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Eliciting specific humoral immunity from a plasmid DNA encoding infectious bursal disease virus polyprotein gene fused with avian influenza virus hemagglutinin gene

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DNA vaccine coding for infectious bursal disease virus (IBDV) polyprotein gene and that for avian influenza virus (AIV) hemagglutinin (HA) gene have been shown to induce immunity and provide protection against the respective disease. The present study was carried out to determine whether an IBDV polyprotein gene-based DNA fused with AIV HA gene could trigger immune response to both IBDV and AIV. After transfection, VP2 and HA were detected in the cytoplasm and at cell membrane, respectively, by immunofluorescent antibody double staining method, suggesting the fusion strategy did not affect the location of protein expression. VP4 cleavage between VP2 and HA was confirmed by Western blot, indicating the fusion strategy did not affect VP4 function in transfected cells. After vaccination in chickens, the DNA construct VP24-HA/pcDNA induced ELISA and virus neutralizing antibodies against VP2 and hemagglutination inhibition antibody against the HA subtype. The results indicated that a single plasmid construct carrying IBDV VP243 gene-based DNA fused with AIV HA gene can elicit specific antibody responses to both IBDV and AIV by DNA vaccination.

1. Introduction

Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae* and genus *Avibirnavirus*. IBDV is an immunosuppressive viral agent due to the fact that the infection causes severe depletion of B lymphocytes in the bursa of Fabricius (Cho, 1970; Wyeth, 1975; Rosenberger and Gelb, 1978). Therefore, the infected chickens have increased susceptibility to secondary infections and decreased vaccination responses (Allan et al., 1972; Faragher et al., 1972; Winterfield et al., 1978). The genome of IBDV contains bipartite double-stranded RNA namely segment A and segment B. There are two partially overlapped open reading frames in segment A (3.3 kb). The first open reading frame encodes the nonstructural protein, VP5 (17 kDa). The second open reading frame encodes the polyprotein VP243 (109 kDa) which is processed through the autoproteolysis of VP4 (28 kDa) to generate pVP2, VP4 and VP3 (Azad et al., 1987; Jagadish et al., 1988). The premature form of the capsid protein, pVP2, is further cleaved at its C-terminus by VP4 and its autoproteolytic activity to form VP2 (37 kDa) (Sánchez and Rodriguez, 1999; Irigoyen et al., 2009). VP2 is the only component in the capsid and thus is the major immunogen for inducing virus neutralizing antibody to protect against IBDV infection (Becht et al., 1988; Fahey et al., 1989; Coulibaly et al., 2005).

Avian influenza virus (AIV) belongs to the family *Orthomyxoviridae* which possesses eight segments of single-stranded minus-sense RNA genome. Hemagglutinin (HA), one of the major surface glycoprotein recognizes and attaches to sialic acid on the cell surface as the receptor for AIV (Webster et al., 1992). Thus the HA-receptor binding specificity determines the viral tropism and host range (Suzuki et al., 2000; Wilks et al., 2012). HA is also one of the major viral proteins responsible for inducing neutralizing antibody and provides protection from AIV infection (Kostolanský et al., 2000; Gao et al., 2006; Swayne, 2009; Varečková et al., 2013). In recent years, high pathogenic H5N1 subtype has become a global concern after the direct transmission from domestic chickens to humans (Claas et al., 1998; Subbarao et al., 1998; Zhang et al., 2013). Generally, wild aquatic birds are considered as natural reservoirs for all AIVs and these viruses must require adaptation to infect and replicate more efficiently in other animal spices (Webster et al.,

Abbreviations: IBDV, infectious bursal disease virus; AIV, avian influenza virus; HA, hemagglutinin; FITC, fluorescein isothiocyanate; TMB, 3,3′,5,5′-tetramethylbenzidine.

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1992; Horimoto and Kawaoka, 2001). It has been suggested that the immunosuppression caused by IBDV in chickens could facilitate the adaptation process for water fowl associated AIV to domestic poultry (Ramirez-Nieto et al., 2010). Thus, it is critical for the poultry industry to prevent both viral infections in the same flock.

DNA vaccines containing VP2 or VP243 gene of IBDV has been shown to provide protection of chickens against IBDV (Chang et al., 2001, 2003; Hsieh et al., 2010; Chen et al., 2011). Vaccination with DNA encoding HA gene has also shown protection against AIV challenge in previous studies (Fynan et al., 1993; Robinson et al., 1993; Kodihalli et al., 2000). The present study was carried out to determine whether an IBDV large segment gene-based DNA fused with AIV HA gene could trigger dual expression of both proteins and induce specific humoral immune responses to both IBDV and AIV by a single plasmid construct.

2. Materials & methods

2.1. Chickens

Specific pathogen free chicks were hatched out after 21 days of incubation from embryos (Charles River Laboratories, Wilmington, MA, USA). After hatching, one-day-old chicks ($n = 30$) were transferred to Horsfall-Bauer isolators with food and water provided ad libitum. The protocol for using chickens in the present study was approved by Purdue University Animal Care and Use Committee.

2.2. Construction of DNA

2.2.1. VP243/pcDNA

VP243 sequence (from variant E strain of IBDV) was PCR amplified from VP243/pCR3.1 (Hsieh et al., 2006) and subcloned with pcDNA3.1/V5-His© TOPO® TA expression kit (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. Primer sequences were VP2 forward (5′-ACGATCGCAGCGATGACAAA-3′) and VP3 reverse (5′-TCACTCAA GGTCCTCATC-3′).

2.2.2. HA/pcDNA

AIV HA gene was subcloned to pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) with restriction enzymes *Bam*HI and *Xho*I (New England Biolabs, Beverly, MA, USA) from cDNA of avian influenza virus stain TK/WI/68 H5, provided by Dr. David Suarez, Southeastern Poultry Research Laboratory, USDA, Athens, GA. Primers used were H5-*Bam*HI-F (5′-GCTCGGATCCATGGAAAGAATAGTGATTGC-3′) and H5-*Xho*I-R (5′-GTACTCTC GAGCTAGATGCAAATTCTGCAC-3′).

2.2.3. VP24-HA/pcDNA

Three steps were performed for preparing VP24-HA/pcDNA, including subcloning of VP243 gene to pNEB193 vector (New England Biolabs, Beverly, MA, USA) with *Kpn*I and *Pme*I sites, inserting HA gene into VP243/pNEB193 with restriction enzymes *Dra*III and *Bsa*AI (New England Biolabs) and subcloning VP24-HA fusion gene into pcDNA3.1 (Invitrogen). Primers used for VP24-HA subcloning were as the following: H5-*Dra*III-SNF (5′-GTGCTCACTCAGTGTAATGGAAAGAATAGTGATTGC-3′) and H5-*Bsa*AI-R (5′-GCATGCACGTAG ATGCAAATTCTGCACTG-3′).

2.3. Protein expression from DNA in Vero cells

Vero cells were transfected with the constructed plasmids by TransIT®-LT1 transfection reagent (Mirus, Madison, WI, USA) following the manufacture's instruction. After incubation for 48 h, cells were fixed with acetone at room temperature for 10 min and subjected to immunofluorescent antibody assay as described previously (Chang et al., 2003) to detect the transient protein expression with antibody R63 (monoclonal antibody against VP2, American Type Culture Collection, Manassas, VA, USA) or anti-HA chicken serum (provided by Dr. Janice C. Pedersen, National Veterinary Service Laboratory, USDA, Ames, IA) as the primary antibody. Goat anti-mouse or anti-chicken IgG conjugated with fluorescein isothiocyanate (FITC) was used as the secondary antibody (KPL, Gaithersburg, MD, USA). The immunofluorescence was examined in an epifluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan).

2.4. Location of expressed proteins from DNA in Vero cells

Vero cells were grown on cover slips in 6-wells and transfection was followed the next day. Forty-eight hours later, cells in 6-wells were stained by anti-HA or R63 antibodies for 30 min directly without acetone fixation. After washing three times with PBS, secondary antibodies were applied for 30 min. After another three-time PBS wash, cells were fixed with acetone. Then immunofluorescent antibody staining for the second protein, either VP2 or HA was followed. For example, the first staining for HA by anti-HA chicken serum without acetone fixation and using FITC-labeled goat anti-chicken antibody as the secondary antibody, followed by the second staining for VP2 by R63 after acetone fixation and using Texas red-labeled goat anti-mouse antibody (KPL) as the secondary antibody.

2.5. Western blotting for expressed proteins from DNA in DF-1 cells

DF-1 cells (ATCC) in 6-wells were transfected with TransIT®-LT1 (Mirus). Dilution of transfection reagent and mix with plasmids were done as described in the manufacturer's manual. After 48 h of incubation, cell extract was prepared in CytoBuster™ (EMD, Novagen®, Gibbstown, NJ, USA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and subjected to SDS-PAGE and Western blotting. The blots were stained with R63 or anti-HA chicken serum first and then followed with goat anti-mouse or anti-chicken secondary antibody conjugated with horseradish peroxidase (KPL). Final incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) membrane peroxidase substrate system (KPL) was done according to the manufacturer's instruction.

2.6. DNA vaccination

Four groups of one-day-old SPF chickens were inoculated in the thigh muscle with 500 μg of the plasmids pcDNA vector (without any gene insert) ($n = 10$), VP243/pcDNA ($n = 5$), HA/pcDNA ($n = 5$), or VP24-HA/pcDNA ($n = 10$), respectively, as described previously (Chang et al., 2003; Chen et al., 2011). Same dose boosters were performed when chickens were 7, 14 and 28 days of age, respectively.

2.7. ELISA antibody titer to IBDV

Blood was withdrawn from the wing vein of chickens ($n = 30$) on day 14, 21, 28, 35 and 41 after the first inoculation with DNA. Serum ELISA antibody titers were determined by using commercially available ELISA kit for IBD (IDEXX, Portland, ME, USA) following the manufacturer's instruction. The ELISA plates were read at the wavelength of 595 nm in an ELISA reader (VMax™, Molecular devices, Sunnyvale, CA, USA).

2.8. Virus neutralizing antibody titer to IBDV

Chicken sera were obtained as described above. The virus neutralizing titer was determined by using a protocol reported previously from our laboratory (Chen et al., 2011). Briefly, the serum was 4-fold serial diluted and transferred to the well in a 96-well