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Effects of avian infectious bronchitis virus antigen on eggshell formation and immunoreaction in hen oviduct

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ABSTRACT

The aim of this study was to determine the mechanism by which the avian infectious bronchitis virus (IBV) affects eggshell formation. Attenuated IBV (aIBV group) or vehicle (control group) was injected into the oviductal magnum lumen of White Leghorn laying hens. The changes in the expression of genes related to eggshell formation (*collagen types I and V*, and *CaBP-D28K*), densities of cytotoxic cells ($CD8^+$ and TCR-$\gamma\delta^+$ T cells), and gene expression of molecules related to cytotoxic immunoreaction (*B-NK*, *perforin*, *granzyme*, and *IL-2*) and proinflammatory cytokines (*IL-1β*, *IL-6* and *IFN-γ*) were examined by quantitative reverse transcriptase polymerase chain reaction or immunohistochemistry in the isthmus and uterus. Gene expression of *IL-1β* and *IL-6 receptors* in the tubular gland cells of the isthmus and uterus was analyzed by reverse transcriptase polymerase chain reaction. Gene expression of *collagen type I*, but not *collagen type V*, in the isthmus and *CaBP-D28K* in the uterus was decreased in the aIBV group compared with that in the control. The frequencies of $CD8^+$ cells and TCR-$\gamma\delta^+$ T cells in the isthmus and uterus were significantly higher in the aIBV group than in the control group. The expression of cytotoxic molecular and proinflammatory cytokines was also higher in the aIBV group than in the control. The expression of *IL-6 receptor*, but not *IL-1β receptor*, was identified in the tubular gland cells in the isthmus and uterus. These results suggest that IBV infection causes disorder of eggshell formation by disturbing gene expression of *collagen type I* in the isthmus and *CaBP-D28K* in the uterus, probably via the effects of substances from cytotoxic cells and proinflammatory cytokines.

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1. Introduction

The eggshell membrane and matrix are formed in the isthmus and uterus of hen oviduct, respectively. The eggshell membrane is composed of inner and outer layers, and the inner layer is rich in collagen types I and V, whereas the outer layer contains collagen type I [1]. Calcium ion for shell formation is secreted from the tubular gland cells of the uterus, and calbindin (CaBP)-D28k plays a primary role in Ca^{2+} transportation [2,3].

Avian infectious bronchitis virus (IBV) belongs to the Coronaviridae family that infects the bronchial tubes, and causes respiratory disease [4]. This virus is also capable of infecting the epithelial surfaces of other organs, such as the trachea, kidney, proventriculus, and oviduct [5,6,7]. Infection by IBV in the oviduct leads to disorder of eggshell formation and reduction of egg production [8,9].

Natural killer (NK) cells, a member of the group of cytotoxic cells expressing B-NK receptor (B-NK) and CD8 antigen on their surface, play an essential role in the innate immune response at the early phase of virus infection [10,11]. The NK cells discriminate virus-infected cells, which are deficient in terms of major histocompatibility complex class I expression, and eliminate them via cytotoxic proteins such as perforin and granzyme [12–14]. In mammals, the cytotoxic ability of NK cells is enhanced by interleukin (IL)-2, which is secreted by $CD4^+$ T cells [15]. In addition, NK cells secrete interferon (IFN)-γ, which enhances acquired immunity by activating T cells and macrophages [16,17]. IFN-γ also causes

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inflammation in combination with other proinflammatory cytokines such as IL-1β and IL-6.

$CD8^+$ cytotoxic T cells recognize antigens presented on infected cells through major histocompatibility complex class I, and kill those infected cells. Although the biological functions of chicken TCR-$\gamma\delta^+$ T cells have not been established, they may have cytotoxic activity, and most of them in the intestinal epithelium express CD8 antigen [18,19]. It has also been reported that the cytotoxic ability of TCR-$\gamma\delta^+$ T cells is enhanced by IL-2 [20,21].

Although it is known that IBV infection in hen oviduct causes disruption of egg formation, its mechanism is not well understood [9]. We hypothesized that an immune response caused by infection may affect the function of egg formation. The goal of this study was to determine the mechanism by which eggshell formation is disrupted by IBV in hen oviduct. Thus, considering the attenuated IBV (aIBV) may exert effects that mimic those of IBV in the immune response [7,22,23], we examined the effects of aIBV on the gene expression related to egg formation (*collagen types I* and *V*, and *CaBP-D28K*), densities of cytotoxic cells ($CD8^+$ and TCR-$\gamma\delta^+$ T cells), and the expression of molecules of cytotoxic cells (*B-NK*, *perforin*, and *granzyme*) and cytokines (*IL-1β*, *IL-6*, *IFN-γ* and *IL-2*) in the isthmus and uterus. The expression of *IL-1β* and *IL-6 receptors* in the tubular gland cells of the isthmus and uterus was also analyzed.

2. Materials and methods

2.1. Experimental birds

White Leghorn laying hens aged approximately 170 days old were kept in individual cages under a daily light regimen of 14-hour light:10-hour dark, and provided with food and water *ad libitum*. They were divided into five experimental groups (n = 5 each): no treatment (0-hour group), and 24 or 48 hours after treatment with vehicle (control group) or aIBV. The magnum of hens allotted to treatments was surgically exposed under anesthesia with pentobarbital (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan) at 1 hour after oviposition (approximately 30 minutes after ovulation), and 20 mL of $10^{6.5}EID_{50}$ aIBV solution (attenuated live vaccine; Kaketsuken, Kumamoto, Japan) or vehicle was injected into the magnum lumen using a syringe (Terumo Co., Tokyo, Japan) with a 21-ga needle (Terumo Co.). The vehicle consisted of 120 mg of lactose hydrate (Sigma-Aldrich, St. Louis, MO, USA), 150 mg of peptone (Sigma-Aldrich), 60 mg of D-sorbitol (Sigma-Aldrich), 75 mg of L-arginine hydrochloride (Sigma-Aldrich), 30 mg of gelatin (Nacalai Tesque, Kyoto, Japan), 300 units of benzylpenicillin potassium (Cosmo Bio, Tokyo, Japan), and 300 μg of streptomycin hydrosulfate (Cosmo Bio). The birds of each group were euthanized under anesthesia with Somnopentyl, and the isthmus and uterus were collected. Mucosal samples from these oviductal segments were processed for paraffin and frozen sections and also total RNA samples. The normality of the shells of eggs laid within 24 or 48 hours was examined morphologically (normal or soft-shelled egg). The eggshell membranes separated from normal shelled eggs and soft-shelled eggs were processed for paraffin sections. The thickness of those eggshell membranes was measured using image analysis software (ver. 3.2; NIS-Elements; Nikon, Tokyo, Japan) under a light microscope. Handling of birds was carried out in accordance with the regulations of Hiroshima University Animal Research Committee.

2.2. Histologic analysis of immune cells

2.2.1. Tissue preparation and staining

The isthmus and uterus tissues and also the eggshell membrane were fixed with 10% (vol/vol) formalin in PBS, and processed for paraffin sections. Some of the tissues of isthmus and uterus were also embedded in a medium for frozen tissue specimens (Optimal Cutting Temperature compound; Sakura Co., Tokyo, Japan), and snap-frozen in isopentane and solid CO_2 mixture to prepare frozen sections. The paraffin sections were stained with Hansen's hematoxylin and eosin for histologic observation. Frozen sections (10 μm in thickness) were prepared using a cryostat (Bright Instrument Co. Ltd., Huntingdon, England), and used for identification of $CD8^+$ and TCR-$\gamma\delta^+$ cells. They were air-dried on slides and fixed with acetone on ice for 30 minutes, and then washed with PBS, followed by incubation with 10% (vol/vol) normal rabbit serum (blocking solution) for 30 minutes. They were incubated overnight with mouse monoclonal antibodies to chicken CD8 (Santa Cruz Biotech., Inc., Santa Cruz, CA, USA) or chicken TCR-γδ (Southern Biotech, Birmingham, AL, USA) diluted at a concentration of 1 μg/mL with PBS. After washing the sections with PBS (3 × 5 minutes), the immunoreaction products were detected using Histofine SAB-PO (M) kit (Nichirei Co., Tokyo, Japan). Briefly, the sections were incubated with biotinylated antimouse Immunoglobulin (Ig) G + IgA + IgM and streptavidin-peroxidase for 1 hour each, with washing in PBS (3 × 5 minutes) after each step. Immunoprecipitates were visualized by incubating the sections with a reaction mixture of 0.02% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride and 0.005% (vol/vol) H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). The sections were counterstained with Hansen's hematoxylin.

2.2.2. Image analysis for immune cell frequencies

Sections were examined under a light microscope with Nomarski filter (Eclipse E400; Nikon, Tokyo, Japan) connected to image analysis software (NIS-Elements, Nikon). The number of $CD8^+$ and TCR-$\gamma\delta^+$ cells within the mucosal epithelium of the isthmus (3.2×10^4–3.9×10^5 μm^2) or uterus (9.7×10^4–6.6×10^5 μm^2), and lamina propria of the isthmus (2.5×10^5–2.6×10^6 μm^2) or uterus (1.1×10^5–2.4×10^6 μm^2) was counted. The frequencies of the T cells were calculated as the number of positive cells in an area of 1×10^5 μm^2. The analysis was performed in triplicate in one tissue, and the average was used for the value of that tissue.

2.3. Reverse transcriptase polymerase chain reaction analysis for expression of genes related to egg formation and immune response

Total RNA extraction from the mucosal tissues of the isthmus and uterus was performed using Sepasol-RNA I Super (Nacalai Tesque). The extracted total RNA samples were dried and dissolved in Tris EDTA buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA). They were treated with 1 U RQ1

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