**INFECTION, HEALTH, AND DISEASE**

Altered pro-inflammatory cytokine mRNA levels in chickens infected with infectious bronchitis virus

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**ABSTRACT** Infectious bronchitis virus (IBV) replicates primarily in the respiratory tract and grows in various organs in chickens, with or without pathological effects. The diversity of this virus has been verified by sequence analysis of the S1 glycoprotein gene, but this method must be supplemented with further analysis for characterization of the agent. To increase our understanding of the pathogenesis of the disease caused by this virus, we investigated the response of chickens to 2 IBV with different genotypes, KIIa and ChVI. The clinical signs induced by the viruses were observed. In addition, the mRNA levels of the pro-inflammatory cytokines, IL-6, IL-1β, and lipopolysaccharide-induced tumor necrosis factor-α factor and the serum levels of α1-acid glycoprotein, which is a major acute phase protein, were measured. The KIIa genotype (Kr/ADL110002/2011) induced clinical signs accompanied by the excessive production of pro-inflammatory cytokines and a higher viral load. In chickens infected with this isolate, simultaneous peaks in the viral copy number and cytokine production were observed at 7 dpi in the trachea and 9 d postinoculation in the kidney. On the other hand, the chickens infected with the ChVI genotype (Kr/ADL120003/2012) did not show a response other than a mild upregulation of cytokines at 1 d postinoculation, which appears to indicate the invasion of the virus. In summary, we confirmed a differential innate response following infection with distinct IBV. We hypothesize that an excessive innate response contributes to the scale of the pathophysiologic effect in chickens.

**Key words:** infectious bronchitis virus, immunopathogenesis, acute phase response, pro-inflammatory cytokine, acute phase protein

2013 Poultry Science 92:2290–2298
http://dx.doi.org/10.3382/ps.2013-03116

**INTRODUCTION**

Infectious bronchitis (IB) is a common, highly contagious, and acute viral disease of poultry. The disease is caused by a coronavirus known as IB virus (IBV). The virus initiates infection via the respiratory tract and replicates in various types of epithelial cells, including those of the kidney, gonads, and alimentary tract (Villegas, 1998; Saif et al., 2011). After the identification of the virus, a high diversity among isolates was reported, not only in antigenicity, but also in pathogenicity, and the nature of this virus motivated researchers to develop an efficient way to classify the isolates (Hofstad, 1958; Gelb et al., 1991; Lee et al., 2008).

Genotyping is a relatively recent method of viral classification that is preferred today because of its ability to rapidly detect new strains. It is based on sequence analysis of the S1 portion of the S protein gene, which encodes the virus-neutralizing epitope and is responsible for viral attachment to cells (Ma et al., 2012). At least 3 genotypes of IBV have been identified in Korea by S1 sequence analysis (Lim et al., 2012). The KII type, one of the antigenic clusters of the Korean isolates, has frequently been isolated in field outbreaks and is related to nephropathogenic Chinese IBV strains (e.g., the ch/QXIBV strain; Lee et al., 2008). The rapid detection of variant isolates is the strongest aspect of the S1 sequence analysis method. However, because the presumed tissue tropism from this system is not always in agreement with the actual tissue tropism (Sapats et al., 1996), a more detailed understanding of the immunopathogenesis of IB infections is required. Such an understanding will enable scientists to be prepared for the emergence of variants with unusual tissue tropisms (Raj and Jones, 1997).

The study of immunopathogenesis has been used to verify disease progress in diverse viral infections. The
correlation between immunopathogenesis and disease progress is widely accepted in diverse studies. In the case of severe acute respiratory diseases, such as SARS, immunopathogenesis has been suggested to contribute to disease progression, providing the rationale for therapy with corticosteroids (Muñoz-Fernandez, 2004). During H5N1 influenza infection, an excessive cytokine level in the blood and the innate immune response were suggested to play a role in disease progression (To et al., 2001; Peiris et al., 2004). In poultry, a role for cytokines in disease progression was suggested in Marek’s disease, mycoplasmosis, salmonellosis, and coccidiosis (Laurent et al., 2001; Swaggerty et al., 2004; Withanage et al., 2004; Jarosinski et al., 2005; Mohammed et al., 2007).

Especially in IB infections, analyzing the correlation between immunopathogenesis and disease progression appears to be valid and effective, considering that the severity of IB infection depends significantly on host factors such as immune status, age, and breed. In a previous study, the renal disease induced by an IB isolate was reproduced at different scales according to the age of the infected chickens (Dolz et al., 2012). The discrepancy in pathogenicity among different genetic lines of specific pathogen-free (SPF) chickens following the same nephropathogenic strain has also been documented (Ötsuki et al., 1990; Ignjatovic et al., 2003).

However, little is known about immunopathogenesis during IB infection in chickens (Asif et al., 2007). One report investigated the overexpression of IL-6 induced by the T strain and its role in exaggerated pathogenicity in S-line chickens (Asif et al., 2007). This report demonstrated that the overexpression of IL-6 by the host may be responsible for the pathological sequelae, but it remains to be verified whether similar dysregulation could be induced by distinct pathotypes, including nonpathogenic strains (Asif et al., 2007).

In this study, we observed changes in the transcriptional levels of 3 pro-inflammatory cytokines that are known to be involved in the innate immune response in chickens (Hong et al., 2006; Davison et al., 2008) after inoculation with 2 IB isolates. Because these 2 isolates are distinct in genotype, we expected to confirm that the scale of change in the cytokines in the trachea and kidney would be correlated with pathogenicity. From this observation, we expected to suggest a strategy to reduce loss from IB infection and to provide helpful information about the immunopathogenesis of IB infection (Cook and Huggins, 1986).

**MATERIALS AND METHODS**

**Viruses**

Among IBV isolated from chickens showing significant clinical signs, 2 field isolates were chosen for this study (Table 1). Briefly, the isolate of the KIIa genotype (kr/ADL110002/2011) was isolated from kidneys of 3-wk-old broilers suffering from severe nephritis, respiratory signs, and increased mortality (1% per day). According to S1 sequence analysis, this isolate belongs to the KIIa type, which is closely related to the QX-IB strain. The isolate of the ChVI genotype (kr/ADL120003/2012) was derived from 38-wk-old layers with a slight decrease in egg production. This genotype (ChVI) does not belong to major clusters of Korean IB isolates but appears to be derived from a Chinese strain (Liu et al., 2006). The virus stock for the challenge study was produced by inoculating field isolates into embryonated SPF chicken eggs via the allantoic cavity and collecting the infectious allantoic fluid 72 h post-inoculation. Reverse-transcription (RT) PCR targeting the IBV S1 gene was carried out, and each 630-bp S1 amplicon was sequenced for genotyping by the direct sequencing method. The median embryo infective dose (EID_{50}) was determined by the Reed and Munch formula. The isolates were shown to be free from contamination by other avian pathogens by inoculating 3-wk-old SPF chicks and testing their sera at least 2 wk later using the procedures for the accreditation of SPF flocks. In this preliminary experiment, the chickens inoculated with the isolate kr/ADL110002/2011 showed severe nephritis after prolonged infection (>14 d), whereas no significant clinical symptoms were observed in chickens inoculated with the same dose of the isolate kr/ADL120003/2012.

**Experimental Design**

During the experiment, the chickens were raised in a HEPA-filtered isolation system. All procedures, including euthanasia, followed the guidelines of the Institute
of Laboratory Animal Resources (confirmation number: CBNUA-446-12-01). Forty-five 3-wk-old SPF chickens (VALO BioMedia North America, Adel, IA) were used and were divided into 3 groups of 15: an unchallenged control group (group 1), a group challenged with an isolate of the KIIa genotype (Kr/ADL110002/2011, group 2), and a group challenged with an isolate of the ChVI genotype (Kr/ADL120003/2012, group 3; Table 1). The chickens were inoculated with an eye drop of 0.2 mL of 10^5 EID_{50} of the IB isolates or 0.2 mL of sterilized PBS. At 1, 3, 5, 7, and 9 dpi, 3 birds in each group were bled before euthanasia, followed by necropsy and sample collection. The blood was kept at room temperature for approximately 3 h, and the serum was separated and stored at −20°C.

During necropsy, tissues of the trachea and kidney were collected and immediately treated with TRIzol reagent (Invitrogen, Carlsbad, CA) for total RNA isolation; tissue samples were taken from the upper one-third of the trachea by the horizontal long axis and the upper left lobe of the kidney.

**Total RNA Isolation**

Total RNA was isolated from tissues obtained from necropsy using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Homogenized tissue samples were treated with TRIzol reagent followed by phenol-chloroform phase separation. The RNA was precipitated using 2-propanol, washed with 75% ethanol, dried, and resuspended in 100 μL of diethylpyrocarbonate-treated water. The RNA was quantified by measuring the absorbance at 260 nm on a spectrophotometer, and the purity was assessed using the 260:280 nm ratio.

**Semiquantitative PCR**

The viral copy number was determined by semiquantitative RT-PCR. Semiquantitative RT-PCR was conducted as previously described, with slight modifications (Liu et al., 2008). Briefly, RNA samples and primers targeting the N gene of the IB virus (Table 2) were added to Maxime RT-PCR PreMix tubes (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea) and brought to 20 μL with sterilized distilled water. The RT-PCR reaction was performed as follows: one cycle of 30 min at 45°C and 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. Upon completion of the last cycle, the reaction mixtures were incubated at 72°C for 5 min. Then, 10 μL of each product was separated by 2% agarose gel electrophoresis. The band intensity of the PCR products was calculated using the Image J 1.37 v program (http://rsb.info.nih.gov/ij). To generate a standard curve, 10^5 EID_{50} of viral stock was serially diluted 10-fold, and RNA was extracted from each diluent. The RT-PCR, agarose gel electrophoresis, and image analysis were conducted as described above, and the results were plotted against the dilution factor. For comparison, the corresponding value of a 10^5 dilution of the viral stock on the x-axis was normalized to 1, and the relative viral load for each sample was calculated according to the standard curve (Figure 1).

**cDNA Synthesis and Quantitative Real-Time PCR**

The cDNA synthesis from the RNA samples was performed using the PrimeScriptRT reagent kit (Perfect Real Time, TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer’s recommendations. Briefly, 10 μL of reaction mixture (PrimeScript Buffer, PrimeScript RT Enzyme Mix I, 25 pmol of Oligo(dT) Primer, and 0.01 μg of total RNA) was incubated at 37°C for 15 min and 85°C for 5 s. Then, qRT-PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA). All reactions were performed in 3 independent samples derived from 3 chickens in 25-μL reaction mixtures containing 12.5 μL of 2 × SYBR Premix Ex Taq II (TliRNaseHPlus, TaKaRa), 9.5 μL of distilled water, 2 μL of cDNA template, and 0.1 μM of each primer (Table 2). The PCR conditions were the same for each targeted gene: 30 s at 95°C, followed by 30 s at 59°C, and 30 s at 72°C, with an additional melting curve analysis.

### Table 2. Primers used in this study1

<table>
<thead>
<tr>
<th>Primers used in this study</th>
<th>Accession no.</th>
</tr>
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<tbody>
<tr>
<td>N gene of IBV⁡</td>
<td>NC_001451</td>
</tr>
<tr>
<td>GAPDH</td>
<td>K01458</td>
</tr>
<tr>
<td>IL-6</td>
<td>AJ250838</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AJ245728</td>
</tr>
<tr>
<td>LITAF³</td>
<td>AY765397</td>
</tr>
</tbody>
</table>

1F = forward; R = reverse. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
2Mardani et al., 2006. IBV = infectious bronchitis virus.
3Hong et al., 2006. LITAF = LPS-induced TNF-α factor.
by 40 cycles of 95°C for 5 s and 62°C for 80 s. Cycling was terminated after 40 cycles, and dissociation curves of the products were generated by increasing the temperature incrementally from 55 to 100°C as the final step of the real-time PCR. To calculate the fold-change over the control chickens treated with PBS, the ratio of the concentration of the target gene to a reference gene (GAPDH) in samples from PBS-inoculated controls (calibrator) was normalized. The normalized ratio was then compared with the ratio of the concentration of the target gene to the reference gene in individual samples from IB-isolate inoculated chickens. The Mann-Whitney U test (SPSS 12.0 K for Windows) was used to determine significant differences between fold change values. The SD was calculated using the fold-change values of 3 replicates for each gene measured.

**Measurement of Alpha-1 Acid Glycoprotein in Serum**

Alpha-1 acid glycoprotein (AGP), an acute phase protein, was measured in serum using a commercially available ELISA kit (chicken α 1-acid glycoprotein (OGCHI) ELISA kit, Cusabio Biotech. Co. Ltd., Wuhan, Hubei, China). The ELISA was conducted according to the manufacturer’s instructions. To determine the AGP level in the serum, a standard curve was generated, and a regression assay was conducted. To determine significant differences, a 2-tailed independent Student t-test was used (SPSS 12.0 K for Windows, SPSS Inc., Chicago, IL).

**RESULTS**

**Development of Clinical Signs and Pathologic Lesions**

Until 7 dpi, no clinical sign was observed in chickens in the unchallenged control group (group 1) or the challenged groups (group 2 and 3). However, in chickens of group 2 (KIIa genotype), apparent clinical signs began to be observed; at 8 dpi, all 3 chickens infected with IBV of KIIa genotype sat crouched while the water consumption increased 200% ($P < 0.05$). The mortality (1 out of 3 birds) was observed only in group 2 at 9 dpi. During necropsy of chickens of group 2, however, we could not observe gross lesions related with these symptoms in kidneys or other organs. In the microscopic examination of chickens of group 2 (KIIa genotype), acute response lesions, such as the loss of cilia, acute necrosis of tracheal epithelial cells, and multifocal areas of acute tubular necrosis without any cellular infiltration in the kidney, were observed (Figure 2). On the other hand, no mortality or clinical sign observed in chickens of group 2, such as increase in water consumption and depression, was observed in chickens of group 3 (ChVI genotype).

**Viral Copy Number Estimated by Semiquantitative PCR**

The N-gene of IBV was amplified by RT-PCR, and the band intensity was rated on a scale of 1 to 10,000 (Figure 3). Until 3 dpi, the viral gene was not detected by RT-PCR. In the tracheae of chickens in group 2 (KIIa genotype), the viral copy number was first detected at 5 dpi ($1,859.9 \pm 642.9$), and the peak ($2,954.7 \pm 1,021.4$) was observed at 7 dpi. In the kidneys of chickens in the same group, the viral copy number was first detected at 7 dpi ($97.24 \pm 21.3$), and the peak copy number was observed at 9 dpi ($3,626.1 \pm 724.8$). In group 3 (ChVI), the viral gene was first observed at 7 dpi, whereas the viral copy number remained at approximately 100 in the trachea and kidney. These results indicate that the copy number was higher in the chickens from group 2, and their viral replication peaked in the trachea and the kidney at 7 and 9 dpi, respectively.

**Transcription Profile of Pro-inflammatory Cytokines**

The changes in 3 pro-inflammatory levels are illustrated in Figure 4. At 1 dpi, a significant increase in IL-6 and IL-1β transcription levels was observed in the
kidney and trachea of group 2 (KIIa), whereas in group 3 (ChVI), an increase was observed only in the kidney IL-6 level \((P = 0.049; \text{Figure 4A})\). In the case of lipopolysaccharide-induced tumor necrosis factor (TNF)-\(\alpha\) factor (LITAF) in the kidney, the arithmetic mean of triplicates in the challenged groups was higher compared with the control group, but a significant difference was not observed among the 3 groups. At 3 dpi, the levels returned to the range of the unchallenged control (Figure 4B), and the levels of IL-6 in the trachea were suppressed. The LITAF transcription level in the tracheae of both challenged groups was significantly decreased compared with the control group \((P = 0.049)\) at 3 dpi. At 5 dpi, the mRNA levels of IL-6 and LITAF were higher in the kidneys from group 2 (KIIa genotype; Figure 4C), whereas no obvious change was observed in the tracheae at 5 dpi. At 7 dpi, the LITAF levels were significantly increased in the tracheae of both challenged groups (Figure 4D). At the same time, the IL-6 levels of the tracheae appeared to be higher; however, a significant difference was not observed. At 9 dpi, the levels of all of 3 pro-inflammatory cytokines were substantially increased in the kidneys in chickens from group 2. Considering the viral copy numbers (Figure 3) and the transcription profiles at 7 and 9 dpi, the peak viral copy number occurred simultaneously with peak cytokine production in each organ.

**AGP in Serum**

Table 3 shows the serum AGP level after inoculation with IB isolates. In the unchallenged control group, the AGP level was in the range of 37 to 45 \(\mu\)g/mL. The AGP level of both challenged groups was compared with the unchallenged group. No significant difference was observed among all 3 groups at 1 dpi. At 3 dpi, the level of AGP in chickens from group 2 (KIIa genotype) had increased to approximately 44 \(\mu\)g/mL \((P = 0.02; \text{Table 3})\). At 5 dpi, the AGP levels in group 2 (KIIa genotype) reverted to the range of the unchallenged control, and no further increase was observed until 7 dpi. At 9 dpi, the AGP level increased almost 3-fold in both challenged groups \((P < 0.001)\). These findings appear to reflect viral invasion but do not appear to be associated with pathogenicity.
DISCUSSION

In this study, we confirmed that the pro-inflammatory response could be induced on a diverse scale depending on the IBV genotype. The KIIa genotype (kr/ADL110002/2011), which is closely related to the nephropathogenic strain, induced clinical symptoms of depression and a stronger pro-inflammatory response. On the other hand, an active infection of the ChVI genotype isolate kr/ADL120003/2012, which resulted in an increase in serum AGP level at 9 dpi (Table 3), evoked only a limited range of pro-inflammatory responses. According to the data collected at 9 dpi, the excessive production of pro-inflammatory cytokines was temporally associated with clinical signs related to renal damage, such as an increased drinking of water and lesions observed in the kidney, including renal tubular necrosis. These data suggest that excessive pro-inflammatory cytokine production contributes to disease severity. In particular, the excessive production of IL-6 in IB infections was already suspected to contribute to the exaggerated disease severity in specific genetic lines (Asif et al., 2007). Our data have different implications than the results of that study; we have demonstrated that specific IB genotypes induce pro-inflammatory cytokines and that differences in the scale of the induction could contribute to the disease severity induced by IBV of diverse genotypes.

In addition to the IL-6 profiling, it was meaningful to observe the pattern of change in the levels of 2 cytokines, IL-1β and LITAF, during IB infection because these immune mediators do not act alone in vivo (Perrone et al., 2008). In chickens, LITAF has been investigated as a substitute for TNF-α in viral infections (Ruby et al., 2006; Esnault et al., 2011), even though its involvement in viral infections was not demonstrated. In mammals, LITAF was reported to regulate TNF-α gene expression (Myokai et al., 1999) and to modulate pro-inflammatory cytokines (Tang et al., 2005b). It is still debatable whether LITAF could be used as an indicator during viral infection, but our findings at 9 dpi demonstrate the upregulation of LITAF following IB infection, so a subsequent increase in transcription of TNF-α could be anticipated. In mammals, IL-1β and TNF-α are known to have tissue-damaging potential, including inducing apoptosis (Dinarello, 1996; Eizirik, 2001; Hehlgans and Männel, 2002) and local damaging effects on the kidney (van de Kar et al., 1992; Jo et al., 2002; Malaponte et al., 2002). In this study, these features, which were not previously reported in chickens, suggest that these cytokines may contribute to the pathological changes of renal disease in IB infection.

At 7 and 9 dpi, temporal associations between peak viral copy number and peak cytokine production were observed. However, the kinetics of viral load were not precisely in accord with those of the pro-inflammatory cytokines. In previous studies of influenza viruses, the differential hyper-induction of cytokines was not explained by differences in viral replication kinetics (Chan et al., 2005), but the accumulation of double-
stranded RNA within the H5N1-infected cell appears to induce pro-inflammatory cytokines in the absence of productive virus replication (Chan et al., 2012). In case of SARS-CoV infection, the upregulation of the pro-inflammatory cytokine IL-6 was observed during in vivo studies (Wong et al., 2004), but a similar finding was not observed in in vitro studies of productively infected cells (Tang et al., 2005a). Instead, the induction of IL-6 was more significant in macrophages primed by bacterial lipopolysaccharide (Tseng et al., 2005). Thiel and Weber (2008) speculated that the pro-inflammatory response appears to be a secondary response of immune activation, not a direct response of cells against viral infection (Thiel and Weber, 2008). Based on these
reports, we assume that the pro-inflammatory response is not a direct sequela of viral infection but is instead a secondary response, and the discordance in the kinetics is consistent with that assumption.

In conclusion, we confirmed that a different pro-inflammatory response was associated with isolates of IBV with different genotypes. The induction of excess cytokine production appears to have a detrimental effect on tissues, and these findings suggest the need for an anti-inflammatory strategy to address the loss from IB infection.

REFERENCES


