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Immunization with avian metapneumovirus harboring chicken Fc induces higher immune responses

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ABSTRACT

In this study, we evaluated the immune responses of avian metapneumovirus harboring chicken Fc molecule. Stable Vero cells expressing chicken Fc chimera on its surface (Vero-cFc) were established, and we confirmed that aMPV grown in Vero-cFc incorporated host derived chimera Fc into the aMPV virions. Immunization of chicken with aMPV-cFc induced higher level of antibodies and inflammatory cytokines; (Interferon (IFN)-γ and Interleukin (IL)-1β) compared to those of aMPV. The increased levels of antibodies and inflammatory cytokines in chicken immunized with aMPV-cFc were statistically significantly ($p < 0.05$) to that of aMPV and control. The aMPV-cFc group also generated the highest neutralizing antibody response. After challenges, chickens immunized with aMPV-cFc showed much less pathological signs in nasal turbinates and trachea so that we could confirm aMPV-cFc induced higher protection than that of aMPV. The greater ability of aMPV harboring chicken Fc to that of aMPV presented it as a possible vaccine candidate.

1. Introduction

Avian metapneumovirus (aMPV), previously known as turkey rhinotracheitis virus or avian pneumovirus, causes an acute respiratory disease in turkeys. It is characterized by coughing, nasal discharge, tracheal rales, foamy conjunctivitis, and sinusitis in turkeys of all ages (Njenga et al., 2003) and is also associated with "swollen head syndrome" in broilers and broiler breeders (Cook, 2000). In laying birds, it causes a transient drop in egg production, along with mild respiratory tract illness (Jones, 1996). The virus was first isolated in South Africa in 1978 and subsequently in other parts of the world. The aMPV isolates are currently classified into four subgroups, subgroups A, B, C, and D based on antigenic and genetic characterization. Subtype A was first isolated in South Africa and England, whereas subtype B virus was initially isolated in continental European countries including Hungary, Spain and Italy. Subtype C was isolated in France (Muscovy ducks), USA (turkeys, and wild birds), and recently in China (chickens and Muscovy duck) and in Korea (pheasants) while the presence of an additional subtype D was reported in France (turkey) (Brown et al., 2014).

Mainly attenuated live and inactivated vaccines are used to protect against aMPV type A and B field infections in Europe (Broor and Bharaj, 2007; Easton et al., 2004). A cold-adapted strain of aMPV C resulted in good protection against virulent aMPV C infection in turkeys (Patnayak and Goyal, 2004a,b). DNA vaccine expressing the F protein of aMPV reduced the clinical symptoms in challenged birds (Kapczynski and Sellers, 2003). A virosome vaccine containing both the F and G protein, have conferred successful protection against virulent aMPV (Kapczynski, 2004) too. In spite of different available vaccines, still the aMPV has major impact on the poultry industry. Therefore, there is an urgent need to develop an effective vaccine for aMPV.

Fc domain bears the recognition signal for specific cellular immunoglobulin G Fcγ receptors (FcγRs) as well as an interaction niche for immune effectors. FcγRs are crucial mediators of cellular immunity and important link between the IgG secreting lymphocytes and phagoctytes, (Ravetch and Bolland, 2001; Allhorn et al., 2008; Figge, 2009). The Fc-FcγRs interaction heralds the occurrence of virus infection and orchestrates other signaling events that lead to T-cell activation (Amigorena and Bonnerot, 1999). Receptors for immunoglobulins (FcR) play an important role in the activation of autoimmune reaction (Ji et al., 2003) and immune reaction for infections of influenza virus, human immunodeficiency virus and

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simian immunodeficiency virus (SIV) (Huber et al., 2001; Perez-Bercoff et al., 2003; Villinger et al., 2003). Other groups reported that hepatitis B surface antigen (HBsAg) complexed to human anti-HBs immunoglobulins induced enhanced immune responses (Wen et al., 1999). It was also reported that a DNA vaccine coding for hepatitis B virus antigen-Fc fusion proteins had enhanced antigen-specific CD4+, CD8+ and B cells (You et al., 2001). Furthermore, the ability of neutralizing antibodies to protect against virus infection is highly compromised when the Fc receptor function is abrogated (Hezareh et al., 2001; Hessell et al., 2007, 2009).

These studies have paved a way to incorporate the Fc fragment of chicken IgG into the aMPV virus so as to enhance immunogenicity. In this study, we constructed genetically modified cells (Vero-cFc) that express chicken immunoglobulin G (IgG) Fc on the cell surface. Propagation of aMPV in Vero cells expressing chicken Fc (cFc) on the surface resulted in taken over cFc on its surface during budding process. These cFc bearing virus particles form a highly efficacious strain enhancing its immunogenicity.

2. Materials and methods

2.1. Cells, and viruses

African green monkey kidney cells (Vero, CCL-81) were maintained in minimum essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Vero cell-adapted avian metapneumovirus subtype B, Korean isolate (Kwon et al., 2010) was propagated in Vero cells cultured in MEM containing 5% FBS. Vero cells were inoculated with aMPV B at a multiplicity of infection (MOI) of 1 and cultured in MEM containing 2% FBS at 37 °C, 5% CO_2 for 72 h.

2.2. Construction of chicken Fc plasmid

The chicken-Fc portion of IgG consisting of CH2 and CH3 domains was amplified from chicken blood. The cloning process inserted chicken Fc portion (1.257 kb) into 5′-*Bgl*II and 3′-*Sal*I terminal restriction sites (5′- AGA TCT CGA TTG TAC CCT CTA TCC −3′ and 5′- GTC GAC TTT ACC AGC CTG TTT CTG −3′) in frame with myc epitope of pDisplay™ vector (Invitrogen, Cat no: V660-20). The chicken Fc sequence is available in the GenBank database under the accession number X07174.1.

2.3. Establishment of chicken Fc cell lines

For the establishment of Vero-cFc cell line, pcFc plasmid was transfected into Vero cells using Attractene transfection reagent® (Qiagen, Hilden, Germany Cat no: 301005). After 24 h transfection, the cells were sub-cultured for a period of three generations. Selection was performed with G418 and several G418-resistant clones were obtained and finally one healthy Vero cell clone, named Vero-cFc was used for the subsequent experiments.

2.4. Confirmation of chicken Fc expression

2.4.1. Immunocytochemistry (ICC)

To detect chicken Fc (cFc) domain on cell surface, immunocytochemistry was performed as follows; Vero-cFc cell lines were seeded at a density of 5×10^5 cells per well in a 6-well plate. After 24 h, cells were fixed by incubating them in 4% (v/v) formaldehyde in PBS for 15 min at room temperature (RT). Unspecific binding was blocked using 1X PBS containing 5% bovine serum albumin for 30 min at RT. Probed with anti-myc antibody, 1:100 (LifeSpan Biosciences, WA, USA, Cat no: LS-C180004) and HRP conjugated anti mouse IgG followed by treatment with 3,3′-diaminobenzidine tetrahydrochloride dehydrate (DAB, Vector Labs Burlingame, CA, USA, Cat no: SK-4100).

2.4.2. Western blotting

To determine cFc expression, a mono-layer of 3×10^6 Vero Fc cells was maintained on T75 flask for 3 days. As a control, non-transfected Vero cell line (Vero-WT) was used. The cells were harvested and collected by centrifugation at 3000*g* for 5 min. The cell pellet was lysed using cell lysis buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF, Sigma). The prepared cell extracts were subjected to electrophoresis through 10% SDS-PAGE gel and transferred onto a polyvinyl difluoride membrane. The antibodies used in this study were anti-myc antibody and HRP conjugated IgG. The bands were visualized using Supersignal West Dura (Pierce Rockford, IL, USA, Cat no: 35075) with LAS-1000PLUS (Fujifilm Fuji Photo Film, Tokyo).

2.5. cFc acquisition assay

Vero-cFc cells were infected with aMPV B or mock, and virus was harvested when 90% CPE was observed. Cultures were stored at −70 °C and the virus released from cells by three times freeze-thawing. Cell debris was then separated from virus by a spin down at 3000*g* for 15 min at 4 °C. Supernatants were subsequently passaged in Vero cells (Vero WT). One hour post infection, aMPV-cFc viruses were probed using antibodies anti-myc (1:100). Expression was confirmed under microscope.

For blotting assay, aMPV and aMPV-cFc of $10^{5.75}$ $TCID_{50}$/ml and $10^{5.50}$ $TCID_{50}$/ml respectively were used and concentrated with polyethylene glycol (PEG, MW 8000). The recovered virus was applied for SDS-PAGE followed by western blotting.

2.6. Immunization

For virus inactivation, formaldehyde solution was added to the viruses; aMPV (aMPV subtype B) and aMPV-cFc (aMPV subtype B carrying cFc). Vaccines, with oil adjuvant, were prepared by emulsifying the $10^{6.0}$ $TCID_{50}$/ml of formaldehyde-inactivated aMPV and aMPV-cFc antigen solution with ISA70 (Seppic, Puteaux, France) at a ratio of 30:70 (wt/wt).

Eighty of four-week-old SPF chickens were purchased, and housed in animal facilities in Chungnam National University (CNU), Korea. They were divided into four groups with 20 chickens in each. Each group was housed in separated positive pressure isolators. Chickens were vaccinated intramuscularly with inactivated aMPV ($10^{6.0}$ $TCID_{50}$/ml) in group I, inactivated aMPV-cFc ($10^{6.0}$ $TCID_{50}$/ml) in group II. Group III is only challenge group and group IV is negative control group followed by boosting after 2 weeks. Chickens in group I, II and III were challenged with live aMPV subtype B, Korean isolate ($10^{6.0}$ $TCID_{50}$/ml) oculo-nasally at three weeks after boosting. Sera were collected at day 0, 14, and 21 and 24 (3 days after challenge). For histopathological study; nasal turbinate and trachea were collected. Collected sera were kept in −20 °C for further analysis. All procedures were conducted with the protocols and regulation approved by Animal Care and Use Committee in CNU, Korea.

2.7. Histopathological staining and evaluation

Nasal turbinates and tracheas were fixed in 10% phosphate-buffered formalin, and tissue sections were stained with haematoxylin and eosin (H&E). Microscopically observed lesions were categorized as negative or positive based on lesions such as conspicuous interstitial heterophilic and lymphocytic infiltration and aggregation, destruction of epithelial and glandular tissue, and