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INFECTIONOUS DISEASE

Histopathological and Immunohistochemical Study of Air Sac Lesions Induced by Two Strains of Infectious Bronchitis Virus

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Summary

Infectious bronchitis virus (IBV) is a highly contagious respiratory coronavirus of domestic chickens. Although mortality is low, infection with IBV results in substantial losses for the egg and meat chicken industries. Despite the economic importance of IBV and decades of research into the pathogenesis of infection, significant gaps in our knowledge exist. The aim of this study was to compare the early progression of air sac lesions in birds receiving a vaccine strain of the virus or a more virulent field strain. The air sacs are lined by different types of epithelia and are relatively isolated from the environment, so they represent a unique tissue in which to study virus-induced lesions. Both the pathogenic and vaccine strains of the virus produced significant lesions; however, the lesions progressed more rapidly in the birds receiving the pathogenic strain. Immunohistochemistry demonstrated that in birds infected with the pathogenic strain of virus, IBV spike protein is detected first in the ciliated cells lining the air sac. These preliminary data provide important clues regarding potential mechanisms for IBV tissue tropism and spread and show that the nature of the virus isolate influences the early progression of IBV infection.

Introduction

Infectious bronchitis virus (IBV) primarily causes an acute respiratory disease in domestic chickens. The disease is characterized by high morbidity, low mortality and substantial loss of production. The respiratory disease caused by IBV was first reported in the USA in 1930 and a single IBV serotype was described (Cavenagh and Naqi, 2003). Since the initial description of the disease, several other syndromes, including renal disease, proventricular disease and egg malformations, have been associated with this virus. Furthermore, over 20 serotypes of IBV are now recognized, with several circulating in the USA at any one time. Massachusetts, Connecticut and California serotypes are currently of the greatest economic importance (Cavenagh and Naqi, 2003). Worldwide, several other significant isolates, unique to particular regions, including Africa, Asia and Europe, have been described (Cavenagh and Naqi, 2003).

For the most part, the serotype of the virus determines both the outcome of the disease and the progression of an outbreak. However, the molecular basis for serotype-specific differences is not fully understood. Some serotypes never replicate outside the respiratory tract, while others can persist in the kidney, proventriculus or oviduct. Laboratory and in-vivo experiments prove that the pathogenicity and tissue tropism varies between serotypes and that significant variation in pathogenicity exists, even between different isolates of the same serotype (Purcell and McFerran, 1972). Understanding the genetic basis for this observation has proven difficult. Virus binding and entry into cells are complex events, which depend on several viral genes, phase of the cell cycle of the target cell and pH (Lukert, 1972; Shen et al., 2004, 2010; Chu et al., 2006; Chen et al., 2007; Ammayappan et al., 2009; Armesto et al., 2009).
Lesions of IBV in natural outbreaks are most commonly reported in the trachea, from where the virus can usually clear the virus after acute illness (Purcell and McFerran, 1972; Purcell et al., 1976; Fulton et al., 1993; Dwars et al., 2009). In more severe cases the virus spreads to the air sacs and causes more significant exudation and tissue damage (Fulton et al., 1993; Cavenagh and Naqi, 2003). Secondary bacterial infections are sometimes cofactors in more serious disease; however, viral serotype may also play a role in disease outcome (Cavenagh and Naqi, 2003; Dwars et al., 2009).

The aim of the present study was to study the morphological progression of infectious bronchitis during early infection of the avian air sacs.

**Materials and Methods**

**Birds**

Seventy-three 1-week-old specific pathogen-free (SPF) white leghorn-type chickens were obtained from the Poultry Research Farm, Cornell University. All birds were homozygous for the N2a major histocompatibility complex (MHC) phenotype (MHC: B1, E-a alloantigen C2, known to be resistant to Marek’s disease virus). The birds were divided into three groups and inoculated with either a vaccine strain of IBV \( (n = 22) \), a virulent strain of IBV \( (n = 29) \) or a sham inoculum of allantoic fluid \( (n = 22) \). The groups were housed in separate isolation rooms and were subjected to similar husbandry practices in accordance with the principles and procedures outlined in the institutional guidelines for the care and use of laboratory animals. Use of the animals in this study was approved by Cornell University’s Institutional Animal Care and Use Committee.

**Virus Isolates**

Two different strains of the Massachusetts serotype of IBV were used. One was a vaccine strain (Mildvac-Ma 5; Intervet Inc., Millsboro, Delaware, USA) and the other a field isolate (a virulent strain, obtained from Dr. J. Gelb, University of Delaware). The viruses were propagated twice in 9- to 11-day-old chicken embryos incubated for 48 h at 37°C. Inoculated allantoic fluid from the second embryo passage (EP2) was used to titrate the virus. The titre was calculated according to the Reed and Muench formula (Swayne et al., 1998).

**Air Sac Inoculation**

Virus inocula were diluted in phosphate buffered saline and a 100 μl aliquot containing 10,000 embryo infective dose 50% (EID₅₀) was injected directly into both the left and right thoracic air sacs. Allantoic fluid from unoinoculated chicken embryos was diluted at the same level as used for the virus and served as the inoculum (100 μl/bird) for the control group.

**Collection and Preparation of Membranes**

Birds were killed by carbon dioxide asphyxiation at regular intervals post infection. Chickens injected with the vaccine strain and control chickens were killed at 0, 1, 3, 6, 9, 12, 24 and 48 h post inoculation (hpi). Birds infected with field virus were killed at 1, 3, 6, 9, 12, 24 and 48 hpi. Two birds from the vaccine and control group were killed at each time point. Four birds from the group inoculated with the field strain were killed at each time point. Tissues from two of these birds were processed for histopathology. Tissues from the additional two birds were used for immunohistochemistry (IHC).

After death the thoracic respiratory air sac membranes were collected and processed. Air sacs were collected as described by Bezuidenhout (2005a). Briefly, the membranes were stabilized by immobilizing them between two stainless steel interlocking rings. This resulted in the edges of the air sac being trapped between the edges of the rings, leaving a central circular portion of the sac exposed in the well formed by the empty centre of the interlocking rings. The exposed area was processed for microscopy. Tissues for light microscopy were fixed in 10% neutral buffered formalin, embedded in plastic and stained with haematoxylin and eosin (HE).

Tissues for IHC were processed by filling the central well with optimum cutting temperature (OCT) compound (TISSUE-TEK, Miles Laboratories, Elkhart, Indiana, USA) and snap-freezing by immersion in isopentane cooled in liquid nitrogen. Frozen sections (6 μm) were air dried, fixed in anhydrous acetone and labelled for the presence of IBV structural proteins by the immunoperoxidase technique (Karaca et al., 1992). No pretreatment to block endogenous peroxidase activity was required as the air sac membrane was found to be free of endogenous peroxidase. Both group-reactive (9:4, S2) and mass-specific (15:88, S1) IBV monoclonal antibodies were employed (Karaca et al., 1992). The group-reactive antibody detects a conserved area of the IBV S2 protein, while the strain-specific antibody detects a specific epitope of the Massachusetts IBV serotype S1 protein (Karaca et al., 1992).

**Results**

**Histopathology**

Sham-inoculated Air Sacs. Inoculation of allantoic fluid without IBV did not elicit significant histological
response in 21/22 of the air sacs examined. Two small foci of macrophages, scattered lymphocytes and heterophils were present adjacent to areas of haemorrhage in one sample collected at 6 hpi. The majority of the normal air sac membrane was lined by simple squamous epithelium interspersed with a patchwork of localized islands of ciliated to tall columnar epithelial cells (respiratory epithelium; Figs. 1A and B). These islands of respiratory epithelium were most dense on and around the septum that separates the cranial and caudal air sacs. Occasional goblet cells were present between the respiratory type cells. A thin layer of connective tissue consisting of collagen and elastin fibres supported the epithelium. Small blood and lymphatic vessels, nerve bundles, fibroblasts and occasional macrophages, leucocytes, adipocytes and mast cells were present in the stroma.

**Vaccine Strain-infected Air Sacs.** Mild lesions were first identified at 12 hpi. In both of these birds the endothelial cells were slightly prominent and in one bird there was mild submucosal oedema. Small numbers of monocytes were adherent to the vascular intima and rare heterophils were present in the vascular lumina.

At 24 hpi (Fig. 1C) there was more significant inflammation. In one bird there was an extensive area of ulceration. The lamina propria beneath the ulcer was infiltrated by a large number of macrophages and scattered heterophils. Proprial oedema was widespread, but was most significant in the area of the ulcer. Smaller and less severe inflammatory foci were present throughout the section. With the exception of the ulcerated focus, the epithelium was normal. In the second bird, lesions were less severe and consisted of small pockets of oedema beneath the respiratory epithelium. Blood vessels in these areas were dilated, with slightly reactive endothelium.

Inflammation was most pronounced at 48 hpi (Fig. 1D). A single focus of moderate inflammation was present in the lamina propria of the air sacs from both birds. These foci were present beneath islands of ciliated epithelium. The overlying epithelium was markedly attenuated, with loss of cilia. However, normal respiratory epithelium was present in the rest of the air sac.

**Field Strain-infected Air Sacs.** Mild lesions were evident in the air sacs infected with the field strain of M41 beginning at 6 hpi. The lamina propria of affected air sacs was expanded by multiple small foci of oedema and dilated blood vessels. Vascular endothelial cells were often hypertrophic and small numbers of foamy monocytes often abutted the endothelial cells. These changes were most evident beneath islands of respiratory epithelial cells. Although much of the respiratory epithelium was normal, small numbers of cells were flattened and elongated and there was patchy loss of cilia. Similar changes were present at 9 hpi (Fig. 1E).

Lesions at 12 hpi were slightly more advanced. Significant oedema was present in multiple foci and small to moderate numbers of macrophages infiltrated the tissue. Additionally, rare heterophils infiltrated the oedematous areas. Respiratory epithelial cells were shorter than normal and there was widespread loss of cilia. Rare epithelial cells were necrotic.

At 24 hpi, air sacs from both birds in this group were moderately affected (Fig. 1F). The lamina propria was infiltrated by moderate numbers of macrophages mixed with fewer lymphocytes and small amounts of necrotic debris. Again the inflammation was most intense beneath the islands of respiratory epithelium. Islands of respiratory epithelium were still recognizable; however, there was widespread attenuation of these cells. Cilia were largely absent and small numbers of epithelial cells were necrotic. The endothelium in the majority of the vessels was reactive and monocytes were frequently present adjacent to endothelial cells.

The most severe lesions were found at 48 hpi, when there was complete loss of ciliated epithelial cells and the entire air sac was lined by plump squamous cells. The lamina propria was infiltrated and expanded by large islands of macrophages. Additionally there were multiple foci of extensive ulceration. The inflammation was most intense in these areas and small numbers of heterophils were present. Blood vessels adjacent to the ulcers sometimes contained thrombi.

**Immunohistochemistry**

IHC was performed on air sacs treated with the vaccine strain, as the epithelium was retained for longer than those receiving the field strain. Results were similar for both the S2 and S1 antibodies. Specific immunolabelling was first seen at 9 hpi and was largely limited to the islands of respiratory epithelium (Figs. 2A and B). This predilection for the respiratory epithelium was noted because the cell shape could be discerned based on the distribution of the chromogen throughout the cellular cytoplasm, and because labelling was most prevalent in the air sac septum, the area in which the respiratory epithelial islands are most extensive. A few squamous cells were immunoreactive with the S1 and S2 antibodies at this time point. These cells were widely scattered across the membrane.

Distribution of epithelial immunoreactivity increased as infection progressed. At 12 hpi increased numbers of respiratory epithelial cells exhibited immunoreactivity for IBV and squamous cell immunoreactivity was more prevalent (Fig. 2C).
Fig. 1. Air sac lesions from birds infected with two strains of IBV and control birds. (A) and (B) Air sacs from control birds 12 and 24 hpi, respectively. Cilia are prominent on the columnar epithelium. There are very few leucocytes in the lamina propria. (C) and (D) Air sacs from birds 24 and 48 hpi, respectively, with vaccine strain of the virus. Note the loss of ciliated cells at 24 h and influx of macrophages and lymphocytes in the lamina propria. The epithelium is denuded by 48 h. (E) and (F) Air sacs of birds infected with the field strain of IBV at 9 and 24 hpi. There is flattening of the columnar epithelium and loss of cilia by 9 hpi and extensive ulceration and inflammatory infiltration by 24 hpi. HE. Bar, 20 µm.
densest labelling of squamous cells occurred adjacent to islands of intact respiratory epithelium; individually positive squamous cells were present throughout the rest of the membrane. By 24 hpi there was diffuse immunoreactivity of all epithelial cells (Fig. 2D).

**Discussion**

The avian air sacs are unique structures that allow for unidirectional air flow through the respiratory system and enhance the ability of birds to utilize inhaled oxygen (Bezuidenhout 2005a, b). The air sacs are poorly vascularized and, due to their blind-ended structure and limited interconnectivity, provide a relatively sequestered environment in which pathogens can flourish (Cook *et al.*, 1987). These anatomical features also make the air sacs ideal membranes on which to study the effects of IBV on respiratory epithelia.

Injection of IBV directly into the air sacs allows a more controlled method of infection than traditional intraocular or aerosol infection. In the latter infection methods, the amount of virus that reaches the target tissue (usually the trachea) cannot be easily controlled. These methods rely on the bird inhaling particles and not sneezing or coughing during the infection procedure. In contrast, direct injection into the air sac allows for controlled delivery of the agent into a specific anatomical area. The procedure is simple, relying on the identification of easily detected landmarks. The injection causes little or no distress to the bird as it is shallow; the air sac lies just beneath the skin and a small needle can be used to minimize pain to the bird. The major drawback of this method is the difficulty in handling the air sacs for histopathological processing. However, the method developed by Bezuidenhout (2005a) allows for relatively easy handling of these thin membranes and the generation of high-quality histological preparations.

Using the sac injection method, the present study has demonstrated three phenomena related to the pathogenesis of IBV infection. Firstly, IBV antigens are initially detected in the islands of respiratory epithelium. Secondly, there is a distinct temporal difference between the progression of disease in birds given the attenuated (vaccine) strain of M41 and the field strain. Thirdly, during early infection, the first cells to respond are macrophages and lymphocytes, with heterophils only becoming prevalent after significant epithelial damage.

Histologically, the air sacs are lined by three morphologically distinct types of epithelia: simple squamous, ciliated columnar and ciliated squamous (Bezuidenhout, 2005a, b). The results of the present immunohistochemical study indicate that IBV infects initially the ciliated epithelial cells and then enters the squamous cells. The IBV S1 and S2 proteins were...
first detected in the air sacs at 9 hpi. During early infection, immunoreactivity was largely confined to the columnar respiratory epithelial cells, with rare labelling of widely scattered squamous cells. Thus, the ciliated respiratory epithelial cells are the early sites of S1 and S2 protein expression. The rare labelling of the squamous cells may in fact represent labelling of the widely scattered ciliated squamous cells; however, the histological detail was not sufficient to clearly identify cilia on the surface of these cells. By 12 hpi, squamous epithelial cells adjacent to the respiratory epithelial islands demonstrated IBV immunoreactivity and by 24 hpi there was uniform labelling of the epithelium.

The initial involvement of the respiratory epithelium is further illustrated by the fact that inflammatory cells were concentrated beneath this tissue early in infection. After infection with the field strain of the virus, vascular changes and cellular infiltrates were most pronounced in areas just beneath the islands of respiratory epithelium. Early vascular lesions were detected at 6 hpi, 3 h before the S1 or S2 proteins could be detected by IHC; hence, the bird was responding to a stimulus prior to the expression of a major viral antigen. Similarly, the intensity of inflammation increased as the infection progressed. Inflammation was most intense beneath respiratory islands and areas of ulceration, but spread to adjacent areas by 9–48 hpi. This pattern preceded the spread of immunoreactivity, but followed the same pattern of spread; beginning near respiratory epithelial islands and spreading to adjacent loci.

Taken together, the immunohistochemical and microscopical observations indicate that there is a morphological difference between the two epithelia and further suggest a difference with regard to their permissiveness to viral infection. More work is needed to determine whether the observed difference could be due to either the ability of the cells to become infected or the ability of the virus to reproduce in the infected cell. Differences in the ability of the cells to become infected would most likely be a receptor-mediated event. The IBV receptor could be present only on the respiratory epithelium, with infection of the squamous cell occurring by cell-to-cell spread. Based on the finding that the squamous epithelial cells adjacent to the respiratory epithelial islands were the first squamous cells to demonstrate significant immunoreactivity, we propose cell-to-cell spread as a possible mechanism of virus transfer.

However, the data do not preclude receptor-mediated events as being the cause of the observed differences in epithelial susceptibility. Higher density of cell surface receptors would favour early virus entry, while it would be prolonged in cells with a low density of receptors. Alternatively, IBV could use multiple methods or receptors to enter cells. The receptors on ciliated cells could be more permissive than those on the squamous cells, thus allowing earlier virus entry. Such variation in cell entry methods has been postulated for the severe acute respiratory syndrome (SARS) coronavirus of man. Angiotensin-2 converting enzyme has been shown to be a receptor for SARS coronavirus, but the virus may also enter via lectins or by direct membrane fusion (Wang et al., 2007). The squamous and ciliated epithelial cells in the avian air sacs express different membrane lectins; therefore, differences in membrane molecule expression may play a role in viral tropism. IBV potentially enters target cells via sialic acid or heparin sulphate receptors; however, other factors may mediate its spread to different epithelial cell types (Winter et al., 2006, 2008; Madu et al., 2007; Abd El Rahman et al., 2009).

Regardless of the mechanism of virus entry, the results of the present study suggest that ciliated epithelial cells play an important role in the pathogenesis of IBV infection. The importance of ciliated epithelial cells in IBV pathogenesis may be similar to that seen in SARS virus infection (Shieh et al., 2005; Han et al., 2006). Using green fluorescent protein-labelled virus Sims et al. (2005) demonstrated that this virus preferentially targeted ciliated cells in vitro, while non-ciliated cells were not infected (Sims et al., 2005, 2006). The infection of the ciliated epithelium is thought to facilitate spread to the lower respiratory system.

The present study has also demonstrated that there are clear temporal differences between the lesions produced by the vaccine strain of M41 and the field strain. Microscopically, the lesions produced by the field strain progressed faster than those caused by the vaccine strain. Significant lesions developed in the field strain-infected tissues at 9 hpi and in those infected by the vaccine strain at 12 hpi. The most pronounced difference in inflammation occurred at 48 hpi. At this time point the vaccine strain had induced inflammation beneath intact island of respiratory epithelium. In contrast, the lesions induced in the birds inoculated with the field strain were more pronounced, with loss of respiratory epithelium, ulceration and more widespread inflammation. One bird receiving the vaccine strain did exhibit significant lesions at 24 hpi; however, the majority of the air sac showed lesions similar to the other bird examined at 24 hpi. Thus, the severe lesion was interpreted to be a result of local damage induced during the injection process.

Previous studies with different strains of IBV have indicated significant differences between the ability of different strains to elicit inflammatory responses. Fulton et al. (1993) demonstrated that IBV-M41 was more effective in stimulating a cellular response...
than was IBV-T. This was demonstrated by the presence of more heterophils in lavage fluid. In the present study, the difference in the severity of inflammation was elicited by different isolates of the same strain of virus. Moreover, the nature of the inflammation did not differ between the isolates; rather the rate of progression of the lesions in the vaccine strain-inoculated birds lagged behind that of the field strain-infected birds. Additionally, inoculation of the field isolate was accompanied by a much greater degree of epithelial necrosis than was infection with the vaccine strain.

The present study has not only demonstrated that the type of inflammatory cell influx varies during very acute infection, but also defines a slightly different type of inflammation than has been previously described. As in previous studies, few inflammatory cells were detected in normal avian respiratory tissue; however, after stimulation there was a rapid influx (Reese et al., 2006). Because we examined birds very early post infection, we were able to document early fluctuations in the type of infiltrating cells. Previous studies that have focused on later events during infection have indicated that air sac lesions are accompanied by a predominantly heterophilic response (Purcell and McFerran, 1972; Purcell et al., 1976). In the present study, macrophages and lymphocytes were the main cells infiltrating the air sacs early in infection, with heterophils only appearing after significant necrosis and ulceration of the air sac epithelium. It is unclear whether the heterophil influx was a result of a specific virus-mediated effect or was simply a response to tissue necrosis. The initial infiltrate of macrophages into the infected tissues indicates that these cells have an important role to play in the pathogenesis of IBV infection. Unlike in mammals, the first line of defence against pathogens in birds is the macrophage (Qureshi et al., 2000).

Taken together, the results of the present study provide some important clues to the pathogenesis of IBV infection. The morphological findings show the importance of ciliated epithelium in virus entry. Moreover, the lesions produced by the two virus isolates were similar; however, the field strain elicited a more rapid inflammatory response. It is possible that the slower progression of vaccine strain-induced infection allows the bird to mount a protective immune response prior to significant damage developing. If the molecular basis for the slower progression of disease can be determined, it is possible that specific viral or immune molecules could be used to develop new vaccines to IBV infection, which may enhance the ability of the bird to respond to infection. The molecular mechanisms for the observed findings are currently being explored. It is hoped that future studies combining molecular and morphological studies of IBV disease progression will one day lead to better control of this important respiratory disease.

**Conflict of Interest**

The authors have no conflicts of interest with regards to the current submission.

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