Virology 500 (2017) 209–217

Contents lists available at ScienceDirect

Virology

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

Comparative studies of infectivity, immunogenicity and cross-protective efficacy of live attenuated influenza vaccines containing nucleoprotein from cold-adapted or wild-type influenza virus in a mouse model

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A R T I C L E I N F O

Keywords:
Live attenuated influenza vaccine
Nucleoprotein
Immunogenicity
Cell-mediated immunity
Cross-protection
Mouse model
Immunodominant epitope

A B S T R A C T

This study sought to improve an existing live attenuated influenza vaccine (LAIV) by including nucleoprotein (NP) from wild-type virus rather than master donor virus (MDV). H7N9 LAIV reassortants with 6:2 (NP from MDV) and 5:3 (NP from wild-type virus) genome compositions were compared with regard to their growth characteristics, induction of humoral and cellular immune responses in mice, and ability to protect mice against homologous and heterologous challenge viruses. Although, in general, the 6:2 reassortant induced greater cell-mediated immunity in C57BL6 mice than the 5:3 vaccine, mice immunized with the 5:3 LAIV were better protected against heterologous challenge. The 5:3 LAIV-induced CTLs also had better *in vivo* killing activity against target cells loaded with the NP_{366} epitope of recent influenza viruses. Modification of the genome of reassortant vaccine viruses by incorporating the NP gene from wild-type viruses represents a simple strategy to improve the immunogenicity and cross-protection of influenza vaccines.

1. Introduction

Influenza A viruses are highly contagious respiratory pathogens that continuously threaten human populations. Almost every year, there are epidemics of 3–5 million cases of severe influenza worldwide, of which at least 250,000 are fatal (Fifth meeting of National Influenza Centers, 2012). The most effective tool for controlling influenza is vaccination. There are two main kinds of influenza vaccine currently in wide use: inactivated influenza vaccine (IIV) and live attenuated influenza vaccine (LAIV).

Immunization with IIV induces mainly humoral immune responses to the viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). However, this immunity is strain-specific and gives little protection against drifted variants of the virus. In contrast, immunization with LAIV induces a wide spectrum of immune responses, including local (mucosal) and T-cell-mediated immunity, in particular CD8+ cytotoxic T-cells (CTLs) (Tamura et al., 2005). CTL-driven immunity is cross-reactive and recognizes conserved epitopes within viral proteins, affording protection against different subtypes of influenza A virus (Kreijtz et al., 2008). One of the major targets for the CTL immune response is a molecule of viral nucleoprotein (NP), which contains multiple immunodominant CTL epitopes (Grant et al., 2013; Thomas et al., 2006).

LAIV strains are routinely prepared by either classical reassortment or reverse genetics, and usually comprise HA and NA genes derived from wild-type influenza virus (seasonal or potentially pandemic). The remaining six genes, including the NP gene, are derived from an attenuated master donor virus (MDV) (the so-called 6:2 genome composition) (Aleksandrova, 1977; Jin and Subbarao, 2015). The most widely used donor viruses – A/Leningrad/134/17/57 (Len/17) in Russia and A/Ann Arbor/6/60 (A/AA) in the USA and Europe – were isolated nearly 60 years ago. It is possible that the nucleoprotein of influenza viruses could have significantly evolved during this period, despite its relatively conserved nature, resulting in a significant change in its antigenic properties. Thus the NP-specific CTL immunity induced by vaccination with classical LAIVs may not be effective against currently circulating influenza viruses. The most straightforward way to overcome this problem would be to include the wild-type NP gene in

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http://dx.doi.org/10.1016/j.virol.2016.10.027
Received 1 September 2016; Received in revised form 23 October 2016; Accepted 27 October 2016
Available online 06 November 2016
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the genome of the LAIV reassortant virus, i.e. to switch from a 6:2 to a 5:3 genome composition (Isakova-Sivak et al., 2016). Such a modification of the vaccine virus genome would require a thorough understanding of the properties of the new LAIV reassortants, including *in vitro* and *in vivo* characterization, as well as their evaluation in clinical trials.

This paper reports a comparison of H7N9 LAIV reassortants with 6:2 and 5:3 genome compositions, with regards to their growth characteristics, induction of humoral and cellular immune responses in C57BL/6 (H2^b) mice, and ability to protect animals against challenge with homologous and heterologous viruses. This particular subtype was selected because of its ability to induce humoral and cell-mediated immunity in C57BL/6 mice, and also because the NP of Len/17 and H7N9 virus have significant differences in the murine immunodominant epitope $NP_{366-374}$, and thus are a good model for comparative epitope-specific cell-mediated immunogenicity studies (Thomas et al., 2006).

2. Materials and methods

2.1. Materials

H7N9 LAIV reassortant virus with a 6:2 genome composition (H7N9 LAIV 6:2) was prepared by classical reassortment in eggs and was tested in preclinical and clinical trials (de Jonge et al., 2016; Rudenko et al., 2016). This virus possesses the HA and NA genes of wild-type (*wt*) A/Anhui/1/2013 (H7N9) strain; the remaining genes come from A/Leningrad/134/17/57 (H2N2) MDV. The corresponding H7N9 LAIV 5:3 reassortant virus with the NP gene inherited from the H7N9 virus rather than the MDV was generated by means of reverse genetics. Both viruses were fully sequenced and found to be identical apart from the NP gene.

Master donor virus A/Leningrad/134/17/47 (Len/17) and its wild-type precursor A/Leningrad/134/57 (H2N2) (Len134 wt) were obtained from the repository of the Institute of Experimental Medicine (Saint Petersburg, Russia). Low-pathogenic avian influenza virus A/mallard/Netherlands/12/2000 (H7N3) was obtained from the repository of the Centers for Disease Control and Protection (CDC) (Atlanta, GA, USA). In addition, two engineered viruses were generated for challenge experiments: an H7N9-PR8 5:3 reassortant, possessing the HA, NA and NP genes of *wt* H7N9 virus and five genes from A/Puerto Rico/8/34 (H1N1) (PR8) virus; and an H1N1 7:1 virus, containing the NP gene from *wt* H7N9 virus and seven genes from the PR8 virus. All viruses were propagated in 10–11-D-old chicken embryos for two days at 33 °C and stored in aliquots at −70 °C.

C57BL6 mice of 8–10 weeks old were purchased from the laboratory breeding nursery of the Russian Academy of Sciences "Stolbovaya" (Moscow region, Russia).

$NP_{366-374}$ peptides (ASNENMDTM and ASNENMEAM) were chemically synthesized by Almabion Ltd (Russian Federation), with a purity of over 95%, as shown by high-performance liquid chromatography (HPLC). Peptides were reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 1 mM and stored at −70 °C in aliquots.

2.2. Methods

2.2.1. CTL epitopes in circulating influenza A viruses

In order to predict the effectiveness of LAIV CTL immunity, we assessed the conservation of selected CTL epitopes in 757 unique NP sequences of influenza A viruses of H1N1 and H3N2 subtypes circulating in 2009–14, using the Immune Epitope DataBase (IEDB). Influenza A virus sequences were obtained from the Influenza Virus Sequence Database of the National Center for Biotechnology Information (NCBI) (Bao et al., 2008). CTL epitopes in the MDV NP sequence were screened with netCTL major histocompatibility complex I (MHCI) peptide binding and netChop proteasome processing prediction algorithms (Larsen et al., 2007). The immunogenicity of CTL epitopes was estimated using the T cell class I peptide MHC (pMHC) immunogenicity predictor algorithm (Calis et al., 2013); peptides with an immunogenicity score above 0 were assumed to be immunogenic. Conservation of immunogenic CTL epitopes was estimated by conservancy analysis, with sequence identity threshold equal to 100% (Bui et al., 2007). For murine experiments, CTL epitope–MHC binding affinity was predicted using the netMHCpan algorithm (Hoof et al., 2009).

2.2.2. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vitro

Temperature-sensitive (*ts*) and cold-adapted (*ca*) phenotypes of the studied viruses were determined by titration at different temperatures in eggs: 38 °C compared with 33 °C for the *ts* phenotype, and 26 °C compared with 33 °C for the *ca* phenotype. The Len/17 and Len134 wt viruses were used as control viruses possessing the opposite *ts/ca* phenotypes. Eggs were inoculated with 10-fold virus dilutions and incubated for either 48 h (at 33 °C or 38 °C) or 6 days (at 26 °C). The growth characteristics of the H7N9 LAIV viruses were analysed in Madin Darby canine kidney (MDCK) cells: cell monolayers were infected with the viruses at a multiplicity of infection (MOI) of 0.01 and 0.001 in triplicate; 150 µl of the media were collected every 12 h and stored at −70 °C prior to titration by 50% tissue culture infective dose ($TCID_{50}$). Virus titers in eggs and MDCK cells were calculated by the Reed and Muench method and expressed in terms of $\log_{10}$ 50% egg infective dose (EID_{50})/ml and $\log_{10}TCID_{50}$/ml, respectively.

2.2.3. Growth characteristics of H7N9 LAIV 6:2 and 5:3 reassortants in vivo

Groups of eight female C57BL6 mice were anesthetized with ether and given 50 µl of virus suspension containing 10^6 EID_{50} by the intranasal (IN) route. The Len/17 and Len134 wt viruses were used as control viruses: the former is characterized by an attenuated (*att*) phenotype, while the latter has a *non-att* phenotype. Nasal turbinates and lungs were collected on days 3 and 6 after inoculation and stored frozen at −70 °C until used for homogenization. Tissue homogenates were prepared in 1 ml of sterile phosphate-buffered saline (PBS) containing antibiotic and antimycotic (Invitrogen, UK), using a small bead mill TissueLyser LT (QIAGEN, Germany). The clarified supernatants were used to determine virus titers by limiting dilutions in eggs, as described above. The limit of detection was 1.2 $\log_{10}$/ml.

2.2.4. Immunogenicity and protection studies

Groups of 54 female C57BL6 mice were given two doses of 50 µl of either H7N9 LAIV 6:2 or H7N9 LAIV 5:3 virus suspension containing 10^6 EID_{50} intranasally, 21 days apart. Control animals received the same volume of PBS. Blood and bronchoalveolar lavage (BAL) samples were collected from six mice in each group on day 21 after each dose, to assess antibody immune responses. Six mice from each group were euthanized on day 7 after each dose and spleen samples were harvested for the assessment of cell-mediated immune responses. *In vivo* killing activity of CTLs was studied in six mice of each study group on day 7 after the second dose. The remaining 24 animals were used for the assessment of protective efficacy of the H7N9 LAIVs.

2.2.5. Antibody responses

Serum antibody titers were determined by hemagglutination-inhibition assay (HAI), and IgG by enzyme-linked immunosorbent assay (ELISA), as described previously (Isakova-Sivak et al., 2014) with some modifications. IgA antibodies were determined in BAL specimens collected on days 21 and 42 using 1 ml of sterile PBS.

For HAI, serum samples were treated with chicken red blood cells to remove nonspecific inhibitors and quantified against four HA units of the following viruses: (i) H7N9 LAIV 6:2; (ii) H7N9 LAIV 5:3; (iii) H7N3 wt; and (iv) H1N1 7:1. ELISA was performed with the same four

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