Vaccinia virus-free rescue of fluorescent replication-defective vesicular stomatitis virus and pseudotyping with Puumala virus glycoproteins for use in neutralization tests

Rommel Paneth Iheozor-Ejiofor, Lev Levanov, Jussi Hepojoki, Tomas Strandin, Åke Lundkvist, Alexander Plyusnin and Olli Vapalahti

Correspondence
Rommel Paneth Iheozor-Ejiofor
rommel.iheozor-ejiofor@helsinki.fi

1University of Helsinki, Department of Virology, Medicum, Helsinki, Finland
2Department of Medical Biochemistry and Microbiology, Microbiology-Immunology, Uppsala University, Sweden
3Department of Virology and Immunology, HUSLAB, Helsinki University Hospital, Helsinki, Finland
4Department of Veterinary Biosciences, University of Helsinki, Helsinki, Finland

Puumala virus (PUUV) grows slowly in cell culture. To study antigenic properties of PUUV, an amenable method for their expression would be beneficial. To achieve this, a replication-defective recombinant vesicular stomatitis virus, rVSV<sup>D</sup>G<sup>+</sup>EGFP, was rescued using BSRT7/5 and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES)-enabled rescue plasmids. Using these particles, pseudotypes bearing PUUV Sotkamo strain glycoproteins were produced, with titres in the range 10<sup>5</sup>–10<sup>8</sup>, and were used in pseudotype focus reduction neutralization tests (pFRNTs) with neutralizing monoclonal antibodies and patient sera. The results were compared with those from orthodox focus reduction neutralization tests (oFRNTs) using native PUUV with the same samples and showed a strong positive correlation (rs<sup>0.82</sup>) between the methods. While developing the system we identified three amino acids which were mutated in the Vero E6 cell culture adapted PUUV prototype Sotkamo strain sequence, and changing these residues was critical for expression and neutralizing antibody binding of PUUV glycoproteins.

Puumala virus (PUUV) is a member of the genus Hantavirus in the family Bunyaviridae and is associated with a mild form of haemorrhagic fever with renal syndrome (HFRS), also known as nephropathia epidemica (NE), with about 5000 reported infections in Europe annually (European Centre for Disease Prevention and Control, 2011). The PUUV genome consists of three segments: large (L), medium (M) and small (S), which respectively encode the RNA dependent RNA polymerase (RdRp), glycoprotein precursor (GPC) and nucleocapsid protein (Plyusnin & Morzunov, 2001; Schmaljohn & Hjelle, 1997). The GPC is expressed as a 1133–1158 aa residue polyprotein, which is co-translationally cleaved after a conserved WAASA amino acid sequence by the host cellular signal peptidase complex in the endoplasmic reticulum (Firth et al., 2012; Löber et al., 2001). The resultant N- and C-terminal fragments respectively mature to viral glycoproteins Gn and Gc (Hepojoki et al., 2012). The nascent Gn and Gc fold cooperatively and, apparently, the cytoplasmic tail of Gn and the downstream Gc signal peptide guide the transfer and localization to the Golgi (Pensiero et al., 2001, 1992; Pensiero & Hay, 1992; Ruusala et al., 1992; Shi & Elliott, 2004). Localization to the Golgi is essential for the maturation of Gn and Gc (Antic et al., 1992; Johansson et al., 2004; Schmaljohn et al., 1986) and for budding of virions (Rowe et al., 2008; Shi & Elliott, 2002). Mature and properly folded Gn and Gc are the specific targets of neutralizing antibodies and thus they have been utilized in the study of glycoprotein folding (Custer et al., 2003; Hooper et al., 1999, 2001; Schmaljohn et al., 1990). The classification of most hantaviruses as biosafety level 3 (BSL3) agents places limitations on their research. The current ‘gold standard’ for serotyping hantaviruses, i.e. the orthodox focus reduction neutralization test (oFRNT), is cumbersome and time consuming (Ogino et al., 2003; Sanada et al., 2012). Hantavirus
glycoproteins have been shown to self-assemble to form virus-like particles (VLPs) (Acuña et al., 2014), but the use of VLPs requires either immuno- or cytochemical detection. Thus generation of vesicular stomatitis virus (VSV) pseudotypes bearing PUUV glycoproteins with the added benefit of a fluorescent reporter would provide a useful tool for receptor and antibody response studies.

VSV (family Rhabdoviridae, genus Vesiculovirus), is a negative sense, single-stranded, enveloped RNA virus (Acha & Szfyres, 2003). The VSV genome consists of N, P, M, G and L genes encoding the nucleocapsid protein, phosphoprotein, matrix protein, glycoprotein and the RdRp, respectively (Lyles & Rupprecht, 2007). One of the unique features of VSV is its phenotypic mixing or pseudotype formation capability, i.e. the virus is able to incorporate heterologous glycoproteins in its envelope when budding (Buonocore et al., 2002; Foley et al., 2002; Kahn et al., 1999; Kretzschmar et al., 1997; Matsuura et al., 2001; Schnell et al., 1996; Takada et al., 1997). Several reverse genetics systems have been developed for rescuing VSV particles from plasmids using either a vaccinia-based reverse genetics systems have been developed for rescuing VSV particles from plasmids using either a vaccinia-based or a vaccinia-free method (Garbutt et al., 2004; Hanika et al., 2005; Harty et al., 2001a; Lawson et al., 1995; Whitt, 2010). The vaccinia-based system, which involves the use of vaccinia virus T7 (vvT7) (Fuerst et al., 1986), has been used to rescue recombinant VSVΔG*EGFP (rVSVΔG*EGFP), i.e. a replication-defective VSV Indiana strain in which the glycoprotein gene is replaced with EGFP (Ramsburg et al., 2005). A crucial safety feature of rVSVΔG*EGFP systems is that its replication is defective and infectious progeny virions are produced only if the glycoprotein responsible for the membrane attachment is provided in trans (Higa et al., 2012; Ogino et al., 2003). In addition, the expression of EGFP by infected cells provides less-tedious means of detection.

In this paper we report 1) the rescue of a replication-defective rVSVΔG*EGFP using a vvT7-free system, 2) restoration of the M segment nucleotide sequence from the PUUV prototype strain Sotkamo adapted to Vero E6 cells (Schmaljohn et al., 1985) towards the consensus sequence in order to attain proper folding and antigenicity, and 3) the production of replication-defective VSV pseudotypes bearing PUUV/Sotkamo glycoproteins and their use as a safe and amenable substitute for native virus.

The coding region of the M segment of a previously described construct, pELVS-PUUVM (Kallio-Kokko et al., 2001), which was made from RNA extracted from Vero E6 cells infected with Vero E6 cell culture adapted PUUV/Sotkamo (Schmaljohn et al., 1985; Vapa-lahti et al., 1992), was further amplified by PCR using primers 5’-TTACTGAATTCTGGAATGGAATGCTGAT-3’ and 5’-TTACTCTCGAGTTAAGGTTTATGATCCTT-3’, with EcoRI and XhoI restriction sites (bold and underlined). The amplified products were cloned into the respective sites on the vector pCAGGS/MCS (Niwa et al., 1991) to form plasmid pCPUUVM (designated clone Pu).

To make the second construct, designated P1, RNA was extracted from Vero E6 cells infected with PUUV/Sotkamo, using the RNase mini kit (Qiagen) and reverse transcription was done using a RevertAid H minus First Strand cDNA Synthesis kit (Thermo Scientific) both according to the manufacturers’ instructions. The coding regions for the M segment of the Vero E6-adapted PUUV/Sotkamo (with about 40 passages for close to 30 years) was amplified using the same primers and cloned as described above. A third construct designated P7 was a synthetic gene, codon-optimized for expression in Homo sapiens, and made from the coding region of the consensus M segment sequence of GenBank sequences JN831951.1, JQ319175.1, KJ994777.1, AB433852.2, AB297666.2, AF442617.1, L08754.1, AY526218.1 and HE801634.1. This was inserted into the EcoRI and XhoI restriction sites on the vector pCAGGS/MCS.

To rescue rVSVGΔ*EGFP, encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) was cloned immediately distal to the T7 promoter of the rescue plasmids pBSN, pBSP and pBSL [encoding VSV nucleocapsid, phosphoprotein and RNA-dependent-RNA polymerase (RdRp)] to yield plasmids pBSIRESN, pBSIRESP and pBSIRESL which, together with pVSV-ΔG*EGFP (the full-length VSV/Indiana strain antigenome with the glycoprotein gene replaced with EGFP, expressed under a T7-promoter) and pVSVG (containing the coding region of the VSV G protein in the vector pCAGGS/MCS) were used in a vaccinia virus-free system (Harty et al., 2001a). Amplification and plaque purification were done using minor modifications of previously described methods (Whitt, 2010) using BSRT7/5 cells (Buchholz et al., 1999; Habjan et al., 2008) and calcium phosphate transfection method. The supernatants were recovered, 0.45 μm filtered, and ultracentrifuged (SW41 rotor, 100 000 r.p.m., 4 °C, 1 h) through a 50 % sucrose cushion, and the resulting pellet was subsequently dissolved in PBS and stored at 4 °C in ice. Titration of the recovered rVSVGΔ*EGFP was done as described previously (Ogino et al., 2003), and fluorescent foci were counted using a fluorescent microscope. The number of infectious units (IU) was estimated by counting the number of EGFP-expressing cells giving 1.4 × 10^14 IU μl^{-1}. Pseudotype production from constructs P1, Pu and P7 was done according to previously described methods (Ogino et al., 2003) using HEK 293T cells and calcium phosphate transfection method. Supernatants were collected after 48 h, filtered, ultracentrifuged and stored as mentioned above. Titration of the ultracentrifuged supernatant gave titres of 4 × 10^3, 5 × 10^5 and 7 × 10^6 IU ml^{-1} for Pu, P1 and P7, respectively. The presence of PUUV glycoproteins, and VSV M and G proteins on the pseudoviruses was confirmed by Western blotting (Fig. S1, available in the online Supplementary Material).

Previously, we had produced an alphavirus-based PUUV/Sotkamo M-segment construct (pELVS-PUUVM) expressing glycoproteins that were recognized by neutralizing mAbs and human sera in immunofluorescence assay
(IFA) (Kallio-Kokko et al., 2001), yet using this same construct as a template to produce Pu construct resulted in constructs and pseudotypes with suboptimal reactivity in IFA (Fig. 1) and neutralization capacity (Fig. S2) with mAbs 4G2 and 1C9. In order to confirm that this difference in activity was not factitious, another construct, P1, was made. A sequence comparison showed that P1 and Pu were the same and both shared nucleotide substitutions 1618G>A, 2465A>G, 2858C>T and 3435A>T resulting in amino acid substitutions Lys540, Gly822, Leu953 and Asp1145, respectively, when compared with the PUUV M-segment GenBank consensus sequence. The Lys540 residue change was unique only to constructs P1 and Pu whereas the presence of Gly822, Leu953 and Asp1145 was also seen in the original sequences from Vero E6 cell culture adapted PUUV/Sotkamo (Fig. 2). Of these two changes, Leu953 caused the most prominent changes on the 3D-structure model of the Gc ectodomain (Fig. 3). Thus we designed a synthetic, codon-optimized Ps7 construct in which three discordant residues – Lys540, Leu953 and Asp1145 – chosen based on their potential significance (Heiskanen et al., 1999; Hepojoki et al., 2010a, b),

*Fig. 1. Detection of Gn- and Gc-infected cells and Vero E6 cells transfected with constructs Pu and Ps7 using indirect immunofluorescence assay as previously described (Kallio-Kokko et al., 2001) using mAbs 5A2 (bank vole anti-Gn), 4G2 (bank vole anti-Gc) and 1C9 (human anti-Gc) as primary antibodies and Alexa Fluor 488-labelled goat anti-mouse and Alexa Fluor 568-labelled goat anti-human (Life Technologies) as secondary antibodies. The slides were viewed using the 40× objective of an Olympus BX51 fluorescence microscope equipped with a DP-70 camera. (a). PUUV-infected Vero E6 cells (infected with approximately 10⁴ f.f.u. ml⁻¹) from left to right stained with mAbs 5A2, 4G2 and 1C9. (b) Pu-transfected Vero E6 cells from left to right stained with mAbs 5A2, 4G2 and 1C9. (c) Ps7-transfected cells from left to right stained with mAbs 5A2, 4G2 and 1C9.*
Neutralization of PUUV pseudotypes

were restored to resemble consensus (putative WT) PUUV (Glu540, Pro953 and Glu1145). Using neutralizing Gn- and Gc-specific mAbs and Ps7-based pseudovirions eventually resulted in good correlation between oFRNT and pseudo-type focus reduction neutralization test (pFRNT) in the absence of complement (Lundkvist et al., 1993). The poor performance of P1- and Pu-based constructs/pseudovirions in IFA and pFRNT with neutralizing mAbs 4G2 and 1C9 indicated that the altered residues are essential for recognition by neutralizing antibodies. Notably, epitopes of neutralizing mAbs 4G2 and 1C9 have been mapped to Gn and Gc of the PUUV M segment with highly reacting sites for 1C9 seen at 822–834 aa and similar highly reacting sites for 4G2 seen between 914 and 1000 aa (Heiskanen et al., 1999) thereby explaining the poor activity of P1 and Pu constructs and pseudotypes in IFA and pFRNT with these neutralizing mAbs. The Ps7-based pseudotypes performed better in both pFRNT and IFA thus highlighting the impact of the observed substitutions. P1- and Pu-based pseudovirions were not used for further pFRNT because they consistently produced neutralization curves with low R² scores and had titres about four logs lower than those produced from Ps7. We speculate that although codon optimization may have an effect, the higher titres seen with Ps7 may be related also to the absence of the Gn Lys540 and Asp1145 (Fig. 2) changes in the Gn and Gc tail residues present in P1 and Pu. This speculation is hinged on observations by Matsuoka et al. (1994, 1996)

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and Andersson et al. (1997) on the importance of the transmembrane domain and cytoplasmic tail regions of members of the family Bunyaviridae in Golgi retention signalling. These changes could also help to explain the relatively low titres of the Vero E6-adapted PUUV/Sotkamo

Fig. 3. Yet Another Scientific Artificial Reality Application (YASARA) (Krieger & Vriend, 2014) view 3D image of PUUV Gc showing all surfaces (Van Der Waals surface, solvent accessible surface and molecular surface). The nucleotide sequences from Pu, P1, Ps7 and Sotkamo 2009 (HE801634.1) were aligned and translated to amino acids on BioEdit (version 7.3). The portion of the GPC coding for Gc and devoid of the last 44 residues (the trans-membrane helix and cytoplasmic tail of Gc) was submitted to SWISS-MODEL (http://swissmodel.expasy.org/) (Arnold et al., 2006) to obtain 3D models. The 3D models were built using SFV E1 (pdb accession code 2ALA) (Roussel et al., 2006) as template. The coloured areas represent 10 amino acid residues with the amino acid change in the middle. (a) Sotkamo 09 (HE801634.1) showing the correct 3D structure of the 953 (purple) and 822 (orange) amino acid residue changes, Ps7 (Pro953, cyan and Glu822, green), P1 and Pu (Leu953, blue and Glu822, magenta. (b) Composite image formed from the superimposition of P1 and Pu, Ps7 on Sotkamo 09 showing the distortive effects of the mutations in relation to one another. The models were also generated with TBEV glycoprotein E (pdb accession code 1SVB) as the templates (Bressanelli et al., 2004) but had a lower Global model Quality Estimation (GMQE) and QMEAN (Benkert et al., 2009) thus making SFV E1 protein models the more acceptable for our purposes. We could demonstrate that the ectodomain residue change at 953 is likely to distort the surface epitopes of the Gc expressed from Pu and P1 as compared with Ps7 or Gc originating from the prototype WT strain Sotkamo 2009 and the presence of the Gly822 residue change on its own did not cause a significant change on the surface of the affected plasmids but may have contributed to further change at the 953 position. The other mutation (Lys540 and Asp1145) was not analysed because they are located in the cytoplasmic tail of Gn and the tail of Gc and so do not form a part of the ectodomain of either protein (Hepojoki et al., 2012).

used in our laboratory and the low titre of recovered P1 and Pu pseudotype viruses. In addition, they could also help explain why we were unable to produce PUUV VLPs using Pu and P1 but were successful using Ps7 (our unpublished data). We had observed fluorescent foci
while titrating the ultracentrifuged culture supernatant from the negative pseudotype controls. In the absence of VSV G protein as confirmed by Western blotting and since VSV has been shown to incorporate the T-cell antigen CD4 (Brown & Lyles, 2005) and other foreign glycoproteins into its envelope (Buonocore et al., 2002; Foley et al., 2002; Matsuura et al., 2001; Ogino et al., 2003), it may have incorporated other cellular glycoproteins. Additionally, the amount of the matrix protein versus PUUV Gn and Gc, together with its presence in supernatants from infected-untransfected and pCAGGS-transfected pseudotyping implies an excess of matrix protein. This anomaly could have resulted from the production of spikeless particles upon pseudotype VSV infection (Mebatson et al., 1996). In effect, we conclude that the poor performance of pseudotypes from Pu and P1 in neutralization assay are due to 1) poor level of expression from these constructs resulting from the absence of the Lys540 and Asp1145 amino acid substitutions leading to low virus titres and/or the amino acid substitutions on the ectodomain (especially Leu953) which resulted in the malformation of the epitopes, and 2) the presence of a high quantity of defective particles leading to lower antibody titres (Handke et al., 2009).

Previous pseudotyping studies had used either ready-made recombinant VSV (Higa et al., 2012; Ogino et al., 2003) or replication-competent particles (Garbutt et al., 2004; Harty et al., 2001a). In addition, Hanika et al. (2005) produced replication-defective particles but amplified the primary rescue using an inducible cell line expressing VSV-G. Though beneficial, these systems are hard to set up and maintain in unspecialized laboratories and, in addition, the replication-competent versions do not optimally meet the biosafety and easy detection needs. As opposed to the previously described vvT7 rescue system, this system removes the requirement of extra biosafety precautions for vvT7 (classified as a BSL 2 agent in places where other orthopoxviruses or recombinants are present) (Chosewood & Wilson, 2009) and removes the inconveniences of inhibiting or removing same vvT7 before further work. The double advantage of this system lies in its safety since WT VSV/Indiana is one of the least pathogenic VSV strains (Krauss et al., 2003; Letchworth et al., 1999). Cloning of the IRES immediately distal to the T7 promoter enabled cap-independent transcription of the rescue plasmids (N, P and L). This was essential for making the rescue conditions as similar as possible to what is obtainable in native virus infections. VSV G protein was essential for the primary rescue because it is incorporated with the highest efficiency compared with other heterologous glycoproteins (Lyles & Rupprecht, 2007) thereby increasing the chances of success.

The oFRNT and pFRNT results showed that at a 95 % level of significance, there is a strong positive correlation (r=0.82) between the results from both methods with all oFRNT-positive and -negative samples being pFRNT-positive and -negative respectively (Fig. S3). In addition, pFRNT results were not affected by complement (Fig. S3). A number of factors could be used to explain differences observed in the neutralization titres from oFRNT and those from pFRNT, with neutralization titres from oFRNT being higher than those from pFRNT (Table S1). Serial dilution factors for both methods differed with oFRNT having the lower (fourfold) dilution series than pFRNT (twofold). Native PUUV has a greater advantage because it has the glycoproteins sufficiently expressed in comparison with pseudotypes which may be partially or fully covered with mixed glycoprotein spikes (defective particles). If partial or mixed glycoprotein spike formation is the case, antibody neutralization of the Gn and Gc will not result in 100 % neutralization. Similar work by Higa et al. (2012) in 2012 had reduced the cut-off point for pseudotypes to 50 % thereby giving pseudotypes an enhanced advantage. In our study, the oFRNT had to be done at a different dilution series (fourfold) and according to standard protocols in order to span a wider dilution range using the six-well plate format and to provide a suitable standard for assessing the pFRNT. This placed limitations on the analysis because of the quantity of serum required to make replicates. As a result, for some of the samples, the oFRNT results only stated if the neutralizing antibody titre was above or below the seropositive threshold titre. Furthermore, some cross-reactivity was observed, particularly with two Seoul virus (SEOV) suspected samples and one Dobrava virus (DOBV) suspected sera, the cause of which will be further investigated upon production of pseudotypes bearing both glycoproteins. A further evaluation of neutralizing titres with larger characterized serum panels from PUUV and other hantavirus infections will be needed to further elucidate the performance of the pFRNT assay.

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References


