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Recombinant duck enteritis viruses expressing major structural proteins of the infectious bronchitis virus provide protection against infectious bronchitis in chickens

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ABSTRACT

To design an alternative vaccine for control of infectious bronchitis in chickens, three recombinant duck enteritis viruses (rDEVs) expressing the N, S, or S1 protein of infectious bronchitis virus (IBV) were constructed using conventional homologous recombination methods, and were designated as rDEV-N, rDEV-S, and rDEV-S1, respectively. Chickens were divided into five vaccinated groups, which were each immunized with one of the rDEVs, covalent vaccination with rDEV-N & rDEV-S, or covalent vaccination with rDEV-N & rDEV-S1, and a control group. An antibody response against IBV was detectable and the ratio of $CD4^+/CD8^+$ T-lymphocytes decreased at 7 days post-vaccination in each vaccinated group, suggesting that humoral and cellular responses were elicited in each group as early as 7 days post-immunization. After challenge with a homologous virulent IBV strain at 21 days post-immunization, vaccinated groups showed significant differences in the percentage of birds with clinical signs, as compared to the control group ($p < 0.01$), as the two covalent-vaccination groups and the rDEV-S group provided better protection than the rDEV-N- or rDEV-S1-vaccinated group. There was less viral shedding in the rDEV-N & rDEV-S- (2/10) and rDEV-N & rDEV-S1- (2/10) vaccinated groups than the other three vaccinated groups. Based on the clinical signs, viral shedding, and mortality rates, rDEV-N & rDEV-S1 covalent vaccination conferred better protection than use of any of the single rDEVs.

1. Introduction

Infectious bronchitis (IB) is a highly contagious viral disease of the upper respiratory and urogenital tracts of chickens that is caused by the infectious bronchitis virus (IBV). The disease is prevalent in nearly all countries with an intensive poultry industry, causing respiratory and renal diseases in chickens of all ages. It also reduces the quality and quantity of eggs produced by mature hens, causing heavy economic losses to the poultry industry. In addition, high mortality often occurs in young chickens infected with nephropathogenic strains as a result of renal pathology (Cavanagh and Gelb, 2008).

The IBV genome consists of a linear, single-stranded, positive-sense RNA, which encodes four major structural proteins, which include the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) phosphoprotein, and the envelope or small membrane (E) protein. The N phosphoprotein is conserved among different IBV serotypes and can induce high titers of cross-reactive antibodies and cell-mediated immunity that protects chickens from acute infection, thus it is used as a target protein in designing vaccines against IB (Williams et al., 1992; Collisson et al., 2000; Seo et al., 1997). The S glycoprotein is responsible for receptor binding and membrane fusion (Hofmann et al., 2004), and consists of the N-terminal S1 and C-terminal S2 subunits (Bosch et al., 2003). Most of the conformation-dependent, neutralizing antigenic, and serotype-specific determinants in IBV have been mapped to S1, while other immunodominant regions are located in the N-terminal regions of S2 (Koch et al., 1990; Kusters et al., 1989; Lenstra et al., 1989). In addition, interactions between the S1 and S2 subunits might affect

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the conformation of the S1 subunit, thereby accounting for differences in serologic protection (Callison et al., 1999). The M glycoprotein of coronaviruses gives the virion envelope its shape. It has been reported that the S glycoprotein interacts with the transmembrane region of the M glycoprotein and the cytoplasmic tail of the IBV E protein is responsible for its interaction with the IBV M glycoprotein (Cavanagh, 2007).

Measures to control IB in poultry rely primarily on vaccination. Multiple live attenuated IBV vaccines are most often required because of poor cross protection between vaccines produced from different IBV serotypes (Liu et al., 2009, 2014). Live attenuated IBV vaccines do not provide adequate protection throughout the lifetime of layers or breeders, whereas inactivated vaccines convey certain advantages, such as slow antigen release and long-lasting immunity throughout the laying period. Unfortunately, inactivated IBV vaccines are not effective when used alone, as birds require one or a series of vaccinations with live-attenuated IBV vaccines (live priming) prior to administration of an inactivated vaccine (Cook et al., 2012). Conventional live IBV vaccines are attenuated by multiple serial passages in embryonated eggs (Gelb and Cloud, 1983; Jackwood et al., 2003; Bijlenga et al., 2004; Huang and Wang, 2006), although this is a time-consuming process.

Genetically engineered vaccines present an alternative to inactivated and attenuated vaccines. In previous studies, a multivalent DNA vaccine expressing S1, N, and M conferred 85% protection (Yang et al., 2009), while a multivalent DNA vaccine combined with an inactivated vaccine booster conferred complete protection (Yan et al., 2013). In addition, a recombinant Newcastle disease virus expressing the S2 protein of IBV was shown to provide broad protection against IBV challenge (Toro et al., 2014).

Duck enteritis virus (DEV) causes duck plague, an acute, contagious, and lethal disease that affects birds of all ages of the order Anseriformes (Davison et al., 1993). DEV is a member of the family *Herpesviridae* with a genome approximately 158 kb in size (Li et al., 2009). Because certain DEV genes are not essential for viral replication in vitro (Wang and Osterrieder, 2011; Liu et al., 2011), DEV has been used as a replicating vaccine vector in chickens to provide rapid protection against the H5N1 influenza virus (Liu et al., 2013a,b). In our current study, we used DEV as a viral vector to construct three recombinant viruses expressing the N, S, and S1 proteins of IBV, and evaluated their protective efficacy in chickens against virulent IBV challenge.

2. Materials and methods

2.1. Viruses and cells

The nephropathogenic IBV strain ck/CH/LDL/091022 is an LX4-type (QX-like) strain that was first isolated in China in 2009 (Sun et al., 2011). The DEV Clone-03 was isolated from a commercial vaccine by plaque assay (Li et al., 2006; Liu et al., 2007). Primary chicken embryo fibroblasts (CEFs) were used for DEV propagation (Li et al., 2006).

2.2. Embryos and chickens

Specific pathogen-free (SPF) white leghorn chickens, chicken embryo eggs and duck embryo eggs were obtained from Harbin Veterinary Research Institute (HVRI; Harbin, China). The birds were maintained in isolators under negative pressure and provided with food and water ad libitum. All experiments were performed in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the Ministry of Science and Technology of the People's Republic of China, and the study protocols were approved by the Committee of the Ethics of Animal Experiments of the HVRI.

2.3. Plasmid construction

DEV genomic DNA was extracted as previously described (Prigge et al., 2004). The left and right homologous arms of the transfer vector were amplified by polymerase chain reaction (PCR) using primers VL1, VL2, VR1, and VR2. The enhanced green fluorescent protein (EGFP) cassette was amplified by primers R1 and R2 (Table 1) from the pEGFP-N1 plasmid and cloned into the pMD18 T-simple plasmid to produce pT-EGFP. The left and right arm PCR products were inserted through the *Cla*I and *Bln*I restriction sites and the *Mlu*I and *Ava*III restriction sites, respectively, of the pT-EGFP plasmid to produce pUS10-EGFP. Complementary DNA (cDNA) of the N, S, and S1 genes of the virulent IBV strain ck/CH/LDL/091022 was synthesized from the viral genomic RNA by reverse transcription (RT)-PCR (Sun et al., 2011). The pUS10-N, pUS10-S, and pUS10-S1 plasmids were produced by inserting the N, S, and S1 PCR products, respectively, between the *Xho*I and *Not*I restriction sites flanking the EGFP open reading frame (ORF) in the pUS10-EGFP plasmid.

2.4. Construction of recombinant viruses

The strategy for the construction of the recombinant DEVs (rDEVs) is depicted in Fig. 1A. Briefly, the genomic DNA of DEV and the pUS10-EGFP transfer vector were cotransfected into CEFs using TurboFect transfection reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). rDEV containing EGFP (rDEV-EGFP) with deletion of the complete US10 gene was selected by plaque assay and used as the parental virus for constructing rDEVs expressing the N, S, and S1 proteins of IBV. The selection of rDEV-N, rDEV-S, and rDEV-S1 was conducted using fluorescence microscopy, and plaques without green fluorescence were purified by plaque assay.

2.5. Identification of the rDEVs

N1F, N1R, SF, SR, S1F, and S1R gene-specific primers (Table 1) were used to confirm the identity of rDEV-N, rDEV-S, and rDEV-S1 by PCR. In addition, the primer pair DF and DR, corresponding to the flanking sequence of the DEV US10 gene (Table 1), were used to differentiate wild-type (WT) DEV from the rDEVs. Western blotting was performed to detect the expression of IBV proteins from the rDEVs (Han et al., 2013). Rabbit anti-GFP IgG (Sigma–Aldrich Corporation, St. Louis, MO, USA), mouse anti-IBV N protein monoclonal antibody (4F10) (Han et al., 2013), and chicken anti-IBV serum were used as primary antibodies for detection of EGFP, N, S, and S1 expressed from rDEV-EGFP, rDEV-N, rDEV-S, and rDEV-S1, respectively. In addition, an anti-chicken β-actin monoclonal antibody of mouse origin (Sigma–Aldrich Corporation) and mouse anti-DEV gL serum (prepared in our laboratory) were used to detect reference proteins and the efficacious replication of DEV or rDEVs in CEFs. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, or anti-chicken IgG (Sigma–Aldrich Corporation) were used as secondary antibodies. An indirect immunofluorescence assay was performed to detect protein expression in infected CEFs. Briefly, CEFs were infected with rDEVs at multiplicity of infection (MOI) of 0.001 and then fixed with 4% paraformaldehyde at 48 h postinfection. The antibodies described in Section 2.5 were used as primary antibodies, while fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-chicken IgG (Sigma–Aldrich Corporation) was used as the secondary antibody. Expression of foreign proteins in recombinant viruses was observed by fluorescent microscopy.