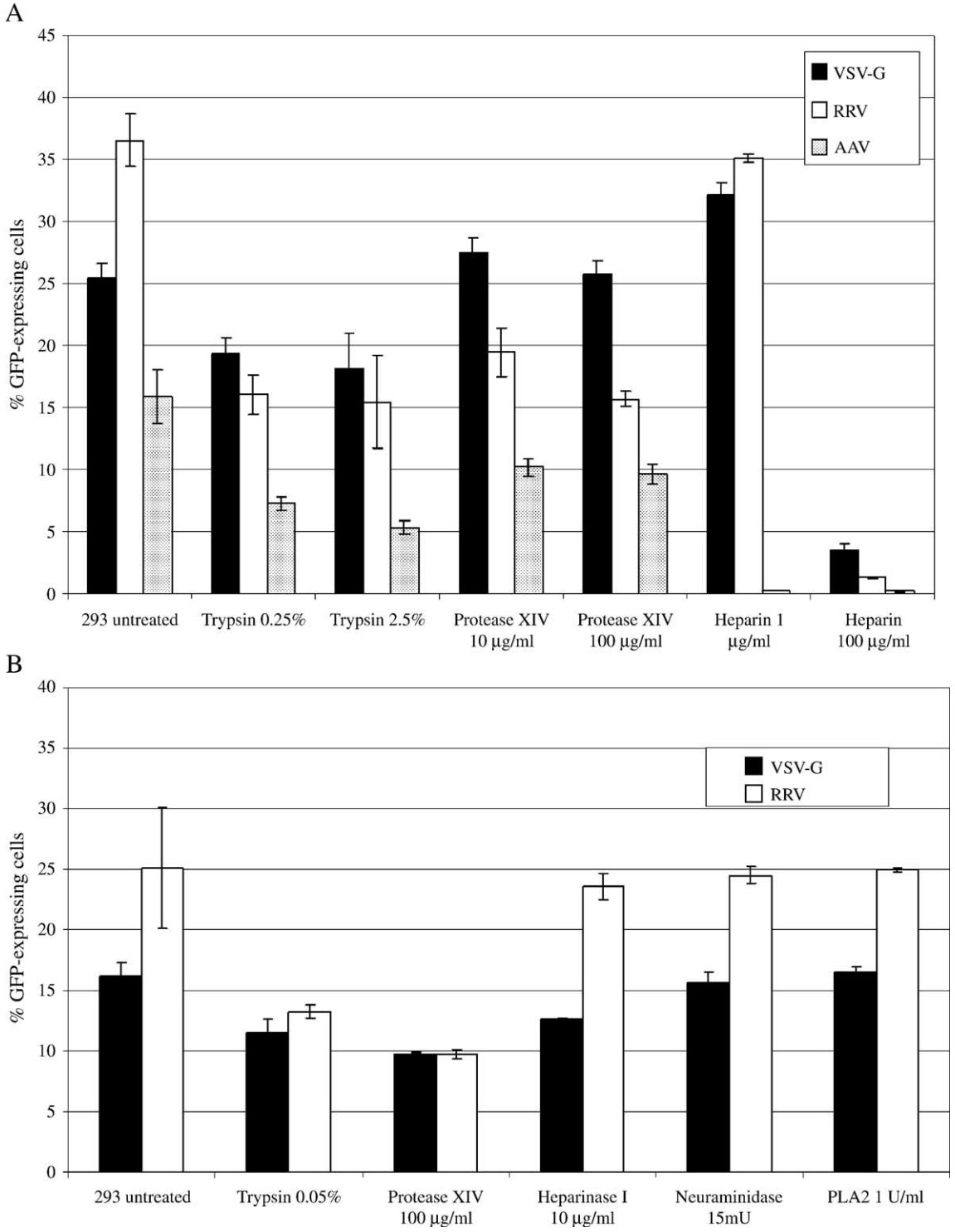

FIG. 5. Effects of chemical modification of 293 cells on transduction with pseudotyped lentiviral vectors and AAV vector. (A) 293 cells were treated with different concentrations of trypsin and protease XIV and then transduced with either pseudotyped lentiviral vectors or AAV vector for 1 h at m.o.i. of 1. Vector supernatants were also mixed with various concentrations of heparin before transduction. (B) 293 cells were treated with trypsin, protease XIV, neuraminidase (Roche), or phospholipase A_2 (PLA_2). Cells were then transduced with VSV-G- and RRV-pseudotyped lentiviral vectors for 4 h at m.o.i. of 1. Transduction efficiency for both experiments was evaluated after 72 h by flow cytometry. All results are given as the means and standard deviations obtained from triplicate transductions.

attachment to cell surface heparan sulfate proteoglycans [27], and these findings also suggest a role for proteoglycans in transduction with VSV-G- and RRV-pseudotyped lentiviral vectors.

To determine if sialic acid molecules or phospholipids may be involved with RRV- and VSV-G-pseudotyped vector transduction, we treated 293 cells with neuraminidase and phospholipase A2. As shown in Fig. 5B, neither of these enzymes inhibited transduction. These results were consistent in a second experiment using enzymes from a different supplier (Sigma, St. Louis, MO, USA; data not shown). Together, the data indicate a role for cell surface proteins and glycoproteins in the viral entry pathway of RRV. While some similarities are noted between RRV and AAV, the enzymes used are nonspecific and affect a wide range of surface molecules. While protease treatment may have specific or nonspecific effects that alter vector transduction efficiency, the marked difference in AAV and RRV-pseudotyped lentiviral transduction efficiencies noted in HEL and K562 cells suggests that distinct receptor molecules are involved with vector uptake.

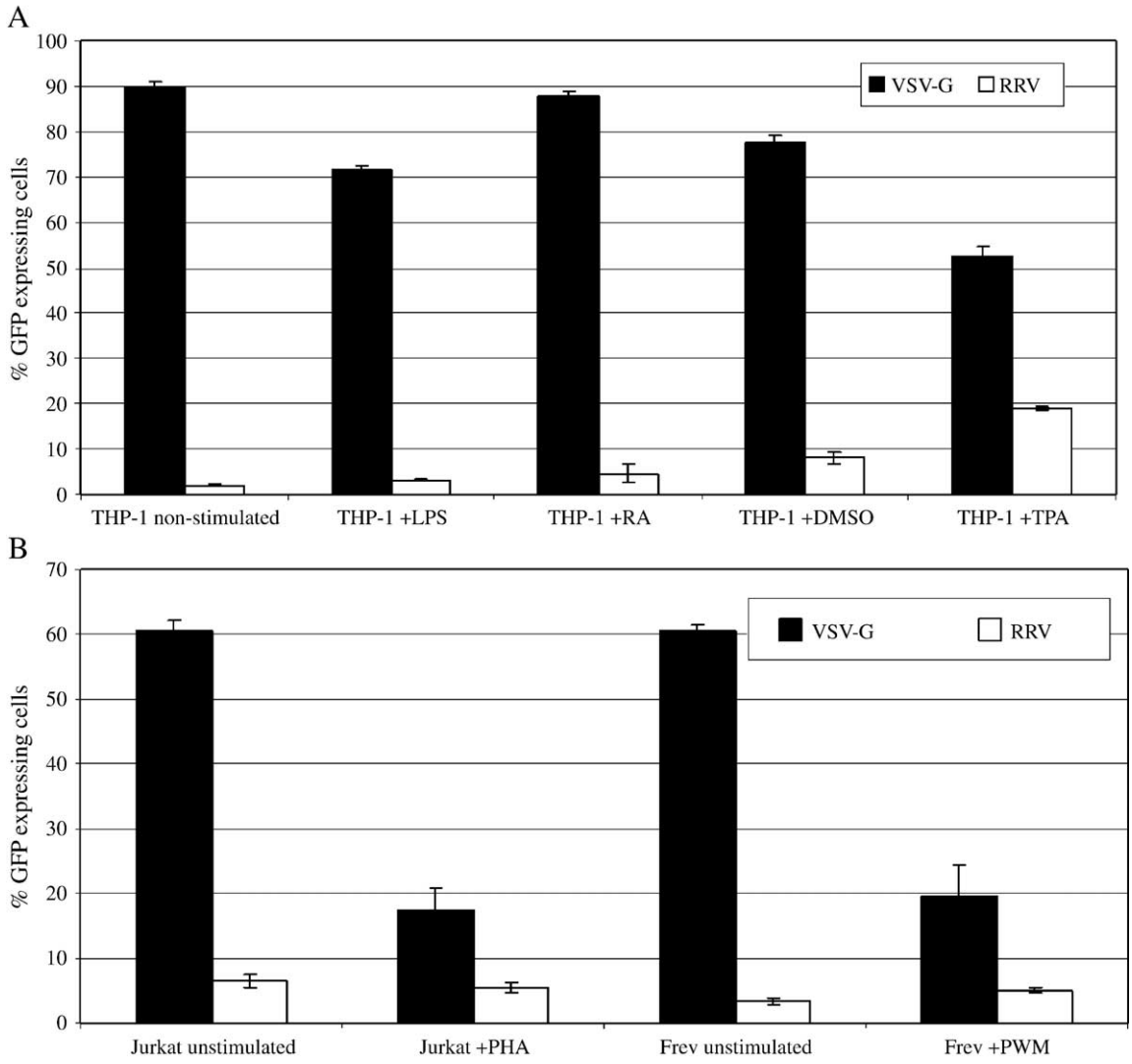

FIG. 6. Effects of cellular differentiation and stimulation on transduction with VSV-G- and RRV-pseudotyped lentiviral vectors. (A) THP-1 cells were treated with 10 ng/ml LPS, 10^{-6} mol/L RA, 1.5% DMSO, or 1 ng/ml TPA for 3 days and then transduced with pseudotyped lentiviral vectors at an m.o.i. of 100 overnight. Treatment continued throughout the time of transduction. (B) Jurkat cells and Frev cells were stimulated with 12.5 μg/ml PHA and PWM, respectively, for 24 h and then transduced with pseudotyped lentiviral vectors at an m.o.i. of 100 overnight. Results are given as the mean percentages of EGFP-expressing cells obtained from triplicate transductions, with the error bars representing the standard deviation.

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Macrophage-like Differentiation Correlates with Increased Transduction by RRV-Pseudotyped Vector, but Cell Stimulation Decreases Transduction by VSV-G-Pseudotyped Vector

Cell stimulation and differentiation can upregulate the expression of a variety of cell surface proteins, including integrins and other candidate molecules that may serve as receptors/coreceptors. Interestingly, monocytes have been shown to be nonpermissive for wild-type RRV, while macrophages were shown to be infectable [16]. We thus hypothesized that permissiveness may depend on cellular differentiation. To test this, we stimulated the monocytic cell line THP-1 with a variety of agents known to induce a macrophage-like phenotype [28]. As shown in Fig. 6A, cells exposed to differentiating agents were transduced to varying degrees with RRV-pseudotyped vector. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a very potent activator of macrophage differentiation, induced a more than 10-fold increase in transduction, whereas other inducing agents were less effective. Differentiation of THP-1 cells with TPA did not increase AAV-mediated gene transfer, providing further evidence that these viruses utilize different entry pathways (data not shown). Interestingly, differentiation moderately decreased transduction with the VSV-G pseudotype (Fig. 6A), suggesting that the increase in transduction observed with the RRV pseudotype is a property of the RRV envelope glycoprotein and not the lentiviral vector core. Based on the literature we would also predict that the observed decrease with the VSV-G pseudotype is envelope related, as treatment of human myeloid cell lines with differentiating agents increases, not decreases, infection with wild-type HIV [28].

To investigate whether the stimulation of lymphoid cell lines would also promote transduction, we stimulated the Jurkat T cell and Frev B cell lines with the mitogens phytohemagglutinin (PHA) and pokeweed mitogen (PWM), respectively. As shown in Fig. 6B, stimulation of the Jurkat and Frev cell lines did not increase RRV gene transfer, yet unexpectedly, there was a marked decrease in transduction by VSV-G-pseudotyped vector. Another experiment evaluating stimulation of the Jurkat T cell line and the Priess B cell line also demonstrated decreased gene transfer with VSV-G-pseudotyped vector, but not with the RRV pseudotype (data not shown).

The surprising and consistent finding was the decrease in gene transfer by VSV-G-pseudotyped lentiviral vectors after cell stimulation in B and T cell lines (Fig. 6B). As noted above, differentiation of THP-1 cells also decreased gene transfer by VSV-G-pseudotyped lentiviral vector (Fig. 6A). To determine if stimulation of primary hematopoietic cells decreases transduction of VSV-G-pseudotyped lentiviral vectors, we transduced murine bone marrow cells without prior stimulation or after 24-h incubation in cytokines. As shown in Fig. 7, cytokine prestimulation decreased gene transfer by approximately 50%. This is consistent with our findings in cell lines and contrasts significantly with attempts to transduce hematopoietic progenitors with retroviral vectors, which generally require cytokine stimulation for efficient gene transfer. These findings indicate that transduction protocols must be tailored not only to the vector but also to the envelope glycoproteins used in vector pseudotyping.

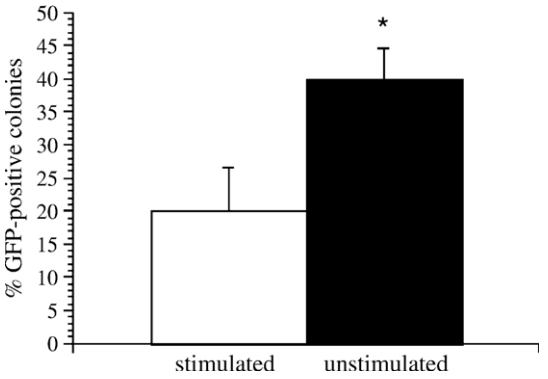

FIG. 7. Effects of cytokine stimulation on transduction of primary murine progenitors with VSV-G-pseudotyped lentiviral vectors. Low-density bone marrow cells were transduced with VSV-G-pseudotyped lentiviral supernatant either immediately or after a 24-h culture period with cytokines. Transduced cells were plated in methylcellulose progenitor assays. Progenitors were scored 7 days later to determine the % GFP^+ colonies. *$P < 0.04$ using an unpaired Student *t* test.

DISCUSSION

Our previous study revealed that glycoproteins derived from the two alphaviruses SFV and RRV can be incorporated into HIV-1-derived lentiviral vectors. These vectors can be concentrated efficiently by ultracentrifugation and transduce epithelial and fibroblast-derived cell lines from various tissues [13]. In the current work, we find that transduction of hematopoietic cells by lentiviral vectors pseudotyped with alphaviral glycoproteins is generally restricted. This restriction was noted in the majority of human cell lines tested and in human primary hematopoietic cells. These results reveal unique characteristics of the alphaviral glycoproteins that distinguish them from the VSV-G, as well as other glycoproteins. Also, we unexpectedly observed that transduction of hematopoietic cells with VSV-G-pseudotyped lentiviral vectors is affected by cellular differentiation and cytokine stimulation.

Alphaviruses have a very broad host range. For example, RRV propagates through mosquitoes after ingestion of virus-containing blood meals [29] and is known to infect an extremely broad host range of vertebrates and invertebrates. It is also known to infect a variety of cell types, such as neurons, macrophages, glial cells, muscle cells, epithelial cells, and synovial cells [12,16,30]. There have been emerging data that despite this large range of hosts and cell targets, alphaviral infection may be restricted in certain hematopoietic cell

populations. A study by Mossman and colleagues revealed that macaque peripheral blood mononuclear cells are resistant to infection by recombinant SFV *in vitro* [31]. Other studies have found that the related Sindbis (SB) virus does not efficiently infect hematopoietic cell lines of various lineages [32,33]. For RRV, macrophages have been shown to be infected with the wild-type virus, whereas lymphoid and monocytic cell lines are relatively resistant [16]. These prior studies were performed with wild-type virus. As our studies were performed with lentiviral vectors pseudotyped with the RRV envelope, our observations suggest that the inability of wild-type RRV to induce a productive infection in hematopoietic cells relates to a receptor-mediated mechanism, as opposed to postinfection mechanisms of resistance.

The receptors for VSV-G and RRV remain to be identified. Both have been shown to mediate viral entry by clathrin-dependent endocytosis [18,21]. The VSV glycoprotein is thought to utilize a ubiquitous phospholipid moiety for entry, although the exact nature of the receptor complex is still elusive, and different phospholipids may be utilized in different cell types [34–36]. Alphaviruses may use proteins as receptors for infection, and it has been speculated that adhesion molecules, such as integrins, may serve as receptors for wild-type RRV infection [12,16,37]. This is supported by our observation that transduction with RRV-pseudotyped lentiviral vector was significantly inhibited by pretreatment of cells with trypsin, but not by removal of sialic acid or treatment with phospholipase A_2 (PLA_2). Our data also suggest a role for cell surface proteins in transduction with VSV-G-pseudotyped lentiviral vector. Prior work with wild-type VSV has provided contradictory information. For example, proteases do not inhibit VSV infection in monkey Vero cells [34,38], while studies in human HepG2 cells have shown moderate inhibitory effects of Pronase on VSV transduction and fusion activity [39,40]. Cell-type-specific differences in the molecules used for viral entry have been suggested for VSV-G [36], which may explain these findings. The possibility that different cells use different molecules to mediate RRV infection must also be considered, especially given the observation that alphavirus SB has been shown to use the high-affinity 67-kDa laminin receptor for entry into baby hamster kidney cells and other mammalian cells, but it utilizes a different receptor for entry into chicken embryo fibroblasts [37].

As investigators have previously found that AAV vectors have a very limited capability for hematopoietic cell transduction [22,23,25], we compared transduction of an AAV-based vector with that of our RRV- and VSV-G-pseudotyped lentiviral vectors. It is unlikely that the major AAV receptor, heparan sulfate proteoglycan, functions as an RRV receptor as RRV does not to bind to cell surface GAG [41]. AAV-2 also requires a coreceptor such as fibroblast growth factor receptor 1 and $\alpha V\beta 5$ integrin [22,24–26,42], but our data suggest that RRV may utilize other molecules. Notably, the leukemia cell lines HEL and K562 were transduced with moderate efficiency with RRV-pseudotyped vector (roughly 30 and 70%, respectively, at m.o.i. of 100), whereas comparable AAV-2 vector preparations provided no significant transduction. While our data suggest different receptors for RRV and AAV, AAV vectors have unique postinfection processing that might contribute to the differences noted [43,44]. Nevertheless, these observations will be useful in future studies aimed at understanding vector processing and in identifying the receptor(s) for wild-type and vector-associated RRV glycoproteins.

Our observation that the monocytic cell line THP-1 is relatively resistant to lentiviral transduction and the resistance can be overcome by exposure to differentiating agents does correlate with the biology of wild-type virus. RRV does not infect primary monocytes but macrophages are directly infectable *in vitro* and are the general agents of disease and muscle pathology in a mouse model of RRV infection [16,45]. Increase in transduction by RRV-pseudotyped lentiviral vectors was not increased in T and B cell lines exposed to mitogens, suggesting stimulation alone was not responsible for the observations in THP-1 cells. Of note, recent work by Klimstra and colleagues has demonstrated that SB virus produced in mosquito cells uses the C-type lectins DC-SIGN and L-SIGN as attachment receptors to mediate infection, whereas SB virus produced in mammalian cells showed very inefficient binding to and infection of DC-SIGN/L-SIGN-expressing cells [33]. DC-SIGN is highly expressed on dermal dendritic cells and macrophages and can serve as an attachment factor for high-mannose oligosaccharides, such as those present on the envelope glycoproteins of arthropod-derived viruses. In contrast, mammalian cells produce glycoproteins with more complex carbohydrate modifications that have a much lower binding affinity for C-type lectins. Thus, species-specific differences need to be taken into account and can influence the host range of a pseudotyped vector. Further studies will reveal whether wild-type RRV spreading from mosquitoes to humans can target macrophages and dendritic cells via attachment to C-type lectins.

An unexpected finding was a threefold decrease in transduction with VSV-G-pseudotyped lentiviral vectors after mitogen stimulation of T and B cells. Differentiation of THP-1 cells was also associated with a marked decrease in transduction and a 24-h incubation of primary murine bone marrow led to a 50% decrease in the number of transduced progenitor colonies. This contrasts with retroviral vectors, in which cell stimulation increases cell cycling and enhances gene transfer. Interestingly, Hagani et al. found that stimulated primary murine T lymphocytes were more efficiently transduced with ecotropic than with VSV-G-pseudotyped oncoretroviral vectors [46], and both human and macaque stimulated T cells

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were more efficiently transduced by simian immunodeficiency virus vectors pseudotyped with the amphotropic or the modified feline endogenous retrovirus envelope (RD114/TR) than with VSV-G [47]. It is thus conceivable that stimulation lowers the expression of the putative VSV-G lipid receptor moiety, or alters its conformation, whereas the protein receptors used by retroviral envelopes are sufficiently expressed on activated cells. It may also be that the level and timing of cell stimulation are important, since studies using VSV-G-pseudotyped lentiviral vectors have found that transduction is significantly increased in activated compared to resting primary human lymphocytes [48,49]. Together, this information suggests that significant differences exist between lentiviral and oncoretroviral transduction beyond the previously published differences related to cell cycling. As oncoretroviral vectors generally do not utilize the VSV-G envelope, we are now investigating whether the differences are vector (oncoretroviral versus lentiviral) or envelope (VSV-G versus ecotropic/amphotropic) specific. Until these mechanisms are better understood, optimization of transduction protocols will require special attention to culture conditions and envelope selection.

While the current work with RRV-pseudotyped lentiviral vectors indicates that they are poorly suited to transduction of hematopoietic cells, this apparent disadvantage could prove useful in certain clinical scenarios. For example, bone marrow transplantation has long explored purging of stem cell products with chemotherapy and immunotherapy agents. To date, the availability of selective agents that do not harm normal hematopoietic tissue has limited this approach. Given the high gene transfer seen with RRV-pseudotyped lentiviral vectors in cells of epithelial origin, the use of these vectors for marrow purging deserves further study. Also, when considering *in vivo* applications of lentiviral vectors, envelopes that prevent transduction of hematopoietic tissues may have some safety advantages. This would be especially true in applications that seek to transduce cancer cells with cytokine and other growth factors that could function in an autocrine fashion if inadvertently introduced into hematopoietic tissues.

MATERIALS AND METHODS

Cell lines. The human embryonic fibroblast cell line 293, human fibrosarcoma cell line HT-1080, and C8166 (human T lymphocyte), MDA-231 (human breast cancer), K562 (human myeloid progenitor), HEL (human erythroleukemia), and Jurkat (human T lymphocyte) cell lines were all obtained from the American Type Culture Collection. The human embryonic fibroblast-derived cell lines 293T and 293T-NA were provided by Cell Genesys (Foster City, CA, USA). Investigators at Indiana University provided the following cell lines: THP-1 (human acute monocytic leukemia) and U937 (human histiocytic lymphoma) cells were kindly provided by Michael Klemsz; Raji and Frev (human B lymphocyte) cell lines were provided by Janice Blum; MOLT-4 (human acute lymphoblastic leukemia) cells were provided by Hal Broxmeyer; HL-60 (human acute promyelocytic leukemia) cells were provided by Robert Hromas; MDA-MB-231 were provided by George Sledge. All cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA) and 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, USA).

Vector production. Lentiviral vectors were produced by transient transfection of 293T cells. Cells (5×10^6) were seeded in 75-cm^2 tissue culture flasks 24 h before transfection in D-10 medium. Cells were refed with fresh D-10 medium 2 h before transfection. Transfection was performed by calcium phosphate precipitation method (Profection Kit; Promega) according to the manufacturer's instructions, using pcDNA-HIV-CS-CGW (18 μg, provided by Phil Zoltick, Children's Hospital, Philadelphia, PA, USA) as the transfer vector to express EGFP and the lentiviral helper plasmids pMDLg (6.6 μg) and pRSV/Rev (3.3 μg; Cell Genesys). The 293T cells were also cotransfected with the appropriate glycoprotein expression plasmid, expressing VSV [pCI-MD.G (5.13 μg)], RRV [pCI-RRV (6.3 μg)], or SFV [pCI-SFV (6.3 μg)] glycoprotein. The construction of the glycoprotein expression plasmids for VSV, RRV, and SFV glycoproteins, which differ only in the coding region, is described elsewhere [13]. Cells were refed with fresh medium 16–18 h posttransfection and vector supernatants were harvested approximately 48 h after transfection. Vector was filtered (0.45 μm) and stored at −80°C. To generate concentrated vector, transient transfections were scaled up to 300-cm^2 flasks. Then, 94 ml of supernatant was loaded per Beckman Quick-Seal ultracentrifuge tube (Beckman Coulter, Fullerton, CA, USA) and spun at 50,000*g* for 1 h in a Beckman Optima XL-100k ultracentrifuge using a 45-Ti fixed-angle rotor. Pellets were resuspended in 1 ml of D-10 and stored at −80°C. Titers of the EGFP-expressing vectors were determined by flow cytometric analysis of transduced 293 cells using our published methods [20].

Transduction using pseudotyped lentiviral vectors and recombinant AAV vector. Transduction of adherent cell lines with lentiviral vectors was performed by plating 10^5 cells per well in 6-well dishes, followed by the addition of 1 ml of vector supernatant and 8 μg/ml Polybrene. After 4 h, supernatants were aspirated and cells were refed with fresh medium. Hematopoietic cell lines were transduced by plating 5×10^4 cells per well in 12-well dishes and adding 0.5 ml of vector supernatant and 4 μg/ml Polybrene. After 18 h, cells were centrifuged and resuspended in fresh medium. All cells were analyzed approximately 72 h posttransduction for EGFP expression or by PCR. Recombinant AAV vector encoding EGFP was generously provided by Arun Srivastava (Indiana University) and was stored in aliquots at −80°C. For transduction, an aliquot was thawed and all dilutions of the vector were done in IMDM (Iscove's modified Dulbecco's medium; Gibco BRL) without FCS, to prevent competition for binding of AAV vector to its cellular coreceptor by fibroblast growth factor contained in the serum. Transductions with AAV vector were performed as described for lentiviral vectors, but without the addition of Polybrene.

Preparation and transduction of primary hematopoietic cells. Umbilical cord blood (UCB) samples were collected under a research protocol approved by the Indiana University Medical Center Institutional Review Board. UCB was processed as previously described [50] and CD34$^+$ cells were isolated using the CD34 Progenitor Cell Isolation Kit and the AutoMACS (Miltenyi Biotech, Inc., Auburn, CA, USA) according to the manufacturer's instructions. The range in purity of CD34$^+$ cells was 85–95%. Cells were then grown for 3 days in IMDM (Gibco BRL) supplemented with 10% FCS (Hyclone), 2 mM L-glutamine (Gibco BRL), and 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL) and containing the cytokines G-CSF, SCF, and MGDF. All cytokines were obtained from Amgen (Thousand Oaks, CA, USA) and used at 100 ng/ml each. The day before transduction, wells in a 12-well plate were coated with 4 μg/cm^2 of the recombinant fibronectin fragment CH-296 (Takara Shiuzo Co. Ltd.) and kept at 4°C overnight. The cytokine-stimulated cord blood cells were then plated at 10^5 cells per coated well in triplicate, and 0.5 ml of concentrated pseudotyped lentiviral vector supernatant with 4 μg/ml Polybrene was added per well. Transduction proceeded for 18 h, after which cells were counted with a hemocytometer. A portion of the cells

were grown for an additional 3 days in fresh medium supplemented with 100 ng/ml each of G-CSF, SCF, and MGDF and then analyzed for EGFP expression by flow cytometry. The remaining cells were plated in methylcellulose (Methocult GF H4434; Stem Cell Technologies), and 10–14 days later progenitor colonies were analyzed for EGFP expression as previously described [50].

Human G-CSF-mobilized peripheral blood stem cells were obtained from a normal donor protocol approved by the Indiana University Institutional Review Board. $CD34^+$ cells were purified by immunomagnetic methods using the Isolex300i cell selection device according to the manufacturer's instructions (Baxter Immunotherapy, Irvine, CA, USA). Isolated $CD34^+$ cells (95% purity) were aliquoted and stored in liquid nitrogen. For this experiment, cells were thawed and incubated in cytokines (100 ng/ml each G-CSF, SCF, and MGDF) for 2 days. Transductions using VSV-G- or RRV-pseudotyped lentiviral vectors were performed on CH-296-coated plates prepared as described above, with 4.5×10^4 $CD34^+$ cells and 0.5×10^4 MDA-MB-231/neo cells added to each well of a 12-well dish. Mock-treated cells were treated in an identical manner, except no vector was utilized during the transduction procedure. Cells were harvested after a single 18-h transduction and then replated in tissue culture treated dishes overnight to allow the majority of the MDA-MB-231/neo cells to adhere. The adherent population was subsequently selected for MDA-MB-231/neo cells by passage in cytokine-free medium with 500 μg/ml G418 (active) and then assessed for GFP expression on day 6. The nonadherent population was analyzed in two ways: a portion was placed in methylcellulose and assessed for GFP expression in a manner identical to that described above for $CD34^+$ cord blood cells, and the remaining portion was maintained in suspension in the presence of cytokines and assessed on day 6 for GFP expression by FACS.

C57BL/6J mice were maintained in a breeding colony approved by Indiana University's Laboratory Animal Research Committee. Bone marrow low-density mononuclear cells were prepared (Ficoll–Hypaque, density 1.119; Sigma) as previously described [51]. Low-density cells were transduced either immediately or after a 24-h culture period in IMDM supplemented with 20% FCS, 200 units/ml interleukin-6, and 100 ng/ml murine SCF (Peprotech, Rocky Hill, NJ, USA). For the transduction procedure, low-density cells (10^6) were placed in a six-well dish containing IMDM, 20% FCS, 200 units/ml interleukin-6, and 100 ng/ml murine SCF and were transduced with concentrated lentiviral supernatant (m.o.i. of 100) in the presence of Polybrene, similar to studies with human $CD34^+$ cells. Transduced cells were plated in methylcellulose for the growth of hematopoietic progenitors and scored on day 7 of culture as previously described [51].

Determination of DNA titers. To detect viral DNA sequences, transduced cells were grown for 1–2 weeks. Genomic DNA was extracted from transduced cells using the Puregene Kit (Promega) according to the manufacturer's instructions. Samples were then amplified by quantitative PCR (TaqMan) and compared to a serially diluted plasmid standard (pcDNA-HIV-CS-CGW) as previously described [20].

Induction of cellular stimulation and differentiation. The differentiating agents retinoic acid (RA), DMSO (dimethyl sulfoxide, endotoxin free), bacterial lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:b4), and phorbol ester TPA were all purchased from Sigma and stored at −20°C. Before use, RA and TPA stock solutions were diluted with ethanol, and LPS was diluted with culture medium to obtain the desired concentrations. To induce cellular differentiation, 10^6 THP-1 cells/ml were treated for 3 days in culture medium supplemented with 10 ng/ml LPS, 10^{-6} mol/L RA, 1.5% DMSO, or 1 ng/ml TPA. Differentiation was determined by monitoring morphological changes, such as cell clustering, and spreading and cellular adhesion to the plastic wells. The stimulating agents PHA and PWM were kindly provided by the Indiana University Histocompatibility Laboratory. To induce cell stimulation, 10^6 Jurkat or Frev cells/ml were treated for 24 h in culture medium containing 12.5 μg/ml PHA or PWM, respectively. All treated and untreated cells were monitored using a hemocytometer and trypan blue exclusion. Cells treated with either differentiating or stimulating agents had lower cell numbers and some decrease in cell viability compared to untreated controls. Cells were then plated at 5×10^4 viable cells per 12-well plate. Transduction was performed as described above for hematopoietic cell lines, except that treatment continued throughout the transduction (18 h). After transduction, cells were resuspended in fresh medium without stimulating or differentiating agents and grown for another 72 h before EGFP expression was assessed by flow cytometry.

Enzymatic and chemical modification of cells. Trypsin, Pronase E (protease XIV), and PLA_2 were all purchased from Sigma. Neuraminidase was obtained from both Sigma and Roche. To obtain the desired enzymatic concentrations, neuraminidase and PLA_2 were resuspended in PBS digestion buffer (PBS with 0.1% bovine serum albumin, 0.1% glucose, 0.2% gelatin), whereas protease XIV was resuspended in PBS. 293 cells (5×10^4 per well in tissue culture treated six-well plates) were incubated with various concentrations of either PLA_2 or neuraminidase solution for 1 h, whereas treatments with trypsin and protease XIV lasted 15 min. Incubation times were derived from previously published protocols [34,38,39]. After enzymatic treatment, cells were washed three times with PBS and transduced with vector supernatants at m.o.i. of 1 for either 1 or 4 h. To determine the effects of heparin on transduction, vector supernatants were supplemented with the appropriate concentration of heparin and incubated at 37°C for 1 h before transduction. The percentage of EGFP-positive cells was determined by flow cytometry 72 h posttransduction as described above.

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