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# Innate antiviral immune response against infectious bronchitis virus and involvement of prostaglandin E2 in the uterine mucosa of laying hens

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ABSTRACT

Infectious bronchitis virus (IBV) is an enveloped RNA virus that causes deformities in eggshells. The aim of this study was to investigate the innate immune response to IBV, and to determine whether prostaglandin (PG) E2, which is synthesized during inflammation, is involved in the innate immune response in the uterine mucosa. The effects of intra-oviductal inoculation with attenuated IBV (aIBV) on the expression of viral RNA recognition receptors and innate antiviral factors were examined by real-time PCR and immunohistochemistry, and on PGE2 levels by ELISA. Then, the effects of PGE2 on the expression of innate antiviral factors in cultured uterine mucosal cells were examined. The results showed that the expression of RNA virus pattern recognition receptors (*TLR3*, *7*, and *MDA5*), antimicrobial peptides (avian β-defensins, including *AvBD1, 2, 4–6* and cathelicidins, including *CATH1* and *3*), and interferons (*IFNα, β, γ, λ*) were upregulated, and the expression of cyclooxygenase 2 (PG synthase) and the level of PGE2 were increased in the uterine mucosa following aIBV inoculation. The number of AvBD2-positive cells in the mucosa also increased in response to aIBV. In cultured mucosal cells (mainly epithelial), the expression of *AvBD4, 10–13* and *IFNα, β, and λ* was upregulated following incubation with 500 nM PGE2. These results suggest that the expression of viral RNA-recognition receptors, *AvBDs*, *CATHs*, and *IFNs* and PGE2 are induced by the IBV antigen, and that the expression of a different set of *AvBDs* is also induced by PGE2 in the cultured uterine mucosal cells. These antiviral factors may play a role in the protection of the uterine mucosa from IBV infection.



## 1. Introduction

Eggshell is an important barrier against pathogens on the surface of eggs [1,2], and its quality is associated with hatchability and production of healthy chicks [3]. Infectious bronchitis virus (IBV) is a common infectious virus that causes a significant decrease in eggshell quality [4]. IBV is an enveloped single-stranded RNA (ssRNA) virus related to the *Coronaviridae* family, and has a high ability to raise new resistant strains due to its high rate of mutation. Thus, traditional vaccination alone is not always sufficient for the prevention of IBV disease [5]. Innate antiviral immunity has been proposed as an alternative way of protecting against IBV [6]. The development of potent inducers of human antimicrobial peptides (AMPs) has been investigated, and the successful induction of some AMPs, such as LL37 (a member of the human cathelicidins family), has been reported [7,8]. We recently reported increased proinflammatory cytokine expression and influx of cytotoxic leukocytes following inoculation with live attenuated IBV (aIBV), which is expected to mimic the antigenicity of wild IBV [9], suggesting the induction of an immune response and inflammation in response to IBV infection.

The innate immune system represents a first line of defense against various pathogens. Induction of an innate antiviral immune response is initiated by the recognition of viral antigens by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), including TLR3 and 7, and melanoma differentiation-associated protein 5 (MDA5), which are present in chicken immune and epithelial cells and facilitates the recognition of viral RNA [10,11]. The resultant innate antiviral immune response leads to the

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production of antiviral factors such as AMPs, including the avian β-defensins (AvBDs), cathelicidins (CATHs), and interferons (IFNs). AvBDs and CATHs are small cationic peptides that exert direct or indirect antiviral action against enveloped viruses [12]. We have shown that *AvBDs* are expressed in the hen oviduct [13,14], and their expression in the vagina is upregulated in response to bacterial and viral TLR ligands [13,15,16]. *CATHs* are also expressed in chicken [17], and we recently showed their expression to be modulated by proinflammatory cytokines [18]. Type I IFNs (IFNα and IFNβ) and type III IFNs (IFNλ) are mainly antiviral, whereas type II IFNs (IFNγ) are immunomodulators, and are also involved in mediating protection against viral infection [19]. Recently, IFNλ was reported to play an important role in mucosal antiviral activity in chickens [20].

Prostaglandin (PG) E2 is a metabolite of PGH2 and is produced by the action of cyclooxygenases (COXs), including the constitutive COX1 and inducible COX2 isoforms [21]. We recently reported that both COXs are expressed in the uterus of laying hens, and that COX2 expression is higher in the uterus than in other segments of the oviduct [22]. Furthermore, PGE2 can modulate both inflammatory and immune responses [23,24], and may be exploited by viruses during viral pathogenesis [25]. However, it is unknown whether PGs affect the synthesis of innate antiviral factors.

We hypothesized that an innate immune response would be induced by IBV antigen and that PGE2 may be one of the factors involved in this response. It has not yet been established whether IBV induces the expression of antiviral factors and PGs in the uterus, or whether PGE2, in turn, affects the expression of these antiviral factors. Thus, the aim of this study was to investigate the innate immune response against IBV, and determine whether PGE2 affects the expression of AMPs and IFNs in the uterus of laying hens. We examined the expression of viral PRRs specific for RNA viruses (*TLR3*, *7*, and *MDA5*), antimicrobial peptides (*AvBDs* and *CATHs*), and interferons (*IFNα*, *β*, *γ*, and *λ*), as well as the synthesis of PGE2 in the uterine mucosa following intra-oviductal injection of attenuated IBV (aIBV) in the uterus. Since the expression of five *AvBDs*, including *AvBD2,* was higher in the aIBV-injected group compared with the control group, immunohistochemistry for AvBD2 was performed to identify the cells responsible for AvBD2 expression. Then, the effect of PGE2 on the expression of *AMPs* and *IFNs* was examined in vitro.

## 2. Materials and methods

### 2.1. Effects of intra-oviductal inoculation of aIBV on the expression of innate antiviral receptors and factors, and synthesis of PGE2

#### 2.1.1. Bird treatment and sample collection

White Leghorn hens (approximately 40-weeks-old), laying five or more eggs in a sequence, were kept in individual cages under a lighting regimen of 14-h light: 10-h dark and provided with commercial feed and water *ad libitum*. Hens were divided into two groups (n = 7/group), namely, the aIBV and control groups. Birds were anesthetized 1 h after oviposition by intravenous injection of sodium pentobarbital (approximately 0.8 ml/bird) (Somnopentyl®; Kyoritsu Shoji Co., Tokyo, Japan). Aseptically, the isthmus was surgically exposed and injected with 2 ml of $2 \times 10^{5.5}$ $EID_{50}$ of live aIBV vaccine (Kaketsuken, Kumamoto, Japan) (aIBV group), or with the vehicle (control group.) The vehicle (1 ml) consisted of 12 mg of lactose hydrate, 15 mg of peptone, 6 mg of D-sorbitol, 7.5 mg of L-arginine hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA), 3 mg of gelatin (Nacalai Tesque Inc., Kyoto, Japan), 30 units of benzylpenicillin potassium, and 60 μg of streptomycin hydrosulphate (Cosmo Bio Co., Tokyo, Japan). After 25 h of the injection, hens were euthanized as mentioned above, and the uterine mucosal tissues were collected. This study was approved by the Hiroshima University Animal Research Committee (No. C11-4).

#### 2.1.2. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis of innate antiviral factors

Total RNA was extracted from the uterine mucosae of birds in both groups using Sepasol-RNA I Super (Nacalai Tesque Co. Inc.), according to the manufacturer's instructions. Pelleted RNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and kept at -80 °C until use. The RNA samples were treated with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA) at 37 °C for 45 min, and then at 65 °C for 10 min and the concentration and purity were measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). The RNA samples were reverse transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan), according to the manufacturer's instructions. Reverse transcription (RT) was performed at 42 °C for 30 min, followed by heat inactivation at 99 °C for 5 min using the programmable thermal controller (PTC-100; MJ Research). The expression of *TLR3/7, MDA5, AvBDs, CATHs, IFNs, COXs,* and *RPS17* was examined by qRT-PCR using a Roche Light Cycler Nano System (Roche Applied Science, Indianapolis, IN, USA) following the MIQE guidelines [26]. All samples from the aIBV and control groups were examined by qRT-PCR to confirm the presence of IBV-specific RNA. All samples from the aIBV group contained IBV RNA, while it was not detected in samples from the control group (data not shown). Target gene expression was examined by qRT-PCR using chicken-specific primers, as previously described (Table 1). The PCR mixture (10 μL) consisted of 0.5 μL cDNA, 1 × Thunderbird SYBR qPCR Mix (Toyobo Co. Ltd.), and 250 nM of each primer. Target genes were amplified under the following conditions; heating at 95 °C for 120 s, 50 cycles of 95 °C for 10 s with annealing temperatures as shown in Table 1, and extension at 72 °C. For *MDA5*, *AvBD5*, *IFNα, COXs,* and *RPS17*, a single temperature was used for annealing and extension, as shown in Table 1.

The qRT-PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method [27] to calculate the relative expression in each sample, using the *RPS17* expression as the internal control for normalization. All qRT-PCR products were confirmed by electrophoresis on 2% (w/v) agarose gel with 0.6% ethidium bromide and observed on a transilluminator (NTM-10E; UVP LLC, Upland, CA, USA). A sample from the control group was used as a standard sample for all analyses.

#### 2.1.3. Prostaglandin E2 assay

To extract prostaglandins, samples of uterine mucosal tissue (approximately 100 mg) from the aIBV and control groups (n = 7/group) were each homogenized in 1 ml of phosphate-buffered saline and kept in a water bath at 37 °C for 20 min, as described by Veerabagu et al. [28]. Then, samples were centrifuged at 9000 × g at 4 °C for 1 min and the supernatants were stored in -80 °C until analysis.

To determine PGE2 concentrations, a competitive ELISA was performed in 96-well plates, in a similar way in our previous study that examined the 13,14-dihydro-15-keto PGF2α [29]. Wells were coated with an anti-PGE2 antibody (Abcam Inc., Cambridge MA, UK; Ab2318). After washing, 10-times diluted samples were added to the wells and incubated with PGE2 conjugated to horseradish peroxidase (Sigma-Aldrich Co.) using the mixed anhydride reaction [30], as previously described [31]. The standard curve ranged from 0 to 5000 pg/ml 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was used for color development by measuring the optical density at 650 nm. The inter- and intra-assay coefficients of assay variability were