JOURNAL OF VIROLOGY, Jan. 2005, p. 756–763 Vol. 79, No. 2
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.2.756–763.2005

Efficient Functional Pseudotyping of Oncoretroviral and Lentiviral Vectors by Venezuelan Equine Encephalitis Virus Envelope Proteins

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Received 7 July 2004/Accepted 13 August 2004

Murine oncoretroviruses and lentiviruses pseudotyped with envelope proteins of alphaviruses have shown great potential in providing broad-host-range, stable vectors for gene therapy. Unlike vesicular stomatitis virus G protein-pseudotyped vectors, they are not neutralized by complement and do not appear to cause significant tissue damage. Here we report the production of murine oncoretroviral and lentiviral vectors pseudotyped with the envelope proteins of Venezuelan equine encephalitis virus (VEEV). When optimized, these pseudotypes achieve titers of 10^6 CFU/ml, which is 5- to 10-fold higher than for previous vectors pseudotyped with envelope proteins from other alphaviruses. They can also be concentrated or stored frozen without significant loss of infectivity. Consistent with the tropism of the envelope donor, they transduce a broad array of human cell types, including lung epithelial cells, neuronal cells, lymphocytes, and fibroblasts. Infection is blocked by agents that inhibit endosomal acidification and by neutralizing antibodies against VEEV. These observations indicate that the pseudotypes present native epitopes on their surface and enter through a VEEV envelope-dependent, pH-sensitive mechanism. The fact that the pseudotypes are unaffected by sera reactive to other alphaviruses indicates that they may be useful when successive gene therapies are required in the presence of an active immune response. In this case, having an array of alphavirus-based vectors with similar cell tropisms would be highly advantageous. These vectors may also be useful in diagnostic assays in which infectious VEEV is undesirable but immune reactivity to native epitopes is required.

The production of novel pseudotyped vectors is particularly useful for the expansion of tissue types that can potentially be targeted by virus-based gene therapy vectors (29). Retroviral vectors permit the efficient transduction of a broad array of cell types. Originally, retroviral vectors based on murine oncoretroviruses were used in a number of human gene therapy trials with some success (6). These vectors have the disadvantage that they require actively dividing cells for efficient transduction. Lentiviral vectors have been developed to overcome this limitation and can efficiently deliver genes to nondividing cells. For each vector, the outcome of infection is the same: a packaged gene becomes permanently integrated into the host genome. In the case of gene therapy, the gene may be corrective or may encode a dominant negative factor to down regulate specific genes and cellular pathways.

The cell tropism of these retroviral vectors is predominantly defined by the envelope proteins that coat the particle. Both discrete and broad-specificity vectors have their advantages. The first broad-specificity vector utilized the amphotropic murine leukemia virus (MLV) envelope protein. Later, however, it was shown that MLV vectors could be efficiently pseudotyped with vesicular stomatitis virus (VSV) envelope proteins (4). These chimeric particles comprised the core of an MLV, coated with the foreign envelope protein. Although the VSV G protein (VSV-G) provides high titers, typically exceeding 10^6 to 10^7 CFU/ml, its use has been problematic due to significant cell toxicity and neutralization by human complement. Recently, other novel, broad-specificity pseudotypes have been produced. These include pseudotypes of lymphocytic choriomeningitis virus (LCMV), Ebola virus, and a number of alphaviruses, namely, Semliki Forest virus (SFV), Sindbis virus (SINV), and Ross River virus (RRV). Pseudotypes of Ebola virus (36) and SFV (11) yielded low-titer viruses that reach only 10^3 to 10^4 CFU/ml, but LCMV (1), RRV (30), and SINV (21) have been more promising, with 10- to 100-fold-higher titers with murine oncoretroviral vectors. In the case of SINV, targeting specificity could be enhanced by insertion of the Fc-binding portion of protein A and by the binding of specific antibodies, a property that other alphaviruses may also permit. More recently, RRV envelope lentiviral pseudotypes have been made and applied in a mouse model to show efficient gene transduction of tissues (12). The potential of alphavirus envelope proteins to be efficiently incorporated into both oncoretroviral and lentiviral vectors and transduce many tissue types is particularly useful.

Venezuelan equine encephalitis virus (VEEV) is a distant relative of SINV, RRV, and SFV and is a representative of the New World alphaviruses. It is an arbovirus that is normally maintained in a "silent" enzootic cycle between mosquitoes of *Culex* (*Melanoconion*) spp. and rodent reservoir hosts. However, sporadic outbreaks involving hundreds of thousands of people have been reported since 1938. These outbreaks appear to result from the transmission of the virus to equines (horses, mules, and donkeys), where it becomes efficiently amplified and subsequently infects humans via *Ochlerotatus taeniorhyn-*

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chus and other mosquitoes (34). Humans succumb to a debilitating acute febrile illness with rapid onset, typically lasting up to 2 weeks. It is recognized as a significant and emerging health threat in North America and also as a potential biological weapon. Like other alphaviruses, VEEV has broad cell tropism in infected animals and in tissue culture. However, unlike the other alphaviruses that have been used to produce pseudotypes, which produce an arthralgia syndrome, VEEV is lymphotropic and also causes acute encephalitis, presumably because this virus has greater tropism for the brain. Indeed, in mice, infectious virus can be recovered from the brain as well as from blood, the lungs, and the spleen (24). However, disease is thought to be a product of replication and not directly due to action of the viral envelope proteins.

The broad specificity of the VEEV envelope protein, the potential to access novel tissues from organs such as the brain and lungs, and the lack of antigenic cross-reactivity to most other viruses make pseudotypes of VEEV a useful tool for gene therapy and also an aid for understanding entry into cells. Here we demonstrate the efficient pseudotyping of murine oncoretroviral and human immunodeficiency virus (HIV) lentiviral vectors with the envelope proteins of VEEV strains 3908 and Trinidad donkey. When conditions were optimized, we achieved titers that exceeded those of the RRV and SINV pseudotypes by an order of magnitude. We showed that these vectors have broad tropism for cell types, including human lung, fibroblast, lymphocytic, and neuronal lineages. These particles presented a native epitope profile as determined by neutralization tests and appear to enter cells through a pH-dependent pathway similar to that of wild-type VEEV. We discuss the usefulness of these particles for understanding the VEEV entry mechanism, expansion of the cell tropism of gene therapy vectors, and potential uses in simple diagnostic assays.

MATERIALS AND METHODS

Chemicals. Bafilomycin A_1 and chloroquine were from Calbiochem (San Diego, Calif.). All other chemicals were Sigma Ultragrade (St. Louis, Mo.) unless stated otherwise.

Antibodies. The anti-E2 monoclonal antibodies were provided by John Roehrig, Centers for Disease Control and Prevention, Ft. Collins, Colo. The anti-VEEV polyclonal antibody was from the American Type Culture Collection (ATCC VR-1249AF).

Cell lines and cultivation. All media were supplemented with penicillin and streptomycin. 293 and Huh-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, Calif.). THP-1 and PC-12 cells were grown in RPMI 1640 supplemented with 10% FBS and 5% FBS–10% horse serum, respectively. A549 and SH-SY5Y cells were grown in Ham's F-12 medium with 10% FBS. Mos-55 insect cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS.

Plasmid constructs. All plasmids were prepared with QIAGEN kits (Valencia, Calif.) or by cesium gradient centrifugation following standard methods. A plasmid containing the 3′ portion of the VEEV genome from subtype 1C strain 3908, a human isolate from 1995, was described previously (3); the plasmid encoding the prototype 1943 subtype 1AB strain Trinidad donkey envelope protein was a gift from Ilya Frolov. The VEEV envelope protein genes from the Trinidad donkey strain were amplified by PCR. The VEEV envelope proteins are normally made as a fusion to the C terminus of the viral capsid protein and do not have a typical initiation codon at the junction. One construct incorporated the C-terminal half of the RRV capsid protein as a leader peptide for the envelope proteins to mimic normal protein synthesis and maturation and is described elsewhere (9). This construct was cloned into pCDNA3 by using flanking BamHI and XhoI sites present in the original vector. A second construct placed an artificial initiation codon after a BamHI site (underlined below). The primer was 5′ GATCGGATCC**ATG**TCACTAGTGACCACCATG 3′. The 3′ primer for both was 5′ TGCTGACCAACCAGAAACA**TAA**CTCGAGCAT 3′. BamHI and XhoI restriction endonuclease sites used in cloning are underlined in the 5′ and 3′ primers, respectively, and initiation and termination codons are in bold type. The PCR product was first cloned into pFastbac (Invitrogen, Carlsbad, Calif.), and the fragment comprising the NsiI to NdeI sites (nucleotides 241 to 2739, with numbering starting at the first nucleotide of the BamHI site) was replaced with that from the original vector, which was previously sequenced. The remainder corresponded to the predicted sequence. To construct the 3908 strain expression clone, the same fragment from the plasmid containing the structural genes was replaced. From this vector, the entire BamHI-XhoI fragment for the Trinidad donkey and 3908 strains was inserted into pCDNA3 (Invitrogen). A unique ScaI site, present in the Trinidad donkey sequence at nucleotide 2579 but absent in strain 3908, was used to confirm the identity of each insert.

Production of pseudotyped MLV. Production of pseudotyped MLV is described in previous work (14). Briefly, 293FT cells (Invitrogen) were used as the producer cell line. The cells were transfected by using calcium phosphate (5) with any of three plasmids encoding the MLV Gag and polymerase (pGAG-POL, gift of J. Cunningham, Harvard Medical School; either pψ β-gal, pψ EGFP, or pFB-luc [Stratagene, La Jolla, Calif.], which encodes β-galactosidase, enhanced green fluorescent protein [EGFP], or luciferase, respectively, under control of the MLV long terminal repeat and packaging sequence) and the plasmids encoding envelope proteins from VEEV strains 3908 or Trinidad donkey, VSV (pVSV-G; Clontech, Palo Alto, Calif.), or Friend MLV (pCDNA3 with Friend-57 MLV envelope gene). The VSV-G-encoding plasmid was used at 1 μg per transfection to limit syncytium formation in the producer cells. After overnight incubation, the medium was replaced. After a total of 36 h, at the peak of virus production, the supernatants were collected and filtered through a 0.45-μm-pore-size cellulose acetate filter. Either the filtrate was used directly, or the virus was pelleted by 2 h of centrifugation at 80,000 × *g* and the pellet was used. In some experiments, virus was collected by pelleting it through a cushion of 20% (wt/vol) sucrose–10 mM Tris-HCl (pH 7.4).

Enhancement of virus titer by sodium butyrate. Twelve hours after transfection of cells, the medium was replaced with fresh medium containing 5 mM sodium butyrate (Aldrich, St. Louis, Mo.). The cells were incubated for an additional 12 h, after which the medium was again replaced with normal medium. Virus was collected after 16 h as described above.

Production of pseudotyped lentivirus. Pseudotypes of HIV were prepared as described above with plasmids carrying HIV *gag-pol* (pLP1; Invitrogen), *rev* (pREV; Invitrogen), and a packageable EGFP marker incorporating the *rev* response element. The last plasmid was made by inserting a PCR-amplified EGFP gene into pLENTI6 V5 TOPO (Invitrogen) by topoisomerase-mediated ligation. These plasmids were transfected together with the envelope protein expression plasmids as for the MLV pseudotypes. 293FT (Invitrogen) cells were used. Virus was handled identically to the MLV particles.

Determination of virus titer. Polybrene was not used for experiments described in this article. In other work, we determined that this compound generally increased virus titers 1.5- to 2-fold only. Cells were plated at a confluence of 20% 1 day before experiments were conducted. The following day, culture supernatants were removed and serial fivefold dilutions of virus were added. Two days later, the medium was removed, either infected cells were stained for β-galactosidase activity or EGFP expression was visualized by a Leitz inverted epifluorescence microscope, and colonies were counted. Host range determination was performed using the cell lines A549 (human lung epithelium), THP-1 (human monocyte), 293 (human embryonic kidney fibroblast), PC-12 (rat neuronal precursor), SH-SY5Y (human neuroblastoma), Huh-7 (human hepatocyte), and Mos-55 (*Anopheles gambiae* mosquito cell line).

Antibody neutralization tests. Serial twofold dilutions of antisera were made in DMEM and mixed with 200 CFU of pseudotyped virus. This mixture was applied to 293 HEK cells in a 12-well plate. After 2 days, transduced colonies were counted as described above.

Use of inhibitors of endosomal acidification. Ammonium chloride and chloroquine were dissolved directly in DMEM and incubated with cells for 1 h before and during incubation with virus. Bafilomycin A_1 was first dissolved in dimethyl sulfoxide as a 50 μM stock and diluted in DMEM before use. 293 HEK cells were pretreated for 1 h with bafilomycin A_1, chloroquine, or ammonium chloride at the concentrations indicated. Virus was then added at 1,000 CFU per well. After 3 h, the cells were washed three times with fresh medium and incubated for 2 days. Transduced cells were visualized as described above and counted.

RESULTS

Production of MLV vectors with VEEV envelope pseudotype and their characterization. The broad cell tropism of alphaviruses, which encompasses animal and insect cell types, is dictated by the viral envelope proteins. These proteins are also the major target for neutralizing antibodies from previously infected individuals and laboratory-immunized animals. The envelope proteins are synthesized as a single polyprotein precursor with the capsid protein. The latter is autoproteolytically cleaved immediately after translation. The remaining polypeptide comprises E3, E2, 6K, and E1. E3 functions as a signal sequence for E2, while the transmembrane portions of E2 and 6K serve as the signal peptide for E1, permitting insertion of each into the cell membrane. 6K is removed shortly after the secretion of the E1 protein (17). Only E2 and E1 are incorporated into the viral membranes of most alphaviruses that bud from the plasma membrane (18).

High-level surface expression of virus envelope proteins is known to be important for efficient pseudotype formation (29). We constructed envelope protein expression plasmids to achieve this expression as a first step in producing a viable pseudotype. Our initial constructs were based on the Trinidad donkey strain of VEEV, as it is the most highly characterized in the literature. One construct used a portion of the capsid gene from RRV as a leader peptide (Fig. 1A, upper construct). This strategy had previously been used for efficient production and maturation of the related SINV envelope proteins (9). A second, more conventional strategy added an initiation codon at the start of the E3 sequence (Fig. 1A, lower construct) and was a strategy similar to that employed for making pseudotypes of RRV and SINV. We found that while the capsid leader construct produced high-level expression of the envelope proteins in cell lysates (Fig. 1B, lane 3), these proteins were poorly incorporated into MLV particles (lane 10). Since only one band was detected with the anti-E2 monoclonal antibody, 1A3B-7 (lane 3, lower panel), the capsid leader peptide must have been correctly cleaved. The more conventional expression vector produced a major broad band of the same size when it was stained with polyclonal antiserum against VEEV (Fig. 1B, lanes 1 and 2, upper panel). Unlike the envelope proteins produced with the capsid leader peptide, these were readily incorporated into particles (lanes 6 through 9). This result suggests that, while the capsid leader peptide may enhance the production of the envelope proteins, either they become misfolded and do not reach the cell membrane or the capsid leader peptide itself interferes with envelope protein incorporation into the MLV particle. The E1 and E2 proteins migrated closely on gels and, because of heterogeneous glycosylation, could not be distinguished by size. However, the E2 protein was readily detected with an anti-E2 monoclonal antibody. The polyclonal antibody appeared to detect a similarly sized broad band. It also had reactivity to a band not detected by the monoclonal antibody (Fig. 1B, lane 6), and this band is likely E1. It is also known that the correct processing of E2 requires E1 coexpression; therefore, we expect that both proteins were being expressed correctly. For the construct lacking the leader peptide, we tested the efficiency of envelope protein incorporation into intact and defective particles. To do this, we compared pellets made by centrifugation to ones made through 20% sucrose cushions, which exclude defective particles from pellets. We found that the majority of envelope proteins present in the culture supernatant (Fig. 1B, lane 6) was pelleted through the sucrose cushion (lane 7), and this amount was comparable to the amount seen in material pelleted without a cushion (lane 8). This finding suggested that most of the envelope proteins had been efficiently incorporated into particles.

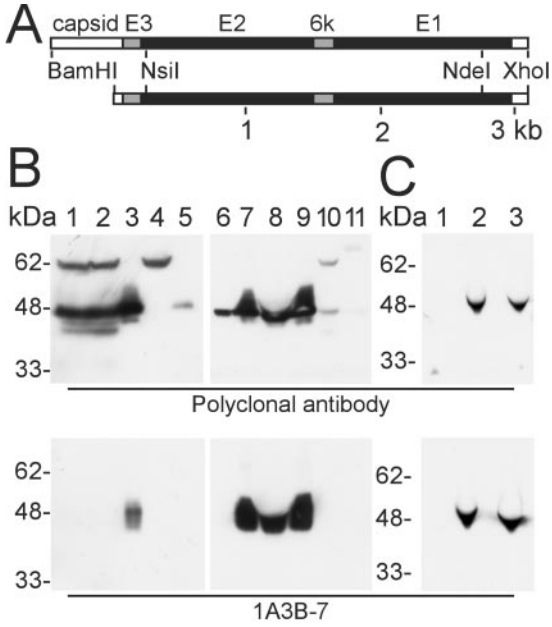

FIG. 1. Construct design and expression of VEEV envelope proteins in 293 HEK cells. (A) Two constructs were made. The first (upper) used the RRV capsid C-terminal domain as a leader peptide to initiate translation of the open reading frame. The second (lower) used an artificial initiation codon that preceded the open reading frame encoding E3 through E1. (B) Western blot analysis of envelope protein expression in 293 cells transfected with expression constructs for VEEV Trinidad donkey envelope protein (left panels) or filtered culture supernatants (right panels) and stained with an anti-VEEV polyclonal antibody (ATCC VR-1249AF) or the E2-reactive monoclonal antibody 1A3B-7. The polyclonal antibody cross-reacted with a band migrating slightly above 62 kDa in cell lysates. Lanes 1 and 2, lysates from cells expressing VEEV Trinidad donkey expression construct without capsid in normal medium and medium supplemented with 5 mM sodium butyrate, respectively; lane 3, construct with capsid leader peptide; lane 4, VSV-G expression vector; lane 5, purified TC-83 virus lysate. The supernatants in the right panels are unconcentrated material (lane 6), virus pelleted through 20% sucrose (lanes 7 and 9), virus pelleted without sucrose (lane 8), pellet from capsid leader construct (lane 10), and VSV-G pseudotype pelleted through sucrose (lane 11). (C) Particles pelleted through sucrose from MLV pseudotyped with VSV-G (lane 1) or with VEEV strain 3908 (lane 2) or Trinidad donkey (lane 3) envelope protein. Each was stained with the same antibodies used as described for panel B.

Our initial characterization focused on the constructs expressing the envelope proteins from the Trinidad donkey strain of VEEV, a prototypical strain and a member of the 1AB serogroup. We then determined if particles could be made containing envelope proteins from the 3908 strain of VEEV, which is a 1C serogroup member and typical of recent field isolates of the virus that is infectious for humans and equines.

TABLE 1. Titers of pseudotyped MLV determined by endpoint dilution[a]

Envelope protein	Titer (CFU/ml) on:	
	HEK 293 cells	293-CAT cells
VEEV capsid leader	$(1 \pm 0.6) \times 10^3$	ND
VEEV E3-1 TRD	$(2.0 \pm 0.5) \times 10^5$	ND
VEEV E3-1 3908	$(1.0 \pm 0.2) \times 10^5$	ND
VSV-G	$(1 \pm 0.5) \times 10^7$	10^7
Ecotropic	0	10^6
None	0	ND

[a] Virus was produced by transfection of 293 HEK cells. Supernatants were collected after 2 days and filtered, and virus titers were determined by endpoint dilution. ND, not determined.

TABLE 2. Inhibition of infection of VEEV strain Trinidad-pseudotyped MLV by neutralizing monoclonal antibodies[a]

Antibody	Specificity	% Reduction in titer (relative to untreated virus)	
		VEEV pseudotype	VSV-G pseudotype
Monoclonal			
1A4B-6	E1	97	0
1A3B-7	E2	44	0
1A3A-9	E2	100	0
1A2B-10	E2	29	0
Polyclonal	Virus	100	2

[a] Monoclonal antibodies were used at a final concentration of 1:200 in medium. Two hundred CFU of pseudotyped virus encoding β-galactosidase activity was added to the antibody-containing medium, which was then overlaid onto 293 HEK cells at 20% confluence. The reduction in titer was then determined by counting colonies after 2 days. Data from one representative experiment are given.

We found that Trinidad donkey and 3908 strain proteins provided similar levels of incorporation into particles (Fig. 1C).

The pseudotypes were then tested for infectivity in human fibroblasts. Virus was made by transient transfection of 293 HEK cells and harvested, and its titers were determined by limiting dilution (Table 1). We found that while the capsid leader construct of Trinidad donkey provided little incorporation of envelope proteins, it infected cells with a low titer of 10^3 CFU/ml. In contrast, constructs lacking the leader typically produced 100-fold-higher titers for both Trinidad donkey and 3908 strains. Background infection was undetectable, as determined by particles pseudotyped with the MLV ecotropic envelope protein (293 HEK cells lack the receptor for this virus).

Effect of sodium butyrate on pseudotype formation. In some experiments, we were able to boost titers up to 10-fold by addition of sodium butyrate (5 mM) to the medium for 12 h before virus harvest (data not shown). This compound is known to increase protein expression from cytomegalovirus promoter-driven vectors by stimulating the enhancer element of the promoter. However, this compound had an effect only when transfection conditions were not optimal. This result suggested that sodium butyrate was useful only when the plasmid was limiting. Indeed, in experiments where transfection was optimized, examination of envelope protein (Fig. 1B, lane 2) and its incorporation into particles (lane 9) from cells treated with sodium butyrate revealed little to no increase in expression levels.

Neutralization of pseudotypes by VEEV-reactive sera. To determine whether the pseudotyped particles entered cells by a VEEV envelope-dependent mechanism, we performed infection inhibition assays using monoclonal antibodies against the Trinidad donkey strain of VEEV. Three antibodies reacted with distinct epitopes on E2 (1A3B-7, 1A3A-9, and 1A2B-10), and one recognized an E1 (1A4B-6) epitope (10, 28). We also tested a neutralizing rabbit polyclonal antiserum reactive to whole virus. We used the monoclonal antibodies at a final dilution of 1:200 (final concentration, 5 μg/ml), which was previously reported to inhibit infection of 100 PFU of several strains of VEEV (including Trinidad donkey) by >70% (28). The polyclonal antibody was tested at 1:100. Two hundred infectious units of pseudotyped virus per well of a 24-well plate was incubated in the presence or absence of antibodies. A VSV-G pseudotype served as the negative control for nonspecific inhibition by the antibodies. Results are shown in Table 2. The three most potent antibodies were 1A4B-6, 1A3A-9, and the polyclonal serum, which all effectively blocked infection. In contrast, the VSV-G pseudotype titer was unaffected. Monoclonal antibody 1A3B-7 reduced infection by half. 1A2B-10, a weakly neutralizing antibody (10), provided only 29% inhibition of infection. Importantly, the neutralization of the VEEV envelope pseudotypes by each monoclonal antibody closely correlated with their reported activity against wild-type virus (10, 28). The exception to this pattern was the reactivity of the anti-E1 antibody. In wild-type virus, this antibody normally interacts with a shielded epitope. We believe that this finding may reflect the fact that the E1 protein in alphavirus particles normally lies at the base of the E2 protein and is protected from antibody interaction through steric hindrance of closely packed E1-E2 heterodimers (23, 25). The pseudotyped particles appear by electron microscopy to have little envelope protein on their surfaces (data not shown), and so the E1 protein is likely accessible to antibody. However, for E2 at least, the data indicate that the pseudotypes present a native set of epitopes on their surface and were efficiently neutralized by the appropriate antibodies. This finding indicates that the VEEV envelope proteins were responsible for permitting entry into the cell.

Inhibition of infection by lysosomotropic agents. We next determined if the pseudotypes were infecting cells through an entry pathway similar to that of VEEV. All alphaviruses are pH sensitive, with receptor engagement aiding attachment to cells. While this property may also trigger some prefusion structural rearrangements of the envelope protein, pH alone is sufficient to initiate virus and cell membrane fusion for SFV (35). Therefore, endocytosis and acidification in the endocytic compartment are likely to be critical for penetration of the nucleocapsid into the cytosol. Compounds that inhibit endosomal acidification also block infection by alphaviruses. To test whether the pseudotypes entered cells through a similar pH-sensitive, endocytic route, we tested their susceptibility to infection after treatment with endosomal acidification inhibitors (Fig. 2). We used VSV-G pseudotypes as a positive control for the action of each inhibitor (Fig. 2). An ecotropic MLV envelope pseudotype served as a pH-insensitive virus-negative control (Fig. 2) and to control for general toxic affects of the

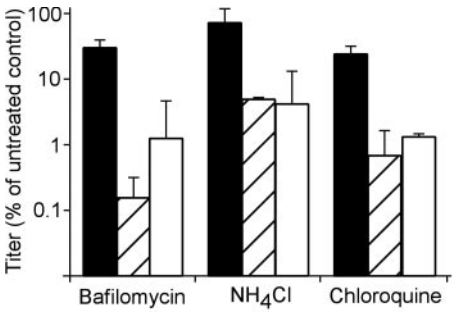

FIG. 2. Inhibition of pseudotype infection by lysosomotropic agents. The titers of ecotropic MLV envelope protein (filled bars), VSV-G (hatched bars), or VEEV strain 3908 envelope protein (open bars) pseudotypes of MLV were determined on 293-CAT cells treated with bafilomycin A_1 (60 nM), ammonium chloride (10 mM), or chloroquine (50 μM). Virus encoding β-galactosidase, with its titer previously determined, was added at 1,000 CFU per well to give a multiplicity of infection of 0.1. After the virus was stained for β-galactosidase activity, the numbers of colonies were counted and given as percentages of colonies relative to those of untreated cells. The graph shows data combined from two independent experiments with error bars showing standard deviations.

inhibitors. Bafilomycin A_1 was the most effective inhibitor, reducing the VEEV envelope and VSV-G pseudotype virus titers 100- and 1,000-fold, respectively. Similarly, ammonium chloride and chloroquine reduced titers >10- and >100-fold, respectively, for both VEEV and VSV-G pseudotypes. For each treatment, ecotropic MLV pseudotype infection was not greatly affected, as reported previously (14). These data strongly indicate that the VEEV-pseudotyped MLV particles enter cells through a pH-dependent pathway, likely involving endocytosis as for native VEEV.

Production of lentiviral vectors with VEEV envelope pseudotype. While MLV vectors are useful for delivery of genes to rapidly dividing cells, they have a major shortcoming in not efficiently infecting senescent cells. In contrast, lentiviral vectors readily infect these cells. Recently, it was reported that pseudotypes of RRV and SINV can be made by using an HIV type 1 lentiviral core (11), potentially expanding the range of target cells that can be transduced with these pseudotypes. To determine if this is a general phenomenon common to other alphaviruses, such as VEEV, we substituted the *gag-pol* and marker genes with those for HIV. Culture supernatants were then tested for infectious particles and yielded an infectious titer of 10^5 CFU/ml on 293 HEK cells for pseudotypes made with the VEEV Trinidad donkey envelope proteins. Titers seen with the strain 3908-derived envelope proteins were within the same experimental range (data not shown). For both, the titers were similar to those for the MLV pseudotypes and demonstrated that the VEEV envelope protein is functional and just as readily accommodated on lentiviral particles. In an effort to increase the titers of the pseudotyped viruses, we used 293FT cells (Invitrogen) in place of the 293 HEK cells. 293FT cells constitutively express high levels of the simian virus 40 (SV40) large T antigen and promote high-level expression of plasmids containing an SV40 origin of replication (22), like pCDNA3. Consistent with this difference in expression, we found that virus titers produced from 293FT cells were approximately 10-fold higher than those produced from 293 HEK cells, for both the MLV- and HIV-derived pseudotypes. These cells were then used to produce virus in the remaining experiments.

Determination of host range. All alphaviruses have extremely broad host ranges and can infect an equally broad range of cells in vivo and in vitro. This property is a major reason that alphavirus envelope pseudotypes have become of interest as potential gene therapy vectors. To define the tropism of these vectors, we screened a range of cell lines derived mostly from human tissues for susceptibility to infection with the VEEV strain 3908 envelope MLV and HIV pseudotypes (Table 3). We tested each type of virus with the same batch of cells so as to compare differences in infectivity for each. VSV-G pseudotypes are known to enter many cells types and were used to control for potential downstream blocks to retroviral infection. Generally, the VEEV-pseudotyped MLV or HIV vectors transduced all cell types tested with an efficiency that was 5- to 10-fold lower than that of the VSV-G control. This phenomenon was due to starting titers and did not reflect a difference in the susceptibilities of the cells. The only exception to this observation was the VEEV pseudotype with the lentiviral core, which infected the liver-derived Huh-7 cells similarly to or better than the VSV-G pseudotype. Interestingly, A549, a lung-derived type II pulmonary alveolar epithelial cell line, and the neuroblastoma-derived SH-SY5Y cells were highly susceptible to VEEV envelope pseudotypes. This

TABLE 3. Comparison of titers for VEEV strain 3908 envelope protein and VSV-G-pseudotyped MLV and HIV-1 lentiviral vectors on different cell lines[a]

Cell line	Cell type and source	Titer (CFU/ml) on:			
		MLV vector		HIV lentiviral vector	
		VSV-G	VEEV	VSV-G	VEEV
293 HEK	Epithelial, human kidney	$(1.6 \pm 0.6) \times 10^7$	$(2.0 \pm 0.8) \times 10^6$	$(3.0 \pm 1.4) \times 10^6$	$(7.0 \pm 1.4) \times 10^5$
THP-1	Monocytic, human	$(6.5 \pm 0.7) \times 10^3$	$(1.2 \pm 0.7) \times 10^3$	2×10^{3b}	3×10^{2b}
HUH-7	Liver, human	$(3.3 \pm 2.4) \times 10^5$	$(3.3 \pm 1.7) \times 10^4$	$(4.3 \pm 0.8) \times 10^4$	$(1.7 \pm 0.3) \times 10^5$
A549	Lung epithelial, human	$(4.0 \pm 0.4) \times 10^6$	$(1.2 \pm 0.3) \times 10^6$	$(4.5 \pm 0.7) \times 10^5$	$(7.8 \pm 1.9) \times 10^4$
SH-SY5Y	Neuroblastoma, human	$(5.0 \pm 4.2) \times 10^5$	$(6.0 \pm 4.2) \times 10^4$	$(5.6 \pm 3.0) \times 10^5$	$(5.0 \pm 3.0) \times 10^4$
PC12	Neuronal, rat	$(4.1 \pm 2.7) \times 10^5$	$(1.0 \pm 0.3) \times 10^5$	$(1.5 \pm 0.7) \times 10^5$	$(9.5 \pm 0.7) \times 10^3$

[a] Virus was produced with 293FT cells (Invitrogen) and provided increased titers compared to 293 HEK cells. All values are averages of data from at least three separate experiments except where indicated. Titers were determined by endpoint dilution using five-fold serial dilutions and counting colonies stained for β-galactosidase activity or for EGFP epifluorescence.

[b] Titer from one experiment.

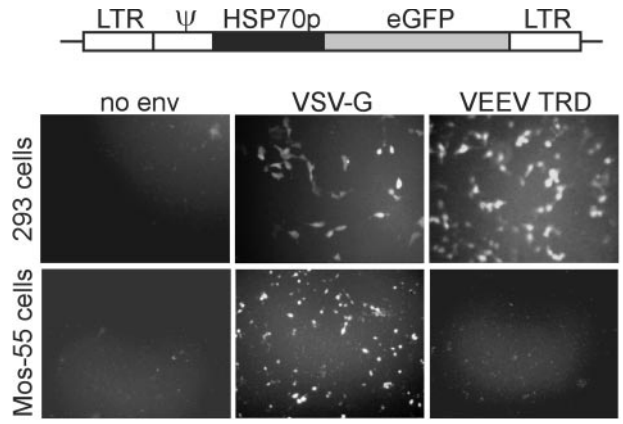

FIG. 3. Mos-55 cells are not susceptible to infection with the VEEV pseudotypes. A retroviral packaging vector was made for the expression of EGFP in insect cells (top diagram). This vector differed from a standard vector by having the EGFP gene (grey bar) under the control of the *Drosophila melanogaster* HSP70 promoter (black bar). The titers of VSV-G and VEEV Trinidad donkey pseudotypes were matched by concentrating the VEEV Trinidad donkey envelope-pseudotyped MLV 50-fold and by concentrating the VSV-G pseudotype 10-fold. Titers of duplicate samples were then determined on 293 HEK cells or Mos-55 cells (about one-quarter the size of a typical mammalian fibroblast). After 2 days, colonies were counted by epifluorescence microscopy. Particles made without envelope proteins were included as a negative control (no env).

finding is consistent with the ability of VEEV to infect lung and brain tissues and indicates that these vectors may be of use for targeting these tissues for gene therapy.

The identities of the receptors for all alphaviruses still remain ambiguous, although reports have indicated roles for the laminin receptor (32), glycosaminoglycans (15), and DC-SIGN in aiding infection (13). The difficulty in identifying the receptors has been due to the observation that nearly all cell types tested are highly susceptible to infection and that virus kills these cells. Classically, genes encoding susceptibility factors are transferred to cells resisting infection and can then be identified through a variety of methods. While VEEV can infect many mammalian hosts, it is much more restricted in its vector host range, which includes several species of *Ochlerotatus* and *Culex* mosquitoes. In contrast, *Anopheles* mosquitoes do not serve as hosts for VEEV or most other alphaviruses (27). Infection determinants in the envelope proteins may be responsible for this tropism (3). Interestingly, cells derived from *A. gambiae* mosquitoes (Mos-55) were reported to be susceptible to infection by VSV-G-pseudotyped MLV vectors (19).

We reasoned that the VEEV-pseudotyped viruses might provide an opportunity to determine whether *Anopheles* cells resist infection due to blocks at entry or other downstream events. For these cells, standard packaging vectors gave no signal indicating entry (8), so we used a modified gene-packaging vector (Fig. 3, upper panel) that drives expression of a reporter gene (EGFP or luciferase) from the *Drosophila* HSP70 promoter element (pLNHX; Clontech). With this vector, we confirmed the previous report that Mos-55 cells were readily infected with VSV-G pseudotypes (Fig. 3) and reached titers of 5×10^5 green fluorescent colonies/ml. This level was approximately 50-fold lower than that on 293 HEK cells, but the colonies were easily visible. We then used the VEEV pseudotype made with the same expression construct. Surprisingly, while this virus had produced a titer of 2×10^6 CFU/ml on 293 cells, no green fluorescent colonies were evident. This experiment was repeated three times with similar results, which indicates that these cells resist infection by the VEEV pseudotype due to a block in entry and that they may therefore lack receptors or some other critical factor required for entry. These results also support the conclusion that the VEEV envelope proteins are expressed on the pseudotyped particles in their native conformation.

DISCUSSION

VEEV envelope proteins yield high-titer oncoretroviral and lentiviral pseudotypes. VEEV is representative of the Venezuelan equine encephalitis complex of alphaviruses. These viruses form a distinct clade of New World alphaviruses that are distantly related to all Old World viruses. VEEV has 40 to 50% amino acid sequence divergence when the more conserved E1 envelope protein is compared to those of SFV, SINV, and RRV (26). The New World viruses also differ from the latter group by causing encephalitis in humans and equines (33). Of the seven subtypes of VEEV, only subtype 1 varieties AB and C (5% sequence divergence in the envelope proteins) generally cause disease in equines (2). In the present work, we chose to make VEEV envelope protein pseudotypes for two representatives of strains that are infectious to both humans and equines. These were Trinidad donkey and 3908 of the epizootic subtypes IAB and IC, respectively. We chose these because they would be best adapted to infect human and other mammalian cells.

Previous work by others has established that MLV and lentiviral pseudotypes could be made with the envelope proteins of the distantly related alphaviruses RRV (11, 30) and SINV (21). Pseudotypes made with RRV envelope proteins have reported titers peaking at 10^4 and 10^5 CFU/ml for MLV and lentiviral cores, respectively. SFV has also been pseudotyped onto lentiviral vectors but produced titers that were 10-fold lower than those of RRV. We therefore wanted to know if the envelope proteins of the two VEEV strains chosen behaved more like RRV or SFV. Our results clearly demonstrate that the Trinidad donkey and 3908 strain envelope proteins produced pseudotype titers that are equal to or higher than those of the RRV-pseudotyped vectors, depending on the method used to produce them. Sodium butyrate, a compound reported to enhance expression from cytomegalovirus promoter-based plasmids, aided in virus production when transfections were not optimal but still produced a titer peaking at 10^5 CFU/ml. In contrast, 293 cells stably expressing the large T antigen of SV40 (293FT) yielded titers that were 10-fold higher. This increase gives these VEEV-pseudotyped particles the highest titers for all alphavirus-derived vectors to date, approaching those typically achieved with VSV-G. Independently of the cell line used, the titers of the oncoretroviral or lentiviral vectors for each strain were approximately equivalent. The MLV particles appeared to be robust and were readily concentrated 50-fold by simple pelleting. These findings indicate that these new pseudotypes may be amenable to gene therapy protocols in which high-titer virus is desired.

VEEV pseudotypes give broad host range. In this study, we examined the ability of the vector to transduce an array of cell types. In each case, the vector was able to deliver and express the marker gene with titers typically ranging from 10^5 to 10^6 CFU/ml. The exception was THP-1, a human monocytic cell type, which produced a titer of approximately 10^3 CFU/ml; this level correlated closely to its susceptibility to VSV-G pseudotype transduction and likely reflects the efficiency of retroviral integration or activity of the endocytic entry pathway. Comparison of the cell tropism of the VEEV vector to the previously described RRV pseudotype is not possible, as a different spectrum of cells was used in the RRV study. However, a similar spectrum of cell types was tested with a recently reported LCMV envelope pseudotype. This vector achieved titers of 10^6 on 293 cells and was able to infect neural (SY5Y) cells with titers similar to those achieved (10^5 CFU/ml) with the VEEV pseudotype. The VEEV vector transduced liver (Huh-7) cells 10-fold better than the LCMV vector. Lung cells (A549) and monocytes (THP-1) were not tested (1). Therefore, the VEEV envelope pseudotype may provide a broad-specificity alternative to the LCMV vector, particularly when multiple therapies are required in the presence of an active immune response. In addition, the high efficiency of the VEEV vector in infecting lung cells may make it better suited for correction of lung disorders such as cystic fibrosis.

Pseudotypes present native, neutralizing epitopes on their surfaces. Our data involving VEEV-specific antisera confirmed the role of the VEEV envelope protein in driving infection. Similarly, the titer of the virus correlated with the level of envelope protein incorporated into the virus particle. One construct that used a leader peptide derived from the RRV capsid produced excellent expression of the envelope proteins but a low virus titer. This may have resulted from an inefficient interaction between the capsid portions and the C termini of the envelope proteins, since RRV capsid does not interact well with heterologous alphavirus envelope proteins (16). Instead, the capsid leader peptide may directly affect envelope protein trafficking or localization, making them unavailable for pseudotype formation. A more conventional construct that initiated translation from a methionine codon placed before the first residue of E3 was effective at raising the pseudotype titer. Our data also indicate that the VEEV envelope protein, and not some other contaminant in the MLV envelope membrane, is directly responsible for infection.

Entry is pH dependent. The pseudotypes appear to enter cells through a pH-dependent entry pathway, being readily blocked by three inhibitors of endocytic acidification. Bafilomycin blocks the vacuolar proton pump in endocytic vesicles, while chloroquine and ammonium chloride behave as weak bases that buffer this compartment against acidification. Each inhibitor was highly effective at inhibiting infection with VEEV or VSV-G pseudotypes and reduced titers 20- to 1,000-fold. Taken together, these data demonstrate that the VEEV particles infected cells through a native infection route requiring endocytosis and acidification in an endocytic vesicle. The pseudotypes thereby offer the potential to study native entry pathways of the parental VEEV in the absence of other cytotoxic effects associated with alphavirus infection. We are now particularly interested in determining if differences exist in the entry pathways used by VEEV and other alphaviruses.

Mosquito cells resist infection due to an early block to infection. We investigated the ability of the VEEV pseudotype to infect mosquito cells. VEEV naturally infects mosquitoes of the *Culex*, *Aedes*, and *Ochlerotatus* genera and others (20), but *A. gambiae* mosquitoes do not serve as natural hosts. It was previously shown that VSV-G pseudotypes of MLV can infect the *A. gambiae*-derived cell line, Mos-55, and stably express β-galactosidase as a marker gene. Here we found that while VSV-G pseudotypes readily infect these cells and express EGFP, they completely resist infection with the VEEV envelope pseudotype. As the only difference between the pseudotyped particles is the source of envelope protein, this finding indicates that the block to infection must be at the point of entry and not at some point downstream. These cells may lack expression of a factor critical for entry, such as receptors, or express a factor that directly blocks entry. Cholesterol has been demonstrated to be important in supporting the entry of SFV and SINV particles and may be required for the entry of other alphaviruses (31). As the cells were incubated in FBS, they are presumably saturated with cholesterol, and the presence of cholesterol is not likely to contribute to resistance unless the cells can preferentially exclude exogenous cholesterol from the membrane. The mechanism of resistance must also be specific to Mos-55 cells, as *Aedes*-derived cells are readily infected with live VEEV. Unfortunately, the *Aedes* cell line C6/36 resisted infection by retrovirus vectors due to postentry blocks to infection, and so we could not test the VEEV pseudotype on them (8). Interestingly, a few alphaviruses can infect anopheline mosquitoes, indicating that alphaviruses can adapt to this host (27). These viruses have many changes in genes encoding nonstructural and structural proteins. Making envelope protein pseudotypes of these viruses will aid in understanding the role of envelope-cell interactions in permitting entry into this host. The further analysis of the pseudotype interaction with Mos-55 cells may also permit identification of new alphavirus entry factors or inhibitors by the transfer of genes from permissive cell lines. This phenomenon could be readily achieved with these retroviral vectors by elimination of resistance factors—by targeted mutagenesis or transfer of genes encoding factors for determining host range from susceptible cells—and by making chimeric virus envelope proteins between VEEV and *Anopheles*-tropic viruses.

Pseudotypes may offer an alternative to live virus in diagnostic assays. In addition to the usefulness of the VEEV envelope pseudotypes in aiding our understanding of factors important in virus entry, they may serve as valuable, alternative diagnostic reagents for detection of protective antibodies in patients and in vaccine trials. Currently, the most widely accepted and sensitive assays (e.g., plaque reduction neutralization) depend on the use of live virus and must be conducted in biosafety level 3 containment laboratories with select agent access. Personnel are also recommended to have been vaccinated with an experimental VEEV vaccine strain. Alternatively, enzyme-linked immunosorbent assays using denatured virus can be used, but denaturation can increase nonspecific interactions and expose core antigens that are not involved in the neutralization of free virus. Virus also needs to be grown in a laboratory and can readily undergo adaptive mutations that change its antigenic properties and interaction with cells (7).

The pseudotypes offer a stable, safe source of native antigen that could be used in such assays.

ACKNOWLEDGMENTS

We thank Ilya Frolov for plasmids encoding the Trinidad donkey envelope proteins and Mardelle Susman for editing this article. We thank John Roehrig for kindly providing the monoclonal antibodies against the VEEV envelope proteins.

This work was supported by a grant from NIAID to R.A.D. through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (NIH grant number U54 AI057156).

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