FULL PAPER *Immunology*

Baculovirus-Derived Hemagglutinin Vaccine Protects Chickens from Lethal Homologous Virus H5N1 Challenge

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ABSTRACT. Since outbreaks of highly pathogenic avian influenza (HPAI) in both human and poultry from 2003, it is critical to have effective vaccines. A cDNA fragment coding the entire hemagglutinin (HA) gene derived from an H5N1 strain (A/duck/China/E319-2/03) was cloned and expressed using the baculovirus system. Two weeks after receiving two doses of recombinant HA (rHA) vaccines, chickens develop high antibody response for hemagglutination inhibition (HI) at titer 7.2 $\log_2$. Challenge studies revealed that vaccinated chickens with HI titers greater than 3 $\log_2$ could have immunoprotection against the same HPAI H5N1 strain virus challenge through intranasal route. Additionally, HI titer of 5 $\log_2$ determined whether the live viruses could not be detected from oropharyngeal, cloacal discharge or in tissues. This result suggests that the rHA expressed from baculovirus system could be a candidate for the development of a safe and efficient subunit vaccine for HPAI (H5N1).

KEY WORDS: baculovirus expression system, H5N1, hemagglutination inhibition, highly pathogenic avian influenza (HPAI), subunit vaccine.

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Since 1997, the H5N1 avian influenza viruses have caused more than 241 fatalities in humans [World Health Organization, WHO; http://www.who.int/en/, Ref. date 30 April 2008]. Prior to 2003, outbreaks of H5N1 viruses in poultry occurred in Asian countries such as China, Thailand, Vietnam and Indonesia [World Organization for Animal Health, OIE; http://www.oie.int]. After 2005, the H5N1 viruses spread from Asia to Europe and Africa through migratory birds or poultry transportation [5, 9]. AI has become a global healthy issue of pandemic disease [3, 30]. Considering the increased risk in poultry industry, instituting a vaccination policy to control the increased number of outbreaks is therefore critical. Several countries including China, Indonesia and Vietnam have preliminarily used vaccines to control the H5N1 epidemic [OIE; http://www.oie.int, WHO; http://www.who.int/en/].

At present, the commercial available vaccines used for chickens were produced by traditional inactivated viruses derived from H5 and H7 viruses [2, 6, 14, 17, 32]. Subunit vaccines of avian influenza composed of hemagglutinin (HA) antigens have been evaluated with H5N2, H5N3, H1, H2, H3, H7 and H9 viruses [4, 11, 12, 20, 22, 29, 33]. Virus vectors such as Newcastle virus and vesicular stomatitis virus encoding influenza antigens are also used for poultry [8] and mammals [8, 13, 23]. These studies clearly indicated that the HA antigen, whether delivered via DNA-plasmid, inactivated virus or composed of subunit formulas, afforded complete protection in virus challenge assays [6, 8, 14, 17, 20]. In addition, subunit vaccines have the advantage to differentiate vaccinated animals from naturally infected animals [2, 14]. The development of a subunit vaccine against avian influenza for poultry may provide an opportunity for humans to control future avian influenza outbreaks.

This study was designed to determine the efficacy of HA subunit vaccine derived from an H5N1 virus. A recombinant (r) HA vaccine was established and tested by measuring the ability to induce antibody response, protection efficacy and viral shedding in vaccinated chickens following the virus challenge.

MATERIALS AND METHODS

Cloning and expression of the hemagglutinin protein: The entire hemagglutinin (HA) encoding region including nucleotides 29 to 1,765 (GenBank accession number: AY518362) with a deletion of the stop codon was amplified by superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and proofreading DNA polymerase (Invitrogen) from an HPAI H5N1 strain (A/duck/China/E319–2/03). This virus was isolated from smuggled Muscovy ducks in Kinmen Island [15]. The genotype of A/duck/China/E319-2/03 virus was classified as clade 2 [34]; and it is similar to the Z subtype which has dominated the outbreaks of avian influenza in China since 2003 [16, 28].

The HA gene was cloned into the pENTR/D-TOPO vector (Invitrogen), and fused into the BaculoDirect™ Baculovirus Expression System (Invitrogen) via homologous recombination. The HA gene was expressed in *Spodopetra frugiperda* Sf9 cells as an HA-V5-His fusion protein according to the manufacturer's protocol (Invitrogen).

Western blot analysis: Twenty microliters of supernatants

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collected from infected cell cultures were analyzed for the presence of the expected proteins via 4–12% SDS-PAGE gradient gels. Proteins were then transferred to nitrocellulose membranes, and non-specific binding sites on the membrane were blocked with 5% non-fat dry milk. The rHA protein was detected by two monoclonal antibodies (YY1 or anti-V5). The YY1 antibody is an H5N1-specific antibody which was produced in our lab. This specific antibody recognizes HA protein of A/duck/China/E319–2/03 virus, which possesses hemagglutination inhibition (HI) and virus neutralizing activities. The recognition of YY1 to rHA is conformation dependent; therefore, samples prepared for SDS-PAGE should be maintained under non-reducing conditions. In contrast, recognition of the V5-tag to rHA is efficient by reducing conditions. Anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Labs, West Grove, PA, U.S.A.) was then applied. The color development was performed using NBT/BCIP reagent (Pierce, Rockford, IL, U.S.A.).

Preparation of subunit vaccines: Two oil emulsion-based vaccines were prepared from baculovirus-expressed rHA antigens. The supernatant obtained from infected cells was chemically treated with 0.002 M binary ethylenimine (BEI) for 16 hr at 37°C to inactivate the baculovirus prior to vaccine preparation [1]. The experimental vaccines were formulated with various amounts of rHA ranging from 0.2 to 10 μg per dose. One of the vaccines based on the water-in-oil-in-water (W/O/W) one step multiple emulsion was processed by adding the antigen to Montanide ISA 206 (SEPPIC, Paris, France) in a ratio of 46:54 (v/v). Another vaccine comprised of water-in-oil (W/O) emulsion was prepared by adding the antigen to Montanide ISA 70 M VG (SEPPIC) in a ratio of 26:74 (v/v).

Animal experiment: *Vaccine dosage determination*: Specific pathogen free (SPF) white Leghorn chickens (4 weeks of age) were used in this challenge study. Forty eight SPF chickens divided into 6 vaccine groups were tested to determine the efficient concentration of vaccine with rHA. Five groups of chickens were vaccinated subcutaneously with 0.2, 1, 2, 5 and 10 μg of rHA formulated into the ISA 206 adjuvant, and negative control group was inoculated with phosphate buffered saline (PBS). Booster was repeated after two weeks. Chicken serum HI titers were then tested at 2, 4 and 7 weeks after the first vaccination.

Vaccine adjuvant selection: Antibody response induced by vaccine with different adjuvants was tested in two groups (n= 8). Each chicken had been vaccinated twice, each containing 10 μg of rHA unit formulated into either ISA 206 or ISA 70. Booster was repeated after two weeks. Chicken serum HI titers were then tested at 2, 4, 7 and 11 weeks after the first vaccination.

In each of the above experiments, microtiter system was used to determine serum HI titer. Serial two-fold dilutions of serum with 0.85% NaCl were performed. Then, an equal volume (50 μl) of 8 HA-unit inactivated H5N1 virus A/duck/China/E319–2/03 was added. One hr after incubation at 37°C, 50 μl of 0.5% chicken red blood cells were added to each well. HI titer was determined after 60 min incubation.

Challenge test: In the challenge study, vaccinated and control chickens were conducted in bio-safety level 3 (BSL-3) facility. Vaccinated chickens have two subgroups. One subgroup was assigned for determining the protection efficacy, and another subgroup was for virus shedding. In the vaccinated group, each bird was intranasally inoculated with $1 \times 10^{8.5}$ ELD_{50} of the A/duck/China/E319–2/03 virus.

To determine the protection efficacy, 18 vaccinated chickens with HI titers from 1 to 8 $\log_2$ and 3 control chickens were challenged. Mortality and clinical signs of infection were monitored daily for 14 days postinfection. In addition, to detect viral shedding, 24 vaccinated chickens with various HI titers and 3 control chickens were challenged, and then sacrificed at 2, 4 and 10 days. Samples were collected from oropharyngeal, cloacal excreta, trachea, liver, heart, spleen, intestine, bursa, kidneys, lungs and brain to examine whether live virus could be isolated from challenged chickens by embryonated eggs inoculation.

Statistical methods: The results of HI antibody response after vaccination were expressed as $\log_2$ mean titers ± standard error of the mean (SEM). For the antigen dosage analysis, means were compared using least significant difference (LSD) test in the general linear model (GLM) procedure of SAS version 9 (SAS Institute, SAS Circle, Cary, NC, U.S.A.). To compare the efficacy of various adjuvants used in vaccines, a *t*-test was conducted for analysis. A $P<0.05$ was considered statistically significant in all cases.

RESULTS

Properties of recombinant hemagglutinin (rHA): Three bands migrated as 70, 140 and 195 kDa were obtained from the rHA protein of recombinant baculovirus expression system. The target rHA protein is 70 kDa. The 140 and 195 kDa bands were similar to homodimers and homotrimers of the rHA protein (Fig. 1). The yield of rHA protein in the supernatant was determined via protein purification procedure with a Cobalt-column. Each fraction was subjected to Western blotting using an anti-HA monoclonal antibody (YY1). Protein concentrations were determined using Bicinchoninic acid (BCA) assay with bovine serum albumin as the standard. A concentration of 20 μg/m*l* in culture medium at 96 hr postinfection was measured.

Deglycosylation of the rHA protein with PNGase F led to the appearance of an approximately 58 kDa band (Fig. 1), demonstrating the function of glycosylation in Sf-9.

Antibody responses and duration of immunizations: The rHA protein obtained from infected culture medium was formulated into adjuvants without further purification. Various rHA units in each dosage ranging from 0.2 to 10 μg formulated into ISA 206 adjuvant were evaluated with 2 vaccine doses. Antibodies elicited by each vaccine were evaluated with HI assay. Greater than 5 μg of the rHA unit per vaccine dose could induce a significant HI response,

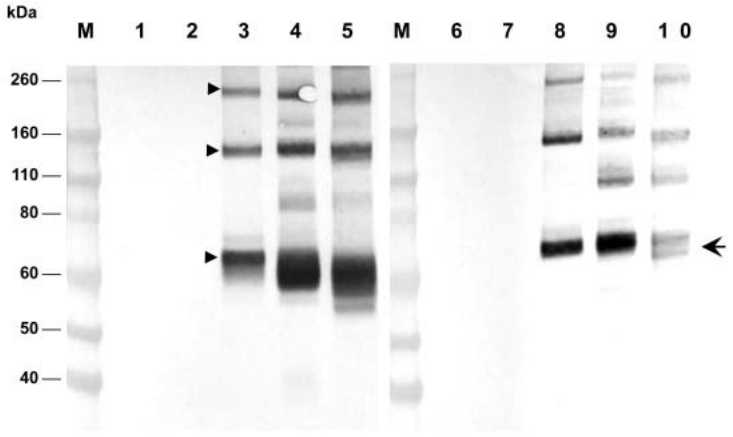

Fig. 1. Western blot result to detect recombinant HA protein from a recombinant baculovirus system. Supernatant (20 μl) (lanes 3 and 8), cell pellet (2×10^4 cells) (lanes 4 and 9) and cell pellets (2×10^4 cells) treated by PNGase F (lanes 5 and 10) from infected Sf9 cells were separated by non-reducing SDS-PAGE. In addition, supernatant (lanes 1 and 6) and cell pellet (lanes 2 and 7) from uninfected cell culture are also used as the control. After transferring to nitrocellulose, lanes 1 to 5 were treated with the YY1 MAb (1:500), and lanes 6 to 10 were treated with the anti-V5 MAb (1:1,000). M indicates the marker. The arrow heads (▶) indicate different types of HA, and the arrow indicates deglycosylated form.

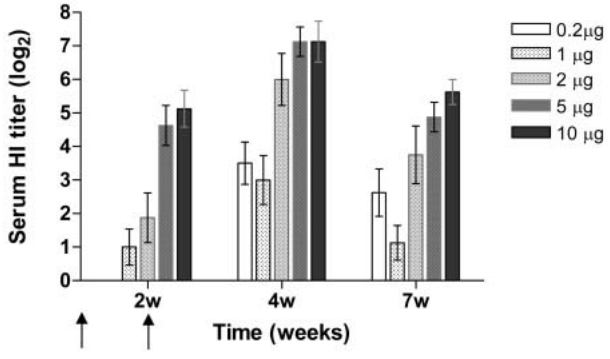

Fig. 2. The HI antibody response in chickens induced by rHA subunit vaccines containing various doses of antigen. Chickens were vaccinated via subcutaneous injection at weeks 0 and 2 with rHA. Arrows indicate the time points for immunization.

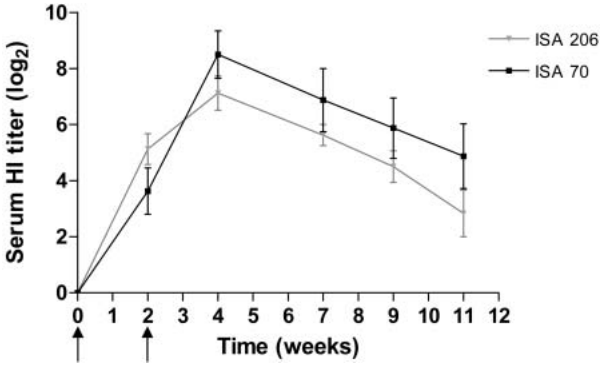

Fig. 3. Evaluation of the duration of serum HI antibody response induced by vaccines formulated with ISA 206 and ISA 70 adjuvants. Chickens were vaccinated via subcutaneous injection at weeks 0 and 2 with 10 μg rHA. Arrows indicate the time points for immunization.

with an average titer of 4.7 $\log_2$ (P<0.05) at 14 days after the first vaccine dose. In chickens that received two doses, each 2 μg of rHA, resulted in a significant HI response with an average titer of 6 $\log_2$.

To compare the efficacy of vaccination based on different adjuvants, vaccines containing 10 μg of rHA unit formulated into ISA 206 (W/O/W) and ISA 70 (W/O) adjuvants were evaluated. At the first vaccination, the adjuvant formulated with ISA 206 had a higher HI response than that of ISA 70. Whereas chickens received two doses with ISA 70 had an average titer of 8.5 $\log_2$ (Fig. 3) at 2 weeks post-vaccination which was maintained at 4.9 $\log_2$ for the entire 9 weeks follow up. The titer with ISA 206 was 7.2 $\log_2$ at 2 weeks post-vaccination, but this titer gradually decreased to 2.9 $\log_2$ at 11 weeks (Fig. 3).

Vaccine efficacy evaluated by H5N1 virus challenge: The rHA vaccinated chickens with serum HI titers ≥ 3 $\log_2$ were completely protected against virus challenge (Table 1). Three vaccinated birds with HI titers between 1 and 2 $\log_2$ demonstrated mild clinical signs including decreased appetite and lethargy. These birds did however fully recover from day 5 postchallenge. In contrast, all control chickens showed severe clinical signs and died between days 2 and 4 following infection.

In terms of viral shedding, live viruses from the oropharyngeal and cloacal excreta were not isolated in chickens with HI titers > 2 $\log_2$ (Table 2). Further, inoculation of homogenized tissue samples from 9 different organs of each infected chicken into chicken embryos showed that live

Table 1. The protection efficacy measured by serum HI titers after rHA subunit vaccination

Serum HI titer (log_2) range	Clinical signs/ No. of birds	Mortality/ No. of birds
1*, 1*, 2*, 2, 3, 3	3/6	0/6
4, 4, 5, 5, 6, 6	0/6	0/6
7, 7, 7, 8, 8, 8	0/6	0/6
0, 0, 0	3/3	3/3

* Indicates the mild clinical signs.

viruses were not detected in tissues from birds with HI titers ≥ 5 log_2 (Table 2).

DISCUSSION

The available evidence indicates that the immunogenicity of hemagglutinin (HA) is an attractive candidate for a subunit vaccine [4, 22, 29], and which has a benefit without handling the highly pathogenic H5N1 viruses. In addition, HA subunit vaccines permit one to discriminate between birds that have been infected and birds that have been vaccinated based on serum antibody panels [2, 14]. Therefore, we developed a rHA subunit vaccine derived from an H5N1 virus (A/duck/China/E319–2/03) belonging to the clade 2 [34] as a backup vaccine for H5N1 viruses.

Proteins expressed from baculovirus system provide an eukaryotic environment that generally engenders similar protein conformation and modification for the biological activity [10, 19]. Our laboratory has characterized the entire rHA protein, whilst maintaining hemagglutinating activity. This protein forms different molecular sizes in gel analysis similar to dimer and trimer complexes in solution and is recognized by a conformation-dependent monoclonal antibody, which is indicative of its true conformation. Although the rHA protein was released into culture medium in the supernatant with a level of 20 μg per m*l*, a substantial amount of protein was found to be associated with cell debris as determined by Western blotting.

Previous studies based on comparison with the H1 and H3 antigens suggested that the rHA vaccines from H5 viruses are less immunogenic than the inactivated vaccines or have a reduced immunogenicity when prepared from mammalian species[18, 24, 27]. We also found that chickens which received one dose of rHA-H5 vaccine with less than 5 μg of antigen had a markedly poorer response (Fig. 2). Priming by 2 dosages containing 2 μg per dose appears to improve the antibody response. Moreover, the vaccinated birds with HI titers lower than 2 log_2 may fail to prevent the clinical signs in infected chickens. Serum HI titers lower

Table 2. Viral replication and shedding in vaccinated chickens challenged by H5N1 virus

Bird number	HI titer (log_2) prior to challenge	Virus shedding at 2 and 4 days postchallenge		Virus isolation in tissues from 9 organs (Days post challenge)
		Oral	Cloacal	
A40	8	–	–	0/9 (D2)
A38	8	–	–	0/9 (D4)
A45	8	–	–	0/9 (D10)
A39	7	–	–	0/9 (D2)
A23	7	–	–	0/9 (D2)
A27	7	–	–	0/9 (D4)
A18	7	–	–	0/9 (D10)
A20	6	–	–	0/9 (D2)
A35	6	–	–	0/9 (D10)
A34	5	–	–	0/9 (D2)
A28	5	–	–	0/9 (D4)
A32	4	–	–	1/9 (D2)
A6	4	–	–	0/9 (D4)
B40	3	–	–	0/9 (D2)
A3	3	–	–	0/9 (D4)
A13	3	–	–	0/9 (D10)
B30	2	–	–	4/9 (D2)
A17	2	–	–	0/9 (D4)
A9	2	–	–	1/9 (D10)
A1	1	–	–	0/9 (D2)
B15	1	–	–	2/9 (D2)
A4	1	–	+	5/9 (D4)
B43	0	–	+	7/9 (D2)
B4	0	+	+	5/9 (D10)
C10	0	–	+	4/9 (D2)
C4	0	+	+	7/9 (D4)
C7	0	+	+	7/9 (D4)

- Each vaccinated birds was challenged with $10^{8.5}$ ELD_{50}.
- Organ samples were collected from trachea, lungs, liver, kidneys, small intestine, brain, bursa, spleen and heart.

than 5 $\log_2$ was unable to cease viral replication in tissues.

ISA 206, a W/O/W emulsion, resulted in a rapid antibody response, but only short antibody duration. However, ISA 70, a W/O emulsion, appeared to induce a delayed type immune response but longer duration of antibody response. As a result, one may conclude that the immune efficacy of the subunit vaccine might be improved by increasing vaccination frequency, antigen units, or the use of different adjuvants [18, 21].

The final question that we explored was whether the virus could continue to replicate in vaccinated birds. Several studies have concluded that most H5N1 vaccines based on inactivated whole viruses, lysed viruses, HA-DNA or recombinant HA antigens were capable of affording completely protection against the virus as well as a significant reduction in viral replication. Nonetheless, these vaccines failed to provide sterilizing immunity [4, 6, 21, 24–26, 31]. Whereas, our study similar to two H5 subunit vaccines evaluated in birds [4, 24] points out the potential to completely prevent viral replication in tissues depending on serum HI titers following vaccination (Table 2). The differences among those studies of HA subunit vaccines for stopping viral replication in birds seem to strongly associate with the challenged viruses that HA vaccines have potential to eliminate viral replication from homologous virus challenge [4, 24]. In addition, it appears that the HI titer of 5 $\log_2$ is a threshold for eliminating viral replication in tissues as determined by using the inoculation of embryonated chicken eggs, a method with the greatest sensitivity to recover the live virus particles. High dosages of antigen from inactivated H5N2 virus also have the capacity to arrest viral replication [7, 32]. These results suggest that subunit vaccines can decrease viral replication.

Between 2003 and 2005, the HPAI H5N1 has genetically diverged into 2 distinct clades in Asia [34], and presents minor differences in antigenicity based on the serum cross-reactions. The A/duck/China/E319–2/03 virus that we used to develop the subunit vaccine was tightly clustered within clade 2 [34], which is closely related (genetically) to the majority of viruses identified in Indonesia and China during 2003 and 2004. At present, it is unclear whether the serum antibodies induced by vaccinating birds with a monovalent rHA antigen would afford broad protection to heterologous H5N1 viruses [8]. Interestingly, the YY1 monoclonal antibody produced from A/duck/China/E319–2/03 virus exhibits high cross-reactivity and virus-neutralization to an influenza virus (NIBRG14-M4), bearing mutant H5-HA, intact NA (A/VN/1194/04; a clade 1 virus) and the internal genes from PR8 (A/Puerto Rico/8/34; H1N1) (data not shown). The YY1 antibody also exhibited high neutralization to a heterotypic H5N3 virus, but not to the H5N2 viruses isolated from Taiwan (data not shown). Although these interpretations were only based on one monoclonal antibody (YY1) without including complete epitopes involved in virus neutralization and hemagglutinin activity, parts of epitopes may be well conserved and maintain sufficient capacity to induce protective antibodies for heterologous viruses. Moreover, the inactivated vaccine based on H5N2 (A/CK/Mexico/232/94) was an efficient vaccine against H5N1 viruses under field conditions in Hong Kong [6]. Also, an inactivated H5N3 vaccine was also shown to protect birds against H5N1 viruses [17].

To conclude, this study supports that the HA subunit vaccine may be sufficient for broadly protecting chickens or beneficial as a backup vaccine for H5N1 viruses. Our results also suggest that subunit vaccines may be considered in high risk areas or regional control areas to prevent virus transmission or can be used to protect the poultry industry from a future mass outbreak.

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