

Retroviral Pseudotypes

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Retroviral pseudotypes are a main research and diagnostic tool of basic and clinical scientists facilitating the detailed study of individual viral genes, receptors and highly pathogenic viruses, without the need to handle live virus. Their use as gene therapy vectors is widely documented, but their other uses are less well known. The switching of envelope proteins expressed on the surface enables them to be used as surrogate viruses in neutralization/antiviral assays and for the study of cell–virus receptor interactions. However, they have recently been used as vaccine immunogens, expressing the antigen either on the pseudotype surface or as the transfer gene for stable or transient expression. Only now is their full potential being realized.

Advanced article

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Introduction

It is important to first answer two questions: What are pseudotypes and why are retroviruses the ideal platform for this technology? The definition of a pseudotype is a viral particle that carries the genome of one virus and one or more proteins of another virus. Retroviruses are named from the Latin for ‘behind’ or ‘backward’ (*retro-*) and this highlights their key fundamental characteristic, the native ability to reverse transcribe their ribonucleic acid (RNA) genome into a double-stranded deoxyribonucleic acid (dsDNA) copy, which is subsequently integrated into the cell chromosome via the use of viral and cellular enzymes. Typical retroviral pseudotypes extensively discussed here carry the retroviral RNA genome coding for marker genes. The RNA genome is packaged by retroviral core proteins but these viral cores bear foreign, often nonretroviral envelope proteins on their surface. Pseudotype infection, i.e. transfer of their genome (containing the transfer/marker gene) to target cells, depends on the function of the foreign envelope proteins and results in integration and subsequent expression of the marker gene. The function of the foreign envelope can therefore be detected/quantified by measuring marker gene expression. **See also:** [Retroviral Replication](#)

Retroviral pseudotypes have proved to be powerful tools employed by molecular biologists and immunologists since their first use in 1965 (Rubin, 1965). The ability of retroviral pseudotypes to stably integrate genes into an infected

cell’s chromosome was a major factor in their development as gene therapy tools. There followed an exponential development period in pseudotyping methods that resulted in several ‘generations’ of vector systems being described. The first generation techniques for the production of helper-free vectors in the late 1970s and early 1980s involved the transfection of the transfer gene into a cell line that constitutively expressed all retroviral proteins required for pseudotype production. However, it became apparent that virus recombination resulted in infectious virus production at high frequency. As this presented major safety issues, it was necessary to minimize the chance of infectious progeny being produced and therefore the *gag-pol* and *env* genes were cloned into separate plasmids. A 3-plasmid transfection system using these packaging and envelope plasmids together with the transfer gene plasmid was established and is widely used today (**Figure 1**). **See also:** [Gene Therapy: Technology](#); [Retroviral Vectors in Gene Therapy](#); [Viral Vectors for Gene Therapy](#)

The majority of retroviral pseudotypes are based on lentiviruses or gammaretroviruses. Lentiviruses (such as human immunodeficiency virus (HIV), simian immunodeficiency virus and equine infectious anaemia virus) can infect nonproliferating cells by virtue of their ability to cross an intact nuclear membrane (Weinberg *et al.*, 1991). Gammaretroviruses (such as murine leukaemia virus, MLV) have the advantage of an advanced packaging system that makes the processes of pseudotype production more efficient. With regards the insertion of the transfer gene, lentiviral-based pseudotypes predominantly insert it within active genes (Schroder *et al.*, 2002) and gammaretroviruses around promoter sites, which can result in activation of an adjacent proto-oncogene with deleterious consequences. This was in evidence recently with the development of leukaemia in a child during a French trial to test X-SCID (X-linked severe combined immunodeficiency) with gene therapy (Hacein-Bey-Abina *et al.*, 2003). Representatives of all seven genera of retroviruses have been employed in

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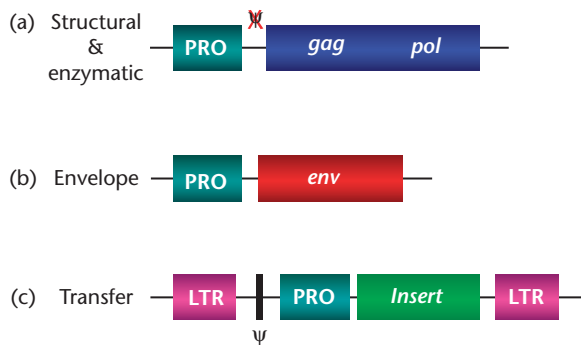


Figure 1 Flexible plasmid platform used to produce pseudotype particles. (a) The retroviral structural (GAG) and enzymatic (POL) proteins are expressed from a single plasmid lacking the packaging signal that has been removed to prevent incorporation into mature particles. PRO=promoter. (b) Envelope (*env*) gene expression is driven by a promoter specific to the envelope. (c) The transfer/marker gene (*insert*) is cloned between flanking LTR regions that play a role in integration and transcription of the pseudotype's RNA genome. A packaging signal (Ψ) and further promoter (PRO) are incorporated upstream of the transfer gene to ensure it is packaged into pseudotype particle and regulates expression once the gene is integrated.

pseudotyping experiments but to date only gammaretroviral or lentiviral pseudotypes are widely used.

There are many advantages in using pseudotype particles over live virus:

1. Binding and entry of target cells is entirely receptor-mediated, therefore, pseudotyped particles can be engineered to specifically target certain cell types, which is of particular use in gene therapy studies. For example, pseudotyping with the vesicular stomatitis virus G protein serves to widen the host range whereas pseudotyping with the hepatitis B envelope confers hepatotropism. As only the foreign envelope is expressed on the pseudotype surface a more specific study of virus-cell receptor interaction can be undertaken in addition to receptor identification.
2. A dsDNA copy of the transfer gene is integrated into the host chromosome resulting in stable and lifelong gene expression.
3. Using the envelope proteins of highly pathogenic viruses in the pseudotype system removes the need for handling these live viruses making it a much safer alternative.
4. The pseudotypes are relatively stable compared to the analogous live virus having a half-life of several hours at 37°C (Higashikawa and Chang, 2001) and of several days at room temperature (personal observation).

General Methodology

Construction of retroviral pseudotypes is achieved through the introduction of a foreign envelope gene, core retroviral genes and the gene of interest (transfer gene) concurrently

into producer cells, normally human embryo kidney (HEK) 293 T cells.

1. *The envelope gene construct.* Envelope sequences of the virus to be studied can be readily cloned into an appropriate expression plasmid that will permit their incorporation into the pseudotype. In general simple RNA viruses require the cloning of the envelope gene alone, whereas more complex viruses require additional accessory envelope sequences, and large DNA viruses with multiple envelope genes will require a combination of these expressed on the pseudotype surface for efficient production/infection. To date, attempts to pseudotype DNA viruses have failed with the exception of hepatitis B virus (not a classical single-stranded or dsDNA virus, but a para-retrovirus) that has been successfully pseudotyped onto lentiviral cores and used to study viral tropism (Chai *et al.*, 2007).
2. *The retroviral gag-pol construct.* These are the two genes that encode the structural proteins (e.g. matrix, nucleocapsid, p7 expressed from *gag*) that comprise the core, and the enzymatic proteins (reverse transcriptase, protease and integrase expressed from *pol*) responsible for processing the structural proteins and ensuring integration of a dsDNA copy of the transfer gene. Additionally in a lentiviral *gag-pol* construct, the *rev* gene is included for efficient processing.
3. *The transfer gene construct.* This is the gene that is stably integrated into the host cell DNA. Where it is expressed via a single plasmid, a packaging signal is engineered upstream of the gene to ensure efficient incorporation of full-length RNA copies of that gene into the pseudotype capsid. Once integrated the gene is expressed via various *cis*-acting transcriptional elements.

It should be noted that particles can be produced by transfection of a *gag-pol* construct and an envelope construct (i.e. the transfer gene construct is not required for production). Also, pseudotypes lacking the envelope will bud but will be noninfectious.

As an alternative to using the 3-plasmid system for producing pseudotypes the necessary genes can be transfected via either 1 or 2 plasmids. Using three plasmids offers the most ease and flexibility (switch of plasmids, no cloning required), and safety (less chance of recombination) as the envelope, core and reporter gene are expressed from different plasmids. The flexibility of the system enables tailoring of the pseudotypes depending on experimental requirements.

An overview of a standard protocol using the 3-plasmid approach is shown in **Figure 2**. Once the cellular transcriptional machinery has transcribed and translated the 3 transfected genes, an RNA dimer of the transfer gene is incorporated into the pseudotype core, which is composed of the GAG proteins, courtesy of the packaging signal. Further signals result in the migration of the capsid to the cell plasma membrane, into which the envelope protein has been inserted, and the pseudotype buds from the cell taking the cell plasma membrane to form its own membrane. Once

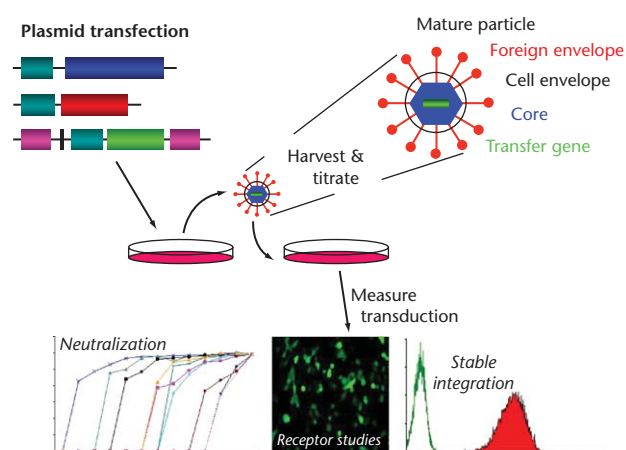


Figure 2 Pseudotype production and uses. Plasmids are transfected into producer cells and the resulting pseudotype particles are harvested 48 and 72 h posttransfection. These particles comprise a core encasing the RNA genome, surrounded by an outer matrix protein complex, with the cell plasma membrane, displaying the foreign envelope proteins, forming the viral membrane. These mature pseudotype particles can then be added onto target cells and the level of transduction/infection measured using the appropriate methods.

fully matured, the virus can be harvested and titrated onto target cells where the level of transduction can be measured by assaying for the transfer gene. Since the transfer gene is the only gene to contain a packaging signal no other nucleic acids are incorporated into the mature pseudotype particle. Consequently, no viral proteins are produced in the target cells so further pseudotype production and propagation does not occur. This is a key feature that renders working with pseudotypes a safe option compared to work with the live virus. Green fluorescent protein (GFP)-based pseudotypes are readily titred using flow cytometry, luciferase pseudotypes by luminometry and beta-galactosidase (β -gal) pseudotypes by colour reaction. The titres obtained by this method are highly dependent on the cell type chosen for the assay as receptor density may differ between cell types. Other methods for the standardization of retroviral pseudotypes are RT-PCR to determine virus particle numbers, ELISA (enzyme-linked immunosorbent assay) to determine GAG levels in the retrovirus cores and the measurement of surface envelope content. As an example, transduction of influenza haemagglutinin (HA)-based retroviral pseudotypes depends upon sialic acid receptor type (α -2,3 sialic acid receptors are used by avian strains and α -2,6 sialic acid receptors are used by human strains) and density (different Madin–Darby canine kidney (MDCK) cell lines have different ratios of 2,3 and 2,6 receptors). Also important is the density of HA on the surface of the pseudotype particle that may differ from strain to strain.

Gene Delivery

The use of retroviral pseudotypes for gene delivery has major implications for gene therapy. They can also be used

to study the effect of introducing foreign proteins into a cell, as pseudotype transduction results in the long-term stable expression of the integrated gene. The foreign gene may be a marker gene (GFP, luciferase or β -gal) as discussed in other sections of this article, a therapeutic gene (as is the case for gene therapy applications) or indeed any gene that is required to be stably expressed in a given cell line/type. To achieve efficient and sustained expression of foreign genes delivered by retroviral vectors, there are several options for the design of the plasmid. The foreign gene may be directly expressed from the promoter in the construct 5'-long terminal repeat (LTR) or from a heterologous promoter (CMV – cytomegalovirus, β -actin) downstream of the 5'-LTR. If expression of two genes is required then a spliced message or an internal ribosome entry site (IRES) may be used to express the second gene (Adam *et al.*, 1991; Buchschacher, 2001). Cell-specific promoters can be used to regulate gene expression and alternative envelopes can be used which allow targeting of specific cells (Pizzato *et al.*, 1998). Also, in addition to the delivery of full-length genes, small interfering RNA (siRNA) can be delivered by retroviral vectors (Clements *et al.*, 2006). **See also:** [Lentiviral Vectors in Gene Therapy](#); [Retroviral Vectors in Gene Therapy](#); [Retroviruses in Human Gene Therapy](#)

Analysis of Receptor Binding and Virus Entry and Exit

Retroviral and lentiviral vectors pseudotyped with foreign envelopes have many advantages over the live virus when used as tools to study viral entry (Sanders, 2002). First and foremost, use of retroviral pseudotypes can have major safety advantages when studying the entry of high-containment viral pathogens such as ebola, severe acute respiratory syndrome coronavirus (SARS-CoV), lyssaviruses and highly pathogenic avian influenza (HPAI) viruses (H5N1, H7N1). Pseudotyped particles can be safely and easily modified; nucleotide substitutions, deletions and insertions in surface protein genes can be introduced and evaluated more readily in the pseudotype system. Also using relevant marker genes, the virus entry process of the pseudotyped virus is easily and reproducibly measured and monitored. Functional studies of the envelope proteins in a foreign context allow the isolation of the viral entry process from the other steps in viral replication.

Retroviral pseudotypes can also be used for the identification of new viral receptors and for the study of receptor binding. Pseudotypes have been used to identify and study cell entry receptors of ebola (Wool-Lewis and Bates, 1998), HIV (Deng *et al.*, 1997) and SARS-CoV (Wang *et al.*, 2004). The first step in the identification of a viral receptor using this technology involves the construction of a retroviral particle pseudotyped with a foreign viral surface glycoprotein and delivering a selectable marker (e.g. neomycin/G418). Secondly, two cell lines need to be identified; a cell line that is refractory to entry by this

pseudotyped virus and another cell line that is susceptible. A complementary DNA (cDNA) library is constructed from the susceptible cells and transfected into the refractory cells. Any resulting cells that contain the receptor gene(s) can be transduced by the pseudotyped virus and selected. The cDNA within these populations can then be sequenced to determine which genes are present. **See also:** [Virus Host Cell Receptors](#)

Elegant examples of the use of pseudotypes to study viral binding and entry are provided by members of the coronavirus family. Retroviral pseudotypes have been used to study receptor-mediated entry of the Feline coronavirus, FCoV (Dye *et al.*, 2007) and human coronaviruses NL63, 229E (Hofmann *et al.*, 2005) and SARS (Simmons *et al.*, 2004; Temperton *et al.*, 2005; Wang *et al.*, 2004). The functional receptor used by SARS coronavirus was identified by expression cloning (Wang *et al.*, 2004). In this study, a series of truncated spike (S) glycoproteins were expressed and it was found that the N-terminus 14–502 amino acid residues were sufficient to bind Vero E6 cells, which are susceptible to SARS-CoV. This soluble spike protein fragment was then used as an affinity ligand to screen HeLa cells that had been transduced with a retroviral cDNA library constructed from the susceptible Vero E6 cells. A HeLa cell clone was identified which could bind the spike protein. HIV(SARS-CoV) pseudotypes were constructed and used to infect this cell clone and the receptor for SARS-CoV was thus identified as angiotensin-converting enzyme 2 (ACE2). For the FCoV study the aim was to use pseudotypes bearing the envelope S glycoprotein of either type I FCoV or type II FCoV to determine if aminopeptidase N (which can act as a receptor for the attachment and entry of type II FCoV viruses which replicate well in cell culture) can also act as a receptor for the type I FCoV viruses which are difficult to isolate and propagate in cell culture. Using these pseudotypes to screen a range of feline cell lines for the expression of a receptor for attachment and entry it was shown that type I feline coronavirus surface S glycoprotein fails to recognize aminopeptidase N as a functional receptor on three feline cell lines, CrFK (Crandell feline kidney), Fcwf-4 (Felis catus whole fetus 4) and FCKU (Feline kidney Colorado University) (Dye *et al.*, 2007). Interestingly, FCoV I pseudotypes could transduce cultures of adherent polymorphonuclear cells (primary cell line) obtained from the blood of pathogen-free cats which suggests that pseudotypes may be useful for the identification and characterization of the cellular receptor for type I feline coronaviruses using methodology described earlier. For the human coronaviruses, NL63, 229E and SARS-CoV, similar studies using retroviral pseudotypes have confirmed that 229E uses the aminopeptidase N as a receptor, whereas NL63 and SARS-CoV both use ACE2 as a receptor (Hofmann *et al.*, 2005; Simmons *et al.*, 2004; Temperton *et al.*, 2005). **See also:** [Severe Acute Respiratory Syndrome \(SARS\)](#)

In addition to virus entry studies retroviral pseudotypes have been widely used to study innate antiviral responses such as the post-entry restriction factors, TRIM5 α (tripartite motif 5 α) (Towers, 2007) and apolipoprotein B

mRNA-editing enzyme catalytic polypeptide (APOBEC) (Holmes *et al.*, 2007) that have been shown to be potent antiviral proteins. In these cases retroviral function is studied by taking advantage of a foreign envelope, VSV-G (vesicular stomatitis virus protein G), which has a wider host range and cell tropism than those of parental retroviral envelopes. By inserting the *gag-pol* genes from HIV-infected patients, resistance to antiretroviral drugs such as protease and integrase inhibitors can be measured (Parry *et al.*, 2008). Recently, retroviral pseudotypes were used to study Tetherin, a cellular protein that inhibits the release of HIV by anchoring the virus to the cell (Neil *et al.*, 2008). **See also:** [Retroviral Replication](#)

Retroviral and lentiviral particles pseudotyped with the envelopes of foreign viruses are readily exploitable for the development of novel entry and exit assays which can be used for the screening of new antiviral compounds and for the study of antiviral drug resistance. Cell-based antiviral assays have been developed for influenza virus that enable the measurement of inhibition of pseudotype virus entry (for HA inhibitors) and pseudotype virus exit (for neuraminidase (NA) inhibitors) (Su *et al.*, 2008; Wang *et al.*, 2008a). Luciferase-based retroviral particles pseudotyped with the HA from influenza A/Viet Nam/1194/04 were produced and used in a high-throughput assay to screen for agents that could inhibit the transduction of target cells by these particles. The entry of these particles into human lung carcinoma A549 cells was shown to be inhibited by α -2, 3-SialGal oligosaccharides, the synthetic peptide elementary body (EB), sera from mice immunized with inactivated NIBRG-14 (a reverse genetics derived virus developed by the National Institutes for Biological Standards and Control) virus and newly identified HA-entry inhibitors of which one in particular, P7B3 was shown to be very potent (Wang *et al.*, 2008a). For the influenza exit assay, the requirement of NA for the release of HA-pseudotyped particles from the surface of producer cells (Temperton *et al.*, 2007) was exploited for the development of an assay to evaluate neuraminidase inhibitor (NAI) activities by measuring the inhibition of pseudotype virus production at different NAI concentrations. This assay was used to determine the IC₅₀ values of Oseltamivir and Zanamivir against N1 group neuraminidases and their H274Y Oseltamivir-resistant mutants. IC₅₀ values obtained using this pseudotype-based assay correlated strongly with those determined using the MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) assay (Su *et al.*, 2008). Similar assays have been developed for HIV and rabies (Saeed *et al.*, 2006). **See also:** [Antiviral Drugs](#)

Virus Neutralization Assays

Neutralization assays allow for sensitive detection of functional antibody responses directed against the surface protein envelopes of many viruses. For high-containment viruses (SARS-CoV, influenza H5N1, rabies, Ebola);

however, these assays are not widely applicable due to the requirement for high biosafety laboratory facilities and specially trained personnel. Using retroviral and lentiviral particles pseudotyped with the envelopes of these high-containment viruses as 'surrogate viruses' for use in neutralization assays is one way of circumventing this issue. Using a pseudotype approach, only the envelope protein (s) of the virus is required, with no possibility of recombination or virus escape. These pseudotypes undergo abortive replication and are unable to give rise to replication-competent progeny.

Safety considerations aside, for virus neutralization assays, pseudotypes are excellent serological reagents as they encode reporter genes and bear the heterologous viral envelope proteins of interest. The transfer of these reporter genes to target cells depends on the function of the viral envelope protein; therefore, the titre of neutralizing antibodies against the envelope can be measured by a reduction in reporter gene transfer. For HIV, SARS, H5N1, hepatitis C, rabies and Ross River virus these assays have been shown to be very sensitive and specific, and the neutralizing antibody titres obtained correlate strongly with the respective live virus assays (Bartosch *et al.*, 2003; Kim *et al.*, 2001; Li *et al.*, 2008; Nefkens *et al.*, 2007; Sharkey *et al.*, 2001; Temperton *et al.*, 2005, 2007; Wang *et al.*, 2008b; Wright *et al.*, 2008). These assays are also readily adaptable to a high-throughput format for use in vaccine evaluation, antiviral compound-screening assays and large sero-surveillance studies. See also: [Animal Viruses Pathogenic for Humans](#)

In order for these pseudotype assays to have wide applicability within different laboratories, the availability of different reporter systems is highly desirable. The HIV GFP reporter module pCSGW described recently for use in viral neutralization assays (Temperton *et al.*, 2005, 2007; Wright *et al.*, 2008) has been modified by polymerase chain reaction (PCR) subcloning to express alternative reporters. These are firefly luciferase (Temperton *et al.*, 2008; Wright *et al.*, 2008), renilla luciferase (Capecchi *et al.*, 2008) and β -gal. Of the three types, the luciferase reporter-based assays are the easiest to use in terms of hands on time, and data analysis. However, due to the high cost of the necessary reagents (luciferase assay kits) and necessity for specialized equipment (luminometer) these assays may have limited applicability for laboratories in resource-poor regions. GFP-based assays do not require any extra reagents but do necessitate specialized equipment (fluorescent microscope or 96-well plate flow cytometry facility). The β -gal-based assays are the most cost-effective as the necessary reagents are readily available at low cost, and specialized equipment is unnecessary. The expression of β -gal can be quantified by counting infected cells using a microscope following staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). However, there are 2 colourimetric substrates, ONPG (*o*-nitrophenyl- β -D-galactopyranoside) and CPRG (chlorophenol red- β -D-galactopyranoside) that allow the assays to be evaluated using a microplate reader (Figure 3).

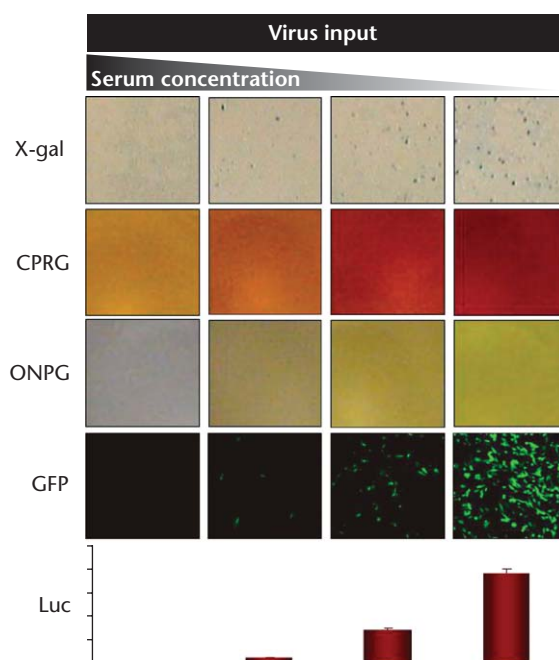


Figure 3 Reporter gene read-outs for use in measuring transduction of target cells. The detection of pseudotypes depends on the reporter-transfer gene incorporated into the particle. This can be (a) *lacZ* and staining directed for infection foci (X-gal) or with a colorimetric substrate such as CPRG or ONPG. (b) A fluorescent protein such as GFP. (c) The light emitted from luciferase oxidizing its substrate luciferin (Luc).

There are major advantages of using pseudotypes for the study of immune responses to viral infections:

1. They are commonly referred to as micro-assays as $<10\mu\text{L}$ of serum is required for each replicate, 5–50-fold less than for live virus assays.
2. There is the choice of reporter to use, luciferase, GFP or β -gal (Figure 3).
3. Using pseudotypes as a surrogate for live virus removes the need to handle dangerous pathogens.
4. Previous studies have shown high sensitivity and specificity and strongly correlating titres when results using live virus or pseudotypes are compared.

However, there are also disadvantages in using pseudotypes over live virus in neutralization assays:

1. The density of envelope protein on the pseudotype surface is unknown and may not be analogous to that found on the respective live virus. If the envelope protein density is significantly lower on the pseudotype it may be more readily neutralized by antibodies. However, as there is a high correlation of neutralizing antibody titres among the assays using live virus or pseudotypes this suggests that this may not be an issue when using polyclonal sera.
2. Some viruses bud internally so that their envelopes are retained on the endoplasmic reticulum (e.g. flaviviruses) (Sandrin and Cosset, 2006). For this reason these

viruses produce low viral titres when their envelopes are used in the pseudotype system.

A recent application of retroviral pseudotypes has been their use as vaccines. Retroviral vectors carrying the surface proteins of influenza (H5N1 and H7N1) and HIV-1 have been shown to function as immunogens resulting in the induction of humoral (influenza and HIV) and cellular responses (HIV) in animal models (Neumann *et al.*, 2006; Szecsi *et al.*, 2006). For the influenza vaccines, retroviral particles were made which incorporated HA, NA and M2 from A/Chicken/FPV/Rostock/1934 (H7N1) and A/Thailand/KAN-1/04 (H5N1). These particles induced rapid, subtype-specific neutralizing antibody production in mice.

For the HIV-1 vaccine a novel MLV(HIV-1) pseudotype vector was constructed and shown to induce HIV-1-specific humoral (HIV-1 ENV-specific antibodies) and cellular immune responses (REV and ENV-specific cytotoxic T-lymphocyte (CTL)-response) in rhesus macaques. These novel vaccine immunogens can be prepared rapidly upon availability of viral RNA and could potentially be used as animal vaccines (influenza; pigs, domestic poultry), or for the generation of panels of neutralizing antibodies targeted against newly emerging viral strains (influenza and HIV).

Notes

'Plasmid' and 'construct' are used interchangeably throughout this article.

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