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Development of a novel equine influenza virus live-attenuated vaccine

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ABSTRACT

H3N8 equine influenza virus (EIV) is an important and significant respiratory pathogen of horses. EIV is enzootic in Europe and North America, mainly due to the suboptimal efficacy of current vaccines. We describe, for the first time, the generation of a temperature sensitive (ts) H3N8 EIV live-attenuated influenza vaccine (LAIV) using reverse-genetics approaches. Our EIV LAIV was attenuated (att) *in vivo* and able to induce, upon a single intranasal administration, protection against H3N8 EIV wild-type (WT) challenge in both a mouse model and the natural host, the horse. Notably, since our EIV LAIV was generated using reverse genetics, the vaccine can be easily updated against drifting or emerging strains of EIV using the safety backbone of our EIV LAIV as master donor virus (MDV). These results demonstrate the feasibility of implementing a novel EIV LAIV approach for the prevention and control of currently circulating H3N8 EIVs in horse populations.

1. Introduction

Influenza A viruses (IAVs) are enveloped viruses in the *Orthomyxoviridae* family that contain a segmented genome made of eight single-stranded RNA molecules of negative polarity (Palese and Shaw, 2007). IAVs are classified by subtypes based on the antigenicity of the two major membrane glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Palese and Shaw, 2007). Equine influenza, caused by equine influenza virus (EIV), is the most common and important respiratory infectious disease of horses. The H3N8 subtype of EIV was first reported from infected horses in Florida in 1963 (Waddell et al., 1963). At the end of the 1980s, H3N8 EIV diverged phylogenetically and antigenically into the American and Eurasian lineages (Daly et al., 1996). The American lineage evolved into Florida, Kentucky and South American sublineages (Lai et al., 2001), and the Florida sublineage has further diverged into the clades 1 and 2 that continue circulating nowadays (Bryant et al., 2009; Murcia et al., 2011). Currently, viruses from the sublineage Florida clade 1 are considered enzootic in the United States (US) but have also produced outbreaks in other parts of the world (Alves Beuttemmuller et al., 2016; Cowled et al., 2009; Woodward et al., 2014; Yamanaka et al., 2008), while the clade 2 viruses of the Florida sublineage are predominant in Europe and Asia (Fougerolle et al., 2017; Qi et al., 2010; Rash et al., 2017; Virmani et al., 2010; Yondon et al., 2013). Based on international surveillance studies, the World Organization for Animal Health (OIE, Office International des Epizooties) recommends including representative viruses from both the sublineage Florida clades 1 and 2 in the composition of H3N8 EIV vaccines (OIE, 2017).

Vaccination is the most effective strategy, alongside isolation, movement restriction and basic biosecurity measures, to prevent H3N8 EIV infections or to limit their consequences (Pica and Palese, 2013; Wong and Webby, 2013). Despite the development and commercialization of vaccines for almost five decades, H3N8 EIV is still circulating and considered endemic in numerous countries around the World, including the US (Cullinane et al., 2010; Paillot, 2014; Paillot et al., 2016). Different vaccine strategies have been available for decades for the control of EIV in horses. These include, mainly, influenza inactivated (IIV) and live-attenuated (LAIV) vaccines. Several vaccination studies have showed that adjuvanted IIVs administered intramuscularly induce humoral immunity, mostly by inducing neutralizing antibodies against the viral HA protein, but are comparatively poor inducers of cellular immunity (Belongia et al., 2009; Osterholm et al., 2012; Paillot, 2014). There is a wide panel of EIV IIVs, but the large majority of them do not contain representative strains of both clades 1 and 2 of the Florida sublineage of H3N8 EIV as recently recommended by the OIE. LAIVs are administered intranasally, mimicking the natural route of viral infection, and are able to induce both cellular and humoral immune responses, providing better immunogenicity and protection than

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IIVs (Belshe et al., 2007; Gorse et al., 1991; Hoft et al., 2011; Paillot, 2014). The only currently available H3N8 EIV LAIV, Flu Avert I.N. (Merck), was developed by passaging the A/equine/Kentucky/1/1991 H3N8 (Kentucky lineage) in embryonated chicken eggs at gradually reduced temperatures to generate a temperature sensitive (ts) variant that replicates efficiently at low temperatures (cold-adapted, ca) (Wilson and Robinson, 2000; Youngner et al., 2001). It has been shown that Flu Avert I.N. induced homologous (Lunn et al., 2001) and heterologous (Chambers et al., 2001) protection against H3N8 EIVs circulating in the 1990s. Thus, if the circulating EIV matches the virus in the vaccine (A/equine/Kentucky/1/1991), Flu Avert I.N. can confer better protection against disease caused by EIVs than the IIV counterparts, inducing faster production of antibodies and broader immune responses (Cullinane et al., 2010; Paillot, 2014; Paillot et al., 2016). For this reason, LAIVs are ideal for their use to prevent and control EIV infections (Cullinane et al., 2010; Paillot, 2014; Paillot et al., 2016). However, although other LAIVs are updated yearly, Flu Avert I.N. has not been updated or modified to match currently circulating EIV strains. Therefore, and because of the ability of influenza virus to undergo antigenic drift, there is not a good match of surface antigens between contemporary EIV strains and the virus present in Flu Avert I.N. The antigenic disparity between the virus in Flu Avert I.N. and currently circulating EIV strains likely results in a significantly lower vaccination impact (Cullinane et al., 2010; Paillot, 2014) and partial protection (Park et al., 2009; Yates and Mumford, 2000). Moreover, Flu Avert I.N. does not contain any representative Florida sublineage clade 1 and 2 H3N8 EIV strains, which is recommended by the OIE to be included in H3N8 EIV vaccines (Paillot et al., 2016).

In order to develop an updated and more effective LAIV for the treatment of currently circulating EIV strains, we used the same strategy that we have recently implemented for the development of LAIVs against H3N8 and H3N2 canine influenza viruses (CIVs) (Nogales et al., 2016b; Rodriguez et al., 2017c). We introduced in the polymerase basic 2 (PB2) and polymerase basic 1 (PB1) viral proteins of A/equine/Ohio/1/2003 H3N8 (Florida sublineage clade 1) the mutations responsible for the ts, ca and att phenotype of A/Ann Arbor/6/60 H2N2 LAIV (Cox et al., 1988; Snyder et al., 1988), the master donor virus (MDV) of the human LAIV (FluMist, MedImmune) and assessed its safety and efficacy in both mice and horses. This is the first description of a ts and att H3N8 EIV LAIV obtained by reverse-genetics technology.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney 293 T cells (293 T; ATCC CRL-11268), Madin-Darby canine kidney cells (MDCK; ATCC CCL-34) and equine dermal cells (E. Derm NBL-6; ATCC CCL-57) were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 µg/ml; L-glutamine, 2 mM) at 37°C with 5% CO_2 (Nogales et al., 2014b).

Recombinant wild-type (WT) and live attenuated (LAIV) H3N8 EIVs were generated using A/equine/Ohio/1/2003 plasmid-based reverse techniques (Martinez-Sobrido and Garcia-Sastre, 2010) and grown in MDCK cells at 33°C. The commercially available A/equine/Kentucky/1/1991 H3N8 LAIV (Flu Avert I.N., Merck) was also grown in MDCK cells at 33°C. The A/equine/Kentucky/2014 H3N8, used in horse challenge experiments, was grown in embryonated hen eggs. For infections, virus preparations were diluted in phosphate buffered saline (PBS) containing 0.3% bovine albumin (BA) and 1% penicillin and streptomycin (PS) (PBS/BA/PS). After 1 h viral adsorption at room temperature (RT), MDCK cells were maintained with post-infection (p.i.) DMEM media supplemented with 0.3% BA, 1% PSG, and 1 µg/ml of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma). Viral titers were determined by immunofocus assay (fluorescent forming units, FFU/ml) in MDCK cells at 33°C as previously described (Nogales et al., 2014b) using the anti-NP monoclonal antibody (mAb) HB-65 (ATCC HB-65, HL16-L10-4R5).

2.2. Plasmids

For the generation of H3N8 EIV LAIV, the PB2 and PB1 genes of A/equine/Ohio/1/2003 H3N8 were subcloned in a pUC19 plasmid (New England BioLabs) to introduce the ts mutations PB2 N265S and PB1 K391E, E581G, and A661T by site-directed mutagenesis. The presence of the introduced mutations was confirmed by sequencing. PB2- and PB1-LAIV viral segments were subcloned from pUC19 into the ambisense pDZ plasmid like the other A/equine/Ohio/1/2003 H3N8 viral genes (PB2- and PB1-WT, PA, HA, NP, NA, M and NS) for virus rescue. pDZ is an ambisense vector that contains a human RNA polymerase I promoter and a mouse terminator sequence that encodes the negative sense genomic RNA and, in opposite orientation to the polymerase I unit, contains a polymerase II transcription cassette (chicken β-actin promoter and polyA) that encode the viral proteins from the same viral gene (Chambers et al., 2009).

2.3. Minigenome assay

To analyze the ability of A/equine/Ohio/1/2003 H3N8 WT and LAIV polymerases to replicate and transcribe at different temperatures (33°C, 37°C, and 39°C) E. Derm cells (12-well plate format, 5×10^5 cells/well, triplicates) were co-transfected in suspension, using Lipofectamine 2000 (Invitrogen), with 0.25 µg of each of the A/equine/Ohio/1/2003 H3N8 WT or LAIV ambisense pDZ-PB2 or PB2-LAIV, pDZ-PB1 or PB1-LAIV, pDZ-PA and pDZ-NP plasmids, together with 0.5 µg of a reporter minigenome (MG) viral (v)RNA-like expression plasmid encoding Gaussia luciferase (Gluc) driven by a murine RNA polymerase I promoter (mpPol-I Gluc), and 0.1 µg of a mammalian expression pCAGGS plasmid encoding Cypridina luciferase (Cluc) to normalize transfection efficiencies (Cheng et al., 2015; Nogales et al., 2016b). Cells transfected in the absence of the pDZ-NP plasmid were included as negative control and empty pDZ plasmid was used to keep the amount of transfected plasmid DNA constant in the negative control. At 48 h post-transfection, Gluc and Cluc expression levels were determined using the Biolux Gaussia and Cypridina Luciferase Assay kits (New England BioLabs) and quantified with a Lumicount luminometer (Packard). Reporter gene activation (Gluc) was normalized to that of Cluc and is reported as fold induction over the level of induction for the negative control (absence of NP). The mean values and standard deviations (SDs) were calculated and statistical analysis was performed using a two-tailed Student *t*-test with Microsoft Excel software. Data are represented as relative activity considering A/equine/Ohio/1/2003 H3N8 WT polymerase activity at each temperature as 100%.

2.4. Virus rescue

Viral rescue of A/equine/Ohio/1/2003 H3N8 WT and LAIV was performed as previously described (Nogales et al., 2014b). Briefly, co-cultures (1:1) of 293 T and MDCK cells (6-well plate format, 1×10^6 cells/well, triplicates) were co-transfected in suspension, using Lipofectamine 2000, with 1 µg of the eight-ambisense A/equine/Ohio/1/2003 H3N8 pDZ-PB2 or PB2-LAIV, -PB1 or PB1-LAIV, -PA, -HA, -NP, -NA, -M, and -NS plasmids. At 12 h post-transfection, the medium was replaced with p.i. DMEM medium supplemented with 0.5 µg/ml TPCK-treated trypsin. Tissue culture supernatants (TCS) were collected at three days post-transfection, clarified, and used to infect fresh monolayers of MDCK cells. Then, at three days p.i., recombinant viruses were plaque purified and scaled up using MDCK cells at 33°C (Martinez-Sobrido and Garcia-Sastre, 2010).