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A single dose of whole inactivated H7N9 influenza vaccine confers protection from severe disease but not infection in ferrets

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ABSTRACT

The H7N9 influenza virus caused significant mortality and morbidity in infected humans during an outbreak in China in 2013 stimulating vaccine development efforts. As previous H7-based vaccines have been poorly immunogenic in humans we sought to determine the immunogenic and protective properties of an inactivated whole virus vaccine derived from a 2013 H7N9 virus in ferrets. As whole virus vaccine preparations have been shown to be more immunogenic in humans, but less likely to be used, than split or surface antigen formulations, we vaccinated ferrets with a single dose of 15, 30, or 50 μg of the vaccine and subsequently challenged with wild-type A/Anhui/1/2013 (H7N9) either by direct instillation or by contact with infected animals. Although ferrets vaccinated with higher doses of vaccine had higher serum hemagglutinin inhibition (HI) titers, the titers were still low. During subsequent instillation challenge, however, ferrets vaccinated with 50 μg of vaccine showed no illness and shed significantly less virus than mock vaccinated controls. All vaccinated ferrets had lower virus loads in their lungs as compared to controls. In a separate study where unvaccinated-infected ferrets were placed in the same cage with vaccinated-uninfected ferrets, vaccination did not prevent infection in the contact ferrets, although they showed a trend of lower viral load. Overall, we conclude that inactivated whole-virus H7N9 vaccine was able to reduce the severity of infection and viral load, despite the lack of hemagglutinin-inhibiting antibodies.

1. Introduction

A novel subtype of avian influenza virus to cause human infections, H7N9, emerged in China in 2013 and has since infected more than 200 humans [1], with unusually high mortality [2]. Unlike highly pathogenic avian strains of human concern, H7N9 is a low-pathogenic avian virus, causing subclinical symptoms in avian species. This presents a considerable obstacle in identification and control of the outbreak sources. Although most H7N9 isolates are susceptible to neuraminidase inhibitors, resistant phenotypes have been identified in patients who received treatment [3,4]. Surveillance studies suggest that the H7 subtype viruses are prevalent in live-bird markets, even in regions outside the reported outbreak areas [5]. Their prevalence and the difficulty of detecting H7N9 circulation in poultry mean that they will likely continue to be a zoonotic threat for the foreseeable future. For these reasons, national and international agencies have begun development of intervention strategies. Vaccination remains an effective strategy to prepare for a pandemic since it provides protection against infection and induces herd immunity to limit virus spread. The World Health Organization (WHO) considers vaccination "a key component in the response and preparedness efforts against a pandemic potential, including avian influenza A (H5N1), A (H9N2), and A (H7N9)" [6]. The reemergence of H7N9 early this year emphasizes the importance in developing an effective and immunogenic vaccine.

For the present situation, an H7N9 vaccine has two major hurdles: vaccines against avian influenza strains typically are poorly

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immunogenic [7,8], and the elderly, who have been disproportionately affected by H7N9, generally respond poorly to influenza vaccines [9]. This presents a compound challenge to developing an effective H7N9 vaccine. Most seasonal inactivated influenza vaccines are comprised of split-virion or surface antigen products due to their lower reactogenicity as compared to inactivated whole-virus vaccines. However, the latter is more immunogenic when compared head-to-head in an unprimed population, eliciting a stronger antibody response with just a single dose [10,11]. Split-virion vaccines for avian influenza viruses, however, are known to be poorly immunogenic in humans (summarized in Table 1 in [12]). Clinical trials with split-virion H7N7 vaccines have reported very low seroconversion rates in vaccinees despite receiving two doses at 90 μg HA each [13] and this vaccine was unable to protect mice from the lethal effects of homologous virus infection (unpublished data). Due to these data, the pending human H7N9 vaccine clinical trials (www.clinicaltrial.gov; study identifier: NCT01995695, NCT01928472, NCT01942265, NCT01938742), and the predictions that similar immunogenicity issues may be apparent with H7N9 based vaccines [14], we sought to determine if an inactivated whole-virus based H7N9 vaccine is able to induce protective antibody levels. As manufacturing burden can dictate the timely supply of vaccine and whole virus preparations are likely to be more immunogenic than the more commonly used split or surface antigen preparations, we chose to evaluate if a single dose of this vaccine is sufficiently protective. The reference vaccine strain was developed by the US Centers for Disease Control and Prevention, Atlanta, and consists of the major antigenic proteins, hemagglutinin (HA) and neuraminidase (NA), from one of the human isolates of H7N9 virus, A/Shanghai/2/2013, that was recommended by the WHO as a candidate vaccine strain, combined with the six internal genes from a high-growth donor strain [15]. Here, we describe the efficacy of an inactivated, whole-virus vaccine based on reverse genetics recombinant H7N9 virus to protect ferrets from infection in a challenge and natural exposure study.

2. Materials and methods

2.1. Viruses and cells

The challenge virus A/Anhui/1/2013 (H7N9) is antigenically similar to the vaccine strain [16] and was received as a kind gift from Dr. Yue Long Shu of the Chinese Centre for Disease Control. Virus stock was prepared by propagation in 10-day-old embryonated chicken eggs at 35 °C for 36 h, aliquoted, and stored at −70 °C. Madin-Darby canine kidney (MDCK) cells used for virus titration were propagated in minimal essential medium (MEM) supplemented with 10% fetal calf serum, vitamins, L-glutamine, and antibiotics in a humidified 5% CO_2 environment.

2.2. Generation of the candidate vaccine

The recombinant vaccine seed strain A/Shanghai/2/2013-PR8-IDCDC-RG32A was developed by and received from the Centers for Disease Control and Prevention. The vaccine strain was a reverse-genetics-based strain bearing the HA and NA from the human isolate of A/Shanghai/2/2013 (H7N9) and six of the internal genes from the A/Puerto Rico/8/1938 strain [17]. Virus stock was prepared at our laboratory by propagation in eggs at 35 °C for 48 h. Virus-containing allantoic fluid was harvested and inactivated with β-propiolactone at a ratio of 1:2000 (v/v) for 72 h. Inactivated virus stock was then concentrated using Amicon ultrafiltration and ultracentrifugation through a 25% and 70% sucrose cushion, pelleted at 76,000 × *g* at 4 °C for an hour, and purified as previously described [18]. The total protein content was determined using the Bradford Assay (Biorad) according to the manufacturer's specifications. To estimate the HA content in the vaccine, vaccine stock and a dilution series of bovine serum albumin (BSA) standards were run on a SDS-PAGE gel and Coomasie-stained. ImageJ software was used to perform densitometry analysis on the protein bands. The HA content was estimated based on the non-linear regression curve generated with the BSA standards (see Supplemental Fig. 1). HA content (total of HA_0 and HA_1) was determined to be between 28 and 31% of total protein. Immunization dose was formulated based on HA content being 25% of total protein content.

2.3. Immunization and challenge

Specific-pathogen-free ferrets, 4 to 6 months old, were purchased from Triple F Farms (Bradford County, Pennsylvania), divided into four groups of nine ferrets each and bled for baseline sera. Ferrets in each group were then vaccinated with 15, 30, or 50 μg of vaccine, diluted in phosphate-buffered saline (PBS) in 0.5 ml volume, by intramuscular injection. A control group received 0.5 ml of PBS only. Three weeks later, sera were collected and tested for antibody titers as described below. Ferrets were subsequently divided into two experiments: 6 vaccinated ferrets were challenged with wild-type H7N9 virus via intranasal instillation, while the remaining 3 were used as vaccinated contacts in a transmission experiment. For the PBS-control group, 3 ferrets were challenged with wild-type virus, 3 served as mock vaccinated contacts, and the remaining 3 served as donor ferrets in the transmission experiment.

For the challenge experiment, ferrets were anesthetized with isoflurane and inoculated intranasally with 1 ml of 10^6 EID_{50} of wild-type H7N9, A/Anhui/1/2013 virus. Clinical signs of infection, weight and body temperature were monitored daily for 7 days post-challenge.

For the transmission experiment, 3 mock vaccinated ferrets were anesthetized with isoflurane and similarly inoculated with wild-type H7N9 virus to serve as donors. One day post-inoculation, vaccinated and mock vaccinated ferrets were placed into the same cage as the donor ferret to serve as direct contacts. The transmission experiment setup consists of 1 infected ferret and 1 ferret from each vaccination group, for a total of 4 ferrets per cage (Fig. 1). Clinical signs of infection, weight and body temperature were monitored daily for 7 days post-challenge.

2.4. Nasal wash and tissue samples

On days 3, 5, and 7 after virus inoculation, ferrets were anesthetized by ketamine (25 mg/kg), and nasal washes were collected in 1 ml of PBS. On day 5, 3 ferrets from each vaccination group in the challenge experiment were euthanized with a mix of ketamine and xylazine according to institutional protocol. Lung tissues were collected from each of the 5 lung lobes and pooled as a single sample, homogenized and titrated on MDCK cells. After three days, a hemagglutinin assay was performed and the virus titer was determined using the Reed and Muench method [19]. The limit of virus detection was <1 $\log_{10}TCID_{50}$/ml.

2.5. Serologic testing

Serum samples were treated with receptor-destroying enzyme (Denka Seiken, Japan), heat-inactivated at 56 °C for 30 min, and tested in an HA inhibition (HI) assay with 0.5% chicken red blood cells [20].

2.6. Statistical tests

Data collected were analyzed and graphed using Graphpad's Prism version 5.03. HI titers were expressed as geometric mean