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Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector

Yongping Jiang, Kangzhen Yu, Hongbo Zhang, Pingjing Zhang, Chenjun Li, Guobin Tian, Yanbing Li, Xijun Wang, Jinying Ge, Zhigao Bu, Hualan Chen *

Animal Influenza Laboratory of the Ministry of Agriculture and National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, People's Republic of China

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Abstract

H5N1 influenza viruses have caused significant disease and deaths in various parts of the world in several species, including humans. Vaccination combined with culling can provide an attractive method for outbreak containment. Using synthesized oligos and overlapping extension PCR techniques, we constructed an H5 HA gene, optiHA, containing chicken biased codons based on the HA amino acid sequence of the highly pathogenic H5N1 virus A/goose/Guangdong/1/96 (GS/GD/96). The optiHA and wild-type HA genes were inserted into plasmids pCI or pCAGGS, and designated as pCIoptiHA, pCAGGoptiHA, pCIHA and pCAGGHA, respectively. To evaluate vaccine efficacy, groups of 3-week-old specific pathogen free (SPF) chickens were intramuscularly injected with the four plasmids. Sera were collected on a weekly basis post-vaccination (p.v.) for hemagglutination inhibition (HI) assays and neutralization (NT) antibody detection. All chickens receiving pCAGGoptiHA and pCAGGHA developed high levels of HI and NT antibodies at 3 weeks p.v., and were completely protected from lethal H5 virus challenge, while only partial protection was induced by inoculation with the other two plasmids.

A second experiment was conducted to evaluate if a lower dose of the pCAGGoptiHA vaccine could be effective, results indicated that two doses of 10 μg of pCAGGoptiHA could induce complete protection in chickens against H5 lethal virus challenge. Based on our results, we conclude that construction optimization could dramatically increase the H5 HA gene DNA vaccine efficacy in chickens, and therefore, greatly decrease the dose necessary for inducing complete protection in chickens.

Keywords: Avian influenza; H5 subtype; DNA vaccine; Codon optimization

1. Introduction

H5N1 avian influenza viruses (AIV) have a recent history of being a disastrous pathogen for domestic poultry and of being a substantial threat to public health. An H5N1 AIV, A/goose/Guangdong/1/96 (GS/GD/96), was first isolated from geese in Guangdong province in China in 1996 (Chen et al., 2004; Xu et al., 1999). In 1997, an H5N1 AIV again caused disease outbreaks in poultry in Hong Kong (Shortridge et al., 1998). A reassortant virus bearing the hemagglutinin (HA) gene of the GS/GD/96-like virus (Chin et al., 2002) was transmitted to humans causing six deaths (Claas et al., 1998; Subbarao et al., 1998). In early February 2003, an H5N1 virus reemerged in a family in Hong Kong (Peiris et al., 2004). Starting from late 2003, H5N1 AIV began to spread and caused disease outbreaks in China, Japan, South Korea, Thailand, Vietnam, Indonesia, Cambodia, Malaysia and Laos, resulting in the deaths of hundreds of millions of poultry, including chickens, ducks and geese (Office International des Epizooties [http://www.oie.int]). In Thailand, Vietnam, Cambodia, Indonesia, China and Turkey, the viruses were transmitted to humans and have caused more than 50% deaths out of more than 200 confirmed cases according to recent reports (World Health Organization [http://www.who.int]). H5N1 AIV also caused disease and death in wild animals, including tigers, leopards and wild birds (Keawcharoen et al., 2004; Chen et al., 2006). These facts emphasize that the H5N1 AIV can infect multiple avian species and humans and spread widely geographically.

* Corresponding author at: Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, People's Republic of China. Tel.: +86 451 82761925; fax: +86 451 82733132.
E-mail address: hlchen1@yahoo.com (H. Chen).

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The culling of infected poultry is the time-honored method to control or eradicate the highly pathogenic avian influenza outbreaks, and also the best-known way to prevent transmission to humans. However, when the viruses are widely spread over a huge area and involved in multiple avian species, culling and physical containment are not likely to be successful. An alternative strategy would be culling plus vaccination. Whole virus inactivated vaccine (Tian et al., 2005), recombinant fowlpox vaccines (Qiao et al., 2003) and recombinant Newcastle disease virus vaccines (Ge et al., 2007) have been used in some countries, DNA vaccines may offer a number of advantages over these vaccine strategies for avian influenza virus control and infection prevention. First, DNA immunization can achieve both humoral and cell mediated immune responses, similar to an attenuated live virus vaccine, and has the safety of a killed or subunit vaccine (Donnelly et al., 2000; Garmory et al., 2003; Liu et al., 1998; Webster, 1999). Second, DNA vaccines are easier to manufacture than inactivated whole virus vaccines. Third, immune responses are generated against the expressed gene product and not the DNA vaccine vector.

A DNA vaccine based on influenza HA gene is attractive for several reasons. HA is the major surface glycoprotein of influenza A virus primarily involved in induction of specific humoral immunity. Antibodies against HA generally neutralize viral infectivity, probably by interfering either with viral attachment to sialic-acid receptors on the host cell surface, or with fusion between virus and endosomal membranes (Brown et al., 1992). HA gene-based DNA vaccines have successfully induced immune responses to influenza viruses in several different species, including chickens, swine, mice and ferrets (Larsen and Olsen, 2002; Robinson et al., 1993). HA gene-based DNA vaccination does not generate antibodies to nucleoprotein (NP), which are often used for avian influenza serological surveillance. HA gene-based DNA vaccines allow for differentiation between vaccinated birds and birds infected with the field virus, which is necessary for outbreak surveillance and trade (Swayne, 2003). These advantages mark HA gene-based DNA vaccines as a potential strategy for avian influenza control. Fynan et al. reported that saline injection of 200–300 μg plasmid DNA could induce 30–63% protection in chickens against the lethal H7N7 virus challenge (Fynan et al., 1993). Suareaz et al. reported that intramuscular injection of two doses of 100 μg plasmid DNA could induce antibody response in 80% chickens tested (Suarez and Schultz-Cherry, 2000). We previously observed that chickens could be protected from lethal H5N1 avian influenza virus challenge after two doses of 50 or 100 μg HA gene DNA vaccine injection (Chen et al., 2001). Although these studies have confirmed the efficacy of the HA gene-based DNA vaccine in chickens, the high dosage (100–400 μg plasmid DNA) needed is a major obstacle for the field application of such vaccines. In the present study, we explored a strategy to decrease the dosage of DNA vaccine required by improving the expression of the target gene. Our results indicate that intramuscular injection of two doses of only 10 μg of plasmid containing the optimized H5 subtype avian influenza virus HA gene in a pCAGGS plasmid vector could completely protect chickens from a lethal dose of highly pathogenic H5N1 AIV challenge. In addition, the protective immune response lasted for more than a year.

2. Materials and methods

2.1. Animals, viruses and cell line

SPF chickens were housed in HEPA-filtered isolators. Chicken embryo fibroblasts (CEF) cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at pH 7.2 and were kept at 37 °C with 5% carbon dioxide. Highly pathogenic H5N1 avian influenza virus GS/GD/96 and A/duck/Shanghai/16/04 (DK/SH/04) were propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs. The 50% chicken lethal dose (LD_{50}) was determined by inoculating serial 10-fold dilutions of virus into 6-week-old SPF chickens intranasally.

2.2. Construction of a codon optimized optiHA gene and its expression plasmids

The synthetic gene for H5 subtype avian influenza HA with codons optimized for chicken usage was synthesized by PCR assembly of long single-strand DNA templates (100 bases in length) (the oligonucleotide sequences are available upon request) as described by Haas et al. (1996). The synthetic optiHA gene shares 100 and 74.7% homology at the amino acid level and nucleotide level, respectively, with the wild-type HA gene of GS/GD/96 as confirmed by sequencing. Both the wild-type HA gene of GS/GD/96 virus and optiHA were cloned into the plasmid vector pCAGGS (kindly provided by Dr. Y. Kawaoka) under the control of the chicken β-actin promoter, and plasmid pCI (Promega) under the control of the cytomegalovirus (CMV) promoter. The four plasmids were named pCIHA, pCIoptiHA, pCAGGHA and pCAGGoptiHA, repectively, and the expression of the HA protein from these plasmids was confirmed by indirect immunofluorescence assay and Western blotting of plasmid transfected CEF cells.

2.3. Immunofluorescence assay

Monolayer of 80–90% confluent CEFs in six-well plates were transfected with 4 μg of the plasmids using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, DNA and transfection reagent were mixed (2.5 μl of Lipofectamine 2000/μg DNA), incubated at room temperature for 30 min, and added to the cells. Six hours later, the DNA-transfection reagent mixture was replaced by DMEM (GIBCO/BRL, Carlsbad, CA) containing 10% fetal calf serum. The medium was aspirated 24 and 48 h after transfection, and the cells were washed once with phosphate-buffered saline (PBS), fixed with 75% ethanol for 30 min at room temperature, and washed once again with PBS. Primary and secondary antibodies were diluted in a 1:10 dilution of blocking solution in PBS. Incubation times were 45 min for each antibody,

after which the cells were washed three times. Primary antibodies used were chicken H5-AIV specific antiserum. Secondary antibodies used were FITC-conjugated rabbit–anti-chicken IgG (Sigma).

2.4. Western blotting

Monolayer of 80–90% confluent CEFs in six-well plates were transfected with 4 μg of the plasmids pCIHA, pCIoptiHA, pCAGGHA or pCAGGoptiHA, as described above. In the mock-treated cells, only the transfecting reagents were used. Cells were lysed at 48 h after the transfection. The lysates of about 10^4 cells were loaded and separated on 10% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (PAGE), and blotted onto polyvinylidene difluoride membranes. For the HA protein detection, the membrane was hybridized with chicken H5-AIV specific antiserum, followed by detection with horseradish peroxidase (HRP)-conjugated rabbit–anti-chicken IgG (Sigma). For the β-actin detection, the membrane was hybridized with the monoclonal anti-β-actin antibody (Sigma), followed by detection with horseradish peroxidase (HRP)-conjugated goat–anti-mouse IgG (Sigma). Proteins were visualized with 3,3′-diaminobenzidine (DAB) reagents (Wuhan Boster Biological Technology Co. Ltd. Products).

2.5. Immunization of SPF chickens with plasmid DNA and challenge

Vaccine trials and challenge experiments in SPF chickens were conducted in HEPA-filtered isolators. Plasmids pCIHA, pCAGGHA, pCIoptiHA, and pCAGGoptiHA were amplified in E.coli JM 109 and extracted using the alkaline lysis method as previously described (Sambrook et al., 1989). After purification by polyethylene glycol precipitation, the plasmids were quantified by ultraviolet spectrophotometry (Ultrospec3000, Pharmacia Biotech Inc.) and resuspended in phosphate-buffered saline (PBS, pH 7.2) at a final concentration of 0.5 μg/μl. The extra elimination of endotoxins in the plasmid preparations was not performed. The plasmids were kept at −20 °C before the animal studies. The plasmids were diluted to the desired concentration and were administered by intramuscular injection in a 200 μl volume at the leg muscle. Chickens were challenged with 1000 LD_{50} of H5N1 viruses in 0.1 ml intranasally. Oropharyngeal and cloacal swabs were collected from all chickens at 3, 5 and 7 days post-challenge (p.c.) for virus titration in eggs, and chickens were observed daily for disease signs and death for 2 weeks. In all the experiments, sera were collected regularly post-vaccination (p.v.) for detection of hemagglutination inhibition (HI) antibody and neutralization (NT) antibody.

2.6. Serological tests

HI assay procedures were performed as previously described (Deck et al., 1997). NT assays were conducted by using Madin–Darby canine kidney (MDCK) cells with a microtiter technique (Robinson et al., 1997). Titers represented the highest dilution capable of preventing replication of a 100 $CCID_{50}$ of GS/GD/96 influenza virus. The presence of replicating virus in 96-well plates was scored according to the cytopathic effect and HA test.

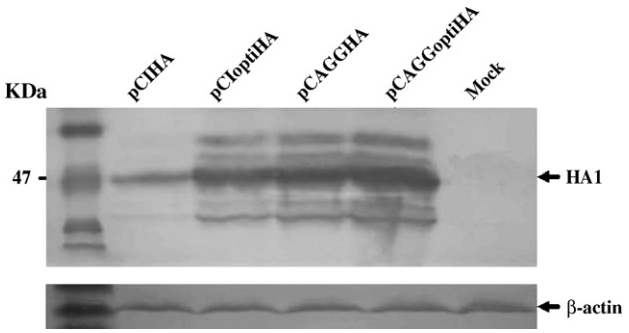

Fig. 1. Western blot analyses of plasmid expressing H5 AIV HA. Lysates of CEFs transfected with different plasmids were incubated with chicken H5-AIV specific antiserum. Binding was visualized with DAB reagent after incubation with peroxidase-conjugated secondary antibodies. Location of marker protein is indicated on the left, and the HA1 of AIV hemagglutinin and β-actin are indicated on the right.

2.7. Nucleotide sequence number

The sequence of the optimized H5 HA gene is available in Genbank under the access number of DQ420166.

3. Results

3.1. Confirmation of the expression of the HA gene by different plasmids

Expression of influenza H5 HA by the plasmids was confirmed by immunostaining the transfected CEFs. Cells transfected with all four plasmids were stained by chicken antisera to H5 AIV HA protein. In contrast, the cells transfected with the plasmid pCI or pCAGG were not stained (data not shown). The expression of HA protein by the plasmids was also confirmed by western blot analysis. As shown in Fig. 1, the majority of the HA protein expressed by these plasmids were cleaved, and the levels of the HA protein expression by the plasmids are different, with the pCIHA showing the weakest signal and the pCAGGoptiHA showing strongest signal (Fig. 1), though no significantly difference were observed among the pCIoptiHA, pCAGGHA and pCAGGoptiHA. As control, the levels of β-actin in all lanes are similar, indicating similar amount of cell protein were loaded on each lane.

3.2. Antibody responses in chickens induced by the plasmid vaccination

HI and NT antibodies are necessary to prevent avian influenza virus infection (Brown et al., 1992). To investigate the antibody responses of the four plasmid constructs, groups of 3-week-old SPF chickens were intramuscularly inoculated with 100 μg of pCIHA, pCIoptiHA, pCAGGHA and pCAGGoptiHA. Serum samples from all of the chickens were collected before and after vaccination on a weekly basis for analysis of the HI and NT

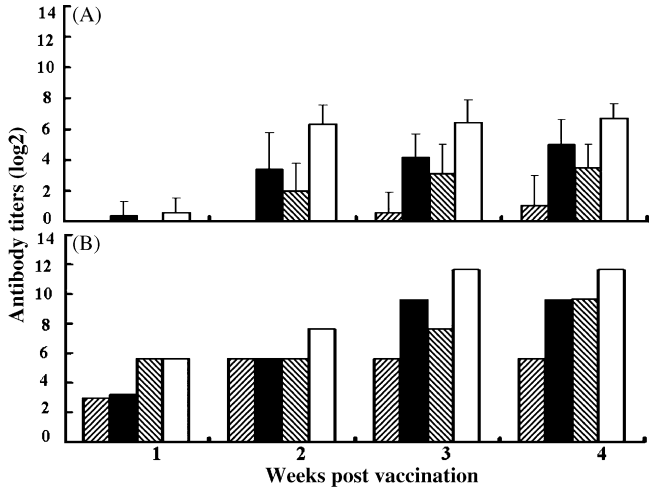

Fig. 2. Antibody titers of chickens induced by different plasmids. Groups of 3-week-old SPF chickens were inoculated intramuscularly with one dose of 100 μg of the plasmid pCIHA (▨), pCAGGHA (■), pCIoptiHA (▧) or pCAGGoptiHA (□) and sera were collected on a weekly basis for antibody detection. The HI antibodies are shown as mean ± standard deviation measurements from groups of five chickens, and the antisera from one group were pooled for the NT antibody detection. (A) Measurement of HI antibody and (B) measurement of neutralization antibody.

antibody titers. As shown in Fig. 2A, two plasmids, pCAGGHA and pCAGGoptiHA, induced detectable HI antibody in chickens 1 week after vaccination, and titers increased sharply at the second week p.v. The pCIoptiHA- and pCIHA-vaccinated chickens only developed detectable HI antibody 2 and 3 weeks p.v., respectively. Four weeks after the vaccination, the average HI antibody titers of the chickens vaccinated with plasmid pCIHA, pCAGGHA, pCIoptiHA, pCAGGoptiHA were 1 log 2, 5 log 2, 3.5 log and 6.7 log, respectively. However, all four plasmids induced high titers of NT antibody in chickens at 1 week p.v., with the titers of the pCAGGoptiHA-vaccinated group being higher than the titers of the other groups in other time points detected (Fig. 2B).

3.3. Protective efficacy of different plasmids in chickens against the highly pathogenic H5 avian influenza virus challenge

To investigate the protective efficacy of the four plasmids, we challenged chickens with 1000 LD_{50} of GS/GD/96 virus 4 weeks p.v. As shown in Table 1, chickens vaccinated with the pCAGGoptiHA and pCAGGHA were completely protected from virus challenge, showing no signs of disease, virus shedding, or death. In contrast, three of five chickens in the pCIHA inoculated group shed virus on day 3, and two of them died during the observation period. One chicken in the pCIoptiHA inoculated group shed virus and died on day 4 p.c. The plasmid pCI, pCAGGS and PBS inoculated control chickens started to show disease signs 3 days p.c. and died within a week after challenge.

3.4. Dose–response relationship of plasmid pCAGGoptiHA in chickens

The plasmid pCAGGoptiHA induced the strongest immune response in chickens, although both of the plasmids in pCAGGS vector induced complete protection after challenge with the highly pathogenic AIV GS/GD/96 (Table 1). Based on these results, we selected pCAGGoptiHA for the further dose–response evaluation. Groups of 3-week-old SPF chickens were inoculated with 100, 10 and 1 μg of pCAGGoptiHA in 200 μl PBS or with 200 ul PBS as the control. They were challenged with 1000 LD_{50} of GS/GD/96 virus 3 weeks p.v. As indicated in Fig. 3, all of the vaccinated chickens developed detectable HI and NT antibodies, with the titers of the antibodies correlating with the dosage of plasmid inoculated. At 3 weeks p.v., the mean HI and NT antibody titers in the chickens inoculated with 100 μg plasmid reached 6.4 log 2 and 11.6 log 2, respectively. The mean HI and NT antibody titers in the 1 μg plasmid inoculated chickens were 2.9 log 2 and 5.6 log 2, respectively. The antibody titers in the 10 μg plasmid inoculated chickens were 4 log 2 and 8 log 2, respectively.

Table 1
Protective efficacy of the different plasmids in chickens against H5N1 virus challenge

Inoculated plasmid	Virus isolation from the swabs on different days p.c.: shedding/total (log_{10} EID_{50})						Survival/total
	Day 3		Day 5		Day 7		
	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
pCIHA	2/5 (2.5)	3/5 (2.4)	1/3 (+)	0/3	0/3	0/3	3/5
pCAGGHA	0/5	0/5	0/5	0/5	0/5	0/5	5/5
pCIoptiHA	0/5	0/5	1/4 (+)	0/4	0/4	0/4	4/5
pCAGGoptiHA[a]	0/10	0/10	0/10	0/10	0/10	0/10	10/10
pCI	5/5 (2.4)[b]	4/5 (2.1)[b]	1/1 (+)	1/1 (1.3)	/	/	0/5
pCAGGS	5/5 (1.9)[b]	5/5 (1.3)[b]	2/2 (1.6)	2/2 (1.0)	/	/	0/5
Control[a]	9/10 (2.4)[b]	9/10 (2.2)[b]	0/1	0/1	/	/	0/10

Groups of 3-week-old SPF chickens were inoculated intramuscularly with 100 μg of each plasmid in 200 μl PBS or with 100 μl PBS as control, and challenged intranasally with 1000LD50 of GS/GD/96 virus in a 100-μl volume 4 weeks post-immunization. The swabs were suspended in 1 ml of PBS, and were titrated for virus shedding in eggs at initial dilutions of 1:10, or undiluted if it was negative at the lowest dilution. (+) Virus was detected in the undiluted samples and (/) all birds died.

[a] Data shown were two repeats with five chickens in each group.

[b] The swabs of chickens which died before 3 days were also titrated and calculated at day 3 p.c.

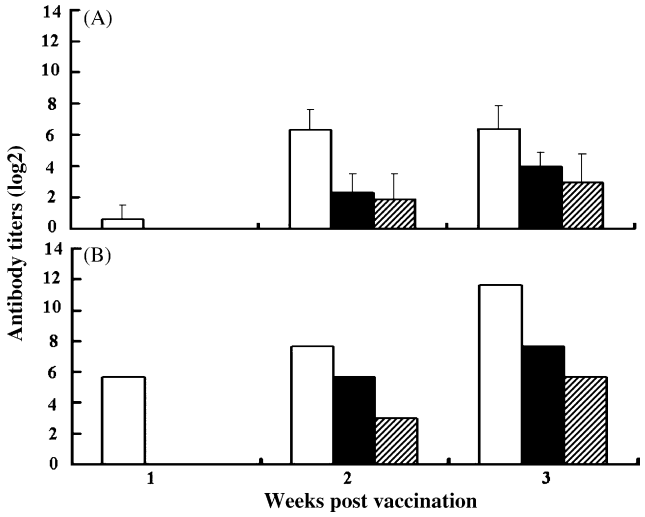

Fig. 3. Antibody titers of chickens inoculated with various doses of plasmid pCAGGoptiHA. Groups of 3-week-old SPF chickens were inoculated with one dose of 100 μg (□), 10 μg (■) or 1 μg (▨) of the plasmid pCAGGoptiHA intramuscularly and sera were collected on a weekly basis for antibody detection. The HI antibodies are shown as mean ± standard deviation measurements from groups of 8 chickens, and the antisera from one group were pooled for the NT antibody detection. (A) Measurement of HI antibody and (B) measurement of neutralization antibody.

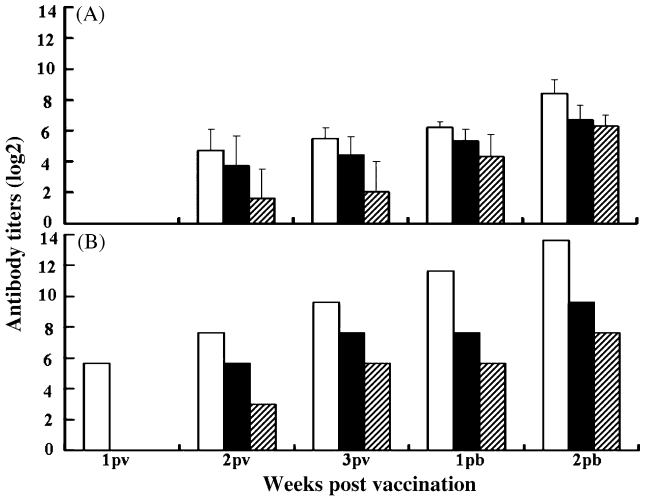

Fig. 4. Antibody titers of chickens inoculated with two shots of different doses of plasmid pCAGGoptiHA. Groups of 3-week-old SPF chickens were inoculated with two doses of 100 μg (□), 10 μg (■) or 1 μg (▨) of the plasmid pCAGGoptiHA in a 3 weeks interval. The HI antibodies are shown as mean ± standard deviation measurements from groups of 8 chickens, and the antisera from one group were pooled for the NT antibody detection. (A) Measurement of HI antibody and (B) measurement of neutralization antibody. pb means post-boost.

When the chickens were challenged with a lethal dose of GS/GD/96 virus, all of the 100 μg plasmid inoculated chickens were completely protected from disease signs, virus shedding and death (Table 2). Two of eight chickens inoculated with 10 μg plasmid shed virus, but none of the birds showed disease signs or died during the observation period. In the 1 μg plasmid inoculated group, two and five chickens shed virus through the oropharynx and cloaca, respectively, on day 3 p.c. and one of them died on day 4 p.c. All of the chickens in the control group shed viruses and died on days 3 and 4 p.c.

The above results indicated that pCAGGoptiHA could induce an immune response at dosages as low as 1 μg, though this low dosage only provided partial protection in chickens from highly pathogenic virus challenge. To investigate if a second shot of the plasmid could improve the antibody response and protective efficacy, groups of chickens were inoculated with two doses of 100, 10 or 1 μg of pCAGGoptiHA in a 3-week interval. As shown in Fig. 4, both the HI and NT antibody titers in chickens increased dramatically after the second shot. Two weeks after the second shot, chickens were challenged with a lethal dose of GS/GD/96 virus. The chickens in the 100 and 10 μg plasmid inoculated groups were completely protected, again displaying no signs of disease, virus shedding, or death. Only two of the eight chickens inoculated with 1 μg of plasmid shed detectable

Table 2
Dose–response relationship of plasmid pCAGGoptiHA in chickens

Dosage	Virus isolation from the swabs on different days p.c.: shedding/total (log_{10} EID_{50})						Survival/total
	Day 3		Day 5		Day 7		
	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
Single shot							
100 μg	0/8	0/8	0/8	0/8	0/8	0/8	8/8
10 μg	2/8 (1.8)	1/8 (1.5)	1/8 (2.8)	0/8	0/8	0/8	8/8
1 μg	2/8 (+)	5/8 (1.3)	0/7	0/7	0/7	0/7	7/8
PBS	8/8 (3.5)[a]	8/8 (2.2)[a]	/	/	/	/	0/8
Two shots							
100 μg	0/8	0/8	0/8	0/8	0/8	0/8	8/8
10 μg	0/8	0/8	0/8	0/8	0/8	0/8	8/8
1 μg	1/8 (1.8)	1/8 (+)	2/8 (1.6)	0/8	0/8	0/8	8/8
PBS	8/8 (3.2)[a]	6/8 (1.9)[a]	1/1 (+)	0/1	/	/	0/8

Groups of 3-week-old SPF chickens were inoculated intramuscularly with one or two doses of different amount of plasmid pCAGGoptiHA in 200 μl PBS or with 100 μl PBS as control, and challenged intranasally with 1000LD50 of GS/GD/96 virus in a 100-μl volume at the time points indicated in the text. The swabs were suspended in 1 ml of PBS, and were titrated for virus shedding in eggs at initial dilutions of 1:10, or undiluted if it was negative at the lowest dilution. (+) Virus was detected in the undiluted samples and (/) all birds died.

[a] The swabs of chickens which died before 3 days were also titrated and calculated at day 3 p.c.

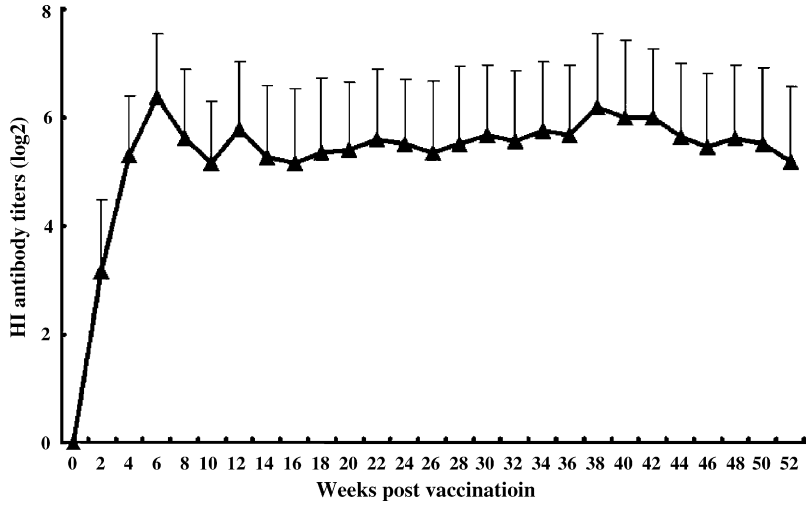

Fig. 5. HI antibody duration of chickens inoculated with plasmid pCAGGoptiHA. Three-week-old SPF chickens were vaccinated with two doses of 10 μg of pCAGGoptiHA in a 3 week interval, and the sera were collected from 10 chickens every 2 weeks for HI antibody detection. Data are shown as mean ± standard deviation measurements from groups of 10 chickens.

virus, and all of them were free of disease and survived the virus challenge.

3.5. Duration of protective immunity of the vaccinated chickens

The duration of the protective immunity is very important for a vaccine for field application. Our previous study indicated that a single dose of inactivated H5N1 avian influenza vaccine could induce 43 weeks protection in SPF chickens (Tian et al., 2005). To determine the duration of protective immunity induced by DNA vaccination, we inoculated the chickens with two doses of 10 μg of pCAGGoptiHA, and checked the HI antibody every 2 weeks. As shown in Fig. 5, the HI antibody titers reached a peak of 6.4 log 2 at 3 weeks after the second dose, and then remained at the level of 5–6 log 2 for at least 50 weeks. We also determined the NT antibody titers of chickens at several time points, and found that the titers were 7 log 2, 8.2 log 2 and 9 log 2 at 2, 4 and 6 weeks p.v. (3 weeks after the second dose), respectively. It is remarkable that the high NT titers of 8 log 2 were detected from the chickens at 20, 30, 40 and 50 weeks p.v. At the end of the observation period, we divided the chickens into two groups and challenged them with 1000 LD50 of the homologous virus GS/GD/96 and a heterologous virus DK/SH/04. The vaccinated chickens were completely protected from GS/GD/96 challenge (Table 3), while all of the chickens in the control group shed virus and died by day 4 p.c. In the DK/SH/04 virus challenged groups, seven chickens were completely protected. One of eight vaccinated chickens shed virus on days 3 and 5 p.c., and then died by day 6 p.c. All of the chickens in the control group shed high titers of virus and died within 3 days after challenge (Table 3).

4. Discussion

We created various plasmid constructs to determine their efficacy as DNA vaccines. One plasmid, pCAGGoptiHA, contained a chicken codon optimized H5 avian influenza HA gene created by the use of synthesized oligos and overlapping PCR techniques. Our results indicate that pCAGGoptiHA is very immunogenic and provides solid HI and NT antibody responses and completely protects chickens against highly pathogenic H5N1 avian influenza virus challenge. Our dose–response rela-

Table 3
Protective efficacy in chickens against H5N1 avian influenza viruses 50 weeks after the plasmid inoculation

Challenge virus	Groups	Virus isolation from the swabs on different days p.c.: shedding/total ($\log_{10}$ EID_{50})						Survival/total
		Day 3		Day 5		Day 7		
		Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
GS/GD/96	Vaccinated	0/8	0/8	0/8	0/8	0/8	0/8	8/8
	Control	8/8 (1.7)[a]	8/8 (1.6)[a]	/	/	/	/	0/8
DK/SH/04	Vaccinated	1/8 (1.0)	0/8	1/8 (+)	1/8 (3.8)	0/8	0/8	7/8
	Control	8/8 (3.5)[a]	8/8 (4.3)[a]	/	/	/	/	0/8

Groups of 3-week-old SPF chickens were inoculated intramuscularly with two doses of 10 μg plasmid pCAGGoptiHA in 200 μl PBS or with 100 μl PBS as control, and challenged intranasally with 1000LD50 of GS/GD/96 virus or DK/SH/04 virus in a 100-μl volume at 50 weeks after the first dose. The swabs were suspended in 1 ml of PBS, and were titrated for virus shedding in eggs at initial dilutions of 1:10, or undiluted if it was negative at the lowest dilution. (+) Virus was detected in the undiluted samples and (/) all birds died.

[a] The swabs of chickens which died before 3 days were also titrated and calculated at day 3 P.C.

tionship study demonstrated that the inoculation of two doses of 1 μg of pCAGGoptiHA could prevent chickens from disease and death after a homologous highly pathogenic virus challenge, though virus shedding could be detected in some of the birds. Intramuscular injection of two doses of 10 μg of pCAGGoptiHA induced a similar antibody response and protective efficacy as inoculation with one dose of 100 μg of pCAGGoptiHA. The duration of protective immunity lasted more than 50 weeks.

The vaccine efficacy of the plasmid pCAGGoptiHA was dramatically improved in comparison with the plasmid pCIHA. The effect of the vector (pCI versus pCAGGS) seems as important as codon optimization and both effects appear to be cumulative. Our experiments demonstrated that the HA gene in the pCAGGS plasmid vector induced a better immune response than the HA gene within the pCI vector, and this may be due to differences in expression caused by differences in the promoter/enhancer elements or other transcriptional elements in pCI and pCAGGS. The pCI vector contains the CMV immediate early enhancer–promoter and intron A, which has been shown to direct high level of transgene expression in eukaryotic tissues (Galvin et al., 2000). The pCAGGS vector contains the β-actin/CMV promoter/enhancer and the bovine growth hormone (BGH) poly(A) signal, which previous studies have indicated leads to stronger expression than RSV, SV40, PGK or CMV promoters (Bu et al., 2003; Xu et al., 2001). Therefore, the differences in promoter activities may have led to differences in gene expression, which again could influence the immune responses elicited by the pCI and pCAGGS-based constructs.

It is also obvious that the antibody response in chickens induced by the plasmids containing the optiHA gene were better than those induced by the plasmids containing the wild-type HA gene. Several factors may account for this difference. As different species have different codon biases for protein translation (André et al., 1998; Holm, 1986; Ikemura, 1985), the use of chicken optimized codons in the HA gene may have allowed for a great increase in the expression of this gene in chickens. The high GC ratio in the optiHA gene may increase its mRNA stability, processing and nucleocytoplasmic transport. Also, the number of CpG motif was increased to 16 in the optiHA, while there are only 2 in the wild-type HA gene, and it is possible that these motifs may also contribute to the increased immunogenicity of the optiHA sequence (Krieg et al., 1998).

Several studies have explored and confirmed the efficacy of the avian influenza HA gene-based DNA vaccination in chickens (Chen et al., 2001; Fynan et al., 1993; Kodihalli et al., 1997; Suarez and Schultz-Cherry, 2000; Robinson et al., 1993). However, the high dosage required by intramuscular injection is an obstacle for the application of this vaccination strategy in the field. Though the gene gun delivery system could reduce the dosage to 1–10 μg plasmid (Kodihalli et al., 1997), this method is not likely to be practical for field use. Our present study indicated that intramuscular injection of one dose 100 μg plasmid pCAGGoptiHA could provide complete protection in chickens against H5N1 avian influenza viruses. Dose–response studies demonstrated that intramuscular injection of two doses of 10 or 1 μg of pCAGGoptiHA could induce HI and NT antibodies and provide complete protection in chickens against highly pathogenic virus challenge, although virus shedding was detected at the 1 μg dose. It is worth noting that the HI antibody titers were directly related to the protective efficacy in our studies, which differs from a previous report (Kodihalli et al., 1997), in which the chickens were protected despite the lack of detectable HI antibody after DNA vaccination.

Influenza virus easily undergoes antigenic drift during circulation in nature, and the antigenic match between vaccine and the circulating viruses is one of the most decisive factors in determining the efficacy of the vaccine in preventing influenza virus replication and transmission. Our results show that an HA gene-based DNA vaccine works better for a homologous virus than a heterologous virus and that a higher dose (10 μg) works better than a low dose (1 μg). Thus, optimum dosing and optimum sequence selection are both critical parameters for the field application of the HA gene-based DNA vaccine

Inactivated vaccines are widely used in China and other Asian countries for the control of H5N1 avian influenza in poultry. Multiples doses are recommended for field application because of the short duration of the antibody response induced by the vaccines. This makes a vaccination campaign extremely expensive and laborious. Our investigation demonstrated that two doses of 10 μg of pCAGGoptiHA inoculation could induce protection lasting for more than a year, which covers the entire time period of layers that were kept in the farm, thus eliminating the need for costly serial vaccinations. Our study demonstrates several advantages of DNA vaccines compared to other vaccine types currently being used in the field, and suggest that the construction optimized HA gene-based DNA vaccine holds promise for commercial development for the prevention and control of highly pathogenic H5N1 avian influenza virus infection.

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