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Intradermal immunization with inactivated swine influenza virus and adjuvant polydi(sodium carboxylatoethylphenoxy)phosphazene (PCEP) induced humoral and cell-mediated immunity and reduced lung viral titres in pigs

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ABSTRACT

Swine influenza virus is endemic worldwide and it is responsible for significant economic losses to the swine industry. A vaccine that stimulates a rapid and long-lasting protective immune response to prevent this infection is highly sought. Poly[di(sodium carboxylatoethylphenoxy)-phosphazene (PCEP) has demonstrated adjuvant activity when formulated as part of multiple vaccines in mice and pigs. In this study we examined the magnitude and type of immune response induced in pigs vaccinated via the intramuscular or intradermal routes with inactivated swine influenza virus (SIV) H1N1 vaccine formulated with PCEP. Intradermal administration of PCEP-adjuvanted inactivated SIV vaccine stimulated significant anti-SIV antibody titres, increased neutralizing antibodies, and significantly reduced lung virus load with limited reduction of gross lung lesions after challenge with virulent H1N1 relative to control animals. These results indicate that PCEP may be effective as a vaccine adjuvant against swine influenza viruses in pigs and should be considered a potential candidate adjuvant for future swine intradermal influenza vaccines.

1. Introduction

Swine influenza A virus (SIV) causes influenza, a highly contagious acute respiratory disease of pigs responsible for significant economic losses to the swine industry. In addition, SIV infections are a threat to public health since transmission from pigs to humans can occur. There has been an increase in genetic diversity of swine influenza A virus (SIV) in North America over the last two decades but the majority of the SIV infections in pigs are caused by subtypes H1N1, H1N2 and H3N2 [1]. The most cost-effective biomedical approach to control SIV infection is through effective vaccination of pigs with current vaccines comprised of inactivated H3N2 and H1N1 [2]. Reformulation of the vaccine to include a potent adjuvant may improve the efficacy of existing SIV vaccines.

Adjuvants are routinely included in vaccines comprised of inactivated virus and/or subunit vaccines to augment the magnitude and quality of immune responses and by enhancing onset and extending duration of immunity. Polyphosphazene adjuvants are high-molecular weight, water-soluble polymers that, when co-administered with many viral and bacterial antigens, have been shown to enhance long-lasting immune responses [3–6] and robust Th1/Th2 type, broad spectrum immune responses in mice [7] and are potent adjuvants for intradermal immunization in pigs [8,9]. PCEP regulates a number of "immune response genes" when administered intradermally and may promote a Th-2 type immune response [10].

In this study, we evaluate the immunogenicity and protective efficacy of PCEP-adjuvanted inactivated SIV vaccine in pigs. We show that PCEP may be effective as a vaccine adjuvant capable of generating neutralizing antibodies against swine influenza viruses in pigs as well as reduce viral load after challenge and should be considered as a a candidate for future intradermal swine influenza vaccines.

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2. Materials and methods

2.1. Swine influenza virus adsorption, purification and inactivation

Confluent Madin-Darby canine kidney (MDCK) cells (T-75 flasks) were inoculated with a Saskatchewan isolate influenza swine H1N1 virus (A/SW/SK 02 H1N1 (H1N1)) at an M.O.I. of 0.001 for 1 h with minimal essential medium (MEM) (Sigma-Aldrich, Oakville, ON, CAN), which was then supplemented with 0.2% BSA (Sigma-Aldrich, Oakville, ON, CAN), 1 μg/mL TPCK-trypsin (MJSBioLynx, Brockville, ON, CAN) and 50 μg/mL gentamicin. Cultures were incubated in a humidified 5% CO_2 atmosphere at 37 °C for 3 days or until 90% of cytopathic effect (CPE) was observed. Viral media were collected and centrifuged at 216*g* for 10 min at 14 °C then ultracentrifuged at 112,700*g* for 2.5 h at 4 °C. Viral pellet was resuspended in TSE buffer, pH 7.4 (20 mM Tris, 2 mM EDTA & 150 mM NaCl) and then layered onto a 30%/60% sucrose gradient followed by centrifugation at 107,170*g* for 2.5 h at 4 °C. An 18 gauge cannula was used to collect the virus (white band) at the interface and later subjected to a TSE wash at 210,053*g* for 1.5 h at 4 °C. Virus was inactivated with 10% formalin at a final concentration of 0.1% and incubated at 37 °C with constant nutation for 48 h. To test and confirm inactivation, an aliquot of the inactivated virus or saline mock controls were diluted at 10^2, 10^3 10^4 in 10% formalin. Virus inactivation was confirmed by the inability of the viruses to replicate in MDCK cells as observed by negligible CPE.

2.2. Adjuvant and vaccine preparation

PCEP was synthesized using methods previously described in [7,11] and dissolved in endotoxin-free, sterile phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, Carlsbad, CA, USA). Inactivated virus vaccines consisted of 128, 256 and 512 Haemaglutination units (HAU) ± 500 μg PCEP diluted with PBS, pH of 7.4. The dose of PCEP was chosen based on the previous experiments in pigs [9,10].

2.3. Immunization and SIV virus challenge experimental design

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and was approved by the Animal Care Committee of the University of Saskatchewan. For all trials, 3–4 weeks old Landrace cross pigs were immunized on day 0 and a booster vaccination was given at day 21. Sera were collected in SST tubes at 0, 21, 28, 35 and/or 40 days post vaccination. Challenged pigs were anaesthetised with 5% isoflurane and anaesthesia maintained using 2% isofluorane then an endotracheal tube was inserted to about 2/3 of the trachea length just above the bifurcation. A solid ended catheter was the inserted through the endotracheal tube to ensure the challenge was aerosolised and the virus was deposited. Animals were euthanized 5 days post-infection using Euthanyl (25 mg sodium pentobarbital) administered intravenously (IV).

2.4. Necropsy and macroscopic examination of the lungs

Each lung lobe was visualised on the dorsal surface and scored individually by the percentage of the lungs affected with purple-red, firm lesions typical of SIV infection [12]. The percentage of area affected by pneumonia was estimated visually for each lung lobe and the total percentage was calculated based on weight proportions of each lung lobe to the total lung volume as described by [13]. Tissue from the right apical, cardiac and caudal lobes were taken for virus isolation and histopathological examination.

2.5. Virus isolation and titration

Virus titers in lung tissues were determined as detailed in "The processing clinical material for virus isolation" in accordance with [14]. Briefly, lung tissue (500 μg) was homogenized using 2.4 mm zirconia beads with a BioSpec mini-beadbeater-1 at 4800 oscillations/min using a 10 s burst, followed by 2 min on ice, with a repeated 10 s burst in MEM. The slurry was centrifuged at 10,000*g* at 4 °C for 10 min then the supernatant was serially diluted and inoculated onto MDCK cells grown in 96-well flat bottom culture plate. $TC_{ID}50$ titres were calculated according to the Spearman and Kärber algorithm [15].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Serum SIV H1N1 antibodies were determined as described by Dar et al. [9] with the following changes: (1) Purified SIV H1N1 was inactivated by being diluted to 0.5 μg/mL in 0.5 M bicarbonate buffer. It was then applied to Immulon® 2 96U plates (Thermo Lab systems #3655) for detection of IgG (H + L) only, and (2) serial (4×) diluted test serum was added to each well.

2.6.1. Porcine IFN-γ & IL-17A enzyme-linked immunospot (ELISPOT) assay

Single cell suspension of prescapular lymph node cell and IFN-γ ELISPOT assay were performed as described in Masic et al. [16] with the following changes: (1) Lymph node cells were seeded at 5×10^5 cells per well, (2) cells were stimulated with Influenza A/SW/SK 02 H1N1 for 8–16 h, (3) Streptavidin Alkaline Phosphatase (SAP) (Jackson- 016–050-084) was used at a 1:500 dilution and (4) spots were counted on AID ELISPOT reader ELR07 (Strassberg, Germany). IL-17A ELISPOT assay was perform as above with the following changes: (1) Coated plates with capture antibody Rabbit Anti-Porcine-IL-17A (Cedarlane- KP0498S-1000), (2) cells were stimulated for 36 h, (3) detection antibody was Rabbit anti-porcine-IL-17A biotinylated (Cedarlane- KPB0499S-050; 0.2 μg/well) for 2 h.

2.7. Swine influenza virus neutralization assay

Virus neutralization assay was performed as described in "WHO manual on animal influenza diagnosis and surveillance" [24] using the Spearman and Kärber algorithm [17] to calculate $TC_{ID}50$ neutralizing antibody virus titres rather than the Reed–Muench method.

2.8. Assessment of intradermal injection site reaction to vaccination

Skin punch biopsy tissues were prepared for histopathological evaluations as follows: Fixation of tissues with 10% formalin; Drying of tissues by incubation of tissues with 70–100% ethanol and 4% xylol to clear the tissues; Preparing paraffin block of the tissues, preparing tissue slices with 3–5 μm thickness, Staining using standard Haematoxylin-eosin method as described by [17]. The histopathological changes of the skin included were granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation which were scored as follows using light microscope by a pathologist; who was unaware of groups' allocation as follows: (A) No pathological changes = 0, (B) Patchy pathological changes = 1, (C) Moderate pathological changes = 2, (D) Severe pathological changes = 3 described in details by [18].

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