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The large-scale production of an artificial influenza virus-like particle vaccine in silkworm pupae

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ABSTRACT

We successfully established a mass production system for an influenza virus-like particle (VLP) vaccine using a synthetic H5 hemagglutinin (HA) gene codon-optimized for the silkworm. A recombinant baculovirus containing the synthetic gene was inoculated into silkworm pupae. Four days after inoculation, the hemagglutination titer in homogenates from infected pupae reached a mean value of 0.8 million hemagglutination units (HAU), approximately 2,000 μg HA protein per pupa, more than 50-fold higher than that produced with an embryonated chicken egg. VLPs ranging from 30 nm to 300 nm in diameter and covered with a large number of spikes were detected in the homogenates. The spikes were approximately 14 nm long, similar to an authentic influenza HA spike. Detailed electron micrographs indicated that the VLP spike density was similar to that of authentic influenza virus particles. The results clearly show that the expression of a single HA gene can efficiently produce VLPs in silkworm pupae. When chickens were immunized with the pupae homogenate, the hemagglutination inhibition titer in their sera reached values of 2,048–8,192 after approximately 1 month. This is the first report demonstrating that a large amount of VLP vaccine could be produced by single synthetic HA gene in silkworm pupae. Our system might be useful for future vaccine development against other viral diseases.

1. Introduction

In recent years, occasional outbreaks of highly pathogenic avian influenza (HPAI) viruses caused by the H5 and H7 subtypes have occurred in various regions of the world and they have killed a tremendous number of chickens [1–3]. The 1997 H5N1 outbreak in Hong Kong was the first case in which not only chickens but also 18 humans were infected, 6 of whom died [3–5]. Since then, outbreaks of the virus have occurred worldwide, and the threat of H5N1 infection remains.

Because the World Health Organization has highlighted the risk of a human pandemic resulting from the HPAI virus [6], avian influenza vaccines for chickens and humans have been developed or are currently under development [7,8].

Although studies to create more effective and safer vaccines are ongoing worldwide [7–11], the development of a vaccine against HPAI H5 viruses is associated with intrinsic problems such as the low immunogenicity of the virus [12] and the biohazard risk of using an infectious HPAI virus for the vaccine production. To overcome these hurdles, we synthesized an H5HA gene that lacks 4 basic amino acids associated with the pathogenicity of HPAI viruses located between the HA1 and HA2 subunits. The synthesized gene was codon-optimized for the silkworm. We then produced a recombinant baculovirus containing the synthetic HA gene and used it to inoculate silkworm pupae for mass production of the HA protein.

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Using this method, we produced large amounts of the HA protein present in influenza virus-like particles (VLPs).

2. Materials and methods

2.1. Evolutionary analysis

The sequence data used for the analysis are shown in Supplemental Tables 1–5. The synonymous substitution distances were estimated using the Nei and Gojobori method [13]. Phylogenetic trees were constructed using the neighbor-joining method [13,14].

2.2. Cells and viruses

Silkworm Bm-N cells and MDCK cells were maintained in TC-100 medium and minimal essential medium containing 10% fetal bovine serum, respectively. The P6E strain of the *Bombyx mori* nuclear polyhedrosis virus (BmNPV)[15] was used to generate the H5HA-BmNPV recombinant virus. Baculoviruses were propagated in Bm-N cells. The avian influenza viruses used for the hemagglutination inhibition (HI) test (shown in Fig. 4D) were propagated in 10-day-old embryonated chicken eggs (37 °C, 2 days).

2.3. Generation of H5HA-BmNPV

Synthesis of the H5HA gene was carried out by Dragon Genomics (Shiga, Japan). The synthesized gene was inserted into the pBM-8 plasmid [15] using the In-Fusion technique (Clontech, Mountain View, CA, USA) to produce pBM-8-H5HA. Next, pBM-8-H5HA and linearized BmNPV genomic DNA were co-transfected into Bm-N cells, which were then cultured in TC-100 medium without serum. After 5 h, the medium was changed to TC-100 containing 10% fetal bovine serum and the cells were further cultured for 5–7 days. The medium was then harvested and centrifuged at 3000 rpm for 4 min to remove cell debris. The resulting supernatant was used as recombinant virus H5HA-BmNPV.

2.4. Antibodies and Western blot analysis

The anti-FLAG mouse monoclonal antibody M2 and the anti-DYKDDDDK-tag mouse monoclonal antibody were purchased from Sigma (St. Louis, MO, USA) and Wako (Osaka, Japan), respectively. The fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody and the peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). For the Western blot analysis, the peroxidase-conjugated IgG antibody was visualized using an ECL detection system (GE Healthcare, Port Washington, NY, USA), following the manufacturer's instructions.

2.5. Hemadsorption test

Bm-N cells infected with viruses were washed 3 times with phosphate-buffered saline (PBS) and then overlaid with 0.5% (v/v) chicken erythrocytes in PBS. After 30 min, the cells were washed thoroughly with PBS and observed under a microscope.

2.6. Hemagglutination test and hemagglutination inhibition tests

Hemagglutination (HA) and HI tests were performed as described previously [16].

2.7. Production of HA protein in pupae

Silkworm pupae were injected with 100 μL of H5HA-BmNPV in TC-100 (Fig. 4A). Four days later, the infected pupae were homogenized in PBS containing 0.01% formalin and phenylthiourea as an antioxidant; homogenization was performed using a Handy Sonic (Model Tomy UR-20P). Sonication was performed on ice by using 6 pulses, each lasting 2 min. The resultant homogenate was then centrifuged at 6,000 rpm for 30 min. The supernatant was further centrifuged at 16,000 rpm for 30 min. The supernatant from the second centrifugation was used for subsequent experiments.

2.8. Sucrose density gradient centrifugation and electron microscopy

The homogenate of H5HA-BmNPV-infected pupae was centrifuged through a 10–50% (w/w) sucrose density gradient at 25,000 rpm for 120 min using an SW28 swing rotor (Hitachi). The gradient was fractionated and the fractions were examined for HA activity and protein concentration. Six fractions (21–26) that had high HA activity (greater than 8,192) were collected and centrifuged at 25,000 rpm for 4 hr. The pellet was examined under an H-7600 electron microscope (Hitachi) after staining with 2% phosphotungstic acid.

3. Results

3.1. Selection of the target strain used for the anti-H5N1 influenza vaccine

We selected a vaccine strain based on an evolutionary analysis of H5HA genes. Through precise evolutionary analyses of genotype Z viruses, which appeared in 2002 and became the predominant genotype associated with HPAI outbreak in domestic poultry [17], we found that viruses from subclade 2.3, which included the A/tufted duck/Fukushima/16/2011 (H5N1: duck-Fukushima) strain, show discontinuous evolution (i.e., evolution that did not occur at a single rate over time) at the slow rates of 0.002 substitutions/site and 0.008 substitutions/site per year (subclades 2.3 (i) and (ii) in Fig. 1B, respectively). Considering the slow evolutionary rate of wildfowl influenza viruses (0.005 substitutions/site per year, as shown in Fig. 1A), we hypothesized that fast continuous linear evolution would occur in domestic poultry (Fig. 1B red line) and the slow rate of subclade 2.3 would occur in wildfowl. The atypical evolution of subclade 2.3 may be explained if subclade 2.3 viruses are maintained in poultry (Fig. 1B Clade 2.3, red line) but occasionally infect wildfowl, in which they would then evolve at a lower evolutionary rate. If the hypothesis were correct, subclade 2.3 viruses should be the major virus type disseminated; therefore, we selected the duck-Fukushima strain as the target for vaccine preparation.

3.2. Design of the synthetic H5HA gene and the generation of H5HA-BmNPV

The synthetic gene for the production of the H5HA protein in silkworms was designed based on the nucleotide sequence of the HA gene from the duck-Fukushima strain. Duck-Fukushima is a virulent strain that is highly pathogenic to chickens. The polybasic sequence of RERRRKRG, located at the end of the HA1 subunit, is associated with the high pathogenicity of H5N1. Because this sequence is present, the HA0 precursor is cleaved to HA1 and HA2 in almost any cell in which the HA protein is produced. By cleavage, the HA protein obtains membrane fusion activity and is destabilized in acidic environments such as in intracellular transport vesicles [18,19]. Therefore, the RERRRKRG sequence was replaced with RDTRG to avoid this cleavage in insect cells (Fig. 2). By the introduction of RDTRG sequences instead of RERRRKRG, virulent DNA was converted to avirulent DNA.