Nephropathogenesis of Chickens Experimentally Infected with Various Strains of Infectious Bronchitis Virus

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ABSTRACT. Four-day-old specific-pathogen-free chickens were inoculated by eyedrop with four different strains (Gray, JMK, CV56b, and Wolgemuth) of infectious bronchitis virus (IBV). Birds were monitored clinically and euthanatized at 1, 4, 7, and 14 days post infection and tissues were collected for virus isolation, histopathologic examination, in situ hybridization (ISH), and immunohistochemistry (IHC). Clinical disease was severe in chickens infected with Wolgemuth, but no overt disease was observed with the other strains. Virus was isolated from the kidneys of chickens infected with the Gray-, CV56b-, and Wolgemuth-strains of IBV. Histologically, interstitial nephritis was evident in chickens infected with these same 3 strains. However, viral nucleic acid and antigen were detected only with Wolgemuth-infected kidneys by ISH and IHC. These results indicate that the pathological changes in kidneys from chickens infected with Gray and CV56b may not have resulted from the cytopathic action of the virus.

KEY WORDS: immunohistochemistry, in situ hybridization, infectious bronchitis virus, nephritis.

Coronaviruses exhibit a strong tissue tropism in vivo, infecting many mammalian and avian species and causing upper respiratory, gastrointestinal, hepatic, and central nervous system diseases [22]. Infectious bronchitis virus (IBV) was the first coronavirus described and sequenced completely [2]. The genomic RNA of coronaviruses is the largest among RNA viruses, approximately 27 to 30 kb. The genome of IBV is organized into six regions, encoding four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) protein in the 5’ to 3’ direction [15]. Spikes are synthesized in the endoplasmic reticulum and posttranslationally cleaved into peripheral S1 and integral membrane S2 subunits [5]. Although IBV infections are usually initiated in the upper respiratory tract and limited to this tissue, certain strains can spread to the kidney and cause nephritis [4, 8, 10, 25, 26]. The variations in tissue tropism among coronaviruses are largely attributable to variations in the spike glycoprotein. Large insertions or deletions that occur near specific sites in the S1 subunit and substitution mutations in the S2, that alter heptad-repeat sequences, have been associated with altered receptor interactions and change in tissue tropism [9].

The Gray strain was isolated in the 1960s and is characterized as a moderate nephropathogenic IBV (NIBV) [1, 25]. This strain shares 99% nucleotide sequence similarity in the S1 gene with the JMK strain which is strictly a respirotropic strain [14]. CV56b is the prototype strain of the California serotype and was isolated in 1991 [18]. Nephropathogenicity has not been reported with this strain or other California serotype IBVs. However, in our preliminary study, CV56b-infected chickens had urates in the ureter, and virus could be isolated from kidney. The Wolgemuth strain is a relatively new NIBV identified in Pennsylvania in 1998 [10]. The kidneys of chickens infected with NIBV are swollen and pale, with tubules and ureters extended with urates [8]. However, microscopic changes of nephritis may still be present without gross lesions [26]. Viral antigen in the kidneys has been shown by fluorescence antibody (FA) in an earlier report [12]. However, Lucio and Hitchner [17] found no specific fluorescence in the kidneys from chickens infected with several strains of IBV, including the Gray strain. IBV antigen in the kidneys has been identified by immunohistochemistry (IHC) with certain strains [6, 19]. In situ hybridization (ISH) is a technique that allows for precise localization of viral replication in histologic sections and has been used to detect IBV in ovo [3, 16]. To our knowledge, no study has been reported using ISH for detecting IBV in the kidney in vivo. Further, no comparative studies have been done with strains of different tissue tropism or virulence using these techniques.

In this study, we utilized ISH together with IHC, virus isolation and histopathologic data to compare the pathogenesis of two known NIBV (Gray and Wolgemuth) and one potential NIBV (CV56b). The JMK, a respirotropic strain, was also used as a non-nephropathogenic control.

MATERIALS AND METHODS

**Viruses:** Four different strains of IBV were used. Three strains (CV56b, Gray, and JMK) are maintained as allantoic fluid stocks at the University of Georgia. The Wolgemuth strain was received from Dr. J. Gelb, University of Delaware. Viruses were propagated in 9- to 11-day-old specific-pathogen-free (SPF) embryonating chicken eggs (Charles Rivers Spafas, North Franklin, CT) [21]. Titrations were done as previously described [24].

**Chickens:** Eighty 4-day-old SPF chickens were used and
groups of 16 chickens were housed separately in positive-pressure isolators. Chickens in each group were inoculated via eyedrop with 0.1 ml of 1 × 10⁵ EID₅₀ embryo infectious ducts₀₀₀ (EID₅₀) of inoculum of one of the four different strains. One group of 16 birds served as noninoculated controls. Animals were monitored daily by visual observation for clinical signs. Four birds in each group were euthanized at 1, 4, 7, and 14 days postinfection (dpi). Necropsies were performed immediately postmortem and tissues collected into 10% neutral buffered formalin.

**Virus isolation and titration:** Trachea and kidney tissues were collected in a tube that contained 1 ml of PBS with gentamicin sulfate (200 mg/ml). Approximately, 0.5 g of kidney and 1 cm of longitudinal section of tracheal tissues were minced and centrifuged at 1,500 g for 20 min, and 0.1 ml of undiluted supernatant was inoculated into each of three 9- to 11-day-old SPF embryonated chicken eggs. After 2 days of incubation, allantoic fluid was harvested. Confirmation of IBV was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) as described [13]. Kidney suspensions were tested using embryonated chicken eggs as previously described [24].

**Pathology:** The following tissues were collected immediately postmortem and fixed by immersion in 10% neutral buffered formalin for 48 hr: trachea, lung, kidney, bursa, intestine, spleen, and heart. Tissue were routinely processed into paraffin, and 3 µm sections were cut for hematoxylin and eosin staining, ISH, and IHC.

**Dot-immunoblotting assay (DIA) using Mab 9B1B6:** DIA was conducted as described previously with the following modifications [23]. Two hundred µl of allantoic fluid used for the challenge study was dotted onto 0.45 µm NC membrane (MSI, Westboro, MA) using the 96-well hybridot manifold (BRL, Gaithersburg, MO). After vacuum blotting, the membrane was blocked with 2% (W/V) dry nonfat milk in TST [10 mM Tris (pH 8.0), 0.1 5M NaCl, 0.05% Tween 20] at 37°C for 30 min. Then, it was reacted with diluted (1:200) 9B1B6 [20] in TST at room temperature (RT) for 1 hr. The 9B1B6 monoclonal antibody is against the spike protein of a nephropathic strain of IBV described by Parr and Collisson [20]. After washing the membrane with three changes of TST, phosphatase labeled goat anti-mouse IgG (KPL, Gaithersburg, MD) was added and incubated for 2 hr at RT. Following the washing step as above, the membrane was developed with the chromogen/substrate nitroblue tetrazolium and 5 bromo-4 chloro-3 indolophosphate (NBT/BCIP) (KPL) for 15 min at RT. The reaction was stopped by rinsing with distilled water.

**ISH and IHC:** ISH was conducted with 20 ng of antisense digoxigenin-labeled universal riboprobe. The sequence of the probe is complementary to the 452 bp carboxyl terminal region of the membrane gene of the CV56b strain. The ability of this probe to bind to multiple strains of IBV has been described previously [16]. For ISH, sections were deparaffinized, digested with 30 µg/ml Proteinase K, and hybridized overnight at 42°C. Following stringent washes, hybridization was detected using alkaline phosphatase-labeled goat-anti-DIG antibody (Roche) followed by NBT/BCIP (Roche). Chromogen development was stopped by dipping briefly in distilled water. Slides were counterstained with hematoxylin and coverslipped for a permanent record.

The immunostaining was performed with DAKO EnVision™ System, Peroxidase (DAKO Corporation, Carpinteria, CA) with automated immunostainer (LEICA ST5050, Leica instruments GmbH, Nussloch, Germany). All of the reagents except hydrogen peroxide: 3% W/W solution (Sigma, St. Louis, MO), Proteinase K: ready-to-use (DAKO corporation), and Mab 9B1B6 [20] were provided with the kit.

The immunostaining was conducted by 5 min of blocking with peroxidase blocking reagent, 10 min of treatment with hydrogen peroxide, 5 min of digestion with Proteinase K, 30 min of incubation with diluted (1:100) Mab, 30 min of incubation with peroxidase labelled polymer, and 5 min of developing with substrate and diaminobenzidine tetrahydrochloride (DAB) chromogen. Sections were lightly counterstained with hematoxylin.

**RESULTS**

**Clinical disease and gross pathology:** Disease was severe in chickens infected with the Wolgemuth strain, and no overt disease was observed with the other 3 strains. Birds infected with the Wolgemuth strain were severely depressed by 2 dpi, and 2 birds died at 4 dpi. In birds infected with CV56b, Gray and Wolgemuth strains, kidneys were slightly swollen and ureters were extended with urates at 4 dpi. No gross kidney lesions were observed in JMK and uninfected control chickens.

**Virus isolation and titration:** IBV was isolated from the trachea of all inoculated chickens sampled at 1, 4, and 7 dpi. Virus was recovered from the kidney at 1, 4, and 7 dpi in chickens infected with CV56b and Wolgemuth, and at 4 and 7 dpi in Gray-infected chickens. Virus was not recovered from the kidney of birds infected with the JMK strain. In Wolgemuth-infected chickens, virus was isolated from trachea and kidney at 14 dpi. Based on RT-PCR/RFLP, there was no cross-contamination among groups. We also titrated the virus isolated from the kidney. At 1 dpi, a low titer (less than 10¹⁵ EID₅₀) was observed in CV56b- and Wolgemuth-infected chickens. At 4 dpi, the titer increased three fold compared to 1 dpi and Wolgemuth-infected chickens had a higher titer (10¹⁵ EID₅₀) than Gray- and CV56b-infected chickens. At 7 dpi, CV56b-infected chickens showed the highest titer (10¹³ EID₅₀). The titer decreased at 14 dpi compared with 4 and 7 dpi. Virus isolation and titration results are summarized in Table 1.

**Histopathology:** There were no infection-associated changes in intestine and heart. In spleen and bursa, changes were similar with all of the strains. Compared with controls, bursas of infected birds showed stress-associated changes followed by follicular development. In spleens, there was increased prominence of Schweigger-Seidel sheaths at 4
Table 1. Virus isolation and virus titer in kidney

<table>
<thead>
<tr>
<th>Strain</th>
<th>1 dpi</th>
<th>4 dpi</th>
<th>7 dpi</th>
<th>14 dpi</th>
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<tbody>
<tr>
<td></td>
<td>Trachea</td>
<td>Kidney</td>
<td>Trachea</td>
<td>Kidney</td>
</tr>
<tr>
<td>CV56b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(2.18)</td>
<td>(3.58)</td>
<td>(1.50)</td>
</tr>
<tr>
<td>Gray</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>(3.44)</td>
<td>(2.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JMK</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(3.77)</td>
<td>(3.0)</td>
<td>(1.18)</td>
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</table>

a) Log<sub>10</sub> titer (EID<sub>50</sub>) of virus per 0.1 ml.

Changes in the trachea were severe with JMK, Gray, and Wolgemuth strains with focal degeneration and loss of cilia at 1 dpi, progressing to focal erosions and mononuclear cell infiltration at 7 dpi. Hyperplasia of the epithelium was noted at 7 dpi in all three strains, and at 14 dpi this hyperplasia was still evident in birds infected with JMK and Gray strains. In contrast, birds infected with strain CV56b had mild focal hyperplasia and mononuclear cell infiltration at 4 dpi, but at 7 and 14 dpi there were no histologic abnormalities.

In the lung, there was no histologic changes observed at 1 and 4 dpi. At 7 and 14 dpi, some chickens infected with Gray strain had focal ulcerative bronchitis. Moreover, at 14 dpi, two of three chickens infected with CV56b had focal areas of pneumatic consolidation.

Histologic findings in kidney were as follows. In chickens with the JMK strain, there were no lesions in the kidney at any time point except that 1 of 4 chickens at 1 dpi had small well-contained aggregates in the interstitium adjacent to proximal tubules. Infection with the Gray strain resulted in variably sized well-contained interstitial lymphoid aggregates near proximal tubules at 4 dpi in 2 of 4 birds (Fig. 1. By 7 dpi, these lymphoid aggregates had expanded, occasionally obliterating proximal tubules (Fig. 1b). Some of these aggregates contained developing lymphoid follicles at 14 dpi. In chickens infected with the Wolgemuth strain, at 4 dpi, two of four birds had severe and extensive degeneration and nephrosis of distal tubules and collecting ducts (Fig. 1c). These structures had widened lumens and occasionally heterophils within. By 7 dpi, there were abundant mixed mononuclear cell infiltrates, predominantly around distal tubules and collecting ducts (Fig. 1d). At 14 dpi, dissecting mononuclear cell aggregates were present in the interstitium around distal and collecting tubules, occasionally with the formation of lymphoid follicles. With the CV56b strain, at 4 dpi there were multifocal well-contained lymphoid aggregates in the interstitium, primarily around proximal tubules. At 7 dpi, one of three birds had severe dissecting interstitial nephritis around distal tubules and collecting ducts. At 14 dpi, one of three birds had numerous interstitial lymphoid aggregates, some with follicular development.

**ISH and IHC:** DIA conducted using Mab 9B1B6 showed equal reactivity of this Mab against the 4 strains used in this study (Fig. 2). This Mab was used as a primary antibody for IHC.

ISH and IHC were done on serial sections from the same block. Results obtained were the same with either method (Table 2). Staining was localized to the cytoplasm of infected cells, and no differences in sensitivity between the two techniques was observed. With all 4 strains, there was widespread viral nucleic acid in epithelial cells of trachea at 1 and 4 dpi (Fig. 3a, 3c). Less staining was observed at 7 dpi and no staining on 14 dpi. The extent of staining was correlated with the severity of histopathological lesions. In Wolgemuth-infected chickens, extensive staining was detected in the kidney at 4 and 7 dpi (Fig. 3d). Staining was restricted to cytoplasm of epithelial cells of distal tubules and collecting ducts. No staining was observed in kidneys infected with the other 3 strains (Fig. 3b). A few positive staining epithelial cells were observed in the bursa of chickens infected with either Gray, CV56b, or Wolgemuth strains. No positive staining was observed in lung, intestine, spleen, and heart with any of the strains.

**DISCUSSION**

The results of this study support previous evidence that Gray and Wolgemuth strains produce renal damage. Further, CV56b, which has not been characterized as a nephropathogenic strain, also caused an interstitial nephritis. It is interesting that the renal lesions were so strikingly different with the various strains of virus. That is, the Wolgemuth strain produced nephrosis, with destruction of tubular cells early in the infection, and subsequent mononuclear cell inflammation surrounding those tubules. These renal lesions were severe enough to kill some birds infected with this isolate. In contrast, in chickens infected with Gray and CV56b, the renal lesions consisted of small mononuclear cell aggregates in the interstitium that progressed with time to impact the proximal tubules in almost a space-occupying manner. In agreement with previous reports, we found no evidence of glomerular disease [6, 7].

By ISH and IHC, the presence of the virus was also strikingly different in the strains. These two techniques highlighted viral replication and viral protein production in the renal tubules of the chickens infected with Wolgemuth.
Fig. 1. Kidney. a) Chicken infected with Gray strain 4 days previously; a small aggregate of mononuclear cells is present in the interstitium. b) Chicken infected with Gray strain 7 days previously; an extensive infiltrate of mononuclear cells in the interstitium is obliterating some proximal tubules. c) Chicken infected with Wolgemuth strain 4 days previously; distal and collecting tubules are dilated and have degenerating and necrotic epithelium. d) Chicken infected with Wolgemuth strain 7 days previously; aggregates of mononuclear cells are present in the interstitium adjacent to distal and collecting tubules. HE stain. All figures are same magnification. Bar = 100 µm.

Fig. 2. Sensitivity of Mab 9B1B6 for detection of IBV in allantoic fluid. Dot immunoblotting was conducted with the allantoic fluid from embryonated eggs infected with four different strains of IBV. Allantoic fluid from uninoculated embryonated eggs was used as a control. Dot A; CV56b, dot B; Gray, dot C; JMK, dot D; Wolgemuth, dot E; control fluid.
However, in the chickens infected with Gray or CV56b, no evidence of viral infection of kidney was ever found with these techniques.

Consequently, the pathogenesis of the renal lesions is quite different with the various strains of NIBV. Whereas Wolgemuth has a distinct tropism for renal epithelial cells and causes destruction of tubular cells by direct cytolysis action, the pathogenesis of the two other NIBV in kidney is more indirect. Chong and Apostolov [7] reported the development of auto-antibodies against normal tubular brush bor-

**Table 2.** Extent of viral replication detected by *in situ* hybridization and immunohistochemistry

<table>
<thead>
<tr>
<th>Strain</th>
<th>Trachea</th>
<th>Kidney</th>
<th>Bursa</th>
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<tbody>
<tr>
<td>CV56b</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>JMK</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Gray</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Wolgemuth</td>
<td>+++</td>
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</table>

a) Four slide sections from 4 individual birds were examined at each DPI, and average scores are listed. (+++ = >5 positive cells per high-power field (400X); ++ = >1 and <5 positive cells per high-power field; + = 1 positive cell per high power field; – = no positive cells).

**Fig. 3.** *In situ* hybridization and immunohistochemistry. a) Tracheal epithelium, chicken infected with CV56b strain 4 days previously; note cytoplasmic staining in numerous epithelial cells, *in situ* hybridization, b) Kidney, chicken infected with CV56b strain 4 days previously; no staining, *in situ* hybridization, c) Tracheal epithelium, chicken infected with Wolgemuth strain 4 days previously, note cytoplasmic staining in scattered epithelial cells, immunohistochemistry, d) Kidney, chicken infected with Wolgemuth strain 4 days previously; numerous tubular cells have positive staining indicating presence of replicating virus, *in situ* hybridization. All figures are same magnification. Bar = 100 µm.
dors in chronic nephritis. Birds which had high auto-
tibody titers of kidney also had high antibody titers to
IBV. There may be an immune-mediated mechanism for
the renal damage associated with Gray and CV56b strains.
The histologic appearance of numerous lymphoid interstitial
aggregates in the Gray and CV56b strains is consistent with
that hypothesis.

It should be noted that viral isolation results from kidneys
infected with each of these three isolates were positive.
However, viral isolation indicates only that viable virus was
present in or on that tissue and may just be a reflection of
viremia and may not necessarily be present because of viral
replication within cells of that organ. In support of this,
Hofstad and Yoder [11] demonstrated that several stra ins of
IBV, which were not characterized as nephropathogenic,
could be isolated from the kidneys. Thus, it should be
emphasized that tissue tropism of IBV cannot be determined
only by virus isolation. Further studies using ISH, IHC, and
histopathology should be pursued to identify different
mechanisms of nephropathogenicity.

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