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Vaccination with hemagglutinin produced in *Trichoplusia ni* larvae protects chickens against lethal H5N1 challenge

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5N1 viruses in birds and coincident infections in humans have created demand for a cost-effective vaccine to prevent a pandemic of the disease. We report here that *Trichoplusia ni* (*T. ni*) larvae can act as a cost-effective bioreactor to produce recombinant HA5 (rH5HA) proteins as an effective vaccine for chickens. Western blot analysis revealed that the 70 kDa rH5HA protein and partially cleaved products (40 kDa rH5HA1 and 28 kDa rH5HA2) were generated in *T. ni* larvae infected with recombinant baculovirus carrying the H5HA gene. We demonstrated that a single intramuscular injection of homogenates of *T. ni* larvae containing rH5HA proteins into chickens could induce the production of antibodies with hemagglutination inhibition (HI) capability and stimulate neutralization activity against a lethal H5N1 influenza virus challenge. Furthermore, each vAc-HA5-infected *T. ni* larva can vaccinate approximately 20–36 chickens and induce with HI titer values of approximately 5.5 $\log_2$ after 7 weeks implied that this single-shot protocol should be economical and provide adequate protection. These data suggest that the baculovirus-larvae recombinant protein expression system could be a cost-effective platform for vaccine production.

1. Introduction

Influenza is a highly contagious acute respiratory disease caused by viruses belonging to the *Orthomyxoviridae* family. Influenza viruses are single-stranded ribonucleic acid (RNA) viruses spiked with two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), in a lipid-containing envelope. Since 1997, H5N1 avian influenza viruses (AI) have caused more than 262 fatalities in humans [World Health Organization, WHO; http://www.who.int/en/, Ref. date 4 September 2009]. Prior to 2003, outbreaks of H5N1 viruses in poultry occurred in Asian countries such as China, Thailand, Vietnam and Indonesia. After 2005, H5N1 viruses were transferred via migratory birds or poultry transportation and spread from Asia to Europe and Africa (Ducatez *et al.*, 2007; Gilbert *et al.*, 2006). At present, many countries in Asia, Europe and Africa have reported outbreaks of the highly pathogenic avian H5N1 influenza virus among poultry flocks. H5N1 has thus caused alarm for global human health and the poultry industry. Vaccines for H5N1 are critical for preventing, or at least limiting, potential H5N1 pandemic influenza outbreaks.

The use of licensed technologies such as inactivated (Treanor *et al.*, 2006) or attenuated viral vaccines (Lu *et al.*, 2006) is the most expeditious means of generating H5N1 vaccines. However, there are several practical and scientific challenges to the development of H5N1 vaccines using the traditional egg-based method (Wang *et al.*, 2006). These include the need for high containment facilities; the high pathogenicity of wild-type H5N1 influenza viruses, which results in reduced yields of candidate vaccine viruses in fertilized eggs as compared to yields of human influenza viruses; and the limited manufacturing capacity (Wang *et al.*, 2006). Therefore, alternative vaccine production strategies such as DNA vaccines, adenovirally expressed HA and recombinant antigens purified from baculovirus-infected insect cells have been explored (Subbarao and Luke, 2007). Of these approaches, the baculovirus-insect cell expression system is a promising candidate, as rHA influenza

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vaccines produced using this approach have been tested in several Phase I and Phase II human clinical trials and were found to be safe, immunogenic and efficient (Wang *et al.*, 2006).

Although cultured insect cells have been recognized as an excellent platform for the production of recombinant proteins (Kost *et al.*, 2005; Possee, 1997), limitations associated with culturing insect cells on a large scale present the major bottle-neck for the commercial production of recombinant proteins (Kovaleva *et al.*, 2009). Thus, many researchers have demonstrated that recombinant Autographa californica multiple nucleopolyhedrovirus (AcMNPV) can produce recombinant proteins in insect larvae, like cabbage looper, *Trichoplusia ni* (*T. ni*) larvae (Hale *et al.*, 1999; Medin *et al.*, 1990; Price *et al.*, 1989) and other lepidopteran hosts. In addition, for production of vaccines for the poultry industry, economic factors are also important issues. We also introduced an aerosol inoculation strategy that can efficiently infect *T. ni* larvae (Jinn *et al.*, 2009). This novel baculovirus aerosol infection strategy could facilitate the use of baculoviruses to produce recombinant proteins in insect larvae.

In previous studies, we employed baculovirus-infected insect cells to produce rH5HA vaccines that could protect chickens from lethal HPAI virus challenges (Lin *et al.*, 2008). However, the cost of producing rH5HA vaccines by insect culture system for poultry flocks was not economical. In the present study, we aimed to produce rHA vaccines in *Trichoplusia ni* (*T. ni*) larvae.

2. Materials and methods

2.1. Cells, viruses, and transfection

The *Spodoptera frugiperda* IPBL-Sf9 (Sf9) cell line was cultured in TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum (Chen *et al.*, 2005). Sf9 monolayers were used for virus propagation. All viral stocks were prepared and titers were determined according to standard protocols described by O'Reilly *et al.* (1992). For transfection, Cellfectin™ (Invitrogen) was used according to the protocols provided by the manufacturer.

2.2. Preparation of recombinant baculovirus with hemagglutinin gene

The A/duck/China/E319–2/03 virus was isolated from smuggled Muscovy ducks in Kinmen Island (Lee *et al.*, 2007). The virulence of this virus in chickens indicates that it is highly pathogenic. The genotype of the A/duck/China/E319–2/03 virus was classified as being in clade 2 and was found to be similar to the Z subtype, which has dominated avian influenza outbreaks in China since 2003 (Lee *et al.*, 2007). The entire hemagglutinin (HA) coding region, including nucleotides 29–1765 (GenBank accession number: AY518362) with a deletion of the stop codon, was amplified from the A/duck/China/E319–2/03 virus and cloned into the pENTR/D-TOPO vector (Invitrogen). The HA gene was directly transferred into BaculoDirect linear DNA (Invitrogen) with homologous recombination and transfected into Spodopetra frugiperda Sf9 cells. The recombinant baculovirus expressing the HA protein was fused to the V5 and 6xHis epitope tags. The V5 epitope tag facilitates the identification of the recombinant baculovirus that can produce the rH5HA proteins. The successfully identified recombinant viruses were named vAc-HA5.

2.3. Insect larvae and virus inoculation

Fourth instar *Trichoplusia ni* (*T. ni*) were used to express rH5HA to generate vaccines against H5N1 influenza in chickens. *T. ni* larva were reared under specific pathogen free (SPF) conditions and were provided by the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTSRI), Council of Agriculture (Taichung, Taiwan). There are an isolated compartment and automated procedure to maintain *T. ni* larva with an artificial diet in TACTSRI. The artificial diet contained 0.1% sorbic acid and p-methyl-benzoate for antibacterial and antifungal purposes. The insects were reared at 25 ± 1 °C with 70 ± 5% relative humidity and a 14:10-h (light:dark) photoperiod. Pupae and eggs were washed for 10–15 min in 4% formalin solution and then rinsed for 15 min with water, followed by air drying.

T. ni larva were infected with 1×10^9 pfu/ml of recombinant baculovirus vAc-HA5 using the aerosol method as reported previously (Jinn *et al.*, 2009). After infection, larvae were fed regularly with a fresh non-contaminated diet. The infected larvae were collected at 96 h post-infection (hpi) and frozen at −20 °C until being used. The frozen larvae were thawed and homogenized in phosphate-buffered saline (PBS) with a pH of 7.2. The weight of larvae:volume of PBS was 1:10 and then homogenized with Glass/Teflon Potter Elvehjem homogenizers (30 ml). The homogenate was centrifuged at $10{,}000 \times g$ for 15 min at 4 °C and the supernatant was then further clarified using a 0.22 μm filter membrane. The total soluble proteins were obtained from infected larvae and stored at −80 °C. The identity of the samples was determined by SDS-PAGE and Western blot analysis.

2.4. Western blot

The homogenate expressed from the *T. ni* larvae system and the supernatant collected from infected cell cultures were analyzed for the presence of the expected proteins via 4–12% SDS-PAGE gradient electrophoresis and followed by trans-blotting onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and antigens were detected by two monoclonal antibodies (YY1 or anti-V5). The YY1 antibody is an H5N1-specific antibody that possesses hemagglutination inhibition (HI) and virus neutralizing activities. The recognition of rH5HA by YY1 is conformation-dependent; therefore, samples prepared for SDS-PAGE should be maintained under non-reducing conditions. In contrast, recognition of the rH5HA C-terminal by the V5-tag is efficient under reducing conditions. Anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Labs, West Grove, PA, U.S.A.) and color development with NBT/BCIP reagent (Pierce, Rockford, IL, U.S.A.) was then applied. The rH5HA protein was purified with a cobalt column and concentrations were determined with bicinchoninic acid (BCA) assays using bovine serum albumin as the standard.

2.5. Preparation of subunit vaccines and animal experiments

Specific pathogen free (SPF) white Leghorn chickens (4-week old) were used in this study. Fifteen SPF chickens divided into two vaccination groups and control group. The homogenate derived from infected *T. ni* larvae and supernatant obtained from Sf-9 were chemically treated with 0.1% formalin for 16 h at 37 °C to deactivate the baculovirus prior to vaccine preparation. In our previous study, we had compared the efficacy of vaccination for chicken based on different adjuvants, formulated with ISA 206 (W/O/W) and ISA 70 M VG (W/O). The result indicated theat ISA 206 induced a rapid antibody response, but only short antibody duration. However, ISA 70 M VG appeared to induce a delayed type immune response but longer duration of antibody response (Lin *et al.*, 2008). Thus, the experimental vaccines were formulated with 10 μg of rH5HA per dose in which comprising water-in-oil (W/O) emulsion was prepared by adding the antigen to Montanide ISA 70 M VG (SEPPIC, Paris, France) using a ratio of 26:74 (v/v), and the negative control group was inoculated with phosphate-buffered saline (PBS). The booster was repeated after 2 weeks. Chickens of all groups were observed for 6 weeks after vaccination and the

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following parameters were evaluated: general or local reactions attributable to the vaccine, the number of animals with clinical signs, and the number of losses. In addition, blood samples were drawn and serum HI titers were tested at 2, 4 and 6 weeks after the first vaccination.

The routine necropsy was performed at the 14 days after vaccination. There was no obvious change observed at the injection site. Furthermore, no toxic or allergy reactions were notified during immunization period. Chickens of all groups were observed for 6 weeks after 1st vaccination and the following parameters were evaluated: general or local reactions attributable to the vaccine, the number of animals with clinical signs, and the number of losses. In addition, blood samples were drawn and serum HI titers were tested at 2, 4 and 6 weeks after the first vaccination. To determine the HI titers, 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 hour 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.

In the challenge study, vaccinated and control chickens were evaluated in a bio-safety level 3 (BSL-3) facility. In the vaccinated group, each chicken was intranasally inoculated with $1 \times 10^{8.5}$ ELD_{50} of the A/duck/China/E319–2/03 virus. Mortality and clinical signs of infection were monitored daily for 14 days post-infection.

3. Results and discussion

The insect larvae of baculovirus hosts such as the cabbage looper *T. ni* may be explored as an alternative to cell culture for cost-effective recombinant protein production (Hale *et al.*, 1999; Medin *et al.*, 1990). To produce hemagglutinin from *T. ni* larvae, we first constructed the recombinant baculovirus vAc-HA5, which contains the hemagglutinin (HA) gene derived from an H5N1 strain (A/duck/China/E319–2/03). In vAc-HA5, the expression of H5HA is controlled by the very late strong polyhedron promoter (Fig. 1). Recombinant H5HA produced by vAc-HA5 possesses the V5 and His tags at the C-terminal. The V5 tag allows the expression of recombinant H5HA proteins to be monitored by Western blot probed with anti-V5 antibodies. H5HA is a trimeric protein; we showed that rH5HA proteins produced in Sf9 cells appear as ladder bands after non-reducing SDS-PAGE (Lin *et al.*, 2008). To facilitate analysis of recombinant HA production, YY1 monoclonal antibodies were used as probes. The YY1 antibody can recognize the

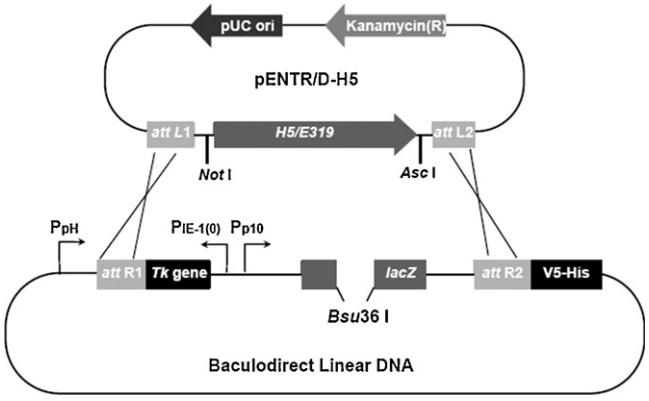

Fig. 1. Generation of the recombinant baculovirus vAc-HA5. The entire hemagglutinin (HA/E319) gene coding region with a deletion of the stop codon was cloned into the PENTR/D-H5 entry clone vector. The HA/E319 was transferred into BaculoDirect Linear DNA and the C-terminal was fused with an H5-V5-His fusion tag using an *in vitro* homologous recombination reaction (LR reaction). The resulting bacmids were then transfected into insect cells and recombinant baculoviruses were selected with Gancyclovir.

HA protein of the A/duck/China/E319–2/03 virus, which shows hemagglutination inhibition (HI) and virus-neutralizing activities (Lin *et al.*, 2008). Thus, the use of YY1 can allow samples to be prepared for SDS-PAGE under non-reducing conditions. We found that the vAc-HA5 *T. ni* larvae produced three bands that migrated at 70, 140 and 195 kDa as in vAc-HA5-infected Sf9 cells. In contrast, uninfected *T. ni* larvae did not produce these recognized protein bands when probed with YY1 monoclonal antibodies by Western blot (Fig. 2A). The H5HA protein contains 568 amino acids with a predicted molecular weight of approximately 64 kDa. The 70 kDa protein may therefore represent the monomeric and glycosylated H5HA protein produced in *T. ni* larvae. The 140 and 195 kDa bands would thus be homodimers and homotrimers of the H5HA protein (Fig. 2). This result indicates that the rH5HA proteins produced in *T. ni* larvae are similar to those produced in Sf9 cells under non-reducing SDS-PAGE. To examine the yields of rH5HA proteins produced in *T. ni* larvae, quantitized rH5HA proteins (360, 36 and 3.6 ng) purified from vAc-HA5-infected Sf9 cells were analyzed by Western blot analysis and probed with two monoclonal antibodies, YY1 and anti-V5 (Fig. 2B and C). The rH5HA proteins produced were estimated at approximately 360 μg per *T. ni* larvae. The yield of rH5HA per larvae was about the same as that produced from 20 ml cultured Sf9 insect cells infected with vAc-HA5. Thus, if 1000

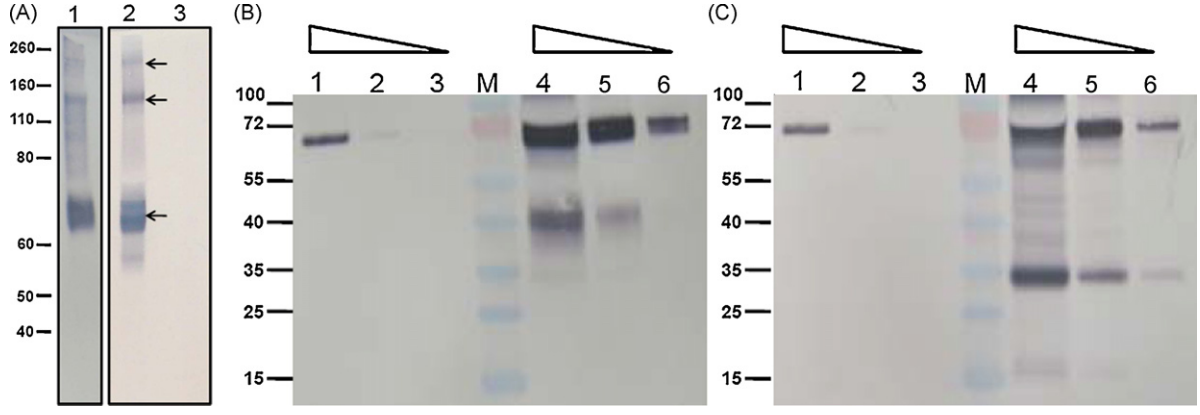

Fig. 2. Expression of recombinant HA protein in *T. ni* larvae. (A) Western blot of recombinant HA protein from infected Sf-9 cells (lane 1) and the homogenate derived from infected and control *T. ni* larvae (lanes 2 and 3). Detection with H5-specific mAb: YY1 MAb (1:500). (B) and (C) Determination of the protein concentration of rH5 derived from *T. ni* larvae. Purified rH5 derived from Sf-9 was used as a standard. Lane 1, 360 ng; lane 2, 36 ng; lane 3, 3.6 ng. Homogenates of infected *T. ni* were serially diluted and compared with the standards. Lane 4, 10 μl; lane 5, 1 μl; lane 6, 0.1 μl. Recombinant rH5HA proteins were probed with anti-H5 mAb YY1 (B) and with ant-V5 mAb (C). M indicates the molecular weight marker.

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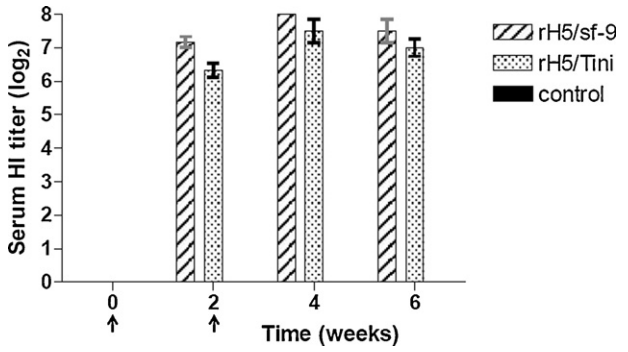

Fig. 3. Evaluation of the duration of serum HI antibody response induced by rH5HA derived from Sf9 cells and *T. ni* larvae. 10 μg purified rH5HA proteins produced from Sf9 cells and 1000 μl*T. ni* larvae homogenates were formulated with ISA 70 adjuvants and were used to vaccinate one chicken. Chickens were vaccinated and boosted via subcutaneous injection at weeks 0 and 2, and arrows indicate the timing of immunization.

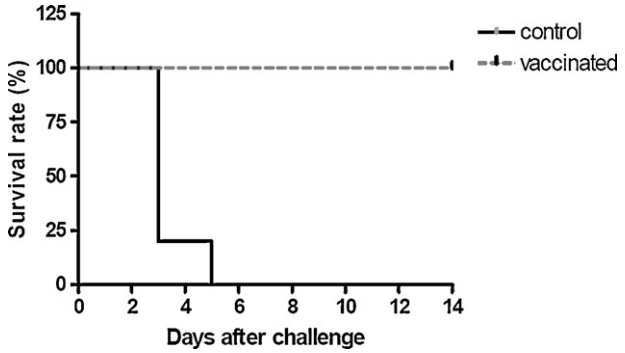

Fig. 4. Survival rates of chickens after challenge with the A/duck/China/E319–2/03 virus. Chickens were vaccinated intramuscularly with rH5HA subunit vaccines derived from *T. ni*, while PBS was used for control chickens. All chickens were challenged intranasally with $1 \times 10^{8.5}$ ELD_{50} of the A/duck/China/E319–2/03 virus. Survival rates and clinical signs were observed every 24 h for 14 days.

T. ni larvae were used, the yield of rH5HA proteins would be approximately 20 l of cultured Sf9 insect cells. This would not only be cost-effective, but would also avoid the complex scale-up required for insect culture processes. Furthermore, the larvae can be homogenized and centrifuged to obtain rH5HA proteins that can be used directly for vaccinating chickens, without requiring that a large volume of insect cells be homogenized and without complex cell lysis processes.

Interestingly, in addition to the major 70 kDa rH5HA proteins, we also found a 40 kDa rH5HA1 band when probing with YY1 monoclonal antibodies (Fig. 2B) and a 28 kDa rH5HA2 band when probing with anti-V5 monoclonal antibodies (Fig. 2C). This result indicates that rH5HA proteins were also spontaneously cleaved in vAc-HA5-infected *T. ni* larvae similarly to when rH5HA proteins were expressed in Sf9 insect cells (Lu *et al.*, 2007; Shen *et al.*, 2008; Wang *et al.*, 2006). However, the molecular weight of rH5HA1 generated in *T. ni* larvae was approximately 40 kDa, which is in contrast to the previous finding that 50 kDa rH5HA1 was produced in Sf9 cells (Shen *et al.*, 2008; Wang *et al.*, 2006). The limited deglycosylation of rH5HA produced in Sf9 cells resulted in seven distinguishable rHA1 bands ranging from 50.9 to 39.2 kDa as revealed by treatment with the deglycosylation enzyme PNGase F (Wang *et al.*, 2006). Thus, similarity of the 40 kDa rH5HA1 in *T. ni* larvae to the complete deglycosylation product implies that the glycosylation of rH5HA in *T. ni* larvae was less extensive than in Sf9 cells. Interestingly, rH5HA2 appeared in the Western blot probed with anti-V5 but not YY1 monoclonal antibodies, and rH5HA1 appeared in the Western blot probed with YY1 but not with anti-V5 monoclonal antibodies. These results indicate that the epitopes recognized by the monoclonal antibody YY1 were located in the N-terminal portion of H5HA.

To test whether the rH5HA produced by the *T. ni* larvae can act as a cost-effective vaccine for poultry, the homogenates of one *T. ni* larva infected with vAc-HA5 were used to vaccinate six SPF chickens. The rH5HA was formulated into ISA 70 adjuvant without further purification. Antibodies elicited by the homogenates were evaluated with HI assays and compared with rH5HA produced from Sf9 cells. Fig. 3 shows that the HI antibody response in chickens induced by rH5HA prepared either from Sf9 cells or from *T. ni* larvae was similar at approximately 6–8 $\log_2$. The sera of chickens vaccinated with homogenates of *T. ni* larvae without infected vAc-HA5 did not produce HI antibodies. Meanwhile, the homogenates of *T. ni* larvae did not cause side effects or allergies in the vaccinated chickens. In previous studies, we demonstrated that vaccination with rH5HA produced in Sf9 cells resulted in HI titers $\geqq 3$ $\log_2$ in chickens and could completely protect against virus challenges (Lin *et al.*, 2008). Thus, we challenged the vaccinated birds with the H5N1 virus to evaluate the efficacy of vaccines formulated from homogenates of *T. ni* larvae infected with vAc-HA5. Birds that received the larval rH5HA vaccine was shown to generate resistance to the lethal H5N1 virus challenge, however, all of the control chickens showed severe clinical signs and died between 2 and 4 days after the virus challenge (Fig. 4). And each chickens of the challenged groups showed a rapid booster of antibody response with an average titer of 9.2 $\log_2$ ($P < 0.05$) at 14 days after H5N1 challenge. Furthermore, the sera t Avian Influenza Virus *Plus* Antibody Test Kit (Synbiotics®) were positive. This result implied that nearly all birds were received the challenged viruses. To further confirm that the homogenates of *T. ni* larvae infected with vAc-HA5 could be an economical vaccine for chickens, we vaccinated the chickens once with homogenates containing approximately 10 μg rH5HA, or approximately 50 μl of the homogenates. Under this protocol, each vAc-HA5-infected *T. ni* larva can vaccinate approximately 20–36 chickens. We found that HI titers in vaccinated chickens peaked at an average titer value of 7 $\log_2$ after 4 weeks and with HI titer values of approximately 5.5 $\log_2$ after 7 weeks. Chickens with HI, titers $\geqq 3$ $\log_2$ could completely protect against virus challenges (Lin *et al.*, 2008), and this single-shot protocol should provide adequate protection. Besides, in our previous study, we have elucidated the relationship between HI titers and virus shedding when challenged birds with the rH5HA proteins produced from insect Sf9 cells (Lin *et al.*, 2008). Live viruses from the oropharyngeal and cloacal excreta were not isolated in chickens with HI titers >2 $\log_2$. Further, inoculation of homogenized tissue samples from 9 different organs of each chicken received vaccination and virus challenge into chicken embryos showed that live viruses would not recovered from birds with HI titers $\geqq 5$ $\log_2$ (Lin *et al.*, 2008). In this experiment, the HI antibody response in chickens induced by rH5HA prepared from *T. ni* larvae was approximately 6–8 $\log_2$. According these results, we suggested the HA subunit vaccine produced form *T. ni* larvae may hugely reduce the virus shedding.

To further explore the rH5HA prepared from *T. ni* larvae could be an economical vaccine for chickens, avian influenza vaccine based on H5N2 virus (A/CK/Mexico/232/94 strain) (Avimex laboratories, Mexico) was purchased and compared with the rH5HA subunit vaccine. After receiving two dosages, the rH5HA vaccinated birds had a higher HI response (HI titers $\geqq 6$ $\log_2$) than the inactivated H5N2 vaccine (HI titers $\geqq 5$ $\log_2$). This study supports that the HA subunit vaccine may offer a broadly protection for chickens or used as a backup vaccine for H5N1 viruses. Thus, our results also suggest that H5 subunit vaccines can be considered in a high risk area or

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regional control area to decrease virus transmission or is considered to protect the poultry industry from a mass outbreak. Furthermore, development of oral-administered vaccines to be used for the vaccination of poultry is potentially necessity because the vaccination process of individual bird is time-consuming and costly. Previous studies have shown that chickens were vaccinated through the oral route (particularly via feed) result into protective antibody response after multiple vaccinations (Spradbrow and Samuel, 1991). In addition, oral deliveries of encapsulated recombinant H5 HA or without adjuvant were effective way to prime the immune system against H5N1 infection in mice (Amorij *et al.*, 2007; Prabakaran *et al.*, 2010). For chicken can feed with insect larvae directly, it is possible to develop an oral-administered vaccine based on these larvae and this will also avoid the extraction of HA proteins from *T. ni* larvae. Hence, we will evaluate the efficacy of immune response of oral administer of the vAc-HA5-infected *T. ni* larvae to the chicken in the future.

4. Conclusion

Our data suggest that the baculovirus-based larval recombinant protein expression system could provide a cost-effective vaccine to prevent outbreaks of highly pathogenic avian influenza. *T. ni* larvae can be considered a safe and cost-effective means of producing vaccines for chicken poultry as well as an inexpensive but highly productive bioreactor for subunit vaccine production. To further reduce the costs of virus production, we plan to generate the vAc-HA5 recombinant virus in *T. ni* larvae in the future. The establishment of this baculovirus and associated recombinant proteins as a *T. ni* larval production platform will facilitate the development of economical vaccines for poultry.

References

Amorij, J. P., T. A. Westra, W. L. Hinrichs, A. Huckriede, and H. W. Frijlink, "Towards an Oral Influenza Vaccine: Comparison between Intragastric and Intracolonic Delivery of Influenza Subunit Vaccine in a Murine Model," *Vaccine*, **26**, 67 (2007).

Chen, Y. J., W. S. Chen, and T. Y. Wu, "Development of a Bi-cistronic Baculovirus Expression Vector by the *Rhopalosiphum Padi* Virus 5′ Internal Ribosome Entry Site," *Biochem. Biophys. Res. Commun.*, **335**, 616 (2005).

Ducatez, M. F., C. M. Olinger, A. A. Owoade, Z. Tarnagda, M. C. Tahita, A. Sow, S. De Landtsheer, W. Ammerlaan, J. B. Ouedraogo, A. D. Osterhaus, R. A. Fouchier, and C. P. Muller, "Molecular and Antigenic Evolution and Geographical Spread of H5N1 Highly Pathogenic Avian Influenza Viruses in Western Africa," *J. Gen. Virol.*, **88**, 2297 (2007).

Gilbert, M., X. Xiao, J. Domenech, J. Lubroth, V. Martin, and J. Slingenbergh, "Anatidae Migration in the Western Palearctic and Spread of Highly Pathogenic Avian Influenza H5NI Virus," *Emerg. Infect. Dis.*, **12**, 1650 (2006).

Hale, C. C., J. A. Zimmerschied, S. Bliler, and E. M. Price, "Large Scale Expression of Recombinant Cardiac Sodium–Calcium Exchange in Insect Larvae," *Protein Expr. Purif.*, **15**, 121 (1999).

Jinn, T. R., S. S. Kao, Y. C. Tseng, Y. J. Chen, and T. Y. Wu, "Aerosol Infectivity of a Baculovirus to *Trichoplusia ni* Larvae: An Alternative Larval Inoculation Strategy for Recombinant Protein Production," *Biotechnol. Prog.*, **25**, 384 (2009).

Kost, T. A., J. P. Condreay, and D. L. Jarvis, "Baculovirus as Versatile Vectors for Protein Expression in Insect and Mammalian Cells," *Nat. Biotechnol.*, **23**, 567 (2005).

Kovaleva, E. S., K. P. O'Connell, P. Buckley, Z. Liu, and D. C. Davis, "Recombinant Protein Productions in Insect Parvae: Host Choice, Tissue Distribution, and Heterologus Gene Instability," *Biotechnol. Lett.*, **31**, 381 (2009).

Lee, M. S., M. C. Deng, Y. J. Lin, C. Y. Chang, H. K. Shieh, J. Z. Shiau, and C. C. Huang, "Characterization of an H5N1 Avian Influenza Virus from Taiwan," *Vet. Microbiol.*, **124**, 193 (2007).

Lin, Y. J., M. C. Deng, S. H. Wu, Y. L. Chen, H. C. Cheng, C. Y. Chang, M. S. Lee, M. S. Chien, and C. C. Huang, "Baculovirus-Derived Hemagglutinin Vaccine Protects Chickens from Lethal Homologous Virus H5N1 Challenge," *J. Vet. Med. Sci.*, **70**, 1147 (2008).

Lu, X., L. E. Edwards, J. A. Desheva, D. C. Nguyen, A. Rekstin, I. Stephenson, K. Szretter, N. J. Cox, L. G. Rudenko, A. Klimov, and J. M. Katz, "Crossprotective Immunity in Mice Induced by Live-attenuated or Inactivated Vaccines Against Highly Pathogenic Influenza A (H5N1) Viruses," *Vaccine*, **24**, 6588 (2006).

Lu, L., L. Yu, and J. Kwang, "Baculovirus Surface-displayed Hemagglutinin of H5N1 Influenza Virus Sustains Its Authentic Cleavage, Hemagglutination Activity, and Antigenicity," *Biochem. Biophys. Res. Commun.*, **358**, 404 (2007).

Medin, J. A., L. Hunt, K. Gathy, R. K. Evans, and M. S. Coleman, "Efficient, Low-cost Protein Factories: Expression of Human Adenosine Deaminase in Baculovirus-infected Insect Larvae," *Proc. Natl. Acad. Sci. USA*, **87**, 2760 (1990).

O'Reilly, D. R., L. K. Miller, and V. A. Luckow, *Baculovirus Expression Vector: A Laboratory Manual*, W.H. Freeman, NY, USA (1992).

Possee, R. D., "Baculovirus as Expression Vectors," *Curr. Opin. Biotechnol.*, **8**, 569 (1997).

Prabakaran, M., S. Madhan, N. Prabhu, G. Y. Geng, R. New, and J. Kwang, "Reverse Micelle-Encapsulated Recombinant Baculovirus as an Oral Vaccine Against H5N1 Infection in Mice," *Antivir. Res.*, **86**, 180 (2010).

Price, P. M., C. F. Reichelderfer, B. Johansson, E. D. Kilbourne, and G. Acs, "Complementation of Recombinant Baculoviruses by Coinfection with Wild-type Virus Facilitates Production in Insect Larvae of Antigenic Proteins of Hepatitis B Virus and Influenza Virus," *Proc. Natl. Acad. Sci. USA*, **86**, 1453 (1989).

Shen, S., G. Mahadevappa, H. L. Oh, B. Y. Wee, Y. W. Choi, L. A. Hwang, S. G. Lim, W. Hong, S. K. Lal, and Y. J. Tan, "Comparing the Antibody Responses Against Recombinant Hemagglutinin Proteins of Avian Influenza A (H5N1) Virus Expressed in Insect Cells and Bacteria," *J. Med. Virol.*, **80**, 1972 (2008).

Spradbrow, P. B. and J. L. Samuel, "Oral Newcastle Disease Vaccination with V4 Virus in Chickens: Comparison with other Routes," *Aust. Vet. J.*, **68**, 114 (1991).

Subbarao, K. and C. Luke, "H5N1 Viruses and Vaccines," *PLoS Pathog.*, **3**, e40, doi:10.1371/journal.ppat.0030040 (2007).

Treanor, J. J., J. D. Campbell, K. M. Zangwill, T. Rowe, and M. Wolff, "Safety and Immunogenicity of an Inactivated Subvirion Influenza A (H5N1) Vaccine," *N. Engl. J. Med.*, **354**, 1343 (2006).

Wang, K., K. M. Holtz, K. Anderson, R. Chubet, W. Mahmoud, and M. M. J. Cox, "Expression and Purification of an Influenza Hemagglutinin—One Step Closer to a Recombinant Protein-based Influenza Vaccine," *Vaccine*, **24**, 2176 (2006).

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