Veterinary Immunology and Immunopathology 157 (2014) 97–104

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

Egg yolk IgY against RHDV capsid protein VP60 promotes rabbit defense against RHDV infection

Li Zai Xin, Hu Wei Dong, Li Bing Chao, Li Tian You, Zhou Xiao Yang, Zhang Zhi *

Key Laboratory of Pharmaceutical Engineering Technology and Application, School of Chemical and Pharmaceutical Engineering, Sichuan University of Science and Engineering, Zigong 643000, China

ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form
26 September 2013
Accepted 8 October 2013

Keywords:
Rabbit hemorrhagic disease virus (RHDV)
Egg yolk antibody (IgY)
VP60 capsid protein

ABSTRACT

VP60 capsid protein is the major structural and immunogenicity protein of RHDV (Rabbit hemorrhagic disease virus, RHDV), and has been implicated as a main protein antigen in RHDV diagnosis and vaccine design. In this report, egg yolk antibody (IgY) against N-terminal of VP60 was evaluated and developed as a new strategy for RHDV therapy. Briefly, N-terminal of VP60 (~250aa) fragment was cloned and inserted into pET28a expression vector, and then the resultant plasmid, pET28a/VP60-N, was transformed into *E. coli* BL21(DE3) for recombinant VP60-N protein (rVP60-N) expression. Next, the rVP60-N was purified by Ni^{+}-affinity purification chromatography and identified by Western blotting with RHDV antiserum. After immunizing the chickens with rVP60-N, the anti-rVP60-N IgY was isolated, and the activity and specificity of the IgY antibody were analyzed by ELISA and Western blotting. In our results, the rVP60-N could be expressed in *E. coli* as soluble fraction, and the isolated anti-rVP60-N IgY demonstrated a high specificity and titer (1:22,000) against rVP60-N antigen. For further evaluation of the IgY efficacy *in vivo*, rabbits were grouped randomly and challenged with RHDV, and the results showed that anti-rVP60-N IgY could significantly protect rabbits from virus infection and promote the host survival after a sustained treatment with anti-rVP60-N IgY for 5 days. Taken together, our study demonstrates evidence that production of IgY against VP60 could be as a novel strategy for the RHDV therapy.

1. Introduction

Rabbit hemorrhagic disease (RHD) is highly contagious and has been characterized as high morbidity and mortality in adult rabbits (Schirrmeier et al., 1999; Liu et al., 1984). The disease was first discovered in China in (Liu et al., 1984) and soon spread worldwide (Gregg et al., 1991; Mitro and Krauss, 1993; Toledo et al., 1995; Bouslama et al., 1996; Embury-Hyatt et al., 2012). The etiological agent is a single-stranded positive-sense RNA virus from the family of Caliciviridae named rabbit hemorrhagic disease virus (RHDV) (Parra and Prieto, 1990). The virions are non-enveloped and icosahedral calicivirus, and genome of which has two open reading frames (ORFs), including ORF1 and ORF2. ORF1 encodes a polyprotein that is cleaved into non-structural components and the major structural protein, the capsid protein VP60 (Parra et al., 1993), which is the main target of the host immune defense against RHDV and plays an important role in virus diagnosis and vaccine design. In the past years, the capsid protein VP60 has been successfully expressed in several heterologous systems and shown to induce full protection of rabbits against a lethal challenge with RHDV (Bertagnoli et al., 1996a,b; Fischer et al., 1997; Castanon et al., 1999; Fernandez-Fernandez et al., 2001; Perez-Filgueira et al., 2007). And also, the antigenic structure of VP60 has been well analyzed by using anti-RHDV monoclonal antibodies (mAbs) and anti-RHDV

* Corresponding author. Tel.: +81 3 5505605; fax: +81 3 5505605.
E-mail address: zhangzhi02@gmail.com (Z. Zhang).

0165-2427/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2013.10.002

serum respectively, suggesting that the N-terminal of VP60 (1-250aa) is the most antigenic region (Viaplana et al., 1997; Martínez-Torrecuadrada et al., 1998).

Antibody-based passive immunization is effective in prevention and treatment of infectious diseases (Keller and Stiehm, 2000), in which the availability of large amount of specific antibodies is the key. Immunoglobulin Y (IgY), the egg yolk antibodies generated as a passive immunity to embryos and baby chicks, can be a good source of such antibody. IgY can be easily produced and purified with high yields from egg yolks of immunized hens by variable methods, which has been used as a safe and inexpensive strategy to control and prevent bacterial and viral infections in domestic farm animals (Chalghoumi et al., 2009b; Vega et al., 2011). However, the possibility of using IgY to treat against RHDV infection in rabbits *in vivo* has not yet been studied.

In present study, we generated a large amount of anti-VP60 polyclonal IgY by using the recombinant N-terminal of VP60 (1–250 aa) as antigen. We successfully produced high titers of specific IgY in egg yolks from immunized chickens and showed neutralizing activity of IgY on RHDV *in vivo*. Our results provide solid evidence that production of IgY could be used as a novel strategy for therapeutic treatments against RHDV.

2. Material and methods

2.1. Materials

The pTNT™ vector containing a full-length RHDV VP60 gene was kindly provided by Prof. Guangqing Liu (Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China). RHDV LQ, a virulent strain of RHDV, and antiserum against RHDV were obtained as a gift from SICHUAN HUAPAI BIO-PHARMACEUTICAL CO.,LTD.

2.2. Amplification of VP60-N

The N-terminal of VP60 (VP60-N) was amplified by PCR with forward primer VP60-N-F (5′-CCGGAATTCATGGAGGGCAAAGCCCGCA-3′) and reverse primer VP60-N-R (5′-CCGCTCGAGATTGCCAACACCAGTGA-3′), which contained *EcoR I* and *Xho I* sites respectively. Next, the PCR product was cloned into the dephosphorylated *EcoR I* and *Xho I* sites of pET28a (Novagen, USA), and the resultant plasmid named pET28a/VP60-N. The recombinant plasmid was confirmed by sequence analysis.

2.3. Preparation of VP60-N antigens

pET28a/VP60-N plasmid was transformed into *E. coli* BL21(DE3), and the recombinant VP60-N (rVP60-N) was expressed with an induction of 0.5 mM IPTG (Merch, Germany) at 25 °C overnight. Next, the His-tagged rVP60-N fusion proteins were purified by using Ni^{+}-affinity purification chromatography column according to the manufacturer's instructions (Novagen, USA). The protein concentration was determined by using the Bradford Protein Assay, and 200 μg of rVP60-N antigens in 300 μL of phosphate buffered saline (PBS) were used for i.m immunization.

2.4. Chickens and immunization

Six-week old Leghorn chickens were housed in individual cages under a regimen of 12 h of light and 12 h of darkness, at room temperature (23 ± 2 °C) and humidity of 75 ± 5%. Water and commercial chicken food were offered daily. The chickens were immunized intramuscularly with rVP60-N protein mixed with Freund's adjuvant (Sigma, USA) at different sites of the breast at 21 weeks old. Three hundred microlitre of protein solution (containing 200 μg rVP60-N) were emulsified with an equal volume of complete Freund's adjuvant for the first immunization. Two booster immunizations were followed up using incomplete Freund's adjuvant (Sigma, USA) with four weeks interval with 100 μg of rVP60-N antigens. Eggs were collected daily, marked and stored at 4 °C before being processed for IgY.

2.5. IgY antibody purification

IgY antibody was extracted and purified from egg yolks using water-dilution method as described before (Akita and Nakai, 1993) with modifications. Briefly, 10 mL egg yolk was diluted 8-fold in deionized ultrapure water adjusted to pH 5.0–5.2 with 1N HCl and homogenized thoroughly. After vortex, the sample mixtures were centrifuged at 12,000 × *g* for 20 min at 4 °C to remove the lipid-rich precipitate. The supernatant, consisting of lipid-free fraction, was collected and precipitated with the addition of 40% ammonium sulfate (w/v). After centrifugation (12,000 × *g*, 20 min, 4 °C), the pellet, the IgY enriched fraction, was dissolved in 10 mL PBS. To eliminate residual salt, the isolated IgY was further purified by ultrafiltration using Millipore Amicon Ultra-15 (100 kD) according to the manufacturer's instructions (Millipore, USA). The final IgY retentate was dissolved in 10.0 mL PBS and stored at −20 °C. The purity and quantity of the isolated IgY antibody were determined by SDS-PAGE and Bradford Protein Assay respectively.

2.6. Detection of rVP60-N specific IgY in yolks by enzymelinked immunosorbent assay (ELISA)

Specific activity of IgY antibody against rVP60-N protein was determined by ELISA. Briefly, a 96-well microtiter plate was coated with 100 μL of rVP60-N antigen (5 μg/mL) at 4 °C overnight. After washing three times with PBS containing 0.05% (v/v) Tween 20 (PBST), the nonspecific binding sites were blocked with 3% (w/v) BSA in PBS for 2 h at 37 °C. After three times washing, 100 μL of a serially diluted IgY antibody in PBST was added to the wells as the primary antibody and then incubated for 3 h at room temperature or 4 °C overnight. The plate was washed again and the bound antibody was then incubated with 100 μL of goat anti-chicken IgY–HRP (1:5000) (Sigma, USA) for 1.5 h at room temperature. Finally, the HRP activity was detected by adding 100 μL of TMB substrate reagent (BEYOTIME, China) to each well for 15–30 min at room temperature, and then the reaction were stopped by adding 50 μL of 2 M H_2SO_4 to the wells. The optical density (OD) was read at 450 nm on