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Biodegradable nanoparticle delivery of inactivated swine influenza virus vaccine provides heterologous cell-mediated immune response in pigs

Santosh Dhakal [a,b], Jagadish Hiremath [a,b], Kathryn Bondra [a,b], Yashavanth S Lakshmanappa [a,b], Duan-Liang Shyu [a,b], Kang Ouyang [a,b], Kyung-il Kang [a,b], Basavaraj Binjawadagi [a,b], Jonathan Goodman [c], Kairat Tabynov [d], Steven Krakowka [e], Balaji Narasimhan [c], Chang Won Lee [a,b], Gourapura J. Renukaradhya [a,b,*]

[a] Food Animal Health Research Program, Ohio Agricultural Research and Development Center, 1680 Madison Avenue, Wooster, OH 44691, USA
[b] Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, USA
[c] Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA
[d] The Research Institute for Biological Safety Problems (RIBSP), Zhambylskaya Oblast, Gvardeiskiy 080409, Kazakhstan
[e] The Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, OH, USA

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ABSTRACT

Swine influenza virus (SwIV) is one of the important zoonotic pathogens. Current flu vaccines have failed to provide cross-protection against evolving viruses in the field. Poly(lactic-*co*-glycolic acid) (PLGA) is a biodegradable FDA approved polymer and widely used in drug and vaccine delivery. In this study, inactivated SwIV H1N2 antigens (KAg) encapsulated in PLGA nanoparticles (PLGA-KAg) were prepared, which were spherical in shape with 200 to 300 nm diameter, and induced maturation of antigen presenting cells *in vitro*. Pigs vaccinated twice with PLGA-KAg via intranasal route showed increased antigen specific lymphocyte proliferation and enhanced the frequency of T-helper/memory and cytotoxic T cells (CTLs) in peripheral blood mononuclear cells (PBMCs). In PLGA-KAg vaccinated and heterologous SwIV H1N1 challenged pigs, clinical flu symptoms were absent, while the control pigs had fever for four days. Grossly and microscopically, reduced lung pathology and viral antigenic mass in the lung sections with clearance of infectious challenge virus in most of the PLGA-KAg vaccinated pig lung airways were observed. Immunologically, PLGA-KAg vaccine irrespective of not significantly boosting the mucosal antibody response, it augmented the frequency of IFN-γ secreting total T cells, T-helper and CTLs against both H1N2 and H1N1 SwIV. In summary, inactivated influenza virus delivered through PLGA-NPs reduced the clinical disease and induced cross-protective cell-mediated immune response in a pig model. Our data confirmed the utility of a pig model for intranasal particulate flu vaccine delivery platform to control flu in humans.

1. Introduction

Swine influenza is an acute respiratory infection of pigs caused by influenza A virus (IAV) of *Orthomyxoviridae* family. At present H1N1, H1N2 and H3N2 subtypes of IAV cause majority of infection in pigs. Owing to the presence of both avian (α2,3 Gal) and human (α2,6 Gal) IAV receptors, pigs can potentially act as mixing vessel for different IAV [1,2]. Acute clinical signs in influenza infected pigs include high fever, anorexia, respiratory distress, nasal discharge and coughing. Therefore, influenza in pigs causes significant economic loss to the porcine industry through morbidity, loss of body weight gain, increased time to market, susceptibility to secondary bacterial and viral infections like mycoplasma and porcine reproductive and respiratory syndrome (PRRS), medication and veterinary expenses [3,4]. Some of the swine influenza viruses (SwIV) can also be transmitted from pigs to humans (zoonotic) creating public health risk. For example, the 2009 pandemic H1N1 swine influenza virus infected approximately 20% of the global population and caused around 200,000 deaths [5–9], in addition to approximately 500,000 deaths due to seasonal annual influenza infection [10,11].

Vaccination is one of the most effective means of controlling influenza, and swine influenza vaccines are commercially available to use in pigs. Due to high mutation rates in circulating influenza viruses in animals the efficacy of commercial vaccines in the field is always poor [12,13]. Commercial multivalent vaccines coadministered with an adjuvant intramuscularly as prime-boost strategy provide homologous, but weak heterologous protection. Intramuscular vaccination does not induce the required levels of local mucosal antibody and cell-mediated immune responses; moreover, there are reports of inactivated vaccine

* Corresponding author at: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA.
E-mail address: gourapura.1@osu.edu (G.J. Renukaradhya).

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associated enhanced respiratory disease [14–16]. Thus, persistent economic burden of swine influenza in pig industry and its potential risk of zoonotic transmission to humans warrants the development of broadly cross-protective vaccine platforms.

Biodegradable and biocompatible polymer, poly(lactic-*co*-glycolic acid) (PLGA), based nanoparticles (PLGA-NPs) are being widely used for controlled vaccine and drug delivery [17–19]. PLGA has been approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) for use in humans (including children) as a vehicle. PLGA-NPs encapsulated vaccine antigens are preserved inside the particles from degradation for a long period of time (4–8 weeks) under physiological conditions, which is critical when vaccine is delivered to mucosal sites; while the benefit of slow release of the cargo *in vivo* when administered by parenteral route helps in prolonged immune activation [20]. Moreover, PLGA-NPs assist in internalization of the antigen by professional antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages (Mϕs) and B cells. PLGA-NPs also facilitate antigen processing and presentation by APCs to naive lymphocytes [21]. PLGA-NPs of size up to 500 nm are readily uptaken by APCs and induce production of virus neutralizing antibodies as well as cell-mediated immune response in mice models [22–24]. Antigens encapsulated in PLGA-NPs and delivered by intranasal route are protected from proteolytic degradation at mucosal surfaces and readily uptaken by immune cells at the mucosal sites of the respiratory tract, and thus have the potential of inducing strong mucosal immune response. Thus, particulate delivery of inactivated influenza vaccine can be a better alternative over existing parenteral vaccine delivery platforms to effectively control influenza [25].

A previous vaccine trial carried out in our laboratory has shown that PLGA-NPs encapsulated porcine reproductive and respiratory syndrome virus (PRRSV) coadministered intranasally with a potent adjuvant significantly reduced challenge heterologous virus induced lung pathology, virus load and protected pigs against the disease [26]. Recently, we found that PLGA-NPs encapsulated highly conserved total of five H1N1 IAV T and B cell peptides cocktail administered intranasally without an adjuvant elicited peptide specific T cell response, but not the antibody response, and still helped in the clearance of a heterologous challenge virus from the lungs of pigs [27]. These findings suggested that the whole inactivated influenza viral antigens delivered in PLGA-NPs vehicle will have the potential to further enhance the breadth of immunity and protection against influenza in pigs. Therefore, in this study we prepared and evaluated the immunogenicity and efficacy of PLGA-NPs encapsulated inactivated SwIV (PLGA-KAg) vaccine in a heterologous virus challenge trial in pigs. Our results suggested that intranasal administration of PLGA-KAg vaccine induced strong T cell response against both homologous and heterologous viruses detected at both pre-challenge as well as post-challenge, and substantially reduced the heterologous challenge virus induced clinical disease, lung pathology and virus load in the lungs.

2. Materials and methods

2.1. Cells and viruses

A stable mycoplasma-free Madin-Darby canine kidney epithelial cells (MDCK, CRL-2285, ATCC, VA) were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic-antimycotic (Gibco) at 37 °C in 5% CO_2 incubator. Field isolates of swine influenza virus (SwIV), SW/OH/FAH10-1/10 (H1N2-δ1 lineage) [28] and SW/OH/24366/2007 (H1N1-γ) [29] were used in inactivated virus vaccine preparation and challenge infection of pigs, respectively. The H1N2 virus (SW/OH/FAH10-1/10) has NP and M genes derived from the 2009 pandemic H1N1 [28], and the A/swine/Ohio/24366/07 was a zoonotic virus isolated from swine and also was shown at the CDC to have 100% identical genome sequence to the human virus associated in the Ohio county fair [29]. SwIV stocks (passage 3) were obtained from the repository at FAHRP, Wooster, Ohio. Both viruses were propagated on MDCK cells by infecting at MOI 0.005 and maintaining in serum free DMEM supplemented with 1 μg/ml TPCK-trypsin (Sigma, MO).

2.2. Vaccine preparation

SwIV isolate SW/OH/FAH10-1/10 (H1N2-δ1) culture fluid was harvested and clarified to remove cell debris by centrifugation at 2000 ×*g* for 30 min and subjected to 10-fold concentration using Pellicon-2 cassette filtration (Millipore, MA) followed by ultra-centrifugation using Optima™ L-100XP ultracentrifuge (Beckman Coulter) with 20% sucrose cushion at 107,000 ×*g* (25,000 RPM) for 4 h without break. Virus pellet was suspended in PBS containing protease inhibitor (Sigma, MO), titrated and stored at −80 °C. Virus was inactivated using binary ethyleneimine (BEI) (Sigma, MO) by treating with 10 mM BEI for 6 h at 37 °C followed by treatment with 10 mM sodium thiosulphate (Sigma, MO) for additional 2 h at 37 °C to neutralize the unused BEI, and the virus inactivation was confirmed in MDCK cells. Total protein concentration in the virus pellet was estimated using micro BCA protein assay kit (Thermo Scientific, MA) as per the manufacturer's protocol.

Inactivated SwIV antigen (KAg) was encapsulated in PLGA-NPs by water/oil/water double emulsion solvent evaporation technique as described previously [26,27]. Briefly, 5 mg of KAg in 500 μl PBS and 250 μl of 2% (*w*/*v*) polyvinyl alcohol (PVA) with protein stabilizers, 50 μl of 20% sucrose (*w*/*v*) and 50 μl of 20% Mg(OH)2 (*w*/*v*), were emulsified in 180 mg of PLGA polymer solution in 4.5 ml of dichloromethane using high intensity ultrasonic processor (Sonics and Materials Inc., CT) for 30 s at duty cycle 30% and output control 3. The resulting water-in-oil (w/o) primary emulsion was poured into a mixture of 23 ml 2% *w*/*v* PVA (Sigma) and 2 ml 12.5% (*w*/*v*) polaxmer 188 (Sigma, MO) to form an aqueous solution. The mixture was divided equally into two tubes and emulsified again by sonication for 60 s to obtain secondary w/o/w emulsion, and it was emulsified by magnetic stirring overnight at 400 rpm in cold (4 °C) to allow evaporation of the organic solvents. Resulting polymeric particles were washed thrice using cold sterile Milli-Q water by centrifugation at 10,976 ×*g* (10,000 RPM) (Beckman Coulter, FX6100 rotor) for 30 min. Finally, PLGA-NP pellet was suspended in 5% sucrose in milli Q water, frozen at −80 °C for 30 min, freeze-dried (Labconco, MO) for 18–20 h and aliquots were stored at −20 °C. The inactivated KAg encapsulated in PLGA-NP is henceforth called as PLGA-KAg.

2.3. Characterization of PLGA-KAg

Particle size and morphology was examined by a FEI Quanta 250 scanning electron microscope (SEM, Kyoto, Japan) after coating with 2 nm of iridium using a Quorum Q150TS sputter coater (Lewes, UK). Nanoparticle size distribution was characterized using ImageJ software (National Institutes of Health, MD) with an average of 200 nanoparticles per image. Quasi-elastic light scattering experiments (QELS) were used to measure the ζ-potential of the nanoparticles using a Zetasizer Nano (Malvern Instruments Ltd., Worchester, UK). NPs 100 μg were suspended in cold nanopure water and thoroughly dispersed using a probe sonicator (Ultra Sonic Processor VC 130 PB, Sonics Vibra Cell, CT) before analysis. Three independent measurements were taken in order to get an average ζ-potential value. Protein encapsulation efficiency and *in vitro* protein release profile in PLGA-KAg at days 0,1, 3, 5, 7, 10, 15, 20, 25 and 30 were estimated and expressed as the cumulative percentage release of SwIV antigens at each time point using the methods described previously [26,27].

2.4. In vitro activation of APCs by PLGA-KAg

Monocyte derived dendritic cells (MoDCs) and alveolar macrophages were used as APCs for *in vitro* activation study. To generate