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H5N2 vaccine viruses on Russian and US live attenuated influenza virus backbones demonstrate similar infectivity, immunogenicity and protection in ferrets

Rita Czakó [a], Leatrice Vogel [a], Troy Sutton [a], Yumiko Matsuoka [a], Florian Krammer [b], Zhongying Chen [c], Hong Jin [c], Kanta Subbarao [a,*]

[a] Emerging Respiratory Viruses Section, Laboratory of Infectious Diseases, NIAID, NIH, USA
[b] Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA
[c] MedImmune Vaccines, Mountain View, CA, USA

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ABSTRACT

The continued detection of zoonotic influenza infections, most notably due to the avian influenza A H5N1 and H7N9 subtypes, underscores the need for pandemic preparedness. Decades of experience with live attenuated influenza vaccines (LAIVs) for the control of seasonal influenza support the safety and effectiveness of this vaccine platform. All LAIV candidates are derived from one of two licensed master donor viruses (MDVs), cold-adapted (*ca*) A/Ann Arbor/6/60 or *ca* A/Leningrad/134/17/57. A number of LAIV candidates targeting avian H5 influenza viruses derived with each MDV have been evaluated in humans, but have differed in their infectivity and immunogenicity. To understand these differences, we generated four H5N2 candidate pandemic LAIVs (pLAIVs) derived from either MDV and compared their biological characteristics *in vitro* and *in vivo*. We demonstrate that all candidate pLAIVs, regardless of gene constellation and derivation, were comparable with respect to infectivity, immunogenicity, and protection from challenge in the ferret model of influenza. These observations suggest that differences in clinical performance of H5 pLAIVs may be due to factors other than inherent biological properties of the two MDVs.

1. Introduction

Circulation of seasonal influenza viruses is responsible for an estimated 226,000 excess hospitalizations and 36,000 excess deaths per year in the United States alone [1]. Additionally, several subtypes of avian influenza viruses have caused human illness; of these, H5N1 viruses are particularly notable with respect to incidence, morbidity and mortality, and evidence of increasing genetic diversification [2]. Vaccination remains a critical element of influenza prevention and control strategies. To this end, the development of candidate vaccines for influenza viruses with pandemic potential has been the subject of substantial international effort [3].

The live attenuated influenza vaccine (LAIV) platform is particularly attractive for the development of a pandemic influenza vaccine. In well-controlled efficacy trials in children, despite induction of lower titers of serum antibody, trivalent seasonal LAIV offered superior protection against matched and antigenically drifted influenza viruses compared to trivalent inactivated vaccine [4–9]. LAIVs can elicit diverse and robust T cell responses [10,11], a feature that is associated with increased breadth of protection [5,9,12] and is particularly relevant given the difficulty in predicting which strain may emerge as a pandemic threat. Robust T-cell responses and mucosal immunity elicited by LAIVs are also associated with protection from severe disease [13–19]. The potential advantages of this platform are recognized by its inclusion in the World Health Organization's pandemic preparedness plans [20,21].

LAIVs are derived from genetically stable, attenuated influenza A and B "master donor viruses" (MDV; MDV-A and MDV-B). Two types of MDVs have been independently generated and licensed in Russia and the United States. These MDVs are used as backbones for LAIVs bearing the surface antigens of wild-type (*wt*) viruses and are generated by either classical reassortment or reverse genetics. The internal protein genes of the MDV confer the properties of

* Corresponding author at: Laboratory of Infectious Diseases, NIAID, 33 North Drive, MSC 3203, Bethesda, MD 20892, USA and WHO Collaborating Centre for Reference and Research on Influenza, The Peter Doherty Institute for Infection and Immunity, 792 Elizabeth St., Melbourne 3000, Australia.
E-mail addresses: ksubbarao@niaid.nih.gov, kanta.subbarao@influenzacentre.org (K. Subbarao).

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attenuation (*att*), cold adaptation (*ca*) and temperature sensitivity (*ts*), that limit LAIV replication to the upper respiratory tract in humans [4].

A growing number of LAIV candidates for potential pandemic influenza strains (pLAIVs) based on each MDV, A/Ann Arbor/6/60 cold-adapted (*ca)* H2N2; (AA/6/60) or A/Leningrad/134/17/57 (*ca* H2N2; Len/17/57), have been evaluated in phase I clinical trials [4,22,23]. The development of a robustly immunogenic pLAIV candidate for H5N1 avian influenza viruses has been challenging. An H5N2 pLAIV derived on the Len/17/57 MDV backbone was infectious and immunogenic in 50% of participants, although antibody titers were modest compared to those elicited by seasonal influenza vaccination [23]. In contrast, an H5N1 pLAIV formulated with the AA/6/60 MDV was shed in a minority of participants and was not immunogenic in healthy adults [24]. However, both H5 pLAIVs induced long lasting B cell memory responses, as demonstrated by a rapid and robust recall response following boost with inactivated H5 vaccine [25,26].

It has been speculated that the Len/17/57-based viruses are more infectious and immunogenic than the AA/6/60-based vaccine viruses but the basis for the observed differences in viral replication and antibody response between the AA/6/60- and Len/17/57-derived H5 LAIVs is not known. To directly compare characteristics of H5 pLAIV formulated with each MDV, we generated reassortant viruses by reverse genetics. All vaccine viruses bore surface antigens from the same low pathogenic avian influenza (LPAI) virus, but varied in internal gene composition to represent the formulations of H5 pLAIV candidates that have been evaluated in clinical studies. We compared the biological characteristics of these viruses *in vitro* and evaluated replication, immunogenicity, and protection from challenge with a heterologous highly pathogenic avian influenza (HPAI) H5N1 virus in the ferret model. Ferrets are naturally susceptible to most influenza A viruses, generate robust antibody responses to influenza antigens, and demonstrate clinical signs of infection [27] and are therefore a valuable model for pre-clinical evaluation of influenza vaccine candidates.

2. Methods

2.1. Cells and viruses

293T and Madin-Darby canine kidney (MDCK) cells were maintained as described previously [28]. Primary human nasal epithelial cells (hNEC; PromoCell) and primary human bronchial epithelial cells (NHBE; Lonza) were seeded onto membrane supports (6.5 mm Transwell; Corning, Inc.) coated with collagen type I (Corning, Inc) and maintained following manufacturer's recommendations. After monolayers reached confluence, an air liquid interface (ALI) was established and cells were differentiated for four weeks before infection.

All viruses in this study were propagated in embryonated chicken eggs (Charles River SPAFAS). Viral cDNA of the HA and NA of *wt* A/duck/Potsdam/1402-6/1986 (A/dk/Potsdam; H5N2 LPAI) were generated by RT-PCR following isolation of viral RNA. The unmodified hemagglutinin (HA) and neuraminidase (NA) coding sequences were cloned into pAD3000, a derivative of pHW2000 [28]. Reassortant attenuated vaccine viruses were generated using an eight plasmid reverse genetics system as previously described, by co-transfection of 293T and MDCK cells [29]. Briefly, the two 6:2 reassortant vaccine viruses were rescued with the six internal protein genes of either Len/17/57 or AA/6/60; the 7:1 vaccine viruses were rescued with only the H5 HA paired with the N2 segment from either MDV (Table S1). The 50% tissue culture infectious dose ($TCID_{50}$) was determined as previously described [14] and titer was calculated by the Reed-Muench method [30]. Sequences of pLAIV stock viruses were verified by Sanger sequencing (BigDye v3.1, ABI).

2.2. In vitro analysis of H5N2 pLAIVs

The *ts* phenotype of the recombinant and *wt* A/dk/Potsdam viruses was evaluated by plaque assay, as described previously [29], with duplicate plates incubated at the permissive temperature (33 °C) or the restrictive temperature (39 °C). Results were independently verified by $TCID_{50}$ assay on MDCK cells. Growth curves were generated by inoculating MDCK cells, NHBE cells, and hNEC cells at a multiplicity of infection (MOI) of 0.01, as described previously [31,32]. Supernatant was sampled at serial time points. The amount of infectious virus in each sample was determined by $TCID_{50}$ assay in MDCK cells at 33 °C.

2.3. Ferret studies

All ferret experiments were approved by and conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH). All study procedures and experiments were carried out in biosafety level 3 facilities in accordance with the Select Agent guidelines of the NIH, Centers for Disease Control and Prevention (CDC), and the U.S. Department of Agriculture (USDA). Adult (≥24 week old) outbred male ferrets (Mustela putorius furo; Triple F Farms) that were seronegative for circulating H1N1 and H3N2 viruses by hemagglutination inhibition (HAI) assay were housed in individual cages.

Prior to vaccination, a subcutaneous transponder was implanted to monitor temperature and to facilitate animal identification. Body weight, temperature, and activity level were assessed daily for 6 days following vaccination as well as after challenge. Ferret activity level was scored daily to assess disease-related changes in behavior, as previously described [33]. Ferrets that exhibited ataxia or hind limb paralysis were humanely euthanized within 12 h of first observation of neurological signs.

Six groups of four ferrets each were lightly sedated with isoflurane and vaccinated with a single dose of either one of the four H5N2 LAIVs or infected with *wt* A/dk/Potsdam, by intranasal inoculation of 10^7 $TCID_{50}$ of virus in a volume of 1 mL. Mock-vaccinated ferrets were inoculated with an equivalent volume of Leibovitz's L-15 media (Lonza). Nasal washes were obtained 1, 3, and 5 days post-immunization to evaluate the replication of each candidate LAIV and the *wt* A/dk/Potsdam virus.

Four weeks following immunization, all ferrets (n = 24) were challenged intranasally with 10^6 $TCID_{50}$ of *wt* A/Vietnam/1203/2004 (H5N1 HPAI) in a volume of 1 mL. Nasal washes were collected at 3 and 5 days post-challenge and all ferrets were euthanized on day 5 post-challenge for titration of virus from tissue homogenates of lungs and nasal turbinates (NT), as described previously [29]. To collect nasal washes, ferrets were anesthetized by intramuscular injection of a ketamine hydrochloride cocktail (ketamine (25 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg)). Sterile PBS in a volume of 1 mL was instilled into the nose. A sneeze response was elicited by stimulation of the nostrils. The expelled secretions were collected in a petri dish, transferred to cryovials, and stored at −80 °C until titration.

2.4. Serologic assays

Baseline serum samples were collected prior to and four weeks following vaccination. Antibody titers were assessed by HAI assay using horse erythrocytes (Lampire Biological Laboratories) and by

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