Infection, Genetics and Evolution 64 (2018) 95–104

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research Paper

Safety, immunogenicity and protection of A(H3N2) live attenuated influenza vaccines containing wild-type nucleoprotein in a ferret model

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ARTICLE INFO

Keywords:
Live attenuated influenza vaccine
Influenza A virus
Nucleoprotein
Reverse engineering
Ferrets

ABSTRACT

Live attenuated influenza vaccines (LAIVs) are promising tools for the induction of broad protection from influenza due to their ability to stimulate cross-reactive T cells against influenza pathogens. One of the major targets for cytotoxic T-cell immunity is viral nucleoprotein (NP), which is relatively conserved among antigenically distant influenza viruses. Nevertheless, a diversity of epitope composition has been found in the NP protein of different lineages of influenza A viruses. The H2N2 master donor virus which is currently used as a backbone for the LAIV and donor of the six genomic segments encoding the internal proteins, A/Leningrad/134/17/57 (MDV Len/17), was isolated 60 years ago. As such, NP-specific T-cell immunity induced upon vaccination with classical LAIVs with a 6:2 genome composition containing this older NP might be suboptimal against currently circulating influenza viruses. In this study, a panel of H3N2 LAIV candidates with wild-type NP genes derived from circulating viruses were generated by reverse genetics (5:3 genome composition). These viruses displayed the cold adaptation and temperature sensitivity phenotypes of MDV Len/17 in vitro. LAIVs with both 6:2 and 5:3 genome compositions were attenuated and replicated to a similar extent in the upper respiratory tract of ferrets. LAIVs were immunogenic as high neutralizing and hemagglutination inhibition serum antibody titers were detected 21 days after infection. All vaccinated animals were protected against infection with heterologous H3N2 influenza A viruses. Thus, LAIV with a 5:3 genome composition is safe, immunogenic and can induce cross-protective immunity.

1. Introduction

Influenza A viruses are highly contagious respiratory pathogens that continuously threaten the human population. Influenza epidemics cause severe respiratory disease worldwide, with up to 645,000 annual influenza-associated illness deaths (Iuliano et al., 2018). The most effective strategy to prevent infection with influenza virus is vaccination. Currently, there are three types of influenza vaccines available – inactivated influenza vaccines (IIV), live attenuated influenza vaccines (LAIV) and recombinant influenza vaccines. Immunization with IIV induces a humoral immune response, typically directed to the viral surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA). However, this immunity is strain-specific and offers little protection against drift variants of influenza viruses. Similarly, LAIVs induce humoral immunity against the HA and NA, but LAIVs induce cross-reactive immunity more efficiently than IIV. Infection with LAIV induces no or mild upper respiratory symptoms mimicking a subclinical influenza virus infection (Hoft et al., 2017). Viral infection activates both systemic and localized innate and adaptive immune responses, which provide protection by a variety of mechanisms including viral interference (Wang et al., 2015), antiviral NK cell activation at the site of infection (Laurie et al., 2015), influenza-specific cross-reactive T cell activation and expansion (Schultz-Cherry, 2015), generation of high-avidity mucosal neutralizing sIgA antibody (Mohn et al., 2017) and induction of immunological memory to influenza virus antigens (van Riet et al., 2012).

LAIV strains typically inherit their HA and NA genes from a wild-type influenza virus (seasonal or potentially pandemic) and the

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https://doi.org/10.1016/j.meegid.2018.06.019
Received 6 April 2018; Received in revised form 14 June 2018; Accepted 16 June 2018
Available online 19 June 2018
1567-1348/

Table 1
Phenotypic characteristics of H3N2 LAIVs and Len/17 master donor virus.

LAIV strain	Source of HA and NA (clade)	Source of NP	Distance to Len/17 NP, aa substitutions	Virus titre in eggs, $lgEID_{50}$/mL		
				26 °C	33 °C	38 °C
TX LAIV 6:2	TX/12 (3c.1)	Len/17	–	7.0 ± 1.3	9.2 ± 0.5	2.2 ± 0.7
TX LAIV 5:3	TX/12 (3c.1)	TX/12	27	6.1 ± 0.5	8.1 ± 0.4	1.8 ± 0.3
SW LAIV 6:2	SW/13 (3c3.a)	Len/17	–	6.1 ± 0.4	8.1 ± 0.7	1.7 ± 0.4
SW LAIV 5:3	SW/13 (3c3.a)	SW/13	29	5.2 ± 0.7	7.8 ± 0.5	1.8 ± 0.3
HK LAIV 6:2	HK/14 (3c2.a)	Len/17	–	5.5 ± 0.5	7.9 ± 0.4	1.8 ± 0.5
HK LAIV 5:3	HK/14 (3c2.a)	HK/14	30	5.4 ± 0.7	7.5 ± 0.3	1.5 ± 0.3
Len/17	Len/17	Len/17	–	6.9 ± 0.7	8.9 ± 0.3	1.9 ± 0.3
TX/12	TX/12 (3c.1)	TX/12	27	3.5 ± 0.8	9.0 ± 0.6	9.0 ± 0.5
SW/13	SW/13 (3c3.a)	SW/13	29	3.0 ± 0.8	7.0 ± 0.9	6.3 ± 1.0
HK/14	HK/14 (3c2.a)	HK/14	30	3.5 ± 0.9	8.4 ± 0.6	8.4 ± 1.1

TX/12: A/Texas/50/2012 (H3N2); NP accession number EPI408574; SW/13: A/Switzerland/9715293/2013 (H3N2); NP accession number EPI540519; HK/14: A/Hong Kong/4801/2014 (H3N2); NP accession number EPI614430; Len/17: A/Leningrad/134/17/57 (H2N2); NP accession number EPI555083; Distances were calculated as number of amino-acid substitutions between wild-type and Len/17 NP sequences aligned by the multiple alignment tool of Geneious 6.0 software.

remaining six genes from an attenuated master donor virus (referred to as a 6:2 genome composition) (Swain et al., 2004). The internal proteins, especially the nucleoprotein (NP), are predominant targets for the CD8+ T-cell immune response in humans (Aleksandrova, 1977). While CD4+ T cells regulate the immune response, CD8+ T cells contribute to influenza virus clearance (Grant et al., 2013).

Influenza A(H3N2) viruses have drifted continuously in humans over the past 40 years (Moskophidis and Kioussis, 1998). This has resulted in decreased effectiveness of seasonal vaccines, since the viruses easily escape vaccine-induced antibody immunity to the viral antigens (Hay et al., 2001). The internal proteins of influenza virus are highly conserved over time as compared to the HA and NA proteins, making them attractive targets to improve the efficacy/effectiveness of LAIVs (Glatman-Freedman et al., 2017). Currently, commercially available LAIVs for influenza A are based on two master donor viruses (MDV), A(H2N2) A/Leningrad/134/17/57 (Len/17) - used in Russia and India, and A(H2N2) A/Ann Arbor/6/60 - used in the USA, Canada and Europe; both strains were isolated 60 years ago. Over this time, A(H2N2) influenza viruses were displaced by A(H1N1) and A(H3N2) variants and mutations accumulated in virus proteins (Quinones-Parra et al., 2014). Indeed, only one third of the predicted the human leukocyte antigen (HLA) class I-restricted NP epitopes identified in A(H2N2) MDV Len/17 are conserved in recent A(H1N1) and A(H3N2) isolates (Machkovech et al., 2015). Additionally, we have found that at least 28 HLA class I-restricted epitopes have diverged between Len/17 MDV and recent A(H3N2) influenza virus NP (Isakova-Sivak et al., 2017).

Thus, there is a risk that the T-cell immunity induced upon vaccination with classical LAIVs (with 6:2 genome composition) may be diminished against currently circulating influenza viruses. The most straightforward way to overcome T-cell epitope mismatch is to include wild-type internal proteins in the LAIV reassortant viruses. In the case of Russian LAIV, only the matrix and NP genes can be replaced because they are not involved in attenuation of the MDV Len/17 virus (Korenkov et al., 2018). Thus, we proposed that the generation of a 5:3 genome composition by introducing a wild-type NP gene into the current 6:2 genome vaccine composition might be sufficient to enhance cross-protection (Isakova-Sivak et al., 2011). We generated a panel of reverse genetics-derived 5:3 LAIVs of A(H1N1), A(H3N2) and A(H7N9) subtypes. Comparative studies in mice of A(H1N1) and A(H7N9) 5:3 LAIVs demonstrated that 5:3 LAIVs could induce broader cytotoxic T-lymphocyte (CTL) cross-protective immunity and protection against more distant strains than their 6:2 LAIV counterparts (Isakova-Sivak et al., 2016). Ferrets are the most suitable animal model to study A(H3N2) human influenza infection, due to the inability of recent A(H3N2) viruses to replicate efficiently in mouse respiratory tissues (Isakova-Sivak et al., 2017; Rekstin et al., 2017). Unfortunately, ferrets' immunobiology has not yet been well described to study cross-reactivity of influenza NP-specific T cells. In particular, there are no data about the specificity of influenza-reactive T cells in these animals. In addition, the information regarding the contribution of nucleoprotein-specific T cells in the total T-cell-mediated response in ferrets is lacking. In the present study we did not focus on the details of ferret's T cell responses, however our previous findings from human T-cell in vitro studies justified the necessity of LAIV virus NP renewal (Narasaraju et al., 2009).

In this study, we compared A(H3N2) LAIV reassortants with 6:2 and 5:3 genome compositions with regard to (i) growth characteristics, (ii) safety, (iii) immunogenicity and their (iv) ability to protect animals against challenge with homologous and heterologous viruses. These ferret studies will serve as a basis for conducting clinical trials of 5:3 LAIVs in volunteers, where the impact of NP specific epitopes of recent A(H3N2) influenza viruses on improving CTL immunity can be thoroughly evaluated.

2. Materials and methods

2.1. Viruses

rgLAIV strains with 6:2 or 5:3 genome compositions were generated by reverse genetics (RG). HA, NA and NP genes of A(H3N2) viruses A/Texas/50/2012 (TX/12), A/Switzerland/9715293/2013 (SW/13) and A/Hong Kong/4801/2014 (HK/14) viruses were cloned into dual-promoter plasmids, as previously described (Korenkov et al., 2018). Six genomic segments coding for internal and non-structural proteins of A/Leningrad/134/17/57 (H2N2) were previously cloned into RG vectors (Isakova-Sivak et al., 2011). Viruses were rescued in MDCK/293 T cells as previously described (Isakova-Sivak et al., 2011). The 6:2 LAIV strains prepared from TX/12, SW/13 and HK/14 viruses were designated TX LAIV 6:2, SW LAIV 6:2 and HK LAIV 6:2, respectively. The 5:3 LAIVs were designated TX LAIV 5:3, SW LAIV 5:3 and HK LAIV 5:3, respectively (Table 1). Wild-type influenza A(H3N2) strains A/Hong Kong/4801/2014, A/Switzerland/9715293/2013, A/Texas/50/2012, A/Perth/16/2009 (PE/09) A/Brisbane/10/2007 (BR/07), A/Panama/2007/99 (PA/99), A/California/7/2009-like (H1N1) and B/Brisbane/60/2008-like were acquired from the WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne, Australia. Wild-type and LAIV viruses were passaged in the allantoic cavity of embryonated hens' eggs and stored in aliquots at −80 °C. Temperature sensitive and cold adapted (*ts/ca*) phenotypes of the LAIV A(H3N2) viruses were determined by titration in eggs at different temperatures: 38 °C compared to 33 °C and 26 °C compared to 33 °C for the *ts* and *ca* phenotypes, respectively. Eggs inoculated with serial 10-fold virus dilutions were