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ORIGINAL ARTICLE

A vesicular stomatitis pseudovirus expressing the surface glycoproteins of influenza A virus

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Abstract Pseudotyped viruses bearing the glycoprotein(s) of a donor virus over the nucleocapsid core of a surrogate virus are widely used as safe substitutes for infectious virus in virology studies. Retroviral particles pseudotyped with influenza A virus glycoproteins have been used recently for the study of influenza hemagglutinin and neuraminidase-dependent processes. Here, we report the development of vesicular-stomatitis-virus-based pseudotypes bearing the glycoproteins of influenza A virus. We show that pseudotypes bearing the hemagglutinin and neuraminidase of H5N1 influenza A virus mimic the wild-type virus in neutralization assays and sensitivity to entry inhibitors. We demonstrate the requirement of NA for the infectivity of pseudotypes and show that viruses obtained with different NA proteins are significantly different in their transduction activities. Inhibition studies with oseltamivir carboxylate show that neuraminidase activity is required for pseudovirus production, but not for the infection of target cells with H5N1-VSV pseudovirus. The HA-NA-VSV pseudoviruses have high transduction titers and better stability than the previously reported retroviral pseudotypes and can replace live influenza virus in the development of neutralization assays, screening of potential antivirals, and the study of different HA/NA reassortants.

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Introduction

Pseudotyped viruses bear the glycoprotein of a donor virus over the nucleocapsid core of a surrogate virus. Most often, the virus surrogate is a retrovirus such as murine leukemia virus or a lentivirus. Pseudoviruses can be assembled by expression of plasmids encoding the structural proteins and the transduced genome of the surrogate virus together with a plasmid encoding the donor virus glycoprotein(s). A major advantage of using a pseudotype over a wild-type virus is that the former can be used in low-containment laboratories. Pseudotyped viruses are widely used for study of virus assembly, receptor tropism studies, and antiviral drug discovery and are now more commonly being used for diagnostic assays.

Since 2008, numerous groups have reported the development of retrovirus-based pseudotypes bearing influenza A virus glycoproteins as gene therapy vectors as well as potential diagnostic assays for neutralizing antibodies or antiviral drug identification [1–4]. These pseudotyped particles incorporating the glycoproteins of influenza A virus can be used as a reliable and safe substitute for the infectious virus when studying processes depending on

hemagglutinin and/or neuraminidase. The pseudotyped retroviruses have the same cell entry characteristics as the wild-type influenza virus and dependence on NA activity (either a coexpressed influenza virus NA or an exogenous bacterial neuraminidase). They can thus be used for the study of HA-mediated entry and NA-mediated virus release mechanisms and identification of potential inhibitors of each. Since it is also possible to make pseudotypes with novel combinations of HA and NA proteins, neutralization properties and drug activity can be safely tested for novel reassortants without the need for construction of recombinant influenza viruses that may have increased pathogenicity [5]. Finally, HA-NA-pseudotyped viruses can substitute for infectious influenza viruses in various immunological applications such as microneutralization assays and vaccine development in the academic or clinical laboratory.

While retroviruses pseudotyped with influenza virus HA and NA glycoproteins have been shown to successfully substitute for infectious virus in serodiagnostic assays and antiviral drug screening, they suffer from several shortcomings. Their production requires a high efficiency transfection of producer cells with at least four plasmids, and the resulting pseudoviruses have a low transduction titer and a short half-life at 4°C or room temperature, making their use for the large-scale virus inhibitor screening or testing of large numbers of human sera impractical. Recently, alternative pseudotype cores have begun to be used for assay development. Recombinant vesicular stomatitis virus is one system that shows good activity and ability to adopt a wide range of glycoproteins. This type of pseudotype provides several advantages over retrovirus-based systems. First, they are assembled by transfection of only the plasmids encoding the donor glycoproteins, which for influenza virus are HA and NA only. Second, they achieve transduction activities that are higher than those attained with HA-NA-pseudotyped retroviruses [6, 7]. Third, they are stable at 4°C for several weeks and, fourth, the expression of the transduced reporter gene can be detected in as little as 8 hours postinfection [7, 8] as compared to 36-48 hours for retroviruses [9].

Here, we report, for the first time, the development of vesicular-stomatitis-virus-based pseudotypes bearing the glycoproteins of influenza A virus. A replication-defective VSV with a deletion in the VSV G gene in the virus genome and with firefly luciferase reporter gene was used. We show that pseudotypes bearing the HA and NA of H5N1 influenza A virus mimic the wild-type virus in neutralization assays and sensitivity to entry inhibitors. We also show that the pseudotypes can be stored for at least three weeks at 4°C and subjected to multiple freeze-thaw cycles with only minimal loss of activity. We tested H5 HA in combination with NA proteins of different influenza A viruses and found that the pseudoviruses that were obtained were significantly different in their transduction activities. Neuraminidase activity was found to be critical for pseudovirus production but not for pseudovirus infection of target cells, as was shown by neuraminidase inhibition studies. This work opens up the possibility of using this system as a robust neutralization assay in the field, replacing the use of stocks of wild-type virus, and we show that it can detect new NA reassortments equally well.

Materials and methods

Plasmids

HA ORF from influenza virus A/Egypt/N04395/2009 (GenBank accession no. CY041941) preceded by a Kozak ribosome initiation consensus sequence was synthesized by GenScript and was subcloned into the pcDNA3 vector. The pCI-Neo-N1(California) plasmid expressing the neuraminidase of the influenza virus A/California/27/2009/H1N1 pandemic strain was provided by Dr. B. Labrosse (INSERM U941, France). The pCAGGS-N1(Hamburg) plasmid expressing the neuraminidase of the influenza virus A/Hamburg/05/2009/H1N1 strain was provided by Dr. M. Matrosovich (Institute of Virology, Marburg). A pCAGGS-N1(Kan) plasmid expressing the neuraminidase of influenza virus A/Thailand/1(KAN-1)/2004/H5N1 was obtained by PCR amplification of the NA preceded by a Kozak sequence, from pHW2000-N1(Kan) plasmid, provided by Dr. E. Hoffman (UTMB, Texas, USA).

Pseudotyped virus production

Pseudotypes were produced by transfection of 293FT cells (Invitrogen, USA) with a combination of HA- and NA-encoding plasmids and subsequent infection by a recombinant vesicular stomatitis virus pseudotyped with the native VSV G glycoprotein. This virus is defective for replication, as VSV G has been deleted from the virus genome and replaced with an ORF encoding firefly luciferase (ΔVSV-G-Luc-G), and it was originally provided by Dr. S. Whelan (Harvard Medical School, Boston, USA). Briefly, 293FT cells were grown in medium lacking geneticin, which is included to select for high expression of SV40 large T antigen. Two to three hours prior to transfection, the cells were split to ~ 70-80 % confluence in medium lacking geneticin. Transfections were then performed by the calcium phosphate method using HEPES buffer (pH 7.05). For each 60-mm dish, 5 μg of each HA- and NA-expressing plasmid was used for transfection. At about 12 h post-transfection, the medium was replaced, and the producer cells were infected with 20 μl

(corresponding to $\sim 2 \times 10^8$ RLU) of ΔVSV-G-Luc-G virus. The virus-containing medium was discarded 4 h later, the cells were washed twice, and fresh growth medium was added. The influenza-VSV pseudotypes were collected at 24 and 48 h postinfection, and both portions were pooled, cleared by low-speed centrifugation, filtered through a 0.45-μm syringe filter, and frozen in aliquots until required.

Infection assays

A 70-80 % confluent monolayer of HEK293 target cells was infected with 50 μL of pseudotyped virus in a 24-well plate. In some cases, infection was made by spinoculation in a swinging-bucket rotor by centrifugation of the plate containing cells and virus at 3000 rpm for 1 h. All infection assays were performed in triplicate. Unless otherwise indicated, luciferase activity was measured 24 hours postinfection. For this, Steady-Glo luciferase assay buffer was purchased from Promega. Infected cells were lysed in lysis buffer containing 0.5 % Triton-X100 detergent in 50 mM Tris-HCl, pH 7.4, and the lysate was transferred to a 96-well plate. Luciferase assay buffer was added, and activity was measured in a Glomax plate reader (Promega).

Drug inhibition experiments were performed by replacement of growth medium with medium containing lysosomotropic agents (NH_4Cl and chloroquine) at the indicated concentrations 30 minutes before addition of virus. For neutralization assays, a 5-fold dilution series of anti-influenza-virus antibodies (a monoclonal Ab, 6F3, against H5 hemagglutinin of a Russian virus isolate, A/duck/Novosibirsk/56/05 [10], and a polyclonal serum from a ferret immunized with an H5N1 avian influenza isolate from Western Siberia A/Gs/Krasnoozerskoye/627/05) were preincubated with virus-containing medium at the indicated doses for 30 minutes at room temperature before adding the mixture to target cells. Typically, HEK293 cells were used for all infection assays.

Inhibition studies with a neuraminidase inhibitor, oseltamivir carboxylate, were performed following two different experimental setups. For pseudovirus infection inhibition, 10 nM, 100 nM, 500 nM, or 1000 nM oseltamivir carboxylate was added to HEK293 target cells 60 min prior to the infection. For pseudovirus production inhibition, 500 nM oseltamivir carboxylate was added to the growth medium of virus-producer 293FT cells. Oseltamivir carboxylate was synthesized locally, at the Institute of Organic Chemistry of the Siberian Branch of the Russian Academy of Sciences.

Microscopy

We noticed a correlation between the pseudotype virus titer and cytopathic effect in cells challenged with the ΔVSV-G-Luc-G virus during pseudotype production. This was documented using a Zeiss PrimoVert microscope.

Data analysis

Statistical analysis of experimental data and construction of graphs and diagrams were done using SigmaPlot 12.5 software.

Results

Neuraminidase activity is strictly required for the infectivity of influenza-VSV pseudotypes

It is known that productive infection by influenza A virus particles requires neuraminidase activity to cleave sialic acid from cell proteins during virion formation. We tested the NA-dependence of H5N1 pseudovirus formation by collecting cell supernatants and determining their transduction titers in a luciferase reporter assay. We obtained the ΔVSV-G-Luc capsid pseudotyped with either H5 hemagglutinin of highly pathogenic avian influenza strain A/chicken/Egypt/0626/2006(H5N1) alone or in combination with N1 neuraminidase of the pandemic influenza virus strain A/California/27/2009/H1N1, which has been reported to have superior sialic acid cleavage activity. This H5 protein does not require specific trypsin-like proteases for activation, since it is cleaved by furin and becomes fusogenic as soon as it is expressed on the surface of the producer cells. A "bald" ΔVSV-G-Luc capsid, produced by VSVΔG-G virus infection of 293FT cells transfected with beta-galactosidase plasmid, which does not encode any surface viral protein, was used as a negative control. It appears that neuraminidase activity is strictly required for the establishment of a high-titer infectious influenza-VSV pseudovirus, since the viral preps containing only the fusogenic H5 hemagglutinin did not show any considerable transduced luciferase activity upon infection of target cells. In fact, the RLU counts of H5(Egypt)- ΔVSV-G-Luc-infected and the bald-capsid-infected cells were almost equal (Fig. 1, columns 4 and 5). Conversely, infection with H5(Egypt) + N1(California)- ΔVSV-G-Luc pseudovirus yielded a luciferase signal of 10^6-10^7 RLU (Fig. 1, column 1).

Infectivity of influenza-VSV pseudotypes depends on the type of neuraminidase

We then attempted to study the role of different neuraminidase enzymes coexpressed with hemagglutinin-encoding plasmid in the infectivity of H5N1-VSV pseudoviruses. The three pseudotypes yielded different levels of

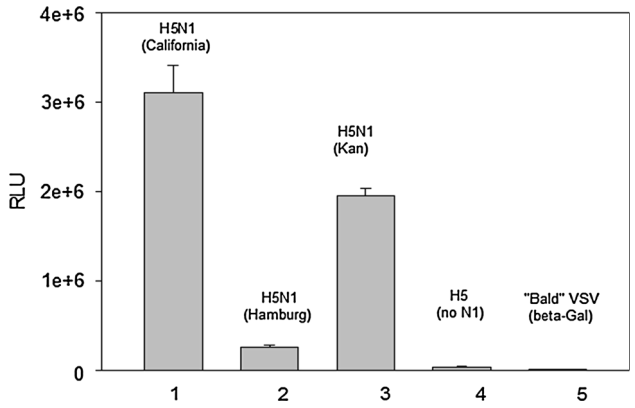

Fig. 1 Infectivities of H5N1-ΔVSV-G-Luc pseudoviruses expressing different neuraminidases. Columns 1-3 represent the transduced luciferase activity in target HEK 293 cells infected with pseudoviruses carrying H5(Egypt) hemagglutinin and co-pseudotyped with N1 protein of influenza virus strains A/California/27/2009/H1N1, A/Hamburg/05/2009/H1N1, and A/Thailand/1(KAN-1)/2004/H5N1, respectively. Column 4 is the infectivity of pseudotyped virus lacking the neuraminidase, and column 5 is the infectivity of a virus lacking both HA and NA

luciferase signal upon infection of target cells. Although slight variation was observed among three independent experiments, the infectivities of (H5 + N1)-ΔVSV-G-Luc viruses pseudotyped with N1(California), N1(Kan), and N1(Hamburg) neuraminidases demonstrated the same pattern, with the N1(California) pseudotype always producing the highest signal, nearly two times higher than the N1(Kan) pseudotype, while the infectivity of N1(Hamburg) pseudovirus was 8-10 times lower (Fig. 1, columns 1, 3, and 2, respectively).

Interestingly, the cytopathic effect (CPE) of pseudovirus production in the producer cell monolayers positively correlated with the infectivity of the pseudovirus preps that were obtained. An almost complete detachment of virus-producing monolayers was observed with N1(California) and N1(Kan) neuraminidases, which also produced the highest pseudovirus titers (Fig. 2, A, C.). Weak CPE was observed with N1(Hamburg) (Fig. 2 B), while CPE was completely lacking in the producer cells expressing either H5 hemagglutinin only or no glycoprotein at all (Fig. 2, D, E).

Neuraminidase activity is essential for the pseudovirus production, but not for infection

Since neuraminidase activity is currently believed to be essential at two points of the infection cycle – for virus particle release and for modulation of the number of sialic acid receptors on target cells available to the infecting virus – we attempted to determine whether NA affects the production/release of our pseudotypes or their ability to infect target cells. We used a neuraminidase inhibitor, oseltamivir carboxylate, to inhibit either the production of pseudotypes by H5N1-transfected 293FT producer cells or the infection of target HEK293 cells by H5N1-VSV pseudoviruses.

We first evaluated the effect of oseltamivir carboxylate (OC) on the infection of target cells by the viruses pseudotyped with H5(Egypt) hemagglutinin and N1(Kan) neuraminidase. Target HEK293 cells were preincubated for 1 h in a growth medium containing 10 nM, 100 nM, 500 nM, or 1000 nM OC before pseudovirus infection. Cells infected with a similar dose of H5N1-VSV(Luc) pseudovirus in the absence of OC were used as a positive control. The transduced luciferase activity in target cells infected in the presence of either OC concentration tested was not significantly different from that of the positive control (Fig. 3A), suggesting that NA activity is not important for infection with H5N1 pseudoviruses.

In order to investigate the role of NA in pseudovirus production, 500 nM of oseltamivir carboxylate was added to the growth medium of virus-producer cells. Briefly, 293FT cells were first transfected with plasmids expressing H5(Egypt) hemagglutinin and N1(Kan) neuraminidase, then infected by VSV-G-VSV(Luc) pseudotype, and after the latter was discarded, OC was added to the cells producing H5N1-VSV(Luc) pseudoviruses. Virus production by similarly modified 293FT cells in the absence of OC was used as a positive control. The collected pseudovirus preps were diluted 1/10 and used to infect target HEK293 cells. We observed a 96 % loss of transduced luciferase activity in the pseudovirus preps produced in the presence of OC (Fig. 3B, column 2) as compared to the positive controls (Fig. 3B, column 1), suggesting that neuraminidase activity is essential for virus particle production, and not for infection with H5N1 pseudovirus. Note that infections by the 1/10-diluted pseudovirus produced in the presence of 500 nM OC actually occurred in the presence of 50 nm OC, unlike in the positive controls. However, this concentration of oseltamivir carboxylate did not affect the efficiency of H5N1 pseudovirus infection in our previous experiments, and thus, this difference may be considered insignificant.

Stability of H5N1-ΔVSV-G-Luc pseudoviruses

In order to evaluate the stability of the pseudoviruses that were obtained, we measured the infectivities of all of the pseudovirus samples that had been stored at 4°C for 3 weeks or subjected to multiple (x3) freeze-thaw cycles. Only 5-10 % loss of transduced luciferase reporter activity was observed in all samples, as compared to the freshly thawed pseudovirus (data not shown), indicating a high thermal stability of H5N1-ΔVSV-G-Luc virus particles.

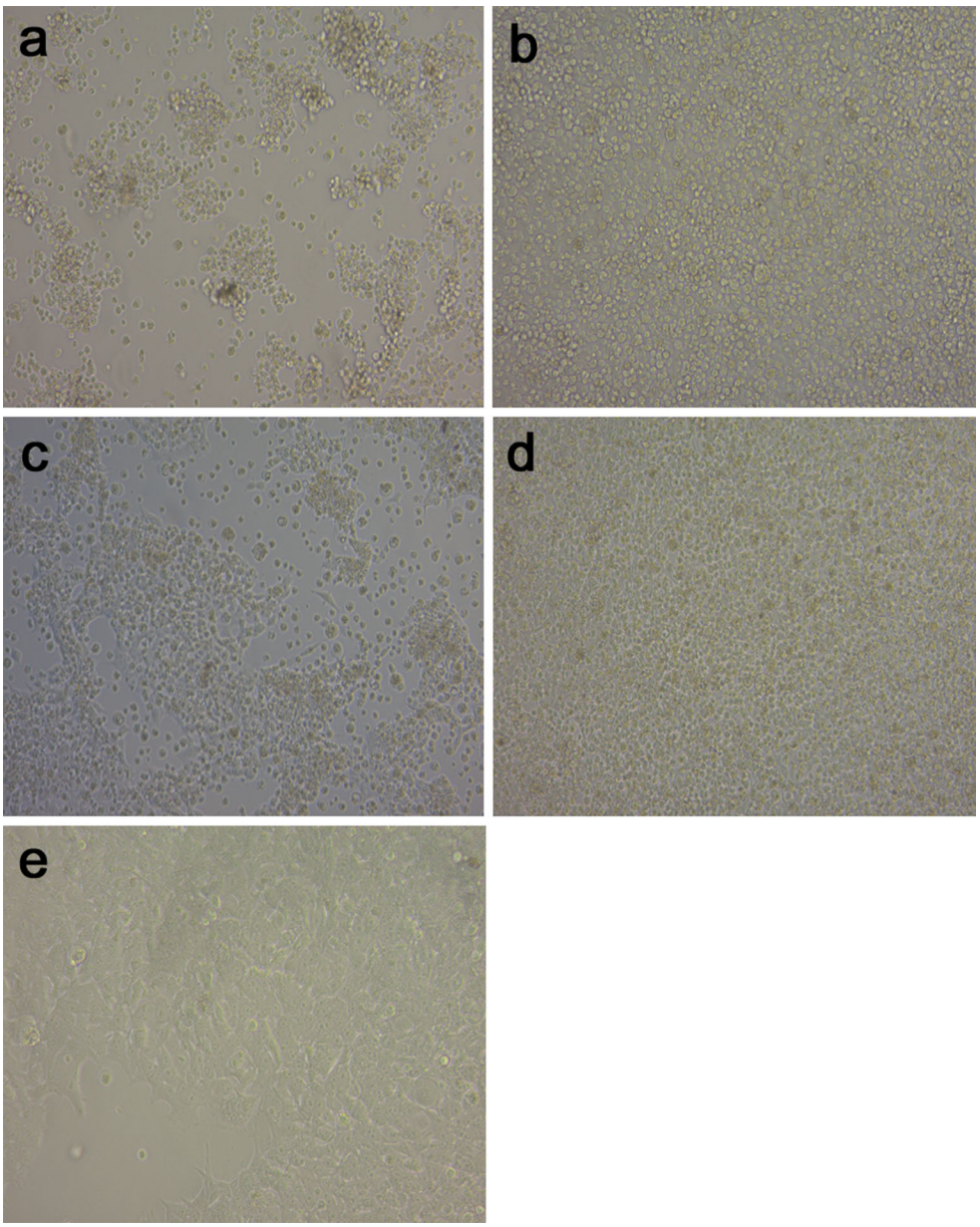

Fig. 2 Cytopathic effect (CPE) of pseudovirus production in producer cell monolayers. Different degrees of CPE were observed in cells expressing H5 hemagglutinin and one of three neuraminidases: N1(California) (A), N1(Hamburg) (B) or N1(Kan) (C). No CPE was observed in cultures expressing only hemagglutinin (D) or no surface glycoprotein at all (E)

Inhibition of H5N1-ΔVSV-G-Luc pseudovirus by lysosomotropic agents and specific antibodies

In order to study the features of H5N1-ΔVSV-G-Luc pseudovirus entry, we investigated inhibition of infection by lysosomotropic agents and specific anti-influenza-virus antibodies (a monoclonal antibody against H5 hemagglutinin and a polyclonal serum against an H5N1 avian virus isolate from Siberia).

Both lysosomotropic agents used (ammonium chloride and chloroquine) efficiently inhibited the H5(Egypt)-N1(California)-VSVΔG pseudovirus, with an IC_{50} value around 10 mM for NH_4Cl (Fig. 4A) and 30 μM for chloroquine (Fig. 4B), thus confirming the requirement for endosomal acidification for productive infection by pseudovirus.

Neutralization tests of H5(Egypt)-N1(California)-VSVΔG infection of target HEK293 cells was performed using anti-H5 monoclonal antibody 6F3 and a polyclonal ferret serum against an H5N1 isolate from Western Siberia (Fig. 5). Each antiserum neutralized H5N1 pseudovirus in a dose-dependent manner with 80 % neutralization

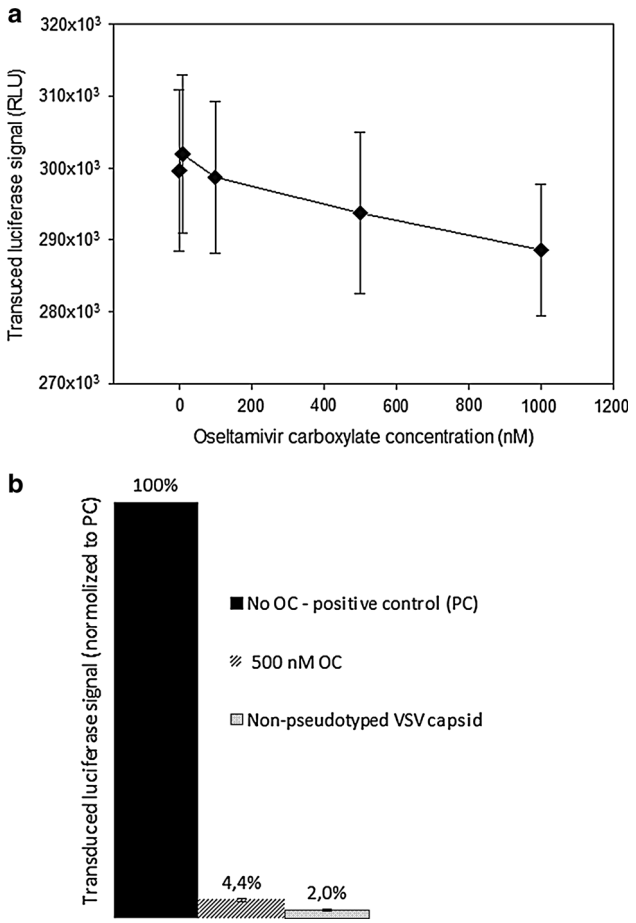

Fig. 3 Effect of oseltamivir carboxylate (OC) on H5N1-VSV pseudovirus infection and production. Pseudovirus infection was not affected by OC (A), while strong inhibition of pseudovirus production by OC was observed (B)

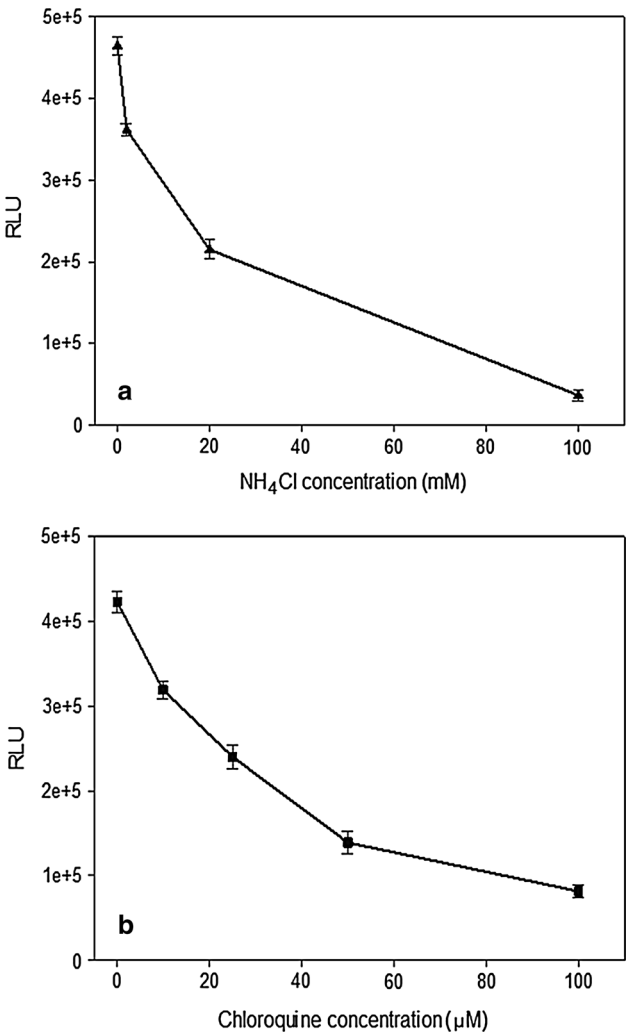

Fig. 4 Inhibition of H5(Egypt)-N1(California)-VSVΔG pseudovirus infection by lysosomotropic agents. a, ammonium chloride, b, chloroquine

achieved at 3×10^6 and 3×10^4 dilutions of the monoclonal and polyclonal antibody, respectively. The control VSVΔG virus pseudotyped with a homologous VSV G glycoprotein was not inhibited by either antibody at the highest concentrations used for H5N1-VSVΔG pseudovirus, thus confirming the specificity of neutralization (data not shown).

The H5N1-VSVΔG pseudovirus that was obtained was thus dependent on H5 hemagglutinin activity for successful entry into the cell, and on neuraminidase activity for its efficient release. It can therefore be used as a safe substitute for infectious influenza virus in a wide range of applications.

Discussion

Pseudotyped viruses bearing the surface proteins of highly pathogenic viruses can serve as a relevant substitute for the infectious virus in a variety of virological, immunological, antiviral drug and vaccine development applications. Two types of pseudovirus platforms are available and used most often: one based on a retroviral/lentiviral capsid and the other employing the vesicular stomatitis virus capsid for pseudotyping.

The possibility of successful incorporation of influenza virus surface glycoproteins into the VSV envelope was suggested by the generation of influenza/VSV pseudotypes observed upon co-infection of cell cultures with vesicular stomatitis virus and H1N1 subtype influenza A virus (A/WSN/1933(H1N1) [11]. However, neither the exact glycoprotein composition nor the infectivity of those naturally occurring pseudoviruses was studied at that time [11]. Later, pseudotypes between recombinant VSV and the same strain of H1N1 influenza virus were obtained purposely, and the incorporation of functional hemagglutinin or neuraminidase proteins into VSV envelope was shown

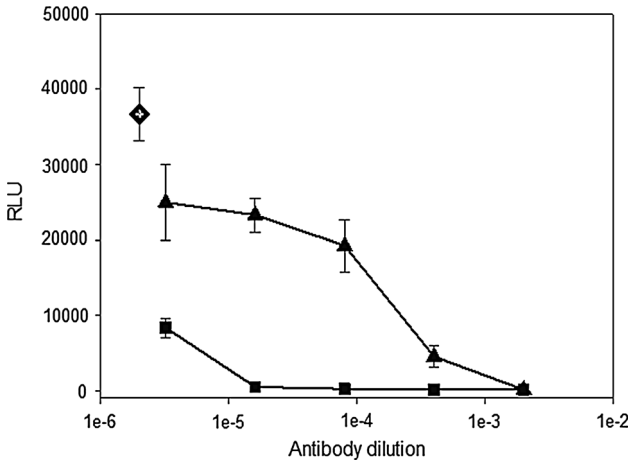

Fig. 5 Neutralization of H5(Egypt)-N1(California)-VSVΔG pseudovirus infection by specific antisera. Crossed diamonds indicate uninhibited infection (positive control), triangles indicate infection inhibition by ferret antiserum against a natural H5N1 influenza virus isolate, and squares indicate infection inhibition by H5-specific monoclonal antibodies

by hemadsorption, hemolysis, NA assays, and immunoelectron microscopy [12]. The glycoprotein envelope of those pseudoviruses was a mosaic between VSV G protein and either H1, or N1 influenza virus surface glycoproteins, which made them unsuitable for both the study of hemagglutinin-dependent entry and the analysis of the infectious properties of different HA/NA reassortants [12]. Also, the approach used for the generation of influenza-VSV pseudotypes in this and a more recent study [13] involved a laborious cloning step in which the VSV G protein gene in a full-length antigenomic VSV construct was replaced by influenza virus glycoprotein genes. We, conversely, employed another strategy in which pseudotypes are generated by co-expression of replication-incompetent VSV capsid and plasmid vectors encoding influenza virus glycoproteins. This approach was successfully used for the production of VSV pseudotyped with influenza C HEF glycoprotein [14] and turned out to be rather convenient for the generation of VSV-influenza A pseudotypes expressing different combinations of HA and NA in our study. Here, we report the establishment of VSV pseudotyped with a combination of H5 hemagglutinin of avian influenza virus and different N1 neuraminidases and analyze its infectious properties.

We attempted to compare the established VSV-based H5N1 pseudotype with the lentivirus-based pseudotypes in terms of virus transduction titer, signal-to-noise ratio, and thermal stability. Luciferase-bearing lentiviral influenza pseudotypes yield luminescence signals ranging from 10^4-10^5 RLU/mL for unconcentrated pseudovirus [15, 16] to 10^6-10^7 RLU/mL and higher when concentrated by sucrose gradient centrifugation [17, 18]. With our influenza-VSV pseudotypes, we routinely obtain transduction activities of 1-5 $\times$ 10^8 RLU. The signal-to-noise ratio of our pseudovirus, determined as the ratio of transduced luciferase signals of H5N1 pseudotyped VSV and the non-pseudotyped "bald" VSV capsid, is higher than 1000:1, which offers a broad dynamic range for the quantitative analysis of pseudovirus infections and their neutralization/inhibition. Also, the H5N1-VSV pseudovirus demonstrates better stability than the lentiviral pseudotype, as we observed little loss of infectivity of the former after 3 weeks of storage at 4°C, compared to the more than 50 % loss of H5-lentiviral pseudotype infectivity reported previously [15].

Another potential advantage of the VSV-based technology over lentiviral pseudotypes is the possibility to establish a multicycle infection model. A specific feature of pseudolentiviruses is that they are single-cycle infection agents, and no virus progeny is released from the infected cells. The VSVΔG-based system can overcome this limitation due to the recently proposed approach of using VSVΔG pseudovirus infections of cell monolayers transfected with viral envelope glycoproteins as a model for viral spread [19]. The multicycle infection model can be a valuable tool in antiviral drug research used to study the cumulative effect of antivirals and/or neutralizing antibodies over a number of viral generations. In contrast, pseudolentiviruses can only be used to evaluate the single-step inhibitory potential of candidate drugs. The use of (HA + NA)-VSVΔG pseudovirus in a multicycle modality is of particular relevance for the study of influenza virus infections, since optimal influenza virus replication is believed to depend on a balance between receptor binding by HA and receptor destruction by NA [20]. Our data (Fig. 1) demonstrate the impact of different types of NA on the infectivity of H5-VSV pseudoviruses, confirming this notion. As HA works at the entry step in target cells, and since NA is required mainly for the release of influenza (pseudo)particles from the producer cells, the effects of different (HA + NA) combinations on the overall infectivity of influenza reassortants can be studied only in a multicycle infection model. Our model is also well suited for the study of neuraminidase inhibitors, which are able to block infection at two different time points of the replication cycle – by reducing virus release and inhibiting reinfection of new target cells due to receptor destruction [20] – and thus require a live virus or multicycle pseudovirus approach in order for the cumulative antiviral effect to be evaluated. The reported (HA + NA)-VSVΔG pseudovirus is a relevant and safe alternative to a wild-type virus for the study of the roles of influenza virus glycoproteins (hemagglutinin and neuraminidase) and their combinations on the infectious properties of influenza

virus, as well as for the search of antivirals targeted at the activities of these viral proteins.

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