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Preparation and characterization of egg yolk immunoglobulin Y specific to influenza B virus

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ABSTRACT

The aim of this study was to prepare egg yolk immunoglobulin (IgY) for use in the prevention and treatment of influenza B viral infections. Laying hens were immunized with inactivated influenza B virus (IBV), and IgY was isolated from the egg yolk by multiple polyethylene glycol (PEG) 6000 extraction and ammonium sulfate purification steps. The titers and specificity of the purified antibodies were assessed. The specific IgY titer increased beginning the second week after the first immunization, with the titer peaking at the fifth week. The yield of IgY was 76.5 mg per yolk, and the purity was 98.2%. The use of western blotting and the hemagglutination inhibition (HI) test demonstrated that IBV-specific IgY binds specifically to influenza B virus proteins, and a plaque reduction assay revealed the neutralization efficacy of IBV-specific IgY at reducing influenza infection in MDCK cells. Furthermore, when mice were treated intranasally prior to or after influenza B virus infection, IBV-specific IgY protected the mice from influenza infection or reduced viral replication in their lungs, respectively. These findings indicate that IgY is an easily prepared and rich source of antibodies that offers a potential alternative strategy for preventing and treating influenza B infections.

1. Introduction

Influenza is one of the most common acute viral diseases, and it causes systemic symptoms of variable degrees, such as mild fatigue, respiratory failure, and even death. Approximately 10–20% of the world's population suffers from seasonal influenza, resulting in 250,000–500,000 deaths every year (World Health Organization, 2003). Influenza B virus is one of the pathogens involved in localized outbreaks of seasonal influenza (Saat et al., 2010). Children recovering from influenza B who receive aspirin treatment may suffer a rare illness, Reye syndrome, which can affect the brain and liver (Ashe, 1975; Corey et al., 1976; Reynolds et al., 1972). Much effort has been made to prevent and cure influenza B viral infections. Antiviral drugs, such as neuraminidase inhibitors, are widely used to treat influenza. However, influenza B virus is increasingly resistant to these agents (Sheu et al., 2008; Sugaya et al., 2007). Although vaccination can provide protection, virtually none of the current vaccines is effective enough for use as a therapy.

Passive immunization based on specific antibodies provides an alternative strategy for the prevention and treatment of infectious diseases (Keller and Stiehm, 2000). The availability of specific antibodies is the key for immunotherapy. Several approaches have been utilized to develop safe, effective, and inexpensive antibodies, including humanized monoclonal antibodies (Mabs) (Goncalvez et al., 2008) and human recombinant antibodies (Sun et al., 2008). Mabs are specific for single epitopes and may not offer full protection clinically, although they can be produced at a high titer.

Immunoglobulin Y (IgY) antibodies, the predominant serum immunoglobulin in birds, reptiles, and amphibians, are transferred from the serum of females to the egg yolk, where they offer passive immunity to embryos and neonates (Patterson et al., 1962). IgY can be extracted at high yield from the yolks of immunized hens by several simple processes. IgY is a safe and inexpensive antibody that has been successfully used to prevent and treat bacterial and viral infections in animals (Nguyen et al., 2010; Wallach et al., 2011). However, the antiviral activity of IgY against influenza B virus has not been extensively investigated.

This study aimed to produce IgY specific to influenza B virus and assess its *in vitro* antiviral activity against influenza B virus. Our results provide a foundation for future alternative prophylactic and therapeutic medicines for influenza.

2. Materials and methods

2.1. Virus and cell lines

The virus strain, B/Guangdong/867/2009, was generously provided by Guangdong CDC (Guangzhou, Guangdong, China) and prepared as described previously (Qiu et al., 1992). Virus stock was diluted 1:500 in 0.9% sodium chloride solution

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and injected into the allantoic cavity of 9-day-old, specific pathogen–free, embryonated eggs (0.2 mL/egg). After incubating for 72 h at 35 °C, the eggs were stored overnight at 4 °C, and, the allantoic fluid was harvested and centrifuged at 2000 *g* for 30 min at 4 °C. Solid polyethylene glycol (PEG) 6000 (Amresco, USA) was added to a final concentration of 8% (w/v) and stirred for 1 h at 4 °C. The precipitate was collected by centrifugation at 30,000*g* for 1 h and resuspended in 0.5 mL of phosphate-buffered saline (PBS). The preparation was centrifuged through 30% sucrose onto a 60% sucrose cushion at 20,000*g* for 1 h at 4 °C. Virus removed from the interface was diluted 1:3 in NTE buffer (10 mmol/L Tris–HCl, 100 mmol/L NaCl, 1 mmol/L ethylenediamine tetra-acetic acid). The diluted virus was layered over a 10 mL 30–60% sucrose gradient and centrifuged at 20,000*g* for 1 h at 4 °C. The virus was collected and diluted 1:5 in NTE buffer followed by sedimenting at 150,000*g* for 1 h at 4 °C. The pellet was dissolved in a small volume of NTE buffer and stored at −80 °C.

Madin-Darby canine kidney (MDCK) cells (Catalogue No. GN023) were obtained from the Cell Bank of the Committee of the Type Culture Collection of the Chinese Academy of Sciences and maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum.

2.2. Immunization of hens

Five high-health-status, 20-week-old Roman laying hens housed at an experimental animal center were immunized intramuscularly with influenza B virus (HA titer of 64) that had been inactivated using beta-propiolactone. For the first immunization, 1 mg of antigen (viral protein) was emulsified with an equal volume of complete Freund's adjuvant (FA) (Sigma, USA), and incomplete FA was similarly used for the subsequent boosting immunizations. The hens were immunized a total of five times at intervals of two weeks. Eggs were collected after the initial immunization. Eggs laid by non-immunized hens were collected for use as the negative control.

2.3. Isolation and purification of IgY

A rapid and simple method adapted from previous studies (Ko and Ahn, 2007; Polson et al., 1980) was used to extract IgY from yolk. Briefly, the yolk was separated from the white by egg separators, and a volume of buffer containing 14% PEG6000 (w/v) equivalent to three volumes of yolk was added. The mixture was stirred at room temperature (RT) for 30 min and was centrifuged at 5000*g* for 20 min at 10 °C. The supernatant was collected and filtered through four layers of sterile gauze. The volume of the filtrate was measured, and PEG6000 was added by gentle stirring to adjust the final polymer concentration to 12% (w/v). The material was centrifuged at 5000*g* for 20 min at 10 °C. The pellet was dissolved to the original volume of yolk in phosphate buffer, solid ammonium sulfate was added to reach 50% saturation, and the mixture was stirred overnight at 4 °C. The precipitate was collected by centrifugation and washed with 33% saturated ammonium sulfate. The precipitate was dialyzed against PBS and freeze-dried, and the powder obtained was stored at −20 °C.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to determine the purity of IgY. A 15% polyacrylamide gel was used with a Mini-PROTEAN® 3 cell (Bio-Rad Laboratories, USA). The analysis was conducted under reducing conditions; the sample was mixed with sample buffer and held for 5–8 min at 100 °C. Ten microliters of the sample was loaded into each well. Pre-stained protein standard (Fermentas, Lithuania) was used as a molecular weight marker. The protein bands were visualized with Coomassie Brilliant Blue R250 (Fluka USA). The gel was analyzed using Bio-Rad image analysis software.

2.5. Enzyme-linked immunosorbent assay

The titer of IgY against influenza B virus was measured by an indirect non-competitive enzyme-linked immunosorbent assay (ELISA) according to previously reported methods with modifications (Zhao et al., 2003; Zhen et al., 2008). A 96-well micro-titer plate was coated with inactivated influenza B virus containing 0.58 mg/mL protein in carbonate–bicarbonate buffer (0.05 M, pH 9.6) at 100 μL/well. Serial dilutions of specific IgY were incubated in the pre-coated and blocked plate for 1 h before the bound IgY was detected with 100 μL/well HRP-conjugated rabbit anti-chicken IgY (1:5000) (Promega, USA). After incubation for 1 h at 37 °C, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST). Next, 100 μL/well 3,3′-5,5′-tetramethylbenzidine (Amresco, USA) substrate was added and incubated for 15 min at 37 °C. The color development was stopped with 2 M sulfuric acid (50 μL/well), and the optical density (OD) was measured on a micro-titer plate reader (Tecan f200, Switzerland) at 450 nm. The reproducibility of the experiment was ascertained by including a blank control (PBS) and a negative control (IgY derived from non-immunized hens) in each plate. IBV-specific IgY titer was defined as the maximum dilution multiple of the sample with an OD value that was 2.1 times that of the negative control.

2.6. Western blotting assay

To confirm the specificity of anti-IBV IgY to influenza B virus proteins, western blotting was conducted using a previously published method with some modifications (Qiu et al., 1992). Briefly, 100 μL of purified influenza B virus containing 1–2 mg/mL protein, as determined by the Bradford method, was mixed with 100 μL of electrophoresis sample buffer. The disrupted virus preparation was subjected to SDS–PAGE in a 14% slab polyacrylamide gel separated by a 4% stacking gel at 100 V for 3.5 h at RT. After electrophoresis, the gel was equilibrated in transfer buffer for 15–30 min, after which the viral proteins were electrically transferred onto a nitrocellulose (NC) membrane (Osmonics, USA) for 1 h at 350 mA at 4 °C. The NC membrane was cut into 0.5-cm strips, which were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat dry milk for 1 h at RT and was incubated overnight at 4 °C with a 1:100 dilution of specific or nonspecific IgY. After incubation, the strips were washed four times with PBS-T and incubated with HRP-conjugated rabbit anti-chicken IgY (Promega, USA) diluted 1:1000. Following 2 h of incubation at RT, the strips were washed three more times. After washing, the strips were incubated in developing buffer containing 4 mg 3,3′-diaminobenzidine tetrahydrochloride (Aladdin, China) in 5 mL Tris–HCl and 15 μL hydrogen peroxide for 3–5 min. This reaction was stopped by rinsing with distilled water. The strips were photographed after development.

2.7. Hemagglutination inhibition (HI) test

HI test was carried out following the standard protocol (Meijer et al., 2006). Twofold serial dilutions of RDE-treated IgY, starting at 1:2, were incubated with equal volumes of inactivated influenza B virus with a titer of 4 HA units in U-bottom 96-well plates for 60 min at RT. At the end of the incubation, 25 μL of a freshly prepared 1% guinea pig (*Cavia porcellus*) erythrocyte suspension was added, and the mixture was homogenized. A virus control (containing virus and erythrocytes alone) and a blank control (containing erythrocytes and buffer alone) were included on the plate. The plates were allowed to stand at RT until a positive hemagglutination reaction developed in the virus control wells and the erythrocyte control showed a button at the bottom of the well, and the reading was subsequently taken. The HI titer was defined as the reciprocal of the highest dilution of IgY that completely prevented viral hemagglutination.

2.8. Plaque reduction assay

To determine the effect of IgY on influenza B virus replication, a plaque reduction assay was conducted as described (Landry et al., 2000). The original stock of influenza B virus was subjected to two additional passages in chick embryo allantoic cavities. The 50% tissue culture infection dose ($TCID_{50}$), and the multiplicity of infection (MOI) of the virus were determined. MDCK cells were seeded in 12-well plates, 2.5×10^5 per well, and cultured for 24 h to ensure the cells were 95–100% confluent. IgY, starting at 2 mg/mL with 2-fold serial dilutions, was incubated with an equal volume of 0.01 MOI influenza B virus at 37 °C for 30 min. Next, 200 μL of the incubated mixture was added to MDCK cell monolayers in the 12-well plates; a cell control (PBS and cells alone) and a virus control (virus and cells alone) were also included. After absorption for 2 h at 37 °C, the wells were overlaid with 1.5 mL of agarose-containing overlay medium. The plates were incubated for 72 h at 37 °C in an incubator with 5% CO_2, and then the overlay was carefully aspirated. The monolayers were fixed in 10% formalin in phosphate-buffered saline and were stained overnight with 1% crystal violet in 50% ethanol. The plaques were counted microscopically at low power. The 50% inhibitory concentration (IC_{50}) of IBV-specific IgY against influenza B virus was calculated by the Reed-Muench method.

2.9. Mouse infection and treatment trials

Mouse experiments were carried out to test the protective effect of the specific IgY in influenza B virus–infected mice. Female BALB/c mice that were 8 weeks old were obtained from the Experimental Animal Center of Guangdong Province (Foshan, Guangdong, China) and were maintained at the animal facilities of Sun Yat-sen University (Guangzhou, Guangdong, China). The mice were randomly divided into four groups of 10. The stock virus (B/Guangdong/867/2009) (25 μL/mouse, $10^{6.5}$ $TCID_{50}$/mL) was used to challenge the mice. Challenge trials in the mice were carried out as follows. The first group was inoculated intranasally with virus (25 μL/mouse, $10^{6.5}$ $TCID_{50}$/mL) and received PBS (25 μL/mouse) treatment 2 h before infection. The second group was infected with virus ($10^{6.5}$ $TCID_{50}$/mL) and were treated with IgY (10 mg/mL, 25 μL/mouse) 2, 26, 50, and 74 h post-infection. The third group received IgY (10 mg/mL, 25 μL/mouse) treatment 2 h prior to infection. The last group was not infected with virus and did not receive IgY treatment. The mice were monitored daily for weight changes for 7 days post-infection. At days 3 and 6 following infection, three mice from each group were sacrificed by inhalation of CO_2, and their lungs were collected. The right lung lobes were fixed in 10% neutral-buffered formalin overnight, embedded in paraffin and stained with hematoxylin and eosin (H&E). The remaining lungs were homogenized in 1 ml of cold PBS and clarified by centrifugation at 4 °C. The clarified homogenates were inoculated into 9-day-old, specific pathogen-free embryonated chicken eggs. After