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Mucosal administration of raccoonpox virus expressing highly pathogenic avian H5N1 influenza neuraminidase is highly protective against H5N1 and seasonal influenza virus challenge

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ABSTRACT

We previously generated recombinant poxviruses expressing influenza antigens and studied their efficacy as potential highly pathogenic avian influenza (HPAI) vaccines in mice. While both modified vaccinia Ankara (MVA) and raccoon poxvirus (RCN) expressing hemagglutinin (HA) provided strong protection when administered by parenteral routes, only RCN-neuraminidase (NA) showed promise as a mucosal vaccine. In the present study we evaluated the efficacy of RCN-NA constructs by both intradermal (ID) and intranasal (IN) routes. Surprisingly, while RCN-NA completely protected mice when administered by the IN route, it failed to protect mice when administered by the ID route. After challenge, significantly less virus induced pathology was observed in the lungs of mice vaccinated with RCN-NA by the IN route as compared to the ID route. Furthermore, IN administration of RCN-NA elicited neutralizing antibodies detected in bronchoalveolar lavage (BAL) samples. We also determined the role of cellular immune responses in protection elicited by RCN-NA by depleting CD4 and CD8 T cells prior to challenge. Finally, we demonstrated for the first time that antibodies against NA can block viral entry in addition to viral spread *in vitro*. These studies demonstrate the importance of mucosal administration of RCN viral vectors for eliciting protective immune responses against the NA antigen.

1. Introduction

Influenza viruses are a significant health concern for animals and humans. The World Health Organization (WHO) estimates that every year seasonal influenza viruses infect up to a billion people, with 3–5 million cases of severe disease and 300,000–500,000 deaths [1]. Recently, highly pathogenic H5N1 influenza viruses have emerged with devastating impacts on avian populations. The global spread of these viruses has continued unrestricted to this day and, as of December 2014, H5N1 viruses have infected 676 humans of which 398 cases have been lethal. In addition to the significant mortality risk, the CDC estimates the cost of a modern influenza pandemic to be $71.3–166.5 billion in the United States alone due to decreased productivity [2]. As a result, many countries are reliant on stockpiled antivirals and inactivated vaccines that elicit a neutralizing antibody response against hemagglutinin (HA) to combat present and future influenza threats. HA is responsible for binding to the host cell by attachment to sialic acid and for penetration of the endosomal membrane to allow the release of viral RNA into the cytoplasm to initiate viral replication [3]. Consequently, HA is susceptible to neutralization by antibodies, and this has been the rationale for the design of most current vaccines for influenza. However, neuraminidase (NA), another influenza surface glycoprotein, also is susceptible to neutralization by antibodies [4] and therefore represents a potential target for the development of a novel influenza vaccine.

The NA protein of influenza virus has opposing function to HA; the enzymatic activity of NA is to remove α-2,3- and/or α-2,6-linked sialic acid moieties from host or viral glycoproteins [5,6]. This activity of NA is necessary to prevent HA from binding to host/viral glycoproteins which causes aggregation of virus and impedes viral release [7]. Evaluation of NA as an immunogen independent of HA for influenza virus generally has not been pursued, probably because of the hypothesis that antibodies against NA would not block viral attachment and penetration and thus would provide incomplete protection. However, we recently demonstrated that a raccoon poxvirus based NA (RCN-NA) vaccine administered *via* mucosal route completely protected mice against H5N1 virus challenge lethality, and no virus was detected in lungs of mice at 5 days post-challenge [8]. Similarly, nonhuman primates (NHPs)

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vaccinated with NDV-NA by the intranasal/intratracheal developed high levels of serum neutralizing and NA inhibiting antibodies, and surprisingly influenza viral replication in the respiratory tract was almost completely prevented [9]. It is possible that mucosal administration of NA could be crucial for eliciting protective immune responses.

Raccoonpox virus (RCN) is an orthopoxvirus isolated from healthy wild raccoons that can be engineered to express viral and bacterial antigens [10–12]. Recombinant RCN vectored vaccines have been administered to a variety of mammalian species, including domestic cats, piglets, dogs, prairie dogs, non-human primates, and recently chickens [12–14]. Furthermore, RCN-based recombinant vaccines can induce systemically protective immune responses when delivered parenterally, as well as by mucosal routes [12,14,15]. Thus RCN is an ideal vector for evaluating parenteral *vs* mucosal route vaccination. In the present study we compared immune responses and protection elicited by parenteral and mucosal administration of RCN-NA based vaccines. We also demonstrate here evidence that NA specific antibodies can prevent infection of cultured cells, in addition to prevention of virus spread.

2. Materials and methods

2.1. Mice

This study was carried out in strict accordance with recommendations set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All animals and animal facilities were under the control of the School of Veterinary Medicine with oversight from the University of Wisconsin Research Animal Resource Center. The protocol was approved by the University of Wisconsin Animal Care and Use Committee (Approval V1312). Female, 6–10-week-old A/J mice were purchased from Jackson Laboratory (Bar Harbor, ME).

2.2. Cells and viruses

Vero and Madin-Darby canine kidney (MDCK) cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin. Cells were maintained in humidified air with 5% CO_2 atmosphere at 37 °C. RCN-NA was prepared as described previously [8]. Inactivated H5N1 influenza (A/Vietnam/1203/2004) vaccine (whole virion, Vero cell derived, non-adjuvanted) was obtained from Biodefense and Emerging Infections Research Resources Repository (NR-12148: BEI Resources, VA). H5N1 HPAI viruses A/Vietnam/1203/2004 (NCBI taxonomy ID: 284218) were propagated on MDCK cells with serum free DMEM supplemented with 1% bovine serum albumin and 20 mM HEPES (influenza media). Seasonal influenza viruses, including A/Puerto Rico/8/34 (PR8; H1N1) and A/Aichi/2/1968 (Aichi; H3N2), were kindly provided by Stacey Schultz-Cherry and Ghazi Kayali (St. Jude Children's Research Hospital, Memphis, TN, USA). Stocks of the propagated viruses were aliquoted, titrated by tissue culture infective dose (TCID) assay and stored at −80 °C until use. HPAI work was conducted in a BSL3+ facility in compliance with the UW Madison Office of Biological Safety.

2.3. Vaccinations

Groups of 5-week-old A/J mice were given 10^7 plaque forming units (PFU) of RCN constructs by the ID, or IN route. For ID vaccinations, 50 μl was injected into the footpad. IN vaccinations were performed by anesthetizing mice with isoflurane and dropwise pipetting 50 μl of RCN into each nostril. Inactivated A/Vietnam/1203/2004 H5N1 influenza vaccine (whole virion, Vero cell derived, non-adjuvanted) was obtained from Biodefense and Emerging Infections Research Resources Repository (NR-12148: BEI Resources, VA) was given at a dose of 5 μg, diluted in pH 7.4 phosphate buffered saline (PBS) by injecting a total volume of 100 μl subcutaneously in the scruff of the neck. Five weeks post-vaccination, mice were given identical booster vaccinations.

2.4. HPAI challenge

Two weeks following boost, mice were challenged with 1×10^4 $TCID_{50}$ units of H5N1 in 20 μl PBS by intranasal instillation under isoflurane anesthesia. All animals were observed over 14 days after challenge to evaluate protection, weight loss and mortality. Lung samples for titration of virus were homogenized using a mechanical homogenizer (MP Biochemicals, Solon, OH, USA). Lung supernatants were used to infect MDCK cells grown to confluency in 96-well plates. Cells were fixed 3 days later with 10% formalin/0.1% crystal violet (v/v) in PBS and viral titers were determined by observing overt cytopathic effects (CPE) with the aid of a light microscope and calculating the $TCID_{50}$ [16]. Lung samples for histopathology were fixed in cold Carnoy's fixative.

2.5. T cell depletions

On days −3, −1 pre-challenge and 1, 3, 5, and 7 post-challenge, mice were administered 100 μg of anti-CD4 mAb (GK1.5) or 250 μg of anti-CD8a mAb (2.43) (Bio X Cell) or both *via* the IP route. Depletion efficacy was determined with flow cytometry by staining with anti-mouse CD4 (RM4-5) and anti-mouse CD8a (53-6.7) mAbs (BD Bioscience). Mice were challenged as described above.

2.6. Serology

Blood samples were collected from mice by saphenous vein bleed prior to challenge and serum was used for immunological analyses. Neutralizing antibody (nAb) titers against HPAI were measured by a microneutralization assay that does not distinguish between antibodies that block viral entry or spread [8]. Briefly, microneutralization. The A/Vietnam/1203/04 virus was diluted to 200 TCID50 units in 50 μl of influenza media, added to 50 μl of serially (two-fold) diluted mouse sera, and then incubated at 37 °C for 1 h. The virus-serum mixture from each dilution was added to duplicate wells of MDCK cells in 96-well plates, incubated at 37 °C for 72 h, fixed, and scored for virus infection based on CPE. Total IgG antibody titers against NA were measured by ELISA. Plates (Costar E.I.A.) were coated by adding 10 μg per well of N1 NA protein (NR-19234: BEI Resources, VA) in PBS and incubating overnight at 4 °C. The remaining ELISA procedure was conducted as described previously [8].

2.7. Histopathology and immunohistochemistry

Lung tissue fixed in Carnoy's fixative were processed by the histopathology laboratory at the School of Veterinary Medicine, University of Wisconsin (Madison, WI, USA), and stained with hematoxylin and eosin. For immunohistochemistry, tissue sections were deparaffinized and rehydrated and stained as previously described [17].

2.8. Statistical analysis

Statistical analyses were performed using GraphPad software (La Jolla, CA). Antibody and viral titers were compared using a non-paired Student's *t* test. Differences in survival were analyzed using a Log-rank (Mantel–Cox) test.