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Protective avian influenza *in ovo* vaccination with non-replicating human adenovirus vector

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Abstract

Protective immunity against avian influenza virus was elicited in chickens by single-dose *in ovo* vaccination with a non-replicating human adenovirus vector encoding an H5N9 avian influenza virus hemagglutinin. Vaccinated chickens were protected against both H5N1 (89% hemagglutinin homology; 68% protection) and H5N2 (94% hemagglutinin homology; 100% protection) highly pathogenic avian influenza virus challenges. Mass-administration of this bird flu vaccine can be streamlined with available robotic *in ovo* injectors. In addition, adenovirus-vectored vaccines can be produced rapidly and the safety margin of a non-replicating vector is superior to that of a replicating counterpart. Furthermore, this mode of vaccination is compatible with epidemiological surveys of natural avian influenza virus infections.

Keywords

Avian influenza; In ovo vaccination; Adenovirus vector; Replication-competent adenovirus

1. Introduction

As the H5N1 highly pathogenic avian influenza (HPAI) virus has devastated the poultry industry in multiple countries during recent years with the capacity of inducing high lethality rate after transmission to humans [1], a pandemic of the same severity as that of the 1918, 1957, or 1968 [2] is conceivable. Strategies to control avian influenza (AI) vary from co-existing with low pathogenic AI virus strains to the other extreme of implementing a slaughter approach in the case of HPAI strains. The radical slaughter approach is feasible when outbreaks of disease occur in restricted areas with adequate veterinary infrastructure, but the costs can be insurmountable if outbreaks are widespread. A more cost-effective approach is mass vaccination to both increase resistance of the chicken population and reduce AI virus shedding. To vaccinate birds in great numbers within a short timeframe in response to an outbreak of AI, vaccines have to be produced rapidly and inexpensively, and elicit protective immunity in a single-dose regimen without involving labor-intensive procedures. Immune responses induced

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by vaccination should allow easy differentiation between infected and vaccinated animals (DIVA).

Poultry have been vaccinated against AI with two types of commercially available vaccines including inactivated whole AI virus [3,4] and fowlpox-vectored vaccines [5]. Whole AI virus vaccines have disadvantages including the requirement of live virus replication in embryonated eggs, incompatibility with DIVA when the vaccine strain is identical to the circulating virus, and withdrawal time of as long as 42 days. The distinct advantage of vectored vaccines is the ability to introduce highly specific immune interventions based on well-defined antigens that can be the focus of specific immune reactivity, without the risk of generating virulent revertants. Evidence shows that vaccination of chickens by delivery of a fowlpox vector encoding an avian hemagglutinin (HA) of a specific AI virus is sufficient to protect immunized birds against lethal AI virus challenges. The homology between the HA of the vaccine and that of the challenge virus is inversely correlated with virus shedding in poultry [5]; however, a broad cross-protection within a subtype between even antigenically diverse AI viruses can be achieved among chickens [6]. Although multiple vectors are available as vaccine carriers, bioengineered avian virus-derived vectors (e.g., fowlpox virus) have shown reduced protection associated with pre-existing immunity to the vector due to pre-exposure of birds to the avian virus [7].

Humans have been safely and effectively immunized by administration of human adenovirus serotype 5 (Ad5)-vectored influenza vaccines [8]. It was recently reported that mice [9,10] and chickens [9] could be protected against H5N1 virus challenges following inoculation of this human Ad5 vector encoding an avian H5 HA. All of the aforementioned Ad5 vectors were produced in human 293-derived cells that intrinsically contaminate Ad5 stocks by generating replication-competent adenovirus (RCA) through homologous recombination between the Ad5 vector and the E1 region in the 293 genome [11]. RCA may induce disease in infected humans, and/or out-replicate the recombinant vectors during large-scale production. In the present studies, we demonstrate that chickens can be protected against HPAI by *in ovo* administration of an RCA-free human Ad5 vector encoding avian H5 HA. This vectored AI vaccine possesses no safety risk due to its replication incompetence, and the *in ovo* mode allows automated mass delivery.

2. Materials and methods

2.1. Construction of the AdTW68.H5 vector

The A/turkey/Wisconsin/68 HA gene was amplified by polymerase chain reaction (PCR) from a plasmid template [12] using the primer pair 5' CACACAAAGCTTGCCGCCATGGAAAGAATAGTGATTGC3' and 5' CACACAGGATCCATCTGAACTCACAATCCTAGATGC3'. These primers contain sequences that anneal to the 5' and 3' ends of the HA gene, a eukaryotic ribosomal binding site immediately upstream from the initiation ATG codon, and unique restriction sites for subsequent cloning. The fragment containing the full-length HA gene was inserted into the HindIII-BamHI site of the shuttle plasmid pAdApt (provided by Crucell Holland BV; Leiden, The Netherlands) to generate the plasmid pAdApt-TW68.H5 with the HA gene under transcriptional control of the human cytomegalovirus (CMV) early promoter. An RCA-free, E1/E3-defective Ad5 vector encoding this H5 HA gene (AdTW68.H5) was subsequently constructed in human PER.C6 cells (provided by Crucell) by co-transfection of pAdApt-TW68.H5 with the Ad5 backbone plasmid pAdEasy1 [13] as described [14]. The AdTW68.H5 vector was validated by DNA sequencing. Titer (infectious units [ifu] per ml) was determined by the Adeno-X rapid titer kit (BD Clontech; Mountain View, CA).

2.2. Experimental design

AdTW68.H5-vectored AI vaccine was administered to specific-pathogen-free (SPF) white leghorn chicken embryonated eggs to evaluate antibody responses and protection against challenge as described in the following trials. *In ovo* inoculation was performed as described [15]. Briefly, embryonated eggs were candled for viability followed by disinfection of the egg shell. A small hole was made through the large end (air cell end) with a drill. Vaccines were injected approximately 1 inch deep into the amnion-allantoic cavity with a 21-gauge needle, followed by sealing the hole and continued incubation of the eggs. Machines widely used for *in ovo* immunization deliver vaccines into the same cavity on day 18 of embryonation (E18) [16]. Post-hatch vaccination was performed intranasally. Hemagglutination-inhibition (HI) antibody titers in serum samples were determined as described [17] against 4 hemagglutinating units of the low pathogenic A/turkey/Wisconsin/68 (H5N9) strain. Titers of $<1.0 \log_2$ were arbitrarily assigned a titer of 1.0 $\log_2$. Birds were reared and handled according to Institutional Animal Care and Use Committee's guidelines at Auburn University as well as USDA Southeast Poultry Research Laboratory.

2.3. Trial 1

AdTW68.H5-vectored AI vaccine was delivered *in ovo* to chicken embryos at a dose of 1.5×10^8 ifu in a volume of 0.3 ml on E10 or E18. The Ad5 vector was purified by ultracentrifugation over cesium chloride gradient and resuspended in Ad buffer as described [14]. Approximately 50% of the hatched chickens were boosted by intranasal instillation with the same dose of AdTW68.H5 on day 15 post-hatch (D15); the remaining chickens did not receive a booster application. Serum samples were obtained on D28 for determination of individual HI antibody titers against the A/turkey/Wisconsin/68 virus from all chicken groups.

2.4. Trial 2

We subsequently expanded the experiment to demonstrate reproducibility as well as to evaluate protection against lethal challenge with HPAI virus strains. Nineteen chickens were immunized *in ovo* on E18 as described in Trial 1. Twelve of 19 chickens were intranasally boosted on D15 and the remaining 7 were not revaccinated. Serum samples from each bird on D23 and D29 were tested for HI antibody titers against the A/turkey/Wisconsin/68 virus. Challenge was performed in a biosafety level 3+ facility by oropharyngeal instillation of 1×10^5 embryo infectivity doses (EID₅₀) of the HPAI virus A/chicken/Queretaro/14588-19/95 (H5N2) [18]. The HA (GenBank accession U79448) of this challenge strain has 94% deduced amino acid sequence similarity with the HA (GenBank accession U79456) of the A/turkey/Wisconsin/68 strain (vaccine strain) expressed from the Ad5 vector. A total of 30 chickens, including 7 birds vaccinated *in ovo* and 12 vaccinated *in ovo* in conjunction with nasal boost, as well as 11 unvaccinated controls, were challenged on D34. Challenged birds were observed daily for morbidity and mortality throughout an experimental period of 14 days. Oropharyngeal swabs from individual birds were obtained for quantitation of AI genomes by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) on days 2, 4, and 7 after challenge. Samples were suspended in 1 ml of brain heart infusion medium (Becton Dickinson and Company; Sparks, MD), and stored at -70°C . RNA was extracted by using the RNeasy mini kit (Qiagen Inc.; Valencia, CA). Real-time RT-PCR was performed with primers specific for type A influenza virus matrix RNA as described [19]. Copy number of viral RNA was interpolated from the cycle thresholds by using standard curves generated from known amounts of control A/chicken/Queretaro/14588-19/95 RNA ($10^{1.0}$ to $10^{6.0}$ EID₅₀/ml).

2.5. Trial 3

To determine whether the AdTW68.H5-vectored AI vaccine can confer protection against a recent H5N1 HPAI virus strain, 31 chickens were vaccinated *in ovo* with the AdTW68.H5

vector at a dose of 3×10^8 ifu on E18. Unlike the vectors produced in Trials 1 and 2, the AdTW68.H5 vector used in Trial 3 was purified by Vivapure AdenoPACK ion-exchange membranes (Sartorius Corp., Edgewood, NY) and subsequently resuspended in A195 buffer [20].

Control groups included 10 chickens vaccinated with an Ad5 vector (AdCMV-tetC) encoding an irrelevant antigen (tetanus toxin C-fragment) [14] and 10 chickens which were not exposed to Ad5 vectors. HI antibody titers were analyzed on D25. Control and immunized birds were challenged through the choanal slit with 10^5 EID₅₀ of the HPAI virus A/swan/Mongolia/244L/2005 (H5N1) on D31 (the HA of this challenge strain has 89% deduced HA amino acid sequence similarity with the HA of the A/turkey/Wisconsin/68 strain).

3. Results

3.1. Trial 1

As shown in Fig. 1, chickens vaccinated *in ovo* on E10 showed HI antibody titers of $2.7 \log_2$ (geometric mean titer [GMT]) against the A/turkey/Wisconsin/68 virus on D28; those vaccinated on E10 with nasal boost showed HI titers of $5.2 \log_2$ [GMT]; those vaccinated on E18 showed HI titers of $5.0 \log_2$ [GMT]; and those vaccinated on E18 with nasal boost had HI titers of $5.2 \log_2$ [GMT]. Overall, *in ovo* administration of this human Ad5-vectored AI vaccine induced a robust antibody response against the H5 HA of AI virus in chickens, whereas intranasal instillation of Ad5-vectored vaccines, as recently reported [21], seemed to be ineffective in chickens.

3.2. Trial 2

Overall, the HI antibody titers against the A/turkey/Wisconsin/68 virus in immunized chickens (Fig. 2A) were similar to the values obtained in trial 1 (Fig. 1). Titers in chickens immunized *in ovo* on E18 increased from $6.0 \log_2$ [GMT] on D23 to $7.0 \log_2$ [GMT] on D29. *In ovo* vaccination in conjunction with nasal boost induced HI antibody titers at the level of $5.3 \log_2$ [GMT] on D23, with subsequent increase to $6.1 \log_2$ [GMT] on D29.

Clinical signs of AI, including swelling of comb and wattles, conjunctivitis, anorexia and hypothermia, were observed two days after challenge with A/chicken/Queretaro/14588-19/95 virus in 10 of 11 control birds. After two more days, most survivors in the control group exhibited comb necrosis, swelling of wattles, diarrhea, dehydration, lethargy, and subcutaneous hemorrhages of the leg shank. No signs of AI were observed in any of the vaccinated chickens. All chickens (19/19) vaccinated *in ovo* with or without booster application survived the challenge with the HPAI virus A/chicken/Queretaro/14588-19/95 strain (Fig. 2B).

Copy number of the A/chicken/Queretaro/14588-19/95 matrix RNA in challenged birds was quantitatively determined by real-time RT-PCR in oropharyngeal swabs collected 2, 4, and 7 days post-challenge. There was a significant reduction of viral RNA in all immunized birds 7 days after challenge (Fig. 2C). Absence of detectable viral RNA in vaccinated chickens provides evidence that *in ovo* vaccination elicited an immune response capable of controlling AI virus shedding within a week, and probably sooner since some of the signals may have been amplified from viral remanence by RT-PCR.

3.3. Trial 3

All of the unvaccinated (10/10) and AdCMV-tetC-immunized (10/10) birds died from AI within 9 days after challenge with A/swan/Mongolia/244L/2005 virus and none of them produced measurable HI antibodies, whereas 68% (21/31) of the AdTW68.H5-vaccinated birds survived without clinical signs 10 days post-challenge (Fig. 3A). *In ovo* immunization induced

antibodies within a range of 1 and 6 log₂ on D25 (Fig. 3B). Notably, 7 birds in the immunized group with HI titers of ≥ 3 log₂ were still killed by this highly lethal H5N1 AI virus. It is conceivable that the survival rate against bird flu may be improved by *in ovo* vaccination with an Ad5 vector encoding an HA with antigenicity more matched to that of the challenge virus.

4. Discussion

In ovo vaccination in chickens on E18 has been routinely practiced for many years in the poultry industry. Currently over 80% of U.S. commercial broilers are vaccinated *in ovo* with a mechanized injector against Marek's disease [15,16]. This automated vaccination method minimizes labor, time, and costs by allowing administration of a uniform vaccine dose into hundreds of eggs per minute.

The results presented herein demonstrated that *in ovo* immunization with an RCA-free human Ad5 vector encoding avian H5 HA elicits protective immunity against HPAI viruses in chickens. Ad-vectored *in ovo* AI vaccines can be produced rapidly and mass-administered into chicken populations within the context of a high-standard safety profile in response to an emerging AI pandemic. Large-scale production of RCA-free Ad5 vectors in the well-characterized PER.C6 cell line in serum-free suspension bioreactors [22] in conjunction with chromatography-mediated purification [23], and buffers that do not require freezers for long-term storage [20], should greatly reduce the production costs of Ad5 vectors. Potency of this novel *in ovo* vaccine conceivably may be further enhanced by codon optimization of the HA gene and/or development of *in ovo* adjuvants. The use of cultured cells instead of embryonated eggs as a substrate for AI vaccine production is significant, particularly during an AI outbreak when embryonated eggs may be in short supply. This Ad5-vectored AI vaccine is in compliance with a DIVA strategy because the vector only encodes the viral HA. Thus, analysis of serum samples both by HI assay and another serologic test detecting a conserved viral protein (e.g., nuclear protein of an AI virus [24]) would allow easy discrimination between chickens infected by field AI viruses and those subjected to vaccination.

Although an aerosol AI vaccine may be developed by expressing HA from a Newcastle disease virus vector [25] or a reassortant influenza virus containing a non-pathogenic influenza virus backbone [26], the RCA-free Ad5-vectored *in ovo* AI vaccine provides a unique platform capable of arresting HPAI virus infections in immunized birds through automated delivery of a uniform dose of non-replicating AI vaccine that is compatible with a DIVA strategy. Unlike replicating recombinant vectors that are associated with the risk of generating revertants and allow spread of genetically modified organisms in both target and non-target species in the environment, the RCA-free Ad5 vectors will not propagate in the field. In contrast to the reassortant AI virus vaccine that may generate undesirable further reassortments with a concurrently circulating wild influenza virus [27], it is not possible for the DNA genome of Ad5 to undergo reassortment with the segmented RNA genome of an influenza virus.

Since most domestic chickens are reared for human consumption (broilers) with a short life span (≤ 2 months in many countries including the U.S.), *in ovo* administration of the RCA-free Ad5-vectored AI vaccine, at minimum, should be able to protect the great majority of chickens against AI and significantly reduce human exposure to AI viruses. In addition to chickens and AI, other domestic birds can also be effectively immunized *in ovo* against other pathogens [28]. Introduction of this new class of *in ovo* vaccine may thus provide a simple and safe solution for mass immunization of poultry in a wide variety of disease settings.

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Fig. 1.

Serum HI antibodies induced by *in ovo* immunization with the RCA-free AdTW68.H5 vector encoding the A/turkey/Wisconsin/68 (H5N9) HA. *In ovo* vaccination was performed on E10 and E18, respectively. Approximately 50% of the *in ovo* vaccinated birds were intranasally boosted on D15 whereas the remaining chickens were not revaccinated. E10, chickens immunized on day 10 of embryonation; E10+, chickens immunized on E10 with nasal boost on D15; E18, chickens immunized on day 18 of embryonation; E18+, chickens immunized on E18 with nasal boost on D15. HI titers against the A/turkey/Wisconsin/68 virus were determined on D28 and are expressed as log₂ [HI titer] in individual birds (circles and triangles); bars represent the log₂ [GMT] for each group. No HI titers (≤ 1 log₂) were detected in 32 naïve control chickens.

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2.

Protection of chickens by *in ovo* immunization with the AdTW68.H5-vectored AI vaccine against A/chicken/Queretaro/14588-19/95 (H5N2) HPAI virus challenge. *In ovo* vaccination was performed on E18. (A) Twelve of 19 of the *in ovo* vaccinated birds were intranasally boosted on D15 whereas the other 7 were not revaccinated. D23 and D29 refer to days of serum collection from *in ovo* immunized chickens; +D23 and +D29 refer to days of serum collection from *in ovo* immunized chickens that were revaccinated intranasally on D15. HI titers against the A/turkey/Wisconsin/68 virus are expressed as $\log_2$ [HI titer] in individual birds (triangles and circles); bars represent the $\log_2$ [GMT] for each group. No HI titers ($\leq 1 \log_2$) were detected in 11 naïve control chickens. (B) On D34, chickens were challenged through the choanal slit with 10^5 EID₅₀ of the HPAI virus A/chicken/Queretaro/14588-19/95. Survival rates were statistically analyzed using the Logrank test (Prism 4.03, GraphPad Software). *In ovo* vaccination with AdTW68.H5 with or without nasal boost significantly protected chickens (100%) against lethal challenge when compared to unvaccinated controls ($P < 0.001$). All data are plotted as percent survival versus number of days after challenge. Numbers in parentheses represent the number of birds in each group. (C) A/chicken/Queretaro/14588-19/95 viral

genomes in individual chickens expressed as $\log_{10}$ copies per ml (triangles and circles) determined by real-time RT-PCR in oropharyngeal samples collected 2, 4, and 7 days post-challenge. Control, chickens without immunization; in-ovo, chickens immunized *in ovo*; in-ovo+, chickens immunized *in ovo* followed by a nasal booster. A significant reduction in viral load was observed in immunized birds 7 days post-challenge.

Fig. 3A

Fig. 3B

Fig. 3.

Protection of chickens by *in ovo* immunization with the AdTW68.H5-vectored AI vaccine against A/swan/Mongolia/244L/2005 (H5N1) HPAI virus challenge. *In ovo* vaccination was performed on E18. (A) Control and immunized birds were challenged through the choanal slit with 10^5 EID₅₀ of the HPAI virus A/swan/Mongolia/244L/2005 (H5N1) on D31. The data are plotted as described in the Fig. 2 legend. (B) Pre-challenge serum HI antibodies on D25 were analyzed. Minus sign (–) indicates birds that succumbed to challenge; plus sign (+) indicates birds that survived challenge. Other symbols and abbreviations are described in the Fig. 1 legend. Survivors had significantly elevated pre-challenge serum HI activity ($P < 0.001$) as compared to those that died (unpaired t-test; Prism 4.03), although a small number of birds with HI titers of 3–5 log₂ were still killed by the H5N1 virus. All naïve control birds (n=10) and birds immunized by the control vector AdCMV-tetC (n=10) produced no measurable HI antibody titers (≤ 1 log₂).