Antiviral Research 147 (2017) 1–10

Contents lists available at ScienceDirect

Antiviral Research

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

Immunoprotection induced by CpG-ODN/Poly(I:C) combined with recombinant gp90 protein in chickens against reticuloendotheliosis virus infection

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ARTICLE INFO

Article history:
Received 1 February 2017
Received in revised form
11 April 2017
Accepted 28 April 2017
Available online 29 April 2017

Keywords:
CpG-ODN
Poly (I: C)
Reticuloendotheliosis virus
Immunoprotection
Subunit vaccine
Chickens

ABSTRACT

The present study is focused on investigating the immunoprotective effects of CpG-ODN/Poly(I:C) combined with the viral glycoprotein gp90 protein against reticuloendotheliosis virus (REV) infection in chickens. REV's gp90 gene was amplified from the REV-infected cells and expressed in *Escherichia coli* (*E.coli*). The expressed products, upon purification, were inoculated into 7-day-old chickens with PBS, CpG-ODN or Poly(I:C) adjuvant; Two booster inoculations were then conducted, and then each chicken was challenged. The presence of REV-antibodies in serum was determined weekly after the first vaccination. The viremia and immunosuppressive effects of REV infection were also monitored after the challenge. The neutralizing effects of the antisera were tested in vitro. The results showed that the recombinant gene containing REV gp90 gene was expressed into the recombinant protein with a size of 51 *Kilo Dalton* (KD), which could be recognized by a monoclonal antibody (MAb) against the gp90 protein. The viremia and immunosuppressive effects of avian influenza virus (AIV) vaccine caused by REV challenge in CpG-ODN group and in Poly(I:C) group were dramatically decreased. REV antibody with low titers was induced in gp90 group and the inoculated chickens were partly protected. Compared with those in gp90 group, the titers and the positive ratios of REV antibody in CpG+gp90 group were significantly increased, whereas the viremia and immunosuppressive effects of AIV vaccine caused by REV infection were significantly decreased. In the Poly(I:C) +gp90 group, the viremia and immunosuppressive effects caused by REV infection were also dramatically decreased, although REV antibody responses were softly increased. The diluted antisera from the vaccinated chickens in both groups could completely inhibit the replication of REV in chick fibroblast cells (CEF). Hence, it can be concluded that CpG-ODN or the Poly(I:C) adjuvant can enhance the antiviral effects of the REV subunit vaccine against REV infection, which may result from different mechanisms.

1. Introduction

Reticuloendotheliosis virus (REV) is a type-C avian retrovirus that can cause growth retardation, immunosuppression, persistent viremia, tumors or even deaths in susceptible chickens (Barbacid et al., 1979; Motha, 1987; Barth et al., 1990; Meng et al., 2006). The immunosuppression caused by REV can affect the antibody titers of vaccines such as avian influenza virus (AIV) and Newcastle disease virus (NDV), and increase the susceptibility to other pathogens such as bacteria or viruses (Sun et al., 2009a; Guo et al., 2006). The epidemiological studies suggest that REV widely distributes not only in chickens(Zavala et al., 2006; Cheng et al., 2006; Barbosa et al., 2007; Qin et al., 2010; Wang et al., 2012; Jiang et al., 2013), also in pigeons, ducks, gooses, quails, peafowl, *etc* (Zhai et al., 2016; Dren et al., 1988; Ludford et al., 1972; Carlson et al., 1974; Miller et al., 1998); Additionally, REV contamination in vaccines has also been reported (Fadly et al., 1996; Fadly and Garcia, 2006; Garcia et al., 2003; Hertig et al., 1997; Liu et al., 2009; Moore

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https://doi.org/10.1016/j.antiviral.2017.04.019
0166-3542/

et al., 2000; Tadese et al., 2008); due to which, the poultry industry has suffered a great loss with billions of dollars and it is very practical and economic to prevent REV infection in poultry (Cui et al., 2000; Sun et al., 2006).

At present, there are no commercial vaccines or effective drugs to control REV. In recent years, the research on REV vaccines has been increasing. The attenuated virus strains could induce the inoculated breeder hens to produce better maternal antibodies and protect the off-spring chickens against early REV-infection (Sun et al., 2007), but it is hardly acceptable to be applied in clinic production because of risk of back-to-virulence. The recombinant gp90 protein expressed in Pichia pastoris could produce the protective immune responses against REV infection (Li et al., 2012, 2013a) and so did the DNA vaccine containing gp90 gene (Li et al., 2013b), but they still can not be applied to control REV infection clinically due to their unstable immunoprotective responses (unpublished data). So it is very important and necessary to find new methods or drugs to control REV infection in poultry.

CpG-ODN and Poly(I:C), as ligands of Toll-like receptors(TLRs), can activate chicken Toll-like receptor 21(TLR21) and TLR3, respectively, and can induce innate immune responses (Keestra et al., 2010; Schwarz et al., 2007). CpG-ODN has been the most widely used to enhance the immunogenicity of vaccines against several pathogens including AIV (Paul et al., 2012). Our studies showed that CpG-ODN demonstrated superior adjuvant potential in eliciting antibody-mediated immune responses to the subunit vaccine of avian leucosis virus (ALV) (Dou et al., 2013; Zhang et al., 2015). Poly(I:C) has also been identified to possess adjuvant potential in enhancing the protective effects of a Marek's disease virus (MDV) vaccine and the administration of Poly(I:C) 3 days after vaccination reduced the incidence of tumors in MDV-infected birds (Parvizi et al., 2012). But little has been known about their protective effects against REV infection. Hence, it is intriguing to determine if CpG-ODN or Poly(I:C) can inhibit the replication of REV as therapeutic agents or improve the immunoprotective effects of the REV subunit vaccine as adjuvant in chickens, which is addressed in the present study.

2. Materials and methods

2.1. Virus, cells, antibodies, plasmids and adjuvant

REV-SNV strain isolated from ducks in U.S.A was sequenced in 2005(GenBank No: DQ003591) (Ji et al., 2005). REV-SNV strain and REV-specific monoclonal antibody (MAb,11B118) (Cui et al., 1986) were generously provided by Prof. Zhizhong Cui and Prof. Shuhong Sun (College of Veterinary Medicine, Shandong Agricultural University). The titer of stock REV-SNV strain was measured by the Reed and Muench method using indirect immunofluorescence assay (IFA) to identify REV-positive virus cultures in DF-1 cells (Bohls et al., 2006a).

The pMD18-T simple vector was purchased from Dalian Takara Biotechnology Co., Ltd and was used for plasmid-cloning experiments. The pET32a expression vector was obtained commercially (Invitrogen, Shanghai, China) and was used for gp90 gene expression. The CpG-ODN containing sequence TCGTCGTTTTGTCGT TTTGTCGTT was synthesized using a phosphorothioate backbone at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd and was diluted appropriately and emulsified with white oil(a mixture of saturated naphthenic and paraffinic) as a sustained releasing adjuvant for subunit vaccines. Poly(I:C) reagent was purchased from Sigma Company (USA, Number: P1530) and also emulsified with white oil as a sustained releasing adjuvant.

2.2. Chickens and humane management

Hy-Line Brown chickens were purchased from Dongyue Poultry Breeding Co., Ltd. (Shandong Province, China) and housed in a clean and comfortable room with free diet. Before the start of the experiment, REV-antibody ELISA (IDEXX USA Inc., Beijing, China) and REV viremia assay were performed to confirm that each chicken was negative for the REV antibody and virus. The treatment inoculations, sample collections and necropsies of the chickens were conducted strictly under the guideline of Animal Ethics Committee at Shangdong Province Animal Protection and Welfare Institute (The Approved Number: SDAU-2014-009). The abnormal chickens were cared for and put on a special diet and treatment. The chickens were sacrificed using the painless method(CO_2 gaseous anaesthetic overdose), which was recommended by the Animal Ethics Committee.

2.3. The preparation of REV subunit vaccine

According to REV gp90 gene sequence published in GenBank (*No.* DQ003591), using *Eco*R I and *Sal I* restriction enzyme sites in the forward and reverse primers, the forward primer (5′-CCGGAATTC TCCTCCATACCTACCTATTACA -3′) and the reverse primer (5′-AGAGTCGACTCA ATTAGGGAGT GCCGTGT-3′) were designed and used to amplify gp90 gene from the REV-infected DF1 cells. The PCR product was gel-purified (Gel/PCR Extraction Kit, Biomiga) and transformed into the pMD18-T vector to generate the recombinant clone vector pET32a(+)-gp90, which was confirmed by DNA sequencing and then amplified in *Escherichia coli* (*E. coli*; DH5α).

The REV gp90 recombinant gene was expressed in Rosetta (DE3) cells using 1.0 mM IPTG at 37 °C. The soluble fraction was harvested and applied to a high-affinity Ni-NTA column. The eluted proteins were further purified by running the eluate through an SD200 gel filtration column twice, with and without 1% nadeoxycholate, to remove the endotoxins. The purified proteins were evaluated by SDS-PAGE in 12% polyacrylamide gels and were analyzed using western blot and REV-specific MAb (11B118). The protein concentration was determined by performing thin-layer chromatography scanning and Bradford's total protein content assay using a Bio-Rad protein assay kit (Bio-Rad); here, bovine serum albumin was used as the standard.

The purified recombinant protein was mixed with CpG-ODN adjuvant or Poly (I:C) adjuvant into the emulsion as subunit vaccines for the vaccinated chickens. The emulsion used for the gp90 protein antigen and CpG-ODN or Poly(I:C) was conducted according to the preparation instructions of most vaccines in production. The components of the emulsion contained an aqueous phase (protein antigen, water and twain −80) and an oil phase (White oil and Span), and they were mixed together into a homogeneous mixture.

2.4. Immunization and sample collection

7-day-old chickens were randomly divided into six groups: a control group (22 chickens), a gp90 group (13 chickens), a CpG + gp90 group (13 chickens), a Poly(I:C) + gp90 group (13 chickens), a Poly(I:C) group (10 chickens), and a CpG group (10 chickens). In the control group, each chicken was intramuscularly inoculated with 300 μL of an emulsion containing PBS; in the gp90 group, each chicken was inoculated with 300 μL of an emulsion containing 100 μg of purified recombinant gp90 protein; in the CpG + gp90 group, each chicken was inoculated with 300 μL of an emulsion containing 100 μg of recombinant gp90 protein and 50 μg of CpG-ODN adjuvant; in the Poly(I:C) + gp90 group, each chicken