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Highly immunogenic prime–boost DNA vaccination protects chickens against challenge with homologous and heterologous H5N1 virus

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ABSTRACT

Highly pathogenic avian influenza viruses (HPAIVs) cause huge economic losses in the poultry industry because of high mortality rate in infected flocks and trade restrictions. Protective antibodies, directed mainly against hemagglutinin (HA), are the primary means of protection against influenza outbreaks. A recombinant DNA vaccine based on the sequence of H5 HA from the H5N1/A/swan/Poland/305-135V08/2006 strain of HPAIV was prepared. Sequence manipulation included deletion of the proteolytic cleavage site to improve protein stability, codon usage optimization to improve translation and stability of RNA in host cells, and cloning into a commercially available vector to enable expression in animal cells. Naked plasmid DNA was complexed with a liposomal carrier and the immunization followed the prime–boost strategy. The immunogenic potential of the DNA vaccine was first proved in broilers in near-to-field conditions resembling a commercial farm. Next, the protective activity of the vaccine was confirmed in SPF layer-type chickens. Experimental infections (challenge experiments) indicated that 100% of vaccinated chickens were protected against H5N1 of the same clade and that 70% of them were protected against H5N1 influenza virus of a different clade. Moreover, the DNA vaccine significantly limited (or even eliminated) transmission of the virus to contact control chickens. Two intramuscular doses of DNA vaccine encoding H5 HA induced a strong protective response in immunized chicken. The effective protection lasted for a minimum 8 weeks after the second dose of the vaccine and was not limited to the homologous H5N1 virus. In addition, the vaccine reduced shedding of the virus.

Introduction

DNA vaccines are new-generation vaccines offering many advantages over conventional ones [13]. They are relatively simple, easy and fast to produce, generate low costs in storage and transport, and are more stable than protein formulations. Numerous data show the effectiveness of experimental DNA immunizations against various viral, bacterial, parasitic and cancer diseases. However, only a few veterinary products have been registered to date in the USA and Canada, and despite several clinical trials, no human DNA vaccine is available [6,10]. Various experimental DNA vaccines have been tested in poultry [15]. The high potential of DNA immunization, particularly in cases requiring a rapid response to an influenza pandemic have led to the development of this technology and increase of report on DNA vaccines for chickens against influenza [4,8,9,11,12,17,19,23].

The influenza virion has several structural and non-structural antigens, namely hemagglutinin (HA), neuraminidase (NA), capsid protein (M1), ion channel protein (M2), nucleoprotein (NP) and the components of the viral polymerase PA, PB1 and PB2 [22]. Although detectable antibody responses are observed against many viral proteins, the major determinants for a protective response are antibodies produced against surface glycoprotein HA, the most prominent antigen of the virus (see the review [20] and references therein). HA is synthesized as a precursor polypeptide H0 and is then cleaved into subunits H1 and H2. The HA cleavage site is the main determinant of the pathogenicity of influenza viruses. In low-pathogenic avian influenza viruses (LPAIVs) the cleavage site can be limited to a single arginine residue recognized

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by extracellular trypsin-like proteases, while in high-pathogenic viruses (HPAIV) the H0 precursor contains a sequence that can be recognized by proteases present in nearly all cell types, which facilitates systemic spread of the virus [20,22].

In the EU, permission to vaccinate poultry against H5N1 HPAI can be granted after the fulfillment of strict requirements laid down in the EU Directive for the Control of AI (Council Directive 2005/94/EC). The directive is concerned with the high risk of a "silent spread" of the virus due to incomplete protection at a flock level, leading to the impossibility of differentiating the infected from the vaccinated individuals in case of usage of inactivated vaccines. Therefore, considering the needs of the DIVA (Differentiating Infected from Vaccinated Animals) strategy, there is a great demand for new-generation vaccines [2,5,18]. It is strongly recommended by the OIE and the EU that preventive and emergency vaccination should be an additional method of controlling and fighting the virus in case of disease outbreak, by protecting valuable flocks and reducing the spread of virus in restriction and buffer zones.

The H5N1 strain of HPAIV which is the object of our studies and caused the Asian epidemic in 2003 was first identified in domestic gees in China in 1996 [27]. After several years of spreading and genetic diverging in South Eastern Asia, some strains have crossed the Russian border and reached the Middle East and Europe [3]. Several local outbreaks appeared in almost all European countries, both on poultry farms and among wild birds. In March 2006, the first disease outbreak was reported in Poland in mute swans [14,25]. Despite the high standards of food and animal trade in the EU due to the intense human and animal movement the risk of virus re-emergence is high. In this study the immunization experiments were conducted with common broiler type chicken grown in a biologically secure poultry-house. The duration of the immunization experiments was 6 weeks, because such is the length of broilers' life. Two intramuscular doses of DNA vaccine were sufficient to stimulate the anti-HA response in sera of immunized chickens. The second series of experiments involved challenge with HPAI H5N1 viruses and were conducted in a P3 laboratory using SPF chickens of laying type, which allowed the time of the experiments to be extended to 8–13 weeks in order to test for the long-term protection. The challenge experiments indicated a high protective potential of the tested DNA vaccine. The immunized SPF chickens were protected in 100% against H5N1 virus from a homologous clade (clade 2.2) and in 80% against the H5N1 virus from a heterologous clade (clade 1).

Materials and methods

Plasmids and vaccine design

Based on the predicted amino acid sequence of HA from H5N1 A/swan/Poland/305-135V08/2006 strain of HPAIV (EpiFluDatabase [http://platform.gisaid.org]; Accession No. EPI156789), a synthetic gene optimized to the domestic chicken codon bias and containing deletion of the proteolytic cleavage site (from Arg-341 to Arg-346) was designed (GenBank Accession No. KC172926). Two variants of the DNA vaccine were prepared: (i) long, codon-optimized HA (aa 1–568) with the original N-terminal signal peptide of 16 amino acids (aa 1–16) and a deletion of the proteolytic cleavage site RRRKKR (Δ341–346) and (ii) short, codon-optimized HA, containing only aa 17–340 (only H1 subunit, without signal peptide). The non-optimal codons in the native HA gene sequence were replaced by codons optimized to chicken codon usage and the sequence was also checked for the absence of cryptic splice sites (commercial service by GenScript USA Inc.). The inserts were cloned into the pCI (Promega) between immediate-early enhancer/promoter from Cytomegalovirus (CMV) and a terminator/polyadenylation signal from SV40. Plasmid DNA was purified using NoEndo JETSTAR Plasmid Kit (Genomed, Germany) and suspended in PBS pH 7.4, and the appropriate amount of DNA (62–250 μg) was mixed with the Lipofectin transfection reagent (Life Technologies, USA) as recommended by the manufacturer. In each trial the same ratio of DNA amount (w):Lipofectin (v), 6:1 was used. The volume of one dose of vaccine was 160 μl.

Influenza viruses and stock preparation

Table 1 lists the used influenza viruses. The HPAIVs were propagated in the allantoic cavities of embryonated chicken eggs (Valo-Biomedia, Germany) in biosafety level 3 conditions of the National Veterinary Research Institute (Pulawy, Poland) and stored in aliquots at −70 °C (for challenge purpose) or inactivated with 0.1% formaldehyde (Sigma–Aldrich, MO, USA) for 2 h at 37 °C (for hemagglutination inhibition test). The LPAIVs were either purchased or kindly provided by others. The viral stocks stored at −70 °C were titrated before use.

Immunization and challenge experiments

Broilers (Ross 308) were housed in poultry-house in cages, in standard commercial conditions including temperature, photoperiod, litter and fodder. Five independent immunizations of broilers were conducted. Depending on the experiment, animals (7–15 per group) were immunized subcutaneously in the neck or intramuscularly in the breast muscle with the indicated amount of DNA complexed with Lipofectin. Blood was collected from the wing veins, allowed to coagulate, and centrifuged. The collected sera were kept at −20 °C.

Specific pathogen free (SPF) White Leghorn chickens, housed in a biosafety level 3 containment of the National Veterinary Research Institute, Pulawy, were immunized intramuscularly twice (using 1-ml syringe with 0.5 × 1.6 mm needle) with the DNA vaccine containing 125 μg of plasmid DNA complexed with Lipofectin. Prior to the challenge, the chickens were placed in separate isolators (Montair Andersen B.V., Holland) equipped with HEPA filters. Three challenge experiments were performed. The immunized chickens (10 birds/group in Experiments 1 and 3, and 5 birds in Experiment 2) as well as control (untreated, fully susceptible chickens, 2–5/group) were inoculated oculonasally with 10^6 50% egg infectious dose (EID_{50}) of the respective virus in the volume of 100 μl (50 μl into the nares and 50 μl into the eye per bird). Approximately 24 h after inoculation, 6-week-old contact SPF chickens (1 or 2 per group) were placed in the same isolators as the vaccinated chickens to monitor virus transmission. Other details are shown in Table 2.

Ethic statements

The experiments were approved by the Second Local Ethical Committee for Animal Experiments at the Medical University of Warsaw, Permit Number 17/2009 (broilers) or the Second Local Ethical Committee for Animal Experiments at the University of Life Sciences in Lublin, Permit No. 26/2012 (SPF chickens). All efforts were made to minimize suffering. The chickens were monitored twice a day (morning and afternoon), including weekends. The immunized chickens were sacrificed (humanely euthanized by decapitation) about 3 weeks after the final immunization (about 6 weeks after hatching).

ELISA

The 96-well polystyrene plates (Nunc, Denmark) were coated overnight at 4 °C with 300 ng of HA antigen (A/swan/Poland/