Antiviral Research 81 (2009) 234–238

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Recombinant fowlpox virus vector-based vaccine completely protects chickens from H5N1 avian influenza virus

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ARTICLE INFO

Article history:
Received 29 May 2008
Received in revised form
27 November 2008
Accepted 1 December 2008

Keywords:
Recombinant fowlpox virus
Vaccine
H5N1
Avian influenza virus

ABSTRACT

With the widespread presence of influenza virus H5N1 in poultry and wildlife species, particularly migrating birds, vaccination has become an important control strategy for avian influenza (AI). In this study, the immune efficacy and hemagglutination inhibition (HI) antibody responses induced by a recombinant fowlpox virus (FPV) vector-based rFPV–HA–NA vaccine was evaluated in SPF and commercial chickens. Four-week old SPF chickens vaccinated with one dose of vaccine containing 2×10^3 plaque forming units (PFU) of virus were completely protected from H5N1 AI virus 1 week after vaccination, and protective immunity lasted for at least 40 weeks. Two-week old commercial layer chickens were vaccinated with the rFPV–HA–NA vaccine and boosted with the same dose of vaccine following an interval of 18 weeks. The HI antibody titers higher than 4 log 2 lasted for 52 weeks after the booster immunization. We also examined the efficacy of the rFPV–HA–NA vaccine in SPF chickens administrated by different routes. The results showed that effective application of rFPV–HA–NA vaccine in poultry may be restricted to wing-web puncture, intramuscular or subcutaneous injection. These results demonstrate that the rFPV–HA–NA vaccine is effective in the prevention of infection of H5N1 AI virus.

1. Introduction

Highly pathogenic avian influenza (HPAI) H5N1 viruses have now spread in poultry and/or wild birds throughout Asia and into Europe and Africa, resulting in the death or culling of hundreds of millions of poultry (Alexander, 2007). During the past 5 years, H5N1 viruses have crossed species barriers and infected humans, and have caused 243 deaths in 385 confirmed human cases according to recent reports (World Health Organization [http://www.who.int]). This demonstrates the significant zoonotic threat of H5N1 AI viruses. These viruses have also been transmitted from poultry to mammals such as tigers, leopards and domestic cats (Amonsin et al., 2006; Thiry et al., 2007).

Inactivated vaccines have been used to control the spread of highly pathogenic H5 and H7 avian influenza viruses (AIV) in several countries (Ellis et al., 2004; Van Der Goot et al., 2005). In China, an inactivated vaccine derived from A/turkey/England/N-28/73 (H5N2) was firstly used for buffer zone vaccination during H5N1 outbreaks in 2004. Then a novel inactivated vaccine composed of a genetically modified reassortant H5N1 low pathogenic AIV (Tian et al., 2005) and a recombinant Newcastle disease virus (NDV)-based vaccine (Ge et al., 2007) have been developed and licensed for the prevention of H5N1 AI. In addition, a DNA vaccine has been developed (Jiang et al., 2007).

Fowlpox virus (FPV), a member of the family Poxviridae, with a large double strand of DNA genome and host range limited to avian species, has been used to successfully express protective immunogen genes from several avian viruses, and vaccination with the recombinant FPV was able to protect chickens from challenge with the corresponding viruses (Paoletti, 1996). The construction and immunogenicity of a recombinant FPV co-expressing H5 hemagglutinin (HA) and N1 neuraminidase (NA) have been reported previously (Qiao et al., 2003). In the present study, the optimal administration route, the time-course of the development of immunity and the duration of immunity induced by an rFPV–HA–NA vaccine were evaluated in specific pathogen-free (SPF) chickens. In addition, we also tested the duration of persistence of hemagglutination inhibition (HI) antibodies induced by the rFPV–HA–NA vaccine in a field trial.

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doi:10.1016/j.antiviral.2008.12.002

2. Materials and methods

2.1. Viruses and chickens

A recombinant FPV co-expressing the HA and NA genes of GS/GD/96 virus, named rFPV–HA–NA, was constructed as reported previously (Qiao et al., 2003). The genetic stability of rFPV–HA–NA was confirmed by serial passage in CEF cells as previously described (Qiao et al., 2004).

A/Goose/Guangdong/1/96 (GS/GD/96) H5N1 HPAI virus was first isolated in China and has been characterized previously (Chen et al., 2004; Xu et al., 1999). This virus was propagated in the allantoic cavities of 10-day old SPF embryonated chicken eggs and the 50% chicken lethal dose (CLD_{50}) was determined by inoculating serial 10-fold dilutions of virus into 6-week old SPF chickens intranasally using standard methods.

White leghorn SPF chickens were obtained from the Experimental Animal Center of Harbin Veterinary Research Institute (HVRI), and housed in negative pressure filtered-air isolation cabinets in a Biosafety Level 3 (BL-3) agriculture facility.

White Leghorn commercial layer chickens, housed in a small-scale farm, were serologically confirmed as AI negative by the agar-gel precipitin (AGP) test.

2.2. Immunization and challenge experiments

Vaccination and challenge experiments in SPF chickens were conducted in a BL-3 facility. Each vaccination was conducted according to the experimental design described below, and challenge was conducted 3 weeks post-vaccination (p.v.) with 0.1 ml of allantoic fluid containing 10^3 CLD_{50} of the GS/GD/96 isolate by the intranasal route. Cloacal swabs were collected from all live chickens at 4 days post-challenge (p.c.) for virus titration in eggs. All the chickens were observed daily for clinical signs and death for 2 weeks, and the mean dead time (MDT) in days was calculated.

2.2.1. Experiment 1

The minimum immunizing dose of the rFPV–HA–NA vaccine was determined as 10^2 PFU in the previous study (Qiao et al., 2006). Here, we selected 20 times the minimum administration dose (2×10^3 PFU) for use as one immunizing dose. To test the effect of administration route on the efficacy of the rFPV–HA–NA vaccine in chickens, 48 4-week old SPF chickens were allocated to 6 groups. Each group of chickens was vaccinated with 2×10^3 PFU of rFPV–HA–NA vaccine via wing-web puncture, eye-drop, intranasal inoculation, intramuscular or subcutaneous injection, respectively. Another group of chickens was kept as a control group for challenge. All the chickens were bled for HI antibody detection and challenged 3 weeks p.v. as described above.

2.2.2. Experiment 2

To determine the time of onset and the duration of immunity, groups of 4-week-old SPF chickens were vaccinated with 2×10^3 PFU of rFPV–HA–NA vaccine by wing-web puncture with a double needle used for commercial vaccination of poultry with FPV. Sera were first collected from 16 chickens at 3 days p.v., and then collected on a weekly basis to check the dynamic changes in the HI antibody titer after immunization. Challenge experiments were carried out on day 3, and weeks 1, 2 and 40 p.v., respectively, as described above.

2.2.3. Experiment 3

We conducted a field study on a small-scale farm to determine the duration of the protective antibody response induced by the rFPV–HA–NA vaccine. Two thousand 2-week old white Leghorn commercial layer chickens were serologically confirmed as AI negative by AGP test. Considering the earlier protections against H5N1 virus infection could be provided under field condition, all the chickens were given a primary vaccination of 2×10^3 PFU of rFPV–HA–NA vaccine by intramuscular injection at 2 weeks old, when the average maternal HI antibody titer had declined to 2 log 2. A booster immunization with the same dosage and by the same administrated route was conducted 18 weeks after the first vaccination. Sera were collected from 30 randomly selected chickens every 2 weeks after vaccination for HI antibody detection.

2.3. Serologic tests and virus re-isolation

HI assays were performed by following the Office International des Epizooties (OIE) standard. Formalin-inactivated GS/GD/96 virus was used as antigen in the HI test. Each swab was washed in 1 ml of cold phosphate-buffered saline (PBS), and virus titration was conducted as described previously (Qiao et al., 2003).

2.4. Statistical analysis

HI antibody titers of the vaccinated chickens were compared using analysis of variance and *t*-test. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Immune efficacy of rFPV–HA–NA vaccine in SPF chickens inoculated by different administration routes

2×10^3 PFU of the rFPV–HA–NA vaccine was administered via wing-web puncture, eye-drop, intranasal inoculation, intramuscular or subcutaneous injection, respectively. All the chickens were bled for HI antibody detection 3 weeks p.v. HI antibody responses were generated following vaccination via wing-web puncture, intramuscular or subcutaneous injection. All the chickens immunized by these routes were completely protected against the challenge with GS/GD/96. Intranasal and conjunctival inoculation failed to induce HI antibodies, and vaccinated chickens died within 2 weeks p.c. Significant differences were observed between the titers of HI antibody and virus shedding in the groups administrated by wing-web puncture, intramuscular or subcutaneous injection and those in groups by eye-drop, intranasal inoculation or unvaccinated control ($P < 0.05$) (Table 1). These results suggest that effective application of the rFPV–HA–NA vaccine for poultry vaccination may be restricted to wing-web puncture, or to intramuscular or subcutaneous injection.

3.2. Determination of the earliest time of induction of protective immunity and the duration of immunity

Groups of 4-week old SPF chickens were immunized with 2×10^3 PFU of the rFPV–HA–NA vaccine. Sera were collected from 16 chickens firstly on day 3, then on a weekly basis, to investigate the dynamic changes in the HI antibody titer. The HI antibodies were first detected 1 week after immunization and the titer rose to about 7 log 2 by 2 weeks p.v. The HI antibody titer remained above 4 log 2 until 30 weeks p.v., then it slowly declined to 3 log 2 by 40 weeks after immunization (Fig. 1).

To determine the protection induced by the vaccine, groups of chickens were challenged with the homologous highly pathogenic virus GS/GD/96 at different time points: 3 days, and 1, 2 and 40 weeks after immunization, respectively. The results shown in Table 2 indicate that all the vaccinated chickens were completely protected from GS/GD/96 challenge, except those given the

Table 1
Protective efficacy in SPF chickens with 2×10^3 PFU of rFPV–HA–NA vaccine administered by different routes.

Administration route	HI antibody titer 3 weeks p.v. (log 2)[a]	Manifestations in chickens	
		Virus shedding 4 days p.c.: shedding/total ($log_{10}EID_{50}$)	No. dead/total (MDT)
Wing-web puncture	6.8 ± 0.8[c]	0/8 (<0.5)[b,c]	0/8 (0)
Intramuscular injection	6.6 ± 0.4[c]	0/8 (<0.5)[b,c]	0/8 (0)
Subcutaneous injection	6.3 ± 0.5[c]	0/8 (<0.5)[b,c]	0/8 (0)
Eye-drop	<1	8/8 (2.1 ± 0.5)	8/8 (6.0)
Intranasal inoculation	<1	8/8 (2.0 ± 0.6)	8/8 (5.8)
Unvaccinated control	<1	8/8 (2.1 ± 0.4)	8/8 (5.9)

Each group of 8 4-week old SPF chickens was vaccinated with 2×10^3 PFU rFPV–HA–NA vaccine by wing-web puncture, eye-drop, intranasal inoculation, intramuscular or subcutaneous injection, respectively. Another group was kept as a control for challenge. All the chickens were bled for HI antibody detection and challenged 3 weeks p.v. Cloacal swabs were collected from all chickens 4 days p.c. for virus titration, and the chickens were observed daily for infection and death for 2 weeks. MDT = mean time to death in days.

[a] The HI antibodies are shown as mean ± standard deviations, and "<1" indicates no corresponding HI antibody.

[b] "<0.5" means no virus was detected from cloacal swabs.

[c] $P < 0.05$ compared with the titers in the groups administrated by eye-drop, intranasal route and unvaccinated control.

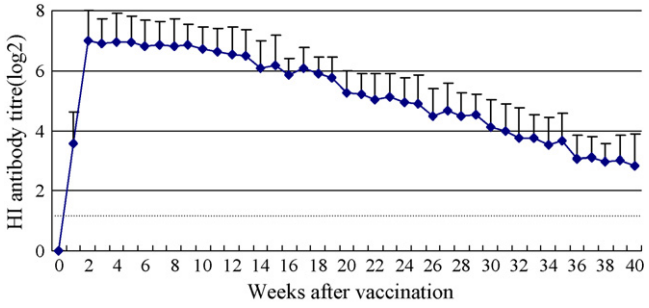

Fig. 1. HI antibody duration induced by rFPV–HA–NA vaccine in SPF chickens. Four-week old chickens were vaccinated with 2×10^3 PFU rFPV–HA–NA vaccine via wing-web puncture. Sera were collected from 16 chickens on a weekly basis to detect HI antibody. The values are shown as mean HI antibody and the bars indicate the standard deviation. The horizontal broken line indicates the minimal detection level for the HI antibody assay.

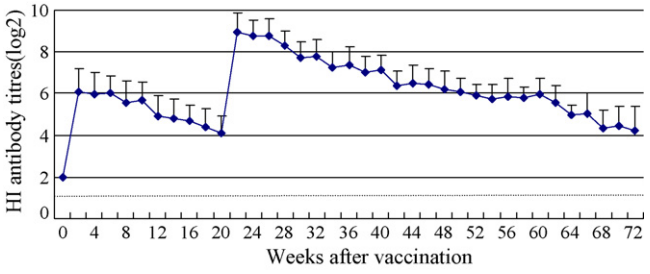

Fig. 2. HI antibody duration induced by rFPV–HA–NA vaccine in commercial layer chickens. Two-week old chickens were primarily vaccinated with 2×10^3 PFU rFPV–HA–NA vaccine by intramuscular injection, and boosted with same dose of vaccine 18 weeks later. Sera were collected from 30 randomly selected chickens every week after vaccination for HI antibody detection. The values are shown as mean HI antibody and the bars indicate the standard deviation. The horizontal broken line indicates the minimal detection level for the HI antibody assay.

first challenge, which was conducted only 3 days after vaccination. Unvaccinated control chickens shed virus from the cloaca and died within 2 weeks after challenge. Significant differences were observed between the titers of HI antibody and virus shedding in the groups of 1, 2 and 40 weeks after vaccination with rFPV–HA–NA vaccine and those in the control groups ($P < 0.05$). These results demonstrate that one dose of rFPV–HA–NA vaccine can induce complete protection against challenge with GS/GD/96 1 week after immunization and that the immunity can last for at least 40 weeks.

3.3. The HI antibody response induced by two-dose immunization in commercial layer chickens

Two-week old commercial layer chickens were given a primary vaccination of 2×10^3 PFU of rFPV–HA–NA vaccine and boosted 18 weeks after the first vaccination with the same dose. The titer of HI antibodies was checked every 2 weeks after vaccination. As shown in Fig. 2, HI antibody was detected at titers as high as 6 log 2 by 2 weeks p.v. An HI antibody level higher than 4 log 2 lasted for 18 weeks p.v. The HI antibody titer increased sharply to 9 log 2 after

Table 2
Protection induced by rFPV–HA–NA vaccine at different time points after vaccination.

Challenge time (p.v.)	Groups	Mean HI antibody titer[a]	Manifestations in chickens	
			Virus shedding 4 days p.c.: shedding/total ($log_{10}EID_{50}$)	No. dead/total (MDT)
3 days	Vaccinated	<1	8/8 (1.8 ± 0.3)	8/8 (6.0)
	Control	<1	8/8 (2.0 ± 0.2)	8/8 (5.8)
1 week	Vaccinated	3.59[c]	0/8 (<0.5)[b,c]	0/8 (0)
	Control	<1	8/8 (2.1 ± 0.1)	8/8 (6.1)
2 weeks	Vaccinated	6.99[c]	0/8 (<0.5)[b,c]	0/8 (0)
	Control	<1	8/8 (1.9 ± 0.3)	8/8 (5.9)
40 weeks	Vaccinated	2.85[c]	0/8 (<0.5)[b,c]	0/8 (0)
	Control	<1	8/8(1.8 ± 0.1)	8/8 (6.2)

Four-week old SPF chickens were vaccinated with 2×10^3 PFU vaccine via wing-web puncture. Sera were collected from 16 chickens firstly on day 3, then on a weekly basis, to detect HI antibody. Challenge experiments were conducted at different time points: 3 days, and 1, 2 and 40 weeks p.v. Cloacal swabs were collected from all chickens 4 days p.c. for virus titration, and the chickens were observed daily for infection and death for 2 weeks. MDT = mean time to death in days.

[a] The values are denoted as the maen HI antibody titers, and "<1" indicates no corresponding HI antibody.

[b] "<0.5" means no virus was detected from cloacal swabs.

[c] $P < 0.05$ compared with the titers in the control groups.

the second immunization, then declined slightly to 4 log 2 by 52 weeks after the second vaccination. These results demonstrate that the rFPV–HA–NA vaccine can induce a long-lasting HI antibody response under field conditions with a two-dose immunization protocol.

4. Discussion

Vaccination against AI has been widely practiced in some countries where infection is common in poultry. Vaccination may be routine or may be used as a means of emergency response during epizootics of HPAI (Karaca et al., 2005). Although inactivated whole-virus AI vaccines have shown efficacy in outbreaks of HPAI, they induce immune responses to the group-specific influenza A nucleoprotein (NP) antigen and thus render differentiation of vaccination from natural infection impossible (Boyle and Coupar, 1988). Many studies have been conducted that were aimed at the development of a novel vaccine strategy (Chamber et al., 1988; Kodihalli et al., 1994, 1999). Live virus vector vaccines provide a very promising vaccine strategy that avoids the above-described pitfall. Previous studies reported the rFPV–HA–NA virus could provide complete protection from homologous H5N1 and heterologous H7N1 HPAI virus challenge in SPF chickens (Qiao et al., 2003). And dose–response analysis was conducted by vaccinating SPF chickens with different doses of rFPV–HA–NA vaccine. Complete protection was induced by 10^2 PFU of the rFPV–HA–NA vaccine (Qiao et al., 2006).

In the present study, we carried out a single inoculation with the same dose of vaccine in SPF chickens by different administration routes. All the chickens inoculated by wing-web puncture, or by intramuscular or subcutaneous injection were completely protected from challenge. However, vaccination by intranasal or conjunctival inoculation failed to induce HI antibodies, and all the chickens vaccinated by these routes died following challenge. These results suggest that effective application of the rFPV–HA–NA vaccine in poultry may be restricted to the wing-web route, or to intramuscular or subcutaneous injection. Others have reported that the most effective and practical administration route for rFPV vaccine appears to be by wing-web puncture, and this is the route currently employed for commercial FPV vaccination (Boyle and Heine, 1994; Beard et al., 1992). In this study, we showed that intramuscular and subcutaneous injections were also effective inoculation routes for rFPV–HA–NA vaccination in addition to the wing-web route.

Challenge experiments were often conducted to test the immune efficacy of a vaccine. In this study, GS/GD/96 was chosen as a challenge virus. The protection was evaluated by death and virus shedding from cloacal swabs. All the chickens immunized with rFPV–HA–NA vaccine were protected against the challenge with GS/GD/96. HI antibodies play a key role in protection against AIV by neutralizing the infective ability of challenge viruses (Brown et al., 1992). For the whole-virus inactivated vaccine, the HI antibody level considered to be the cut-off for susceptibility is 4 log 2. However, this criterion may not be applicable to live virus vectored vaccines. In this study, HI antibodies induced by the rFPV–HA–NA vaccine at titers as low as 3 log 2 could provide complete protection. This result was consistent with report that recombinant vaccinia virus expressing the HA gene of AIV could induce complete protection against lethal challenge, accompanied by an HI antibody titer of 1–2 log 2 (Chamber et al., 1988). These results suggested that cellular immunity is also very important for the protection of chickens against AIV challenge. When the antibody level is insufficient to induce protection, cellular immunity can reduce the morbidity and mortality rates of immunized chickens to a certain extent, although it cannot protect chickens from infection with the challenge virus.

Recombinant FPV vectored vaccines have been shown to induce long-lasting immunity (Boyle and Heine, 1993). In America, a recombinant fowlpox–AI H5 vaccine (TROVAC-H5) expressing the HA of the A/turkey/Ireland/1378/83 H5N8 AI isolate has been used to control H5N2 low pathogenicity AI (Bublot et al., 2006). Bublot et al. (2007) reported that one injection of TROVAC-H5 protects chickens against AI-induced mortality and morbidity for at least 20 weeks. Our data from the present study show that a single inoculation of the rFPV–HA–NA vaccine could protect SPF chickens from lethal dose H5N1 virus challenge for at least 40 weeks. More importantly, the rFPV–HA–NA vaccine was capable of inducing strong antibody responses to the HA, which were detected as early as 1 week after vaccination. However, prior studies using different fowlpox recombinant-AI H5 viruses failed to produce a consistent antibody response (Beard et al., 1991; Swayne et al., 1997). Swayne et al. (2007) have showed recently that failure to produce consistent antibody response of fowlpox-H5 recombinant was due to the use in the HI test of an antigen heterologous to the HA gene inserted in the fowlpox vector; when an homologous antigen is used, consistent HI titers are detected. We hypothesize that one of reasons for the strong immune response was the greater strength of the promoter used in construction of the rFPV–HA–NA virus. Boyle et al. demonstrated that the strength of the promoter used for antigen expression determines the effective antigen dose and thus may influence the level of immune response generated (Boyle, 1992). During construction of the rFPV–HA–NA, the vaccinia virus promoter utilized in the previous studies was replaced by the FPV promoter LP2EP2. Another reason for the results obtained here was that the NA gene was co-expressed with the HA gene in the construction of rFPV–HA–NA. Results of our previous and other studies suggested that supplement of NA protein in the vaccination could offer the prospect of broader heterovariant immunity (Qiao et al., 2003; Johansson et al., 1989; Chen et al., 2000).

One concern about the application of the recombinant fowlpox vaccine is that the vaccine efficacy may be influenced if the chickens were preimmunized with fowlpox vaccine (Swayne et al., 2000). Our unpublished data indicated that the antibody response and the protective efficacy of the rFPV–HA–NA vaccine against H5N1 influenza viruses would not be impaired if it was administrated at the same time with the fowlpox vaccine (Qiao C., unpublished). In the present report, the data from the field study showed that the second dose of the rFPV–HA–NA vaccine administrated 18 weeks after the first dose induced significantly increase of the HI antibody against H5N1 influenza virus. These results suggest that if the application program of these fowlpox vaccine and recombinant vaccines were carefully planned, the negative influence of the fowlpox vaccine on its recombinant vaccines would be minimized.

The recombinant rFPV–HA–NA vaccine has several advantages in comparison with the inactivated vaccine and the recombinant NDV vaccine. The use of rFPV–HA–NA vaccine should prevent confusion between vaccinated birds and infected birds for surveillance purposes, which is a problematic issue with the use of whole-virus influenza vaccines, and also there is no residue problem resulting from the use of adjuvant in the inactivated vaccines. In comparison with the recombinant NDV vaccine (Ge et al., 2007), which is easy to be produced and applied, the recombinant rFPV–HA–NA vaccine induced much longer antibody duration in the vaccinated chickens. Furthermore, the recombinant rFPV–HA–NA vaccine could provide protection against both AI and fowlpox (Boyle and Coupar, 1988).

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. Though the rFPV–HA–NA vaccine could completely protect chickens from the challenge of GS/GD/96-like H5N1 AIV, it remains to be investigated if this vaccine can also provide sound protection to the viruses from other clades.

Acknowledgments

This study was supported by the Chinese National S & T Plan (2004BA519A01) and Chinese Science and Technology Development Program (863) (2001AA213041).

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