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Research Article

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Nanoparticle Vaccine for Avian Influenza Virus: A Challenge Study against Highly Pathogenic H5N2 Subtype

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Abstract

We used a platform of self-assembling protein nanoparticles (SAPNs) to develop a self-adjuvanted vaccine for avian influenza virus (AIV). A SAPNs named SA-MC-Penn was designed to display the ectodomain of influenza matrix protein 2 (M2e) as a tetramer and Helix C (Hel C) of hemagglutinin as a trimer. Flagellin domains expressed within a peptide chain were co-assembled into the SAPN core serving as a built-in adjuvant. SA-MC-Penn was administered every two weeks for three times via the intramuscular (IM) or eye drop (ED) routes in chickens starting at four-weeks of age. Sera were collected from immunized chickens at twelve-weeks of age prior to challenge with a highly pathogenic (HP) H5N2 strain of AIV. ELISA results demonstrated that high titers of serum antibodies were induced in the IM immunized SAPNs. Upon challenge with HP AIV, 63 % and 30% of chickens the SAPNs IM and SAPN ED groups, respectively, survived. A reduction in challenge virus shedding from oropharyngeal swabbings of the SAPNs IM vaccinates was observed on days two and 14-post challenge. These results show the SAPNs vaccine prototype was partially protective against HP AIV.

Keywords

Avian Influenza Virus; Vaccine; Nanoparticle; Flagellin; Adjuvant; SAPNs; Chicken

Introduction

Avian influenza (AI) can be a devastating disease of poultry. Low pathogenic viruses can cause little or no observable disease while highly pathogenic (HP) viruses can induce high mortality, resulting in massive economic losses for the poultry industry due to loss of birds, trade restrictions and disease control and response efforts. The 2014-2015 US HP outbreak cost producers and the US government an estimated 3.3 billion US dollars [1]. In previously rare cases, strains may pose a public health concern [2]. Outside of the US, vaccination has been shown to be an effective measure of controlling AI [3]. However, designing an effective vaccine against AI is challenging due to large number of subtypes, the highly mutable genome of the avian influenza virus (AIV), and the reassortment of genes among different strains [4]. These issues could easily cause mismatch problem between the antigen within the vaccine and circulating field strains, resulting in suboptimal vaccine efficacy [5]. To solve the problem, one practical strategy is to develop a universal vaccine comprised of multiple conserved epitopes against a wide range of AIV strains [4,6].

In recent years, designing novel biodegradable nanomaterials with well-defined structures that modulate immune responses has attracted substantial attention in vaccine development [7]. To improve drug delivery, nanosized lipid or polymer-based particle, liposomes, virosomes and virus-like particles (VLPs) have shown the ability to induce a robust immune response [8-11]. Self-assembling protein nanoparticles (SAPNs) are built by monomeric protein chains composed of coiled-coil oligomerization domains, in which the polymerization of peptide monomers is dictated by the intrinsic property of coiled-coil domains [12,13]. SAPNs are a biodegradable biomaterial with mechanical and chemical stability, which are well suited to repetitively present multiple antigenic epitopes linked to the N or C terminus of the coiled-coil domains, particularly in a conformation-specific manner [12]. SAPNs have the capability of eliciting long lasting antibody and cellular immune responses against *a parasite from the genus Plasmodium*, the causative parasite of malaria [14], but also are able to flexibly carry a built-in protein immune modulator as adjuvant [15]. The potential to induce a long lasting humoral and cellular immune response while displaying self-adjuvanted properties makes SAPNs a powerful platform in designing a universal AI vaccine.

In our previous studies, we have developed two versions of vaccine prototypes for avian influenza based on the SAPNs technology. The first SAPNs version was constructed by co-expressing nanoparticle core sequence and M2e linked to the N-terminus of a tetrameric coiled-coil domain[16], in which M2e is a conserved transmembrane protein across avian and mammal influenza viruses[17]. The SAPNs repetitively displayed M2e on the surface in its native tetrameric conformation state, showing its efficacy in reducing virus shedding in chickens co-administered of the SAPN with Freund's adjuvant after challenge with a low pathogenic avian influenza virus (A/Turkey/CA/D0208651-C/02 H5N2) [16]. To further improve the immunogenicity of the first version of SAPNs [17], we have added our second version SAPNs named SA-MC-Penn with the conserved epitope Hel C from the stalk region of hemagglutinin (HA), an epitope that has been demonstrated to induce broad neutralizing antibodies [18]. In addition, to make SA-MC-Penn self-adjuvanted, we added conserved D0 and D1domains of flagellin molecule from *Salmonella enterica serovar Typhimurium* at the C terminus [15]. Flagellin, a well-characterized molecular adjuvant, has been shown to improve the immunogenicity of vaccines [19,20]. In our SAPNs, the displayed flagellin domains interact with toll-like receptor 5 on the membrane of antigen presenting cells, interconnecting innate immunity and adaptive immunity, thus enhancing the presentation of vaccine immunogens [21]. We have shown our second iteration of design SA-MC-Penn induced high level of cross-neutralizing antibodies in immunized chickens [15]. Moreover, upon immunization of mice with the SAPNs based vaccine, namely SA-MC-PR8 presenting the H1N1 B cell epitopes, mice were completely protected from lethal challenge with H1N1 (A/Human/Puerto Rico/8/1934) [15]. However, the efficacy of SA-MC-Penn to protect chickens from a HP

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AIV challenge has yet to have been determined. Thus, this study was performed to evaluate the efficacy of SA-MC-Penn SAPNs vaccinated chickens to be protected from an H5N2 HP AIV (A/chicken/Pennsylvania/1/1983) challenge.

Materials and Methods

Synthesis and characterization of vaccine prototype SA-MC-Penn nanoparticles

The synthesis of SA-MC-Penn was according to the description of our previous work [15]. Briefly, two fusion genes encoding peptides as building blocks were synthesized and sub-cloned into two plasmids. One gene contained 6X histag at the 5' end followed by an AIV M2e epitope sequence (residues2-23), a tetrameric coiled-coil sequence, a trimeric coiled-coil sequence with universal CD4+ T cell epitope, and an AIV Helix C epitope at the 3' end (the HA stalk region, residues 165-197). It encoded monomeric peptide M2e/Helix C. The other gene encoded monomeric peptide chain so-called flagellin monomer. It contained additional sequences that were derived from the D0 and D1 domains (residues 1-177 and 249-372) of flagellin from *Salmonella enterica* serovar Typhimurium at the 3' end. Genes encoding M2e/Helix C and flagellin monomer were expressed in *E.coli*. The purified gene products were confirmed by SDS-PAGE analysis and were co-assembled into the SA-MC-Penn SAPNs at a molar ratio of 58:2 for M2e/Helix C and flagellin monomers. The final concentration of flagellin molecules in molar mass in the SA-MC-Penn serving as an adjuvant was 3%. Particle size and shape were characterized by dynamic light scattering and transmission electron microscopy.

Virus, animals and immunization

Duplicate groups of 14 specific pathogen free (SPF) white leghorn chickens at four-weeks of age (Charles River Laboratories, Wilmington, MA) were vaccinated three times with the previously optimized dose (immunogenicity study data not shown) of 100 μg of the SAPN SA-MC-Penn via the intramuscular (IM) or eye drop (ED) routes. For comparative purposes, a commercial oil adjuvanted H5N2 vaccine of 0.5mL (Dodge Fort) was given (IM) three times at the same intervals as a control for the SAPN treatments following the manufacturers' recommendations. Two groups of 14, non-vaccinated chickens were included as negative and challenge controls. Serum samples were collected from all birds at day 32 post vaccination. All birds in one of each treatment group were challenged with the HP AIV strain A/chicken/Pennsylvania/1/1983 (H5N2) by intranasal instillation of 10^4 embryo infectious dose$_{50\%}$ (EID_{50}) and were observed for 14 days. Oropharyngeal (OP) and cloacal swabbing were collected 2, 4, 7, 10 and 14 days-post challenge (PC). All chickens had access to feed and water *ad libitum*. Chickens unable to reach food and/or water were euthanized in accordance with the protocol approved by the University of Delaware Institutional Animal and Care Use Committee Gelb (9) 09-09-11R.

Enzyme-linked immunosorbent assay (ELISA) and quantitative real-time RT-PCR

ELISA assay was performed to evaluate the antibody response. Briefly, chicken sera were serial diluted (2-fold dilutions) and raised against the ELISA plates coated with 1 μg/mL of the SAPN SA-MC-Penn and incubated for one-hour at room temperature (RT). Plates were washed 3X with PBST buffer (1× PBS, 0.05% Tween). Secondary antibody, anti-chicken Ig Y (1: 1000 in1×PBS/3%BSA), was added and incubated for one-hour at RT. Plates were washed 3X with PBST. Plates were developed by the addition of TMB developing solution (Thermo Scientific, USA) for 10 min for color development. Reaction was stopped by adding 50 μL of 0.5 M sulfuric acid. Colorimetric changes in each well were detected at 450 nm using a BioTek microplate reader. A cutoff point for a positive reaction by ELISA test was set the OD value at 450nm of a tested serum 3X times the negative control serum.

RNA extracted from OP swabs was quantified by quantitative real-time RT-PCR using the Qiagen one-step RT-PCR kit according to the protocol described by Spackman et al. [22]. Briefly, the primers and probe were used in the reaction as follows: forward primer-5'ACG TAT GAC TAT CCA CAA TAC TCA G3'; reverse primer-5' AGA CCA GCT ACC ATG ATT GC 3'; probe- FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA. The reaction was conducted with a RT profile setting for 30 min at 50°C and 15 min at 94°C, and a H5-specific PCR for 40 cycles as follows: 94°C for 0 s, 57°C for 20 s, and 72°C for 5 s.

Statistics

Data were analyzed using two-way ANOVA with Bonnferoni post-test in Graphpad Prism 6.0. Statistical significance was achieved at levels of P values<0.05 (*), <0.01 (**), and <0.001 (***).

Results

SA-MC-Penn elicited antibody response against AIV-M2e and Helix C

To evaluate the serum antibody responses, sera harvested at 10 weeks of age, 2 weeks after the third vaccination, were evaluated by ELISA in plates were coated with 1 μg/mL SA-MC-Penn. Chickens immunized IM with 100 μg SA-MC-Penn (Figure 1 red line) developed highest level of detectable antibodies compared to chickens vaccinated with the 0.5mL inactivated commercial vaccine IM route (Figure 1 blue line) or the 100 μg SA-MC-Penn ED route (Figure 1 green line). No antibodies were detected in non-vaccinated chickens (Figure 1 red line). The data showed that the IM route was superior to the ED route of immunization with the SAPNs vaccine, as it elicited higher levels of serum antibodies.

SA-MC-Penn nanoparticle vaccine prototype offered partially protection against HPAIV H5N2 challenge

Sixty percent of non-vaccinated chickens succumbed to infection by day 6 PC (Figure 2). In comparison, chickens immunized with 100 μg SA-MC-Penn via the IM or ED route showed a prolonged period of survival after challenge compared to the challenge control chickens. At 14 days PC, 63% and 30% chickens survived in SA-MC-Penn IM and ED route vaccinated groups, respectively, numerically greater survival rates than the challenge control chickens. The survival chickens vaccinated via the IM route were twice that of the ED route and 3X that of chickens in the challenge control group. However, all chickens that received the commercial inactivated vaccine survived challenge.

SA-MC-Penn reduced viral shedding in immunized chickens

Virus shedding from the respiratory (oral) and digestive tracts were evaluated by performing real time RT-PCR screening of OP and cloacal swabbings, respectively (Figure 3). Compared to non-vaccinated and challenged chickens, OP swabs collected from IM or ED SA-MC-Penn vaccinated chickens showed decreased virus shedding 2, 4 and 14 days PC. Oral virus shedding was generally

both found on the surface of AIV, impact the development of effective vaccines for avian and mammalian species. Traditional vaccines are challenged by their effectiveness when the genetic profile of currently circulating strains of AIV change. More importantly, live attenuated vaccines are not utilized due to the possibility of reversion to virulence. In our previous studies, the SAPNs were successfully used as a delivery system for AIV protein. M2e and Helix C, AIV epitopes, can be repetitively displayed on SAPNs surface as a tetramer and trimer, respectively, which are native conformations in the AIV capsid [15]. Importantly, flagellin was co-assembled as a part of the SAPNs SA-MC-Penn to increase interaction with dendritic cells via TLR 5 on cellular membranes and subsequently improve antigen presentation. Inclusion of flagellin gave the SAPNs the self-adjuvanted properties. Our previous studies demonstrated SAPNs vaccine prototypes induced levels of antibodies, especially cross-reactive antibodies, against AIV and more importantly, provided protection against LPAIV in chickens [15,16].

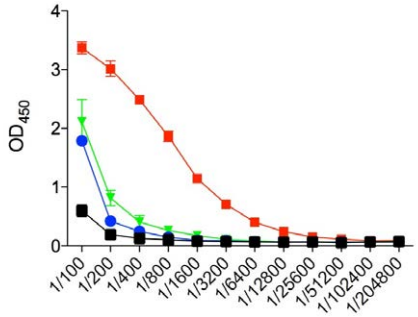

Figure 1: Evaluation of antibody response by ELISA. Antibody detection by ELISA with pooled sera harvest from chickens at two weeks post second boost vaccination; Black line: no vaccine group; Blue line: Chickens group vaccinated with 0.5cc inactivated H5N2 vaccine; Red line: vaccinated chickens with100 µg SA-MC-Penn via IM; Green line: chickens vaccinated with100 µg SA-MC-Penn via ED route

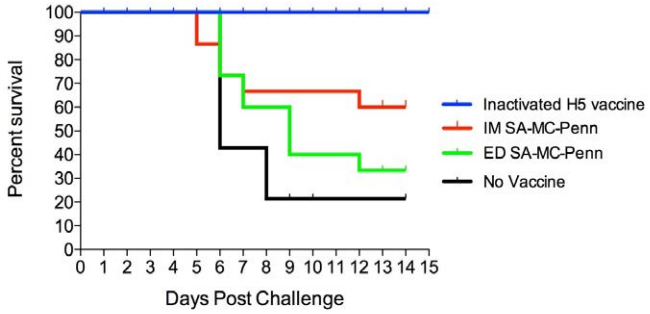

Figure 2: **Survival plot for protection of vaccinated chickens against H5N2 HPAIV subtype challenge**. Chickens survive against HP AI post challenge at 2, 4, 7, 10 and 14 days. Black: survival of unvaccinated chickens. Red: survival of chickens vaccinated IM with100 µg SA-MC-Penn. Green: survival of chickens vaccinated with100 µg SA-MC-Penn via ED route. Blue: survival of vaccinated chickens with 0.5cc inactivated H5N2 vaccine

numerically lower in chickens vaccinated with SA-MC-Penn by the IM route compared to the ED route. A significant ($P<0.05$) reduction of one logarithm was observed in OP swabs collected from chickens vaccinated via the IM route with SA-MC-Penn at 2 days p.c. (Figure 3A). No significant reduction of cloacal virus shedding was observed in chickens vaccinated by either route with SA-MC-Penn (Figure 3B). Chickens receiving the commercial inactivated H5N2 vaccine significantly ($P<0.05$) reduced virus shedding from the respiratory and digestive tracts at all the time points as compared to the no vaccine group and the IM or ED SA-MC-Penn groups.

Discussion

The potential to undergo continuous and rapid evolution of the genes encoding the HA and neuraminidase (NA) envelope proteins,

In this study, we demonstrated SA-MC-Penn partially protected chickens from challenge with an HP H5N2 strain of AIV. Route of administration can directly impact the efficacy of a vaccine [23]. Most currently available vaccines for AIV are inactivated vaccines and are administered parenterally [24]. In contrast, ED route of vaccine administration takes less effort. Moreover, ED administration stimulates the local lymphoid organ called Harderian gland located within the eye sac [25]. It has been proved an effective strategy to administrate inactivated AI vaccine inducing significantly systemic Ig G that protected chickens against HP AI [26]. However, our data showed ED was less antigenic (Figure 1) and protective (Figure 2) than the IM route. One possible reason for the impact

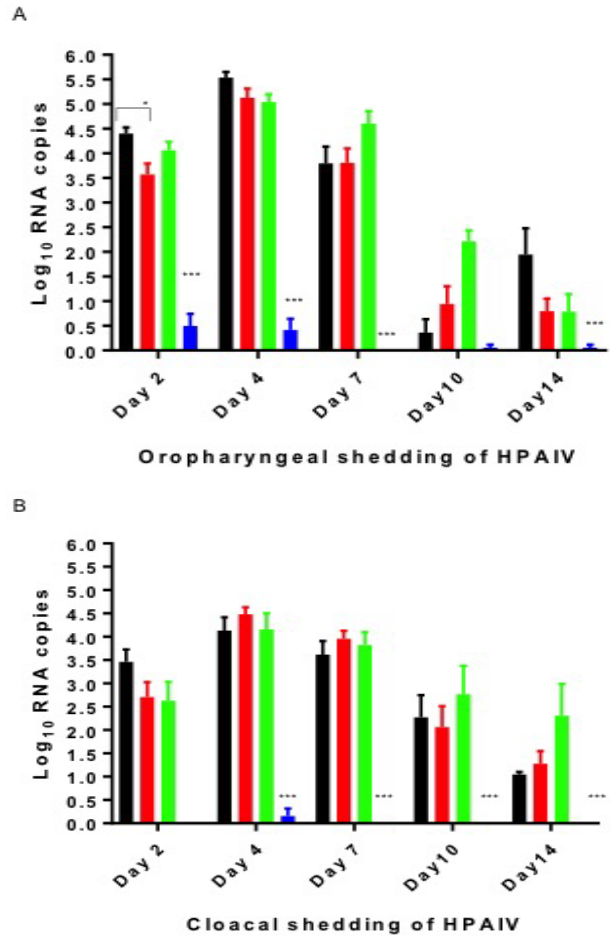

Figure 3: Virus shedding after challenges (A): Oropharengeal virus load and (B): Cloacal virus shedding Evaluation of virus shedding in OP and cloacal swab samples by real time RT-PCR. Black: no vaccine group. Red: Chickens group vaccinated intramuscularly with100 µg SA-MC-Penn. Green: Chickens groups vaccinated with100 µg SA-MC-Penn via eye drop route. Blue: Chickens group vaccinated with 0.5cc inactivated H5N2 vaccine, * was significantly different ($P < 0.05$), ** was significantly different ($P < 0.01$). *** was significantly different ($P< 0.001$)

on the effectiveness of SA-MC-Penn by IM or ED routes of administration could be due to a higher efficiency of vaccine-take of soluble protein antigen SA-MC-Penn via IM route than did ED route at the sites of inoculation. Numerically lower viral shedding from the oral cavity was observed in chickens vaccinated via the IM route vs. the ED route.

Vaccine group treated with whole virus inactivated vaccine with oil-emulsion showed complete protection against HP AIV challenge, and significantly ($P<0.05$) lower OP and cloacal virus shedding as compared to all other treatment group. This finding was not surprising since the whole AIV inactivated vaccine contains all antigenic epitopes capable of inducing a strong immune response. The presence of the virus and adjuvant in the inactivated virus vaccine strongly stimulated humoral immunity and further maximize the adaptive immune responses [24,27]. In comparison, SA-MC-Penn, a viral subunit SAPNs vaccine, achieved 63% livability when it was administered without additional adjuvants. Although the protection less than the whole virus inactivated vaccine, the subunit SAPNs vaccine demonstrated self-adjuvanted characteristics, which was in agreement with the previous malaria SAPNs vaccine prototype [14]. The adjuvant effect might be attributed to hydrophobic coiled-coil domains of protein-based polymers as well as the flagellin domains displayed on the SAPNs [28,29]. Our data also supports the long-standing concept that the application of flagellin as adjuvant in vaccine formulation [19,29].

Results from this study show that further optimization of experimental SAPNs vaccine SA-MC-Penn is required. The data suggest that a higher dose of SA-MC-Penn SPANs may be required to achieve protection similar to the whole virus inactivated vaccine. In addition, cellular immunity is required for the clearance of virus infection [30,31]. Therefore, the SAPNs vaccine may be improved by the addition of conserved T cell epitopes from the AIV nuclear protein. Adding such epitopes may stimulate cellular immunity against AIV thus reducing viral shedding.

To conclude, chickens immunized with the SAPNs SA-MC-Penn prototype vaccine administered without a commercial adjuvant were partially protected from challenge with the H5N2 HPAIV strain A/chicken/Pennsylvania/1/1983.

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Conflict of Interests

PB has an interest in the company Alpha-O Peptides that has patents or patents pending on the technology. The other authors declare no competing interests.

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