Journal of General Virology (2014), **95**, 1634–1639 DOI 10.1099/vir.0.065201-0

Short Communication

Pseudotyping of vesicular stomatitis virus with the envelope glycoproteins of highly pathogenic avian influenza viruses

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Received 28 February 2014
Accepted 6 May 2014

Pseudotype viruses are useful for studying the envelope proteins of harmful viruses. This work describes the pseudotyping of vesicular stomatitis virus (VSV) with the envelope glycoproteins of highly pathogenic avian influenza viruses. VSV lacking the homotypic glycoprotein (G) gene (VSVΔG) was used to express haemagglutinin (HA), neuraminidase (NA) or the combination of both. Propagation-competent pseudotype viruses were only obtained when HA and NA were expressed from the same vector genome. Pseudotype viruses containing HA from different H5 clades were neutralized specifically by immune sera directed against the corresponding clade. Fast and sensitive reading of test results was achieved by vector-mediated expression of GFP. Pseudotype viruses expressing a mutant VSV matrix protein showed restricted spread in IFN-competent cells. This pseudotype system will facilitate the detection of neutralizing antibodies against virulent influenza viruses, circumventing the need for high-level biosafety containment.

The envelope of influenza A viruses contains two major glycoproteins: haemagglutinin (HA) and neuraminidase (NA). HA plays a crucial role in virus entry and is an important target for neutralizing antibodies. As yet, 16 different HA subtypes have been detected in avian influenza viruses (AIVs). Immune sera directed against one subtype normally do not show cross-neutralizing activity against other subtypes. The antigenic drift within a subtype is an important mechanism by which influenza A viruses escape neutralization by antibodies. NA is a sialidase which removes the receptor determinant from cellular and viral glycoproteins, and facilitates release of progeny viruses from the host cell (Palese *et al.*, 1974). At present, nine different NA subtypes have been found in AIVs.

Highly pathogenic AIVs (HPAIVs) are characterized by a multi-basic cleavage site in HA which is recognized by furin and related prohormone convertases (Klenk & Garten, 1994). The ubiquitous expression of these enzymes promotes systemic infection of the host. HPAIVs cause a devastating, economically important disease in domestic poultry. In addition, they pose a considerable threat to public health as exemplified by the numerous fatal cases of human infections with H5N1 in the past. Although H5N1 was not transmitted between humans, further adaptation of these viruses or reassortment with human or porcine influenza viruses may eventually lead to viruses with pandemic potential (Herfst *et al.*, 2012; Imai *et al.*, 2012; Zhang *et al.*, 2013). This risk is not unrealistic as H5N1 viruses continue to circulate and evolve in South-East Asia, leading to new phylogenetic clades and subclades. Recently, human cases of infection with H7N9 demonstrated that the zoonotic potential of AIVs is not restricted to HPAIVs (Gao *et al.*, 2013).

The ongoing evolution of H5N1 highlights the importance of serological surveillance programmes. In addition, the serological relatedness between available vaccines and currently circulating viruses must be determined. However, handling of AIVs with zoonotic potential requires high-level biosafety containment. An attractive alternative is the pseudotyping of harmless viruses with the envelope glycoproteins of zoonotic influenza viruses, as the resulting surrogate viruses can be safely used for detection of neutralizing antibodies. Vesicular stomatitis virus (VSV; family *Rhabdoviridae*), a non-segmented negative-strand RNA virus, is known to incorporate foreign viral glycoproteins in a rather unselective manner (Schnell *et al.*, 1996). Influenza virus HA is also incorporated into recombinant VSV particles (Kretzschmar *et al.*, 1997); however, expression of HA in place of the homotypic VSV glycoprotein did not result in propagation-competent pseudotype virus (Kalhoro *et al.*, 2009). The present study aimed at pseudotyping VSV with the two major influenza virus glycoproteins HA and NA. The recombinant viruses were analysed with respect to incorporation of the foreign viral glycoproteins, competence for autonomous propagation and antibody-mediated neutralization.

The RNA genome of VSV contains five non-overlapping genes encoding the VSV proteins nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G)

and RNA polymerase (L) (Fig. 1a). In previous studies, VSV was genetically commuted into VSV*, which expresses GFP from an additional transcription unit (Schnell *et al.*, 1996). VSV* was further modified by replacing the G gene with either the HA or NA gene of HPAIV A/chicken/Yamaguchi/7/2004 (H5N1) (Halbherr *et al.*, 2013). The resulting viruses VSV*ΔG(HA_{H5}) and VSV*ΔG(NA_{N1}) were propagated on helper cells providing the VSV glycoprotein *in trans* (Hanika *et al.*, 2005). In addition, a VSV vector with seven transcription units was generated to allow the expression of HA and NA together with GFP. The recombinant viruses VSV*ΔG(HA_{H5},NA_{N1}) and VSV*ΔG(HA_{H7},NA_{N1}) contained the HA and NA genes of A/chicken/Yamaguchi/7/2004 (H5N1) and A/turkey/Italy/4580/1999 (H7N1), respectively.

The expression of recombinant antigens was studied by immunofluorescence 12 h post-infection (p.i.) of MDCK cells with either VSV*, VSV*ΔG(HA_{H5},NA_{N1}) or VSV*ΔG(HA_{H7},NA_{N1}) (Fig. 1b). Immune serum specific for HA_{H5} reacted with cells infected with VSV*ΔG(HA_{H5},NA_{N1}), whereas anti-HA_{H7} serum did not bind. Vice versa, anti-HA_{H7} serum bound to VSV*ΔG(HA_{H7},NA_{N1})-infected cells, but not to cells infected with VSV*ΔG(HA_{H5},NA_{N1}). As expected, anti-NA_{N1} serum reacted with cells infected with either VSV*ΔG(HA_{H5},NA_{N1}) or VSV*ΔG(HA_{H7},NA_{N1}) and not with VSV*-infected cells. Thus, both HA and NA antigens were expressed simultaneously from the same vector and transported to the cell surface of infected cells.

VSV*ΔG is a single-cycle vector which cannot spread in non-complementing cells (Takada *et al.*, 1997). Likewise, VSV*ΔG(NA_{N1}) remained confined to primary infected cells 18 h after infection of BHK-21 cells at m.o.i. 0.01 (Fig. 2a). Infection with VSV*ΔG(HA_{H5}) and VSV*ΔG(HA_{H7}) led to the formation of fluorescent cell foci, indicating that HA mediated a slow cell-to-cell spread of the vector. When HA_{H5} or HA_{H7} were expressed together with NA_{N1} from the same vector, the whole cell monolayer became positive for GFP at 18 h p.i., suggesting that virus spread was greatly enhanced by NA.

To further analyse the capacity of the recombinant viruses to propagate on non-helper cells, MDCK cells were infected at m.o.i. 0.01. At different times, cell culture supernatant was collected and infectious virus titres determined (Fig. 2b). No infectious virus was detected in the medium of cells infected with either VSV*ΔG, VSV*ΔG(NA_{N1}) or VSV*ΔG(HA_{H5}). In contrast, VSV*ΔG(HA_{H5},NA_{N1}) and VSV*ΔG(HA_{H7},NA_{N1}) were released from infected cells, leading to increasing infectious titres with time reaching 10^7 focus-forming units (f.f.u.) ml^{-1} at 36 h p.i. These results indicate that while HA_{H5} can mediate cell-to-cell spread of the vector, co-expression of NA is essential for

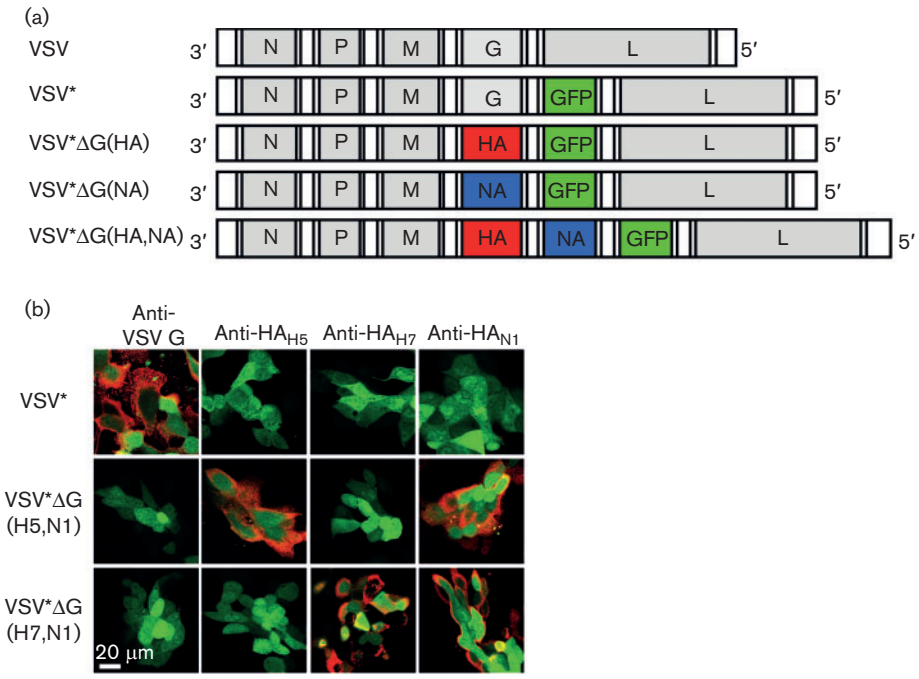

Fig. 1. (a) Genome maps of recombinant VSV vectors. The VSV genome contains five transcription units driving expression of nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase (L). VSV* expresses GFP from an additional transcription unit. In VSV*ΔG(HA) and VSV*ΔG(NA), the VSV G gene was replaced with either HA or NA gene. VSV*ΔG(HA,NA) contains seven transcription units allowing simultaneous expression of HA, NA and GFP. (b) Immunofluorescence analysis of MDCK cells 16 h p.i. with the indicated viruses ('HA' and 'NA' have been omitted from virus nomenclature in the figure). The cells were stained with immune sera as indicated above the images.

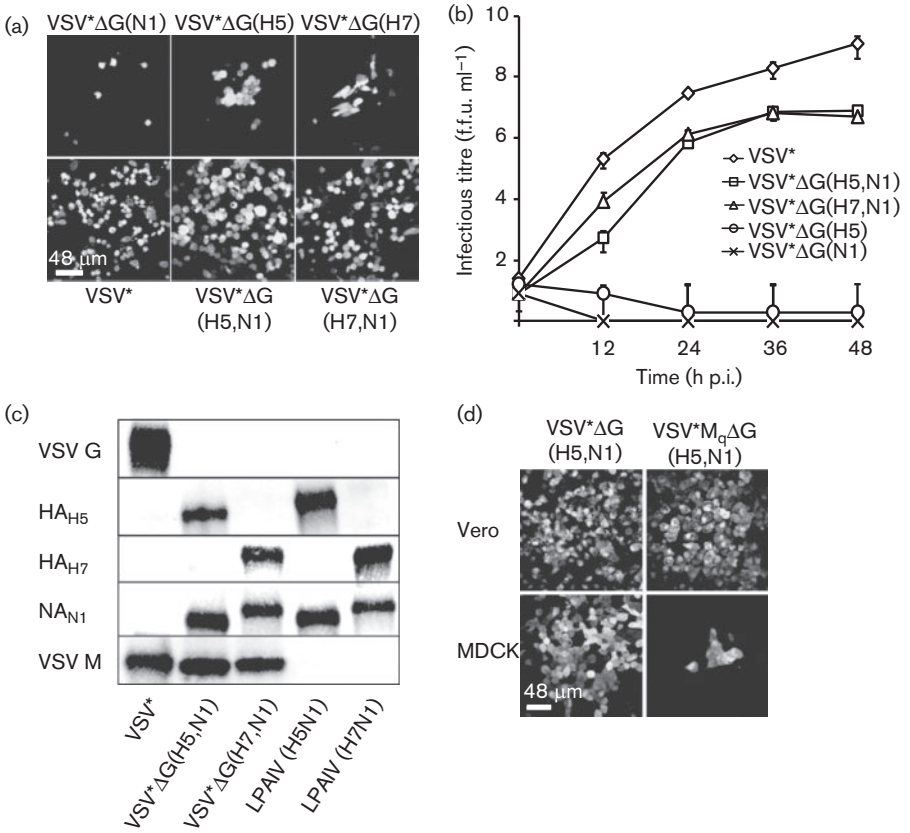

Fig. 2. (a) Analysis of virus spread in cell culture. BHK-21 cells were infected with indicated viruses (m.o.i. 0.01). Expression of GFP was monitored 18 h p.i. by fluorescence microscopy. (b) Virus replication kinetics on MDCK cells. The cells were infected with the indicated viruses (m.o.i. 0.01) and infectious virus titres in cell culture medium determined for the indicated times. (c) Incorporation of foreign glycoproteins into pseudotype viruses. The indicated viruses were pelleted from cell culture supernatant by ultracentrifugation and analysed by Western blot using monospecific immune sera. (d) Spread of VSV*ΔG(HA_{H5},NA_{N1}) and VSV*M_qΔG(HA_{H5},NA_{N1}) pseudotype viruses on Vero and MDCK cells, respectively. The cells were infected (m.o.i. 0.01) and GFP expression was monitored 24 h p.i. ('HA' and 'NA' have been omitted from virus nomenclature in the figure.)

efficient virus release and dissemination. Compared with propagation-competent VSV*, VSV*ΔG(HA_{H5},NA_{N1}) replicated to 100-fold lower titres, indicating that the pseudotype viruses are attenuated.

To study the incorporation of influenza virus glycoproteins into the viral envelope, pseudotype particles were concentrated from the supernatant of infected MDCK cells by ultracentrifugation, separated by SDS-PAGE and transferred to nitrocellulose membranes. Virus particles derived from low-pathogenic AIVs (LPAIVs) served as positive controls. Staining of the protein blots with specific immune sera demonstrated the presence of HA_{H5} and HA_{H7} in VSV*ΔG(HA_{H5},NA_{N1}) and VSV*ΔG(HA_{H7},NA_{N1}) particles, respectively (Fig. 2c). NA_{N1} was detected in VSV*ΔG(HA_{H7},NA_{N1}) as well as in VSV*ΔG(HA_{H5},NA_{N1}) particles. The apparent higher molecular mass of N1 derived from the H7 viruses is believed to be due to differential glycosylation. Using the fluorogenic sialidase substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid, sialidase activity could be detected in VSV*ΔG($HA_{H7}$$NA_{N1}$) and VSV*ΔG($HA_{H5}$,$NA_{N1}$), but not in VSV particles (data not shown). These findings indicate that HA and NA were incorporated into the VSV pseudotype particles.

A virus neutralization test was performed to study the ability of immune sera to inhibit pseudotype virus infection. Serial twofold dilutions of immune sera were incubated for 60 min with 100 infectious units of pseudotype virus. The virus–antibody mixture was then inoculated with BHK-21 cells for 12 h at 37 °C. Infection of the cells was monitored by fluorescence microscopy taking advantage of pseudotype virus-driven GFP reporter expression. The 50 % neutralization dose (ND_{50}) was calculated and expressed as ND_{50} ml^{-1} (Table 1). The neutralization by a given immune serum clearly depended on the glycoprotein incorporated into the pseudotype virus particle. Anti-VSV (serotype Indiana) serum efficiently neutralized VSV*, but was not active against VSV*ΔG(HA,NA) pseudotype viruses. Vice versa, immune sera directed against HA_{H5} or HA_{H7} showed

no inhibitory activity against VSV*. Furthermore, anti-HA_{H7} serum neutralized VSV*ΔG(HA_{H7},NA_{N1}), but not VSV*ΔG(HA_{H5},NA_{N1}), and anti-HA_{H5} serum did not show any inhibitory activity against VSV*ΔG(HA_{H7},NA_{N1}). These results indicate that the pseudotype viruses were neutralized in a HA subtype-dependent manner. Pseudotype viruses with HA from different phylogenetic H5 clades were efficiently neutralized by homotypic immune sera, but less efficiently by immune sera raised against other H5 clades. In particular, the HA of A/peregrine falcon/HongKong/810/2009 (H5N1), which belongs to clade 2.3.4, seemed to differ greatly from all other HA_{H5} clades. Comparative neutralization tests were performed with the AIVs A/Yamaguchi/7/2004 (H5N1) and A/reassortant/NIBRG-14(Vietnam/1194/2004 × Puerto Rico/8/1934) (H5N1) (Table 1). The neutralization patterns of AIVs and pseudotype viruses turned out to be quite similar. Moreover, the neutralization tests performed with pseudotype viruses were at least as sensitive as the neutralization assays performed with authentic AIVs.

VSV is highly sensitive to type I IFN. To block the synthesis of IFN by infected cells, VSV relies on the M protein, which inhibits the export of cellular mRNA from the cell nucleus. Taking advantage of a mutant M protein (M_q) (Hoffmann *et al.*, 2010), we generated VSV*M_qΔG(HA_{H5},NA_{N1}) – a pseudotype virus lacking host shut-off activity. VSV*M_qΔG(HA_{H5},NA_{N1}) propagated efficiently on IFN-defective Vero cells, but was highly attenuated in IFN-competent MDCK cells (Fig. 2d). We believe that this strategy might be useful to further enhance the biosafety of the pseudotype viruses.

The influenza virus HA and the VSV G glycoprotein exhibit similar functions, namely binding to cellular receptor determinants and membrane fusion in a pH-dependent manner. Despite this similarity, expression of HA from a VSVΔG genome did not result in propagation-competent virus. Although infectious progeny was not released into the cell culture supernatant, a slow spread of VSV*ΔG(HA) from cell to cell was observed, which is reminiscent of the previously described HA-mediated cell-to-cell transmission of influenza virus (Mori *et al.*, 2011). Propagation-competent pseudotype virus was achieved only when NA was expressed together with HA, illustrating the importance of NA as a release factor of influenza viruses. It has been shown previously that HA and NA of A/WSN/33 (H1N1) are incorporated into VSV particles (Kretzschmar *et al.*, 1997). In agreement with this observation, Western blot analysis demonstrated the presence of HA_{H5}, HA_{H7} and NA_{N1} in VSV*ΔG(HA_{H5},NA_{N1}) and VSV*ΔG(HA_{H7},NA_{N1}), respectively.

The M2 ion channel protein has been proposed to play a role in virus uncoating as well as in HA maturation by preventing a premature conformational change of the glycoprotein in acidic compartments of the secretory pathway (Schnell & Chou, 2008). This function of M2 was suggested to be particularly necessary for HPAIVs because these viruses are proteolytically activated in the *trans*-Golgi network.

Table 1. Pseudotype virus neutralization by immune sera

Immune serum	Neutralization titre (ND_{50} ml^{-1})							
	VSV*	VSV*ΔG(HA_{H7},NA_{N1})†	VSV*ΔG(HA_{H5},NA_{N1}) containing HA_{H5} of clade‡				AIV (H5N1) of clade§	
			1.1	2.3.2.1	2.3.4	2.5	1	2.5
Anti-VSV	64 000	<400	<400	<400	<400	<400	<400	<400
Anti-H5 (clade 1.1)	<400	<400	8900	450	<400	12 600	3630	4460
Anti-H5 (clade 2.3.2.1)	<400	<400	450	28 840	<400	8300	640	6400
Anti-H5 (clade 2.3.4)	<400	<400	<400	<400	14 450	400	<400	540
Anti-H5 (clade 2.5)	<400	<400	560	800	<400	31 620	560	12 800
Anti-H5 (classical clade)	<400	<400	1120	450	<400	10 000	800	1860
Anti-H7 (Rostock/34)	<400	2240	<400	<400	<400	<400	<400	<400

†VSV*ΔG(HA_{H7},NA_{N1}) was pseudotyped with HA and NA of A/turkey/Italy/4580/1999 (H7N1) (GenBank accession numbers CY021405 and GU052932).

‡VSV*ΔG(HA_{H5},NA_{N1}) was pseudotyped with NA of A/chicken/Yamaguchi/7/2004 (H5N1) (clade 2.5, GenBank accession number AB166864) and HA of either A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (clade 1.1, GenBank accession number BAK39622), A/peregrine falcon/Hong Kong/810/2009 (H5N1) (clade 2.3.4, GenBank accession number BAI39636), A/whooper swan/Hokkaido/4/2011 (H5N1) (clade 2.3.2.1, GenBank accession number BAJ72684) or A/chicken/Yamaguchi/7/2004 (H5N1) (clade 2.5, GenBank accession number AB166862).

§A/reassortant/NIBRG-14(Vietnam/1194/2004 × Puerto Rico/8/1934) (H5N1) (clade 1, GenBank accession numbers GQ454861 and GQ454862) and a cleavage mutant of A/chicken/Yamaguchi/7/2004 (H5N1) (clade 2.5) were used. Neutralization titres obtained with homotypic sera are underlined.

However, this study showed that VSV pseudotypes expressing HA and NA of HPAIVs are infectious in the absence of M2. In addition, a vector expressing the ion channel protein M2 together with HA_{H5} and NA_{N1} did not replicate to higher titres than VSV*ΔG(HA_{H5},NA_{N1}) (data not shown). This suggests that at least some HPAIV strains do not rely on assistance by M2 in order to maintain the metastable pre-fusion conformation of HA.

The VSV pseudotypes proved to be valuable for the detection of neutralizing antibodies against HPAIVs. The antibody-mediated neutralization of the pseudotype viruses occurred in a subtype-specific manner and also reflected the antigenic drift of different H5 clades. Neutralization of the pseudotype VSV was at least as sensitive as neutralization of authentic AIVs, but thanks to the GFP reporter test the results were obtained much faster than with a conventional virus neutralization assay based on cytopathic effects (CPEs). Moreover, a CPE is often more difficult to read and results may vary between different experimenters. Compared with this, the GFP reporter allowed an accurate and reliable reading of test results.

Retroviral pseudotype systems have been used for the detection of neutralizing antibodies against HPAIVs (Alberini *et al.*, 2009; Ao *et al.*, 2008; Tsai *et al.*, 2009; Wang *et al.*, 2008, 2010). These vectors are propagation-incompetent and need to be produced repeatedly by transfection of mammalian cells with a set of plasmids – a cumbersome and expensive procedure. In contrast, the VSV pseudotypes can be propagated on conventional mammalian cell lines and easily standardized according to their infectious titre. Insertional mutagenesis, an inherent risk associated with retroviral vectors, can be excluded since VSV replicates exclusively in the cytosol.

VSV has the potential to infect humans (Fields & Hawkins, 1967). Nevertheless, recombinant propagation-competent VSV pseudotypes expressing the glycoprotein of Ebola virus, a highly virulent haemorrhagic fever virus, were shown to be safe in monkeys and in a human case of emergency vaccination (Geisbert *et al.*, 2008; Günther *et al.*, 2011). This suggests that pseudotyping of VSV with envelope glycoproteins of highly virulent viruses does not necessarily render VSV more virulent. Furthermore, propagation-competent VSV might be attenuated using a mutant M protein which is unable to suppress nucleocytoplasmic RNA transport (Hoffmann *et al.*, 2010). As a consequence, cells infected with these mutant viruses will induce IFN, which hinders virus dissemination by inducing an antiviral state in cells.

In summary, we presented a new viral pseudotype system, which allows safe, fast, sensitive and accurate detection of neutralizing antibodies against influenza viruses. These viruses might also be useful for screening of entry inhibitors, and for studying influenza virus receptor usage and tropism.

Acknowledgements

We are grateful to Yoshihiro Sakoda (Hokkaido University, Sapporo, Japan) for providing chicken immune sera and plasmids. We thank William Dundon (Legnaro, Italy) and James S. Robertson (National Institute for Biological Standards and Control, Potters Bar, UK) for providing viruses. This work was supported by grants from the Federal Food Safety and Veterinary Office (project number 1.13.09) and Swiss National Science Foundation (project number 310030_129669).

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