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VSV-G Pseudotyped Lentiviral Vector Particles Produced in Human Cells Are Inactivated by Human Serum

Nicholas J. DePolo,* Joyce D. Reed,* Philip L. Sheridan,* Kay Townsend,* Sybille L. Sauter,* Douglas J. Jolly,* and Thomas W. Dubensky, Jr.*,[1]

Chiron Corporation, Emeryville, California 94608

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Lentiviral vectors transduce dividing and postmitotic cells and thus are being developed toward therapies for many diseases affecting diverse tissues. One essential requirement for efficacy will be that vector particles are resistant to inactivation by human serum complement. Most animal studies with lentiviral vectors have utilized VSV-G pseudotyped envelopes. Here we demonstrate that VSV-G pseudotyped HIV and FIV vectors produced in human cells are inactivated by human serum complement, suggesting that alternative envelopes may be required for therapeutic efficacy for many clinical applications of lentiviral vectors.

Key Words: **lentivirus vector; retroviral vector; complement; resistance; human sera inactivation; VSV G pseudotyping.**

INTRODUCTION

Two important recent advances in retroviral vector technology are (i) the development of stable human packaging cell lines (PCLs) for production of Moloney murine leukemia virus (MLV) vectors that are resistant to inactivation by human serum complement (1–4) and (ii) the development of lentiviral vectors capable of transducing both dividing and postmitotic cells (5–12). To date, lentiviral vectors derived from human immunodeficiency virus (HIV) and feline immunodeficiency virus (FIV) have been produced by transient transfection or by induction of stable PCLs. These vectors are typically produced in derivatives of the human 293 cell line and are predominantly pseudotyped with glycoprotein G from vesicular stomatitis virus (VSV-G) (5, 10). In contrast to MLV-based vectors, HIV and FIV VSV-G pseudotyped vectors have demonstrated good transduction efficiency in postmitotic cells of many tissues in animals, including retina, respiratory epithelium, muscle, brain, and liver (11, 13–15). VSV-G pseudotyped retroviral vectors are more uniformly infectious over a broad range of tissues and species, compared to vector particles containing the amphotropic or xenotropic envelopes (16, 17). Retroviral vectors produced by transient transfection methods are generally higher in titer when using VSV-G rather than alternative (e.g., amphotropic) envelopes, and VSV-G also provides purification advantages due to the increased stability of the vector particle (16).

Work by us and by others demonstrated that MLV-based retroviral vectors produced in certain human cells are resistant to inactivation by human complement (1–4, 18). Testing of amphotropic MLV vector produced in human HT1080 cells showed that resistance to inactivation by human serum *in vitro* correlated with highly increased *in vivo* systemic stability in chimpanzees following intravenous administration. In contrast, MLV vectors produced in canine cells were rapidly susceptible to inactivation both *in vitro* and *in vivo* (2).

Generally, complement resistance correlates with retroviral vectors produced in cells lacking (α1-3)galactosyltransferase (αGT) activity, particularly certain human cells (19, 20). Complement directed by antibody specific for galactosyl (α1-3)galactosyl (αGal) terminal glycosidic epitopes, synthesized by αGT, plays an integral role in retroviral vector inactivation (20, 21). Other factors besides the producer cell, particularly the envelope glycoprotein, also contribute in determining complement sensitivity (18, 22).

MLV-based amphotropic vectors resistant to inactivation by complement have been an important component in our development of a Factor VIII gene therapeutic for hemophilia A, which is administered intravenously (23). Because lentiviral vectors may become an important vector for this clinical application as well as for others, we decided to determine the relative complement sensitivity of two primary lentiviral vectors, HIV and FIV, produced in human cell lines with either VSV-G or

[1]To whom correspondence should be addressed at Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608. Fax: (510) 923-2586. E-mail: tom_dubensky@cc.chiron.com.

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amphotropic envelopes. Defining the parameters which result in complement resistance is an important step toward developing lentiviral vectors that are broadly useful for human gene therapy applications.

MATERIALS AND METHODS

Cells. BHK-21, HT1080, and 293T cells were maintained in DMEM (Gibco-BRL) containing 10% fetal bovine serum (FBS).

Vectors. All vectors (MLV, HIV, and FIV) were produced by parallel methods through transfection of human 293T cells. Cotransfections of vector, envelope, and gag-pol plasmids were performed as described previously (7, 9, 16). Crude titers ranged between 1×10^5 and 5×10^6 blue colony-forming units per ml (BCFU/ml) in various preparations, with VSV-G preparations averaging 1–2 $\times$ 10^6 BCFU/ml and amphotropic enveloped preparations about 1–3 $\times$ 10^5 BCFU/ml. To standardize input titers and preparation purity, all VSV-G and amphotropic envelope vectors encoding β-galactosidase were purified and concentrated by low-speed centrifugation (24) before resuspension and dilution in growth medium to equivalent titer [10^6 BCFU/ml]. Aliquots were frozen at –70°C, for later use in assays for stability in 80% human sera, for both G and amphotropic envelope preparations of all vectors. Titer recovery was typically in the 50–90% range, and control tests of crude versus concentrated vector showed very similar serum sensitivity.

Titer assays. Vector samples from *in vitro* or *in vivo* assays were titered on HT080 target cells on six-well plates in the presence of 8 μg/ml polybrene (Sigma). BCFU titers were determined following X-gal staining following standard methods, as described previously (2). Determination of VSV titer was by plaque assay of individual serial dilutions of samples (25).

In vitro serum inactivation assays. Human sera used were either (A) pooled complement-active normal human sera (approximately five or more individuals per pool; Quidel, San Diego, CA) or (B) normal human sera from four individuals. Individual human sera were prepared and tested to verify normal complement activity, as described previously (2). Equal input titers of each virus or vector type were used in an experiment. To determine serum inactivation, either viral vector or VSV was diluted fivefold in normal human sera (NHS), heat-inactivated sera (HIS; incubated 1 h at 56°C), or control fetal bovine sera. All incubations were for 1 h at 37°C in 80% test sera with 100- to 200-μl reaction volumes. Sera absorption experiments were performed essentially as described by Beebe *et al*. (26). Briefly, BHK-21 cells (or HT1080 cells which gave similar results) were plated 24 h previously at 1×10^7 cells per T-75 flask and infected or mock infected with VSV-Indiana at an m.o.i. of 10 in 1 ml of medium for 1 h at 37°C and rinsed and fresh medium was added. After 4 h incubation, trypsinized cells were rinsed 2× with growth medium and 2× with PBS and then incubated on ice for 6 h with 0.5 ml of pooled normal human sera, Quidel Lot 2. Cells and debris were removed by two centrifugations for 0.5 h at 15,000*g* in a microfuge at 4°C. Residual VSV was inactivated by UV (30 s in a Stratagene (San Diego, CA) UV Crosslinker, and sera were stored on ice until use in assays.

RESULTS AND DISCUSSION

VSV is sensitive to inactivation by human serum. To determine the serum sensitivity of various VSV-G pseudotyped vectors, it is logical to first measure the sensitivity of VSV grown in nonhuman or human cells, which has been explored by several groups. Beebe *et al*. reported that VSV was equally sensitive when grown in BHK or certain human cells (26), and Welsh *et al*. observed that VSV was equally sensitive to human serum when grown in either αGal(+) or αGal(–) human cells (27), supporting these findings. Conversely, Thiry *et al*. (28) and Takeuchi *et al*. (29) found that human cell-propagated VSV had reduced sensitivity to human sera. In these reports, sera assay concentrations ranged from 10 to 50% and this along with cell line and other assay variations may explain some differences in findings. We have established a standardized higher percentage serum assay that has predictive value for *in vivo* circulation stability in primates, as we have described previously (2). Thus, we first assessed the stability of VSV propagated in BHK-21 hamster or HT1080 human cell lines with 80% serum assays in multiple individuals and two pooled sera lots. HT1080-propagated VSV was substantially inactivated, 100- to 400-fold, while the BHK-21 cell-propagated virus was more strongly inactivated, >10,000-fold (data not shown). These results are in general agreement with the results of Takeuchi *et al*. (29) with VSV propagated in these two cell lines, confirming that VSV grown in human cells retains substantial sensitivity to inactivation by human sera.

VSV-G pseudotyped oncoretroviral and lentiviral vectors are sensitive to inactivation by human serum. We compared the relative resistance to human serum inactivation of matched titer, human 293 cell-produced MLV, and lentiviral (HIV and FIV) vectors containing either VSV-G or amphotropic (4070A) envelope, to determine whether the results observed with VSV also correlated with VSV-G pseudotyped vector serum sensitivity. MLV, HIV, and FIV vectors containing the amphotropic envelope were resistant to inactivation by human serum, while the corresponding VSV-G pseudotyped vectors were nearly completely inactivated by the same experimental conditions (Fig. 1). The relative stability of all vectors observed in HIS suggests that the majority of inactivation in this lot of pooled sera was due to a heat-labile component, presumably complement. We have shown previously with similar matched titer (~2×10^5 BCFU/ml), high serum percentage assays that the level of *in vitro* human serum sensitivity correlated with decreased circulation half-life in primates phylogenetically close to human (2). Thus, these results suggest strongly that VSV-G pseudotyped lentiviral vectors might not be efficacious for human gene therapy applications requiring systemic administration.

The profile of oncoretroviral and lentiviral vector human serum sensitivity reported here is more striking than previous publications which suggested that VSV or VSV-G pseudotyped MLV or HIV vectors produced in αGal-negative cells were substantially more resistant to serum inactivation (29, 30). Several possibilities may explain why we found greater sensitivity to serum, including (i) we compared 80% serum inactivation between vectors all produced by equivalent methods, (ii) we tested at matched low titers to avoid saturation effects (2), and (iii) we evaluated inactivation in multiple sera samples. It seems likely that high percentage sera assays may be more relevant to certain *in vivo* administration routes, such as intravenous. With all these sera, VSV-G pseudotyped MLV, HIV, and FIV vectors produced in

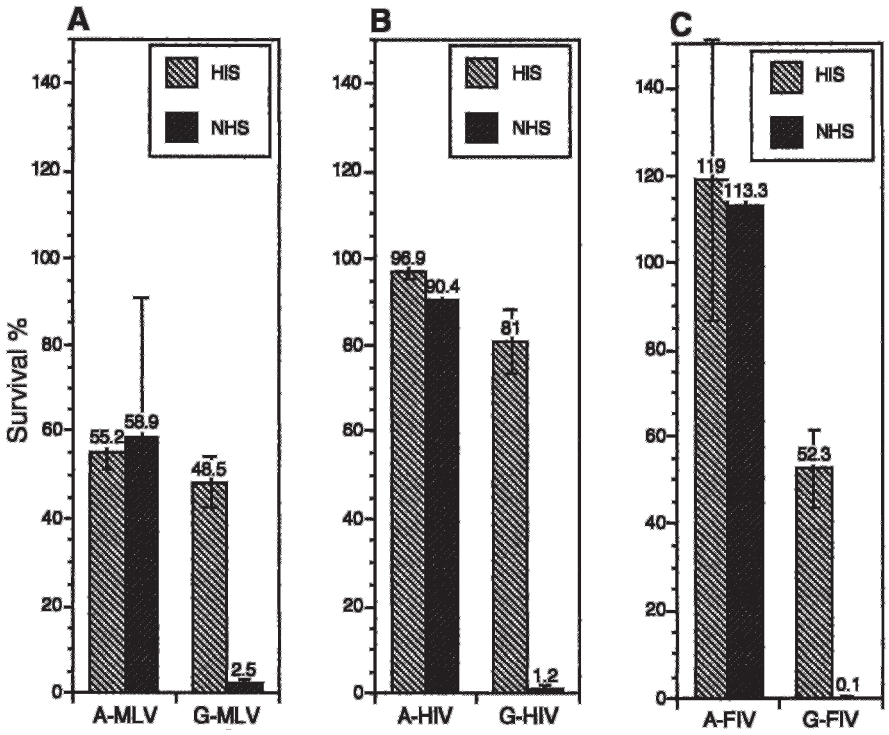

FIG. 1. VSV-G pseudotyped lentiviral and murine retroviral vectors produced in human cells are inactivated by human serum. Inactivation in pooled human sera of MLV, HIV, or FIV vectors containing the VSV-G or amphotropic envelope was measured by titer assays performed on HT1080 cells and staining with X-gal (BCFU/ml) (8). Survival percentage indicates the fraction of titer remaining in the NHS (normal human sera, Quidel pool, Lot 1) or HIS (heat-inactivated sera) versus the FBS control. All assays were repeated a minimum of two times, in triplicate, and mean values (±SE) from representative experiments are shown. A, B, and C represent relative inactivation of MLV, HIV, and FIV vectors, respectively.

293T cells by transient methods were substantially sensitive to serum inactivation. Most importantly, previous studies have not tested matched amphotropic vectors in parallel assays, and the results here indicate their comparatively greater serum stability, even under these stringent conditions.

VSV-G-specific antibodies mediate human 293T cell produced G-vector inactivation. Although the results in Fig. 1 reveal relatively little inactivation of G pseudotyped vector in HI sera from a specific lot of pooled human sera, certain human individual or pooled lots of sera resulted in more heat-stabile (i.e., noncomplement) inactivation. This result was specific to G vectors, as shown in Table 1. Pooled sera Lot 2 showed the highest heat-stabile G-vector inactivation, while a third lot tested was more similar to Lot 1. These results with different pooled human serum lots suggest variable levels of innate neutralizing antibody to VSV-G in human sera. This is similar to the VSV-G innate antibodies in certain mouse strains recently described by Ochsenbein *et al.,* which were low level, but shown to have potent effects on injected virus in their studies (31). As heat-labile inactivation may also be mediated by complement-fixing antibodies (26), it was of interest to determine whether antibodies specific to VSV-G could be preabsorbed. These sera were absorbed with either mock infected or 4-h VSV-infected BHK cells (expressing VSV-G on their surface) as described under Materials and Methods. The native or absorbed sera were tested against either human 293T-produced VSV-G MLV vector or canine D17-produced amphotropic vector (2), as a control for complement activity. The mock-infected BHK absorption caused only a partial reduction in sera inactivation to a similar extent for both G vector and control vector. In contrast, the absorption with VSV-BHK cells nearly eliminated both heat-stabile and heat-labile sera inactivation of the G vector, but not the control. These results suggest that absorbable factors, such as G-specific antibodies, mediate human 293T cell produced G-vector inactivation, both directly and by complement activation.

Because human cells, such as 293, are αGal-negative, it is unlikely that antibodies to this epitope play a role in serum inactivation of these G pseudotyped vectors. Although it is well established that αGal glycosylation epitopes are a primary determinant for targeting oncoretroviral and lentiviral vector inactivation in human sera, it is also apparent that not all mechanisms for this process are fully understood. Complement-resistant retroviral vectors can be produced in nonhuman, nonprimate mink and ferret brain cells, both of which are αGal-positive (18, 22). Furthermore, there is a broad spectrum of relative stability in human sera of vectors

TABLE 1
Amphotropic and VSV-G MLV Vector Sensitivity to Sera Pre- and Postabsorptions

Human sera tested	Survival (%)[a]	
	D17 ampho β-gal	293T VSV-G β-gal
HIS	61 ± 17	0.04 ± 0.02
NHS	0.045 ± 0.02	⩽ 0.01*
HIS-BHK absorbed[b]	74 ± 21	0.67 ± 0.29
NHS-BHK absorbed	0.35 ± 0.15	⩽ 0.01*
HIS-VSV/BHK absorbed	47 ± 12	129± 30
NHS-VSV/BHK absorbed	0.16 ± 0.08	39 ± 8

[a] Survival percentage ± SE is the fraction of vector titer remaining after incubation in test sera (HIS, heat inactivated sera or HS, normal human sera) relative to the FBS control incubation.

[b] Preabsorption conditions for the Quidel Lot 2 pooled sera are described in the text. * indicates samples with no detectable titer observed. Representative results are shown from one of two experiments.

produced in a variety of different human cell lines, all of which are apparently αGal-negative (18, 32). Interaction between vector envelope and producer cell membrane components may also play an interdependent role in conferring complement resistance of the vector (22). Thus, although following certain guidelines such as production in human cells like 293 can help produce vectors that are more stable in human sera, there still remain many other factors that determine complement resistance.

The findings reported here suggest that VSV-G pseudotyping-conferred complement sensitivity is not primarily due to VSV-G specific neutralizing antibody, since only certain HIS samples tested significantly neutralize vector. Our sera VSV G-Ab absorption experiments support the proposal from early VSV experiments that VSV-G specific nonneutralizing IgM class antibodies may bind to a highly conserved epitope(s) and direct classical pathway complement inactivation (26, 28). However, despite repeated efforts, we have so far been unable to generate VSV "antibody-escape mutants" that were able to propagate in human serum (data not shown). These results suggest that there may be critical epitope(s) that are essential for viral function.

Other mechanisms of VSV-G vector inactivation could be involved; for example, VSV-G may interact differently with human cell membrane complement control proteins, like CD-55 or CD-59 (33). VSV-G might either restrict vector membrane incorporation or block function of these cell proteins. According to this mechanism, VSV-G would prevent one part of multiple synergistic complement resistance mechanisms. This notion is supported by the observation that HIV and other enveloped viruses incorporate species-specific membrane complement control proteins (34–37). Whatever the mechanism or combination of mechanisms for inactivation may be, VSV-G pseudotyped lentiviral vectors produced in human cells are inactivated by human serum.

In summary, we have demonstrated that lentiviral vectors containing an amphotropic envelope may have substantial advantages compared to VSV-G pseudotyped vectors for certain human gene transfer applications, particularly those requiring intravenous administration. Although the extent of inactivation of VSV-G pseudotyped vectors across several pooled and individual human sera was somewhat variable, the level of susceptibility was always substantial. This observation has important implications for efficacy *in vivo,* as well as for the design and creation of new lentiviral production and PCL systems.

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