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Polyanhydride nanovaccine against swine influenza virus in pigs

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ABSTRACT

We have recently demonstrated the effectiveness of an influenza A virus (IAV) subunit vaccine based on biodegradable polyanhydride nanoparticles delivery in mice. In the present study, we evaluated the efficacy of ~200 nm polyanhydride nanoparticles encapsulating inactivated swine influenza A virus (SwIAV) as a vaccine to induce protective immunity against a heterologous IAV challenge in pigs. Nursery pigs were vaccinated intranasally twice with inactivated SwIAV H1N2 (KAg) or polyanhydride nanoparticle-encapsulated KAg (KAg nanovaccine), and efficacy was evaluated against a heterologous zoonotic virulent SwIAV H1N1 challenge. Pigs were monitored for fever daily. Local and systemic antibody responses, antigen-specific proliferation of peripheral blood mononuclear cells, gross and microscopic lung lesions, and virus load in the respiratory tract were compared among the groups of animals. Our pre-challenge results indicated that KAg nanovaccine induced virus-specific lymphocyte proliferation and increased the frequency of CD4⁺CD8 α ⁺ T helper and CD8⁺ cytotoxic T cells in peripheral blood mononuclear cells. KAg nanovaccine-immunized pigs were protected from fever following SwIAV challenge. In addition, pigs immunized with the KAg nanovaccine presented with lower viral antigens in lung sections and had 6 to 8-fold reduction in nasal shedding of SwIAV four days post-challenge compared to control animals. Immunologically, increased IFN- γ secreting T lymphocyte populations against both the vaccine and challenge viruses were detected in KAg nanovaccine-immunized pigs compared to the animals immunized with KAg alone. However, in the KAg nanovaccine-immunized pigs, hemagglutination inhibition, IgG and IgA antibody responses, and virus neutralization titers were comparable to that in the animals immunized with KAg alone. Overall, our data indicated that intranasal delivery of polyanhydride-based SwIAV nanovaccine augmented antigen-specific cellular immune response in pigs, with promise to induce cross-protective immunity.

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1. Introduction

Swine influenza A virus (SwIAV) causes considerable economic losses in the pig industry worldwide [1]. Currently, multiple antigenically diverse strains of three major SwIAV subtypes H1N1, H1N2 and H3N2 are circulating in pig populations. Since pigs serve as a mixing vessel for human and avian IAV, numerous distinct SwIAV strains are frequently generated, and some of these have

zoonotic potential [2]. An effective vaccination strategy can prevent economic losses in the pig industry and limit zoonotic transmission of SwIAVs to humans. Vaccination against SwIAV is frequently practiced on pig farms using either bivalent or multivalent whole virus inactivated (WIV) vaccines which protect against homologous virus but are ineffective against heterologous strains [3–6]. Since SwIAV undergoes frequent mutation with antigenic drift and shift, there is an urgent need to develop broadly cross-protective vaccines. Moreover, WIV vaccines do not elicit high levels of antigen-specific secretory IgA antibody response in the respiratory tract where the disease is localized. It is also known that strong mucosal immunity can correlate with cross-protective efficacy against influenza [7,8]. Recently, WIV vaccine

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formulations were reported to enhance the severity of lung lesions in pigs infected with heterologous IAV, raising concerns over judicious selection and use of vaccines [3,4,6]. To overcome these limitations, a novel vaccine delivery platform is needed for prevention and control of influenza in pigs.

Biodegradable and biocompatible polyanhydrides have been widely used for vaccine antigen delivery due to safety [9–11] and their adjuvant properties [12]. The most well-studied polyanhydride copolymers are based on sebacic acid (SA), 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxatane (CPTEG) monomers. Polyanhydrides are surface eroding polymers, which minimize the exposure of encapsulated antigen to moisture providing a better microenvironment for the encapsulated vaccine antigen(s) [12,13]. Polyanhydride nanoparticles retain the structural and biological activity of released vaccine antigens [14–19] and also have pathogen mimicking properties to activate dendritic cells and enhance innate immune response [19–22]. Recent studies have shown induction of high virus neutralizing antibody titer and enhanced cell-mediated immune responses against IAV in mice vaccinated with a hemagglutinin-based polyanhydride nanovaccine [23,24]. In this study, we analyzed the immunogenicity and protective efficacy of 20:80 CPTEG:CPH nanoparticles encapsulating whole inactivated SwIAV vaccine against a heterologous and virulent zoonotic SwIAV H1N1 challenge in pigs. Our results indicated that nanovaccine encapsulation of SwIAV augmented the virus specific cell-mediated immune response and reduced the virus load and fever in pigs.

2. Materials and methods

2.1. Vaccine preparation

The SwIAV isolates, SW/OH/FAH10-1/10 H1N2 a δ lineage virus bearing human like HA and NA genes, swine triple reassortant virus internal genes PB2, PB1, PA and NS and pandemic H1N1 lineage NP and M genes [25], and SW/OH/24366/2007 H1N1 a triple reassortant γ lineage virus having swine origin HA, NA, NP, M and NS genes, human origin PB1 and avian origin PB2 and PA genes [26] were used in vaccine preparation and challenge infection, respectively.

For vaccine preparation, Madin-Darby canine kidney (MDCK) cell grown H1N2 virus culture fluid was concentrated by sucrose gradient ultra-centrifugation, and the virus was inactivated by binary ethyleneimine (BEI). Inactivated/killed SwIAV (KAg) was encapsulated in 20:80 CPTEG:CPH polyanhydride nanoparticles (KAg nanovaccine) as described previously [27,28]. Particle size and morphology were examined by a FEI Quanta 250 scanning electron microscope (SEM, Kyoto, Japan) and size distribution was characterized by using ImageJ software with an average of 200 nanoparticles and with quasi-elastic light scattering experiments (QELS) using a Zetasizer Nano (Malvern Instruments Ltd., Worcester, UK). The SwIAV encapsulation efficiency in the nanoparticles was determined as described previously [14].

2.2. Experimental design and sample collection

Caesarian-delivered colostrum-deprived (CD) and bovine colostrum-fed Large White-Duroc crossbred piglets ($n = 30$) were raised in the BSL2 facility at OARDC as described previously [29]. Piglets were confirmed seronegative for hemagglutination inhibition (HI) antibodies against SwIAV H1N1 and H1N2, and were randomly divided into 4 experimental groups ($n = 7$ or 8 pigs/group) (Table 1). Maintenance of pigs and all experimental procedures were conducted in accordance with the guidelines of the

Institutional Animal Care and Use Committee at The Ohio State University.

Animals were vaccinated at 4–5 weeks, boosted after 3 weeks, and challenged after 2 weeks of boost i.e., day post-vaccination (DPV) 35. For each vaccination dose, pigs intranasally received 10^7 TCID₅₀ equivalent of inactivated H1N2 virions (KAg) or KAg entrapped within the nanoparticles (KAg nanovaccine) suspended in 2 mL DMEM. The challenge SwIAV inoculum consisted of a virulent, zoonotic, and heterologous SwIAV H1N1 (6×10^6 TCID₅₀ in 2 mL) of which 1 mL was administered intranasally and 1 mL intratracheally [26]. Plasma samples were collected at the time of vaccination and necropsy. After challenge the rectal temperature was recorded daily, and nasal swab samples were collected in 2 mL of DMEM at four days post-challenge (DPC). Pigs were euthanized at six DPC and lungs were examined and scored for gross lesions [30]. Broncho-alveolar lavage (BAL) fluid was collected for virus titration and lung lysate (prepared using 1 g of tissue from the right apical lobe suspended in 3 mL of DMEM, which was homogenized and the supernatant was collected) was analyzed for antibody response [31]. Lung tissues were formalin fixed for histopathological and immunohistochemical evaluations. PBMCs were isolated by using density gradient medium Lymphoprep in SepMate-50 tubes (Stemcell, BC, Canada) as per the manufacturer's instructions at DPC 0 and 6 for lymphocyte proliferation and flow cytometry assays.

2.3. Cell proliferation and flow cytometry assays

SwIAV antigen-specific lymphocyte proliferation was assessed using PBMCs and the cell titer 96 aqueous non-radioactive proliferation assay kit (Promega, WI) as per the manufacturer's instructions. One million cells per well were seeded in triplicates in 96 well sterile U-bottom plate (Greiner bio-one, NC) and stimulated with live SwIAV H1N2 at 0.1 MOI or with medium control. After 72 h of incubation at 37 °C in a 5% CO₂ incubator, MTS + PMS solution was added and the OD_{490nm} was measured after 4 h using an ELISA plate reader (Spectramax Plus384, Molecular Devices, CA). Stimulation index (SI) was determined by dividing OD of stimulated PBMCs by OD of cell control of the same pig.

PBMCs isolated at DPC 0 were also analyzed to determine the frequency of different T cell subsets by flow cytometry. At DPC 6, isolated PBMCs were stimulated with live SwIAV H1N2 or H1N1 at MOI 0.1 for 72 h, and cells were immunostained and analyzed by flow cytometry to determine the frequency of activated (IFN- γ^+) T cell subsets as described previously [29]. Antibodies used in the assay were anti-porcine CD3 ϵ , CD4 α and CD8 α (Southern-biotech, AL), CD8 β , δ -chain, and IFN γ (BD biosciences, CA) along with their respective isotype controls.

2.4. Virus titration

Serial tenfold dilutions of BAL fluid and nasal swabs were prepared in DMEM supplemented with TPCK-trypsin (1 μ g/mL) and transferred to MDCK cells grown on 96 well cell culture plates. After 48 h of incubation at 37 °C in a 5% CO₂ incubator, cells were immunostained using IAV nucleoprotein specific primary antibody (#M058, CalBioReagents, CA) followed by AlexaFluor 488 conjugated goat anti-mouse IgG (H + L) antibody (Life technologies, OR). Immunofluorescence was recorded using fluorescent microscope (Olympus, NY) and infectious virus titer was calculated using the Reed and Muench method [29,32].

2.5. Antibody titration

Hemagglutination inhibition (HI) titer and SwIAV-specific antibody responses were determined as described previously [29].

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