PROVENTRICULITIS IN BROILER CHICKENS AND ITS RELATIONSHIP TO INFECTIOUS BURSAL DISEASE VIRUS

by

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(Under the Direction of Thomas P. Brown)

ABSTRACT

Proventriculitis in broilers causes carcass condemnation because of contamination when swollen proventriculi tear during evisceration. Although the cause of proventriculitis is unknown, infectious bursal disease virus (IBDV) has been implicated. To study the role of IBDV in proventriculitis, proventriculi and bursas were collected from chickens with naturally occurring proventriculitis, and from chickens experimentally infected with seven different IBDV strains. All tissues were examined for IBDV using light microscopy, immunohistochemistry (IHC), real time RT-PCR, and for apoptosis by TUNEL method. We concluded that proventriculitis can occur in the absence of IBDV, and that the IBDV strains tested do not directly produce proventriculitis.

Proventriculitis was studied by experimentally reproducing the disease in commercial and specific pathogen free (SPF) broilers. Differences in weight gain, organ weights, and the presence of lesions between these birds and controls were assessed. Bacteria were not identified in histological sections of proventriculi nor were they isolated from affected proventriculi. Attempted virus isolation from affected proventriculi caused stunting in inoculated embryos, and infectious bronchitis virus (IBV) was detected in allantoic fluid. Proventricular homogenates

used to induce proventriculitis were positive for IBDV, IBV, adenovirus, and chicken anemia virus (CAV), but proventriculitis could also be produced in chickens in the absence of these viruses.

Immunosuppression was induced in broiler chickens using chemicals (cyclophosphamide and cyclosporin) or virus (IBDV) to study the effect of immunosuppression on proventriculitis. Cyclophosphamide and IBDV, both B cell suppressors, did not significantly affect the incidence or characteristics of the proventriculitis induced with a proventricular homogenate from a diseased bird. However, an increase in the size of the proventriculus was observed at 7 days post inoculation. Chickens immunosuppressed with cyclosporin, a T cell suppressor, developed more severe lesions and had a higher incidence of proventriculitis than immunocompetent controls. Although both, B and T cells, are involved in the immune response against proventriculitis, it appears that cell mediated immunity plays a more important role. This was also supported by the lymphocytic infiltrate observed in diseased proventricular glands. CD8+ T lymphocytes were the most common cell type and were widely distributed in the proventriculus, whereas CD4+ T cells and B cells tended to form aggregates in the chronic stages of the disease.

INDEX WORDS: Proventriculitis, Infectious bursal disease virus.

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DEDICATION

To my parents, Mario and Mary Jo, for all they have given me. I'm very proud of being their daughter, much of what I have accomplished I owe to them.

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CHAPTER 1

INTRODUCTION

Purpose of the Study

Proventriculitis is a naturally occurring disease that affects commercial chickens. Damaged proventriculi are enlarged, swollen and filled with fluid and feed and often rupture during routine evisceration causing contamination of the carcass (2, 14). The main economic impact of this disease is due to condemnation of these contaminated carcasses, although proventriculitis also has been associated with impaired growth, poor feed conversion, intestinal fragility, stunting syndrome and passage of undigested feed (1, 4, 14, 16, 21, 27). The poultry industry reports sporadic, thought economically important, outbreaks of proventriculitis in broilers (14), and the condition appears more common in younger birds, processed at 4 to 5 weeks of age (2).

Potential noninfectious causes of proventriculitis include oral exposure to biogenic amines (10, 20, 23), mycotoxins (5, 7, 11), lack of dietary fiber (20, 25), and excessive copper sulfate (3, 15). Possible infectious causes include adenovirus (16, 18), reovirus (17, 18, 21), infectious bronchitis virus (30), and megabacterium (12, 13, 19, 22). However, none of these noninfectious or infectious agents have been found in a majority of cases. Electron microscopy has detected viral particles in acute lesions but isolation of this virus from affected proventriculi has been unsuccessful (8, 9, 14).

Infectious Bursal Disease Virus (IBDV) has been implicated as the cause for this disease (2, 14) and IBDV vaccination decreases its incidence (6). Proventriculitis has been reproduced by orally inoculating broilers with homogenized proventriculi collected from affected birds (2, 14, 24). A filterable agent found in these homogenates causes lesions similar to those found in field cases (9, 14) and IBDV has been immunoprecipitated from these homogenates (14). Commercial broilers exposed to this IBDV developed increased proventricular lesion scores but had no increase in proventricular size, a characteristic feature produced by exposure to infectious proventricular homogenates (14).

Infectious Bursal Disease (IBD) is an acute, highly contagious viral disease in chickens which produces necrosis of lymphocytes in the bursa of Fabricius followed by immunosuppression (28). Classical strains of IBDV produced lesions in the proventriculus of specific pathogen free (SPF) leghorns (29). However, using *in situ* hybridization staining with riboprobes specific for the VP2 gene of IBDV, no virus was detected in the proventriculi of 3 week-old chickens experimentally exposed to IBDV strains Delaware A, D78 or Bursavac® and no histologicaly evident proventricular lesions were present (26).

The objective of this study was to determine the role of IBDV in proventriculitis, and better understand the pathogenesis and possible causes of proventriculitis.

Objectives and Originality

Recent investigations have implicated IBDV as a potential cause of proventriculitis. Variant strains of IBDV have been isolated from proventricular homogenates from diseased birds, and SPF leghorns and broilers inoculated with these homogenates, develop a disease typical of IBDV infection, as well as proventriculitis. Furthermore, vaccination against IBDV

reduces the incidence of proventriculitis, but does not eliminate it. Although indirect evidence exists, the definite role of IBDV in proventriculitis has not been determined. It is possible that a new variant IBDV could be the direct cause of the disease, or it may be that IBDV, by its immunosuppressive effect, allows some other agent to produce the disease. This research was designed to investigate the role of IBDV as the causative agent of proventriculitis in chickens.

Our first objective was to determine if IBDV either directly, or indirectly by inducing apoptosis, causes proventriculitis in chickens. To address this we examined the proventriculi and bursas of chickens with naturally occurring proventriculitis as well as those from SPF broilers experimentally infected with multiple strains of IBDV. The presence of IBDV in these tissues was determined by RT-PCR and IHC for viral gene sequences and viral antigen, respectively. The presence of apoptosis was examined by a modified TUNEL method, and lesions induced by the virus were examined by histopathology.

Our second objective was to reproduce proventriculitis and characterize the changes present in the proventriculus and other organs. To accomplish this we examined proventriculi and other organs after experimental induction of proventriculitis in commercial and SPF broiler chickens. In an attempt to identified possible causative agents involved in proventriculitis, including IBDV, molecular, bacteriological, serological, and histopathological methods, and electron microscopy were undertaken.

The purpose of our third study was to investigate if immunosuppression had an effect on the incidence, severity, or character of proventriculitis in broiler chickens. IBDV induces immunosuppression in chickens, which may play a role in the pathogenicity of proventriculitis. To address this objective, one-day-old commercial and SPF broilers were immunosuppressed with cyclophosphamide (B cell suppressor), cyclosporin (T cell suppressor), or IBDV.

Subsequently these chickens were exposed to a proventricular homogenate from affected chickens, and the effect of immunosuppression on proventriculitis was determined.

The main histological finding in transmissible proventriculitis is a marked lymphocytic infiltration of the proventricular glands. The purpose of our fourth study was to characterize this lymphocytic infiltrate to gain insights into the identity of these cells and their potential role in generating a protective immune response in the proventriculus. To accomplish this objective we experimentally infected commercial broiler chickens with proventricular homogenates from affected broilers and studied the proventricular lesions using histopathology. We stained for lymphocyte cell-surface markers, and studied the distribution of different lymphocyte subsets *in situ*.

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CHAPTER 2

LITERATURE REVIEW

Part 1: Proventriculitis

a) The Proventriculus.

The proventriculus or glandular stomach is a fusiform organ lying dorsal to the liver and between the esophagus and the gizzard. It is approximately 4-5 cm long and 2 cm in diameter in adult fowl. The lumen is narrow and the thick walls are composed mainly of masses of compound tubular glands (94). The primary function of the proventriculus is the production and release of the gastric secretions, pepsin, hydrochloric acid, and mucus. The food that passes through the proventriculus is held in the gizzard, where the gastric secretions act (222).

The wall of the proventriculus consists of four layers: the mucous membrane, submucosa, muscular tunic and serosa (154). The mucosal lining of the proventricular lumen forms folds termed plicae. Scattered over the mucosal surface are a number of papillae, through each of which passes a secretory duct of the proventricular glands opening at the apex of the papilla. The mucous membrane is lined by a single layer of columnar cells that secrete mucus. This mucous secretion acts as a protective lining for the surface of the epithelium (154). Underlying the surface epithelium and occupying the center of the mucosal folds is the tunica propria. Within this tunic lymphoid infiltrates are frequently found and large lymphoid foci often occur in association with mucosal papillae (151) . Aggregates of lymphocytes are also found in the

lamina propria of the esophageal-proventricular junction and these lymphoid accumulations have been named the esophageal tonsil (181).

The mass of proventricular glands makes up the greatest part of the thickness of the proventricular wall (94). The glands are composed of numerous rounded or polyhedral lobules which are arranged in small groups, each draining into the lumen through one of the mucosal papillae. Each lobule is composed of numerous straight alveoli radiating out from a central cavity. Groups of several alveoli join together to form first a short common tertiary duct, then a wider secondary duct, and finally a short primary duct passing up through the mucosal papilla and opening into the lumen. Surrounding each lobule are connective tissue septa of collagenous and elastic fibers, a few muscle fibers, and blood vessels and nerves (94).

The primary, secondary and tertiary ducts are all lined with columnar epithelium similar to that covering the mucosal surface. The glandular epithelium consists of a single layer of cuboidal to low columnar oxynticopeptic cells. These secrete both, hydrochloric acid and the enzyme precursor pepsinogen, hence combining the functions of mammalian zymogenic (chief) and parietal cells (222). The gastric juice is composed principally of hydrochloric acid, mucus and the proteolitic enzyme, pepsin. In addition to the oxynticopeptic cell, the epithelium of the tubular alveoli contains a number of glandular endocrine cells (154). As in mammals, stimulation of the vagus provokes the secretion of juice, and also a gastrin mechanism appears to exist (154). Gastrin cells have been described in the pyloric region of the fowl and would seem to confirm a role for gastrin in the proventricular secretion process (107). Other hormonal mechanisms may also be involved in the stimulation of proventricular secretion. Bombesin, present in proventricular endocrine cells, is secreted into the blood and carried to its target areas for stimulation of gastrin release, of pancreatic secretion, and enhancement of gut motility (107).

The submucosal connective tissue consists of a narrow band of white fibrous connective tissue and contains the submucosal nerve plexus. The muscularis externa consists of the inner circular and a much thinner, outer longitudinal layer of smooth muscle fibers. Between them lies a myenteric nerve plexus. Externally there is a thin layer of loose, adventitial connective tissue and a peritoneal coat (94). The celiac artery supplies both the proventriculus and the gizzard. Venous outflow occurs via the gastrointestinal vein which flows into the hepatic portal vein (222). The proventriculus is innervated by branches of the vagi and by perivascular nerve fibers from the celiac and mesenteric plexi (222).

The intermediate zone between the proventriculus and the gizzard is very short, being approximately 0.75 cm in the adult chicken. At the point where the proventriculus narrows to form this isthmus, the proventricular glands terminate abruptly and the plicae become shorter and gradually change over to the gizzard glands. The intermediate zone functions mainly when contracted as a barrier separating the proventriculus from the gizzard (154).

Matsumoto and Hashimoto (151) described the normal distribution and developmental changes of the lymphoid tissues in the chicken proventriculus. Development of lymphoid masses in the proventricular lamina propria occur underneath the surface epithelium and near the duct orifices, which suggests that the local mucosal immune mechanism develops primarily with a dominant participation of T lymphocytes in the early post-hatching period. Lymphocytes infiltrating the gland epithelium are γδ T lymphocytes, which play important roles both in recognition of antigenic substances invading the epithelium and in renovation of damaged epithelial cells. The development of B lymphocytes occurs following the invasion of antigens associated with food intake. No M cells could be detected in the proventriculus suggesting that routes for uptake of intraluminal antigens other than those traditionally attributed to M cells.

b) Transmissible proventriculitis

Definition and economic significance. Transmissible proventriculitis is an infectious disease of chickens of unknown etiology (73). It is characterized by an enlarged, atonic proventriculus that is filled with fluid and feed (11, 74, 79, 99, 122, 193). The gastric isthmus connecting the proventriculus and gizzard is enlarged, with dilation of the constriction present at this juncture.

The economic impact of proventriculitis is mainly due to condemnation of contaminated carcasses subsequent to the rupture of the proventriculus during routine evisceration (11, 99). An estimated 1% of processed birds must be reprocessed need because of gastro-intestinal tearing during mechanical evisceration (230). Contamination is the third most common cause of condemnation of broilers at processing after septicemia and airsacculitis in the US, accounting for about 0.05% of broilers processed in the United States (Poultry Slaughter, 2001).

Proventriculitis is more severe in younger birds (4-5 wks of age) and has been associated with impaired growth, poor feed conversion, intestinal fragility, stunting syndrome and passage of undigested feed (4, 21, 99, 130, 183, 193, 206). The poultry industry reports sporadic, thought economically important, outbreaks of proventriculitis in broilers (99). Although broiler chickens throughout the world are commonly plagued by outbreaks of disease characterized at least in part by proventricular enlargement, lesions consistent with transmissible proventriculitis have been described in detail only in the United States (74, 79, 99), Holland (130), and Australia (193). Definitive prevalence data regarding the global incidence and distribution of proventriculitis are lacking.

Transmission. The route of natural infection is unknown; however, chickens can be infected experimentally by oral inoculation with a homogenate prepared from proventriculi of

chickens with proventriculitis (11, 79, 99, 193). Because the disease is reproduced with proventricular homogenate filtrates (0.2 μm), a virus is suspected as the etiologic agent (79, 99, 193). Consequently, the disease is also termed also transmissible viral proventriculitis (TVP)(74, 79). However, the severity of lesions and the effects on production are more severe in birds treated with unfiltered homogenates, suggesting an additive effect of others concomitant infectious agents (99).

Gross Lesions. Proventriculi of affected chickens are enlarged and the serosal surface of the proventriculus often appears mottled or has irregular white plaques. The proventricular wall is thickened, some glands are distended, and exude viscous white material when compressed (73). The gastric isthmus is distended and flaccid. Some affected commercial birds also have gizzard erosions, fragile and thin intestines, mild to moderate enteritis, and low uniformity in carcass weight (11).

Microscopic Lesions. There is necrosis of the alveolar (oxynticopeptic) pepsinogen- and hydrochloric acid-secreting cells. These cells have an amorphous, granular, or vacuolated cytoplasm and nuclear condensation, fragmentation or lysis (73). Fewer attached or sloughed cells have swollen nuclei with marginated chromatin and clear centers (11, 73, 79). Proliferating hyperplastic and hypertrophic columnar to low cuboidal cells line primary, secondary, and tertiary gland ducts. Cuboidal to low columnar, pale, basophilic, and distinctly vacuolated duct-like epithelium replaces the destroyed alveolar secretory cells (11, 74). Severely affected glands occasionally coalesce. There is a moderate to marked increase in number of lymphocytes infiltrating the connective tissue stroma (tunica propria). Lymphocyte infiltrates in the glandular interstitium develop in areas containing affected glandular epithelial cells. Marked lymphocyte

infiltrates expand the glandular interstitium in the epithelium between the ductular and the glandular epitheliums (79).

Differential diagnosis. Several causes have been associated with proventricular enlargement and proventriculitis. A non-infectious proventriculitis can be produced by oral exposure to toxic chemicals such as biogenic amines (82, 172, 191, 221), and mycotoxins (39, 49, 84, 184, 185), which often contaminate poultry feed. A diet low in fiber has been shown to cause proventricular swelling and proventricular lesions (172, 197). Dietary copper sulfate within levels commonly fed to chickens as a growth stimulant, also causes proventricular hypertrophy (12, 120).

Some avian infectious agents can produce proventricular lesions. Velogenic strains of Newcastle disease virus (NDV) can produce hemorrhages in the proventricular mucosa (2), as can highly pathogenic avian influenza virus (HPAI)(224). Reticuloendotheliosis virus (REV) infection can cause stunting and neoplastic cellular infiltrates resembling nonpurulent inflammation are present in these proventriculi (168). In Marek's disease, diffuse lymphomatous involement and enlargement of the proventriculus occurs. Increased numbers of lymphoid follicles in the tunica muscularis of the proventriculus are pathognomonic for infection with avian encephalomyelitis (AE)(27).

Proventricular Dilation Syndrome (PDD), is a common chronic disease of psittacines birds characterized by dilation of the proventriculus, anorexia, regurgitation, passing of undigested seeds in feces, diarrhea, neurological signs, loss of weight, etc. The cause is not known, but is presumed to be a virus (75). In PPD there is accumulation of lymphocytes and plasma cells in the autonomic nervous system, especially the nerves that supply the muscles in the proventriculus and other digestive organs including crop, ventriculus and small intestine.

Central nervous system signs associated with PDD, which may occur in addition to, or independent of, gastrointestinal signs, may include ataxia, abnormal head movements, seizures and proprioceptive or motor deficits. Dilated thin proventriculi are present in 70% of cases with lymphoplasmacytic ganglioneuritis of splanchnic nerves of crop/esophagus, proventriculus, gizzard, and intestine (75).

A very large, Gram-positive, rod-shaped microrganism has been found associated with proventriculitis in canaries, budgerigars, ostriches and recently in chickens (87, 97, 169, 187). This so-called "megabacterium" is a novel, anamorphic ascomycetous yeast named *Macrohabdus ornithogaster* that colonizes the narrow zone (isthmus) between the proventriculus and gizzard. Proventricular trichomoniais has been reported in budgerigars (87), and filamentous bacteria (232) inhabit the upper gastrointestinal tract and are potential pathogens. Other bacterium, *Helicobacter pullorum*, is also found in the digestive tract of 60% of commercial poultry tested (5). *H. pullorum* belongs to the genus Helicobacter, the same as *H. pylori* which causes ulcerative gastritis in humans and some other mammals (64). The role of these bacteria in proventriculitis of chickens is unknown. They may have some pathogenic effects in the proventriculi, possibly potentiated by other infectious, chemical, or immunosuppressive agents.

Cases of marked necrotizing ulcerative mycotic proventriculitis in ostrich chicks have been associated with Zygomycetes and are accompanied by superficial microcolonies of yeasts (presumably *Candida* spp) (77, 119). Proventricular cryptosporidiosis is common among zoo and pet bird species (19, 233) and has been reported once in chickens (72). Mucosal colonization by *Cryptosporidium* is accompanied by inflammation and exfoliation of parasitized epithelial cells. Purulent necrotizing proventriculitis with intralesional *Toxoplasma gondii* was reported in one flock of chickens in Norway (52).

Outbreaks of *Dispharynx nasyta* have been reported in several avian species including chickens. Infected proventriculi are enlarged, and the mucosal surface is covered with parasites and necrotic debri (70). *Tetrameres americana, T. crami,* and *T. fissispina* also parasitize the proventriculus (176).The females reside deep within a proventricular gland and completely fill and distend its lumen. Heavy infections in chickens can cause emaciation and anemia (176).

Traumatic proventriculitis may also occur after foreign bodies have been ingested and retained (78) and secondary bacterial infection may occur.

History. Initially, transmissible proventriculitis was described as one of the lesions associated with malabsorption syndrome (21). Differing combinations of clinical manifestations resulted in a variety of names for this syndrome: infectious stunting syndrome (21, 194), runting-stunting syndrome (157), pale bird syndrome (71), and infectious proventriculitis (130). These conditions cause growth retardation and poor feed conversion in young broiler chickens. The causative agents of these syndromes have not been clearly identified, and proventriculitis may or may not be present as a lesion in these syndromes. For example, cases of malabsorption syndrome may or may not include proventricular lesions (219). Filterable agents isolated in the Netherlands were originally linked to proventriculitis, causing runting syndrome in broilers (130). These authors suggested the involment of both bacteria and viruses in the etiology of malabsorption syndrome (130, 131). Shapiro and Nir (206) reported both proventricular enlargement and decreased body weights in birds infected with crude homogenate of intestines from broiler chickens affected with stunting syndrome.

Reoviruses have been strongly implicated as a causative agent for concurrent proventricular lesions present in some flocks naturally affected with malabsorption syndrome (131). Proventriculitis was reproduced by inoculation of two reovirus isolates from the intestines

of birds with malabsorption syndrome (183). A homogenate of proventricular and duodenal tissues from stunted birds raised in northwest Arkansas produced proventriculitis and decreased body weight when gavaged into specific-pathogen free birds. However a cell culture adapted reovirus isolated from this same homogenate produced proventriculitis without affecting the body weight (4). The addition of histamine to the feed of broiler chickens orally infected with an avian reovirus vaccine interacted to cause proventricular enlargement and decreased body weight (24).

A comparative study of the pathogenesis of five different malabsorption syndrome homogenates from the Netherlands and Germany distinguished the inoculated groups of chickens by their histopathologic lesions: proventriculitis, lesions in the intestine only, or combination of both (219). Lesions in the small intestine had more impact on weight gain depression than lesions in the proventriculus. Reovirus and enterovirus-like particles were detected in the inoculated groups. Also bacteriophages and bacteria (hemolytic *Escherichia coli, Pasteurella hemolytica*, and *Enterococcus durans* were isolated from inoculated chicks. The individual role that each of these pathogens plays in the pathogenesis of malabsorption syndrome is still unsolved (219).

Mild proventriculitis has also been reproduced experimentally in chickens infected with some isolates of adenovirus (130, 141) however, this virus hasn't been consistently isolated from diseased proventriculi. Infectious Bronchitis Virus (IBV) isolates from naturally occurring cases in China produced proventricular lesions in infected birds. Their proventriculi were enlarged and swollen and the mucosa was thickened and exuded white viscous fluid (255).

A filterable (0.2-μm) agent found in homogenized affected proventriculi can cause lesions similar to the proventriculitis seen in naturally-occurring cases but not to the same degree

as the caused by unfiltered homogenate. This proventriculitis could be produced independently of an effect on growth, and only unfiltered homogenate caused stunting (11). The proventriculitis produced was best detected using histopathology, and was sufficiently severe to produce mural thinning with increased susceptibility to rupture during evisceration at processing.

Goodwin *et al*. (74) reported the presence of intralesional virions in proventriculi from chicks that failed to thrive and had proventriculitis, and suggested a causal relationship between the virus and the lesion in its host. Hexagonal intranuclear and intracytoplasmatic virus particles were described and resembled adenovirus or poliomavirus. However, DNA *in situ* hybridization failed to detect adenovirus or poliomavirus nucleic acids. Huff et al. (99) also reported the presence of similar virus-like particles in the nuclei of many epithelial cells of the proventriculus of chickens experimentally inoculated with homogenate prepared from the proventriculi of chickens with proventriculitis. The particles, nonenveloped spheres of about 100-200nm in diameter, appeared hexagonal and were arranged in semiparacrystalline arrays in the nuclei (99).

Guy and Barnes (79) reproduced proventriculitis by administration of a filtrate (0.2-μm) from a homogenate produced from the proventriculi of chickens with proventriculitis. However, affected chickens had no decrease in body weight. This inoculum was free of avian reovirus, avian group I adenovirus, infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV). Adenovirus-like particles, similar to those observed by Goodwin *et al* (74), were identified by thin-section electron microscopy in nuclei of affected glandular epithelium cells.

Reece (193) reported that flocks with proventriculitis and stunting syndrome were generally characterized by poor feed conversion, reduced growth rate and/or uneven weight at slaughter age. Proventricular homogenates prepared from these birds were highly infectious and transmissible for at least four passages in birds. Treatment of the inoculum with chloroform did

not reduce infectivity, supporting the hypothesis that the putative etiological agent of infectious proventriculitis was a non-enveloped virus. This did not grow in any of a wide variety of primary and established cell culture systems. Chicken embryos were inoculated via various routes, embryo viscera were harvested, and these were inoculated into SPF chickens. No proventricultis was produced. The original inoculum contained chicken anemia virus (CAV), fowl adenovirus type 8, avian nephritis virus and Marek's disease virus (MDV) but did not contain avian leukosis virus (ALV), infectious bronchitis virus (IBV), reovirus, Newcastle disease virus (NDV) or infectious bursal disease virus (IBDV).

c) **Proventriculitis and Infectious Bursal Disease Virus (IBDV).**

Proventriculitis was experimentally reproduced by oral infection of commercial broilers with a 0.2-μm filtrate of an infectious proventricular homogenate (11). Serologic tests for induced IBDV antibody were positive, whereas those for reovirus antibodies were negative, suggesting the possible involvement of IBDV. The presence of IBDV in this homogenate was later confirmed by isolation of immunoprecipitated IBDV virus in embryos, and visualization of IBDV-like particles in the livers of SPF embryos inoculated with a filtrate of this homogenate (99). A challenge study with the IBDV immunoprecipitated from this homogenate increased the proventricular lesion scores at 14 days post inoculation in commercial chickens that received the inoculum at one day of age. However, there was no proventricular enlargement due to IBDV inoculation. Huff *et al.* (99) reported the isolation of a unique bacterial culture (Clostridia sp.) from the same infected proventriculus homogenate suggesting bacterial involvement in this syndrome. Challenge studies in broiler chickens comparing the pathogenicity of this infectious proventricular homogenate, the monoclonal antibody precipitated IBDV and this bacteria isolate, alone or in combination, showed that only the combination of virus and bacteria reproduced

proventriculitis similar to the proventricular homogenate. The homogenate, bacteria alone, and the combination of virus and bacteria each caused poorer feed conversion efficiency compared to the saline control, indicating that the *Clostridium sp.* isolate may be responsible for the poor feed conversion. The severity of lesions and the effects on production were more severe in birds treated with the homogenate, suggesting there were either additional factors involved, or dose-related effects on the pathogenesis. Huff *et al.* concluded that a viral infection, as well as various dietary factors, may facilitate bacterial invasion of the proventriculus, and more than one type of virus may act as facilitator in this disease syndrome.

IBDV produces hemorrhage, necrosis, and heterophilic infiltration, in the proventricular mucosa of SPF white leghorns (213). Proventriculitis experimentally produced by challenge of SPF leghorns with IBDV included gross and histopathological lesions but not the severe proventricular enlargement seen in naturally-occurring cases of this disease (173).

In the past several years, IBDV has been implicated as the cause of proventriculitis in broiler flocks from north Alabama (48). The disease resulted in poor feed conversion, weight reduction, and mortality. A small study using live IBDV vaccines was performed with two commercial IBDV vaccines. SPF birds were vaccinated with either a live intermediate vaccine containing an antigenic standard virus or a combination product, containing both standard and variant IBDV vaccine viruses. Vaccinated and nonvaccinated birds were exposed to a virulent Alabama IBDV isolate implicated in causing proventriculitis. Fifty percent of the nonvaccinated birds showed atrophy of the bursa and proventriculitis. In contrast, only 25% and 10% of the birds that received the combination or standard vaccine alone, respectively, had these lesions. Lesions still occurred suggesting that another agent or agents are involved in the production of proventriculitis. The authors suggested that a variant IBDV may play a role in proventriculitis

and that vaccination of broiler progeny can be helpful in reducing the incidence and severity of the disease (48).

Proventriculitis cases were also reported in Arkansas (122). These birds were reovirus negative and variably positive for chicken anemia virus (CAV) by serologic tests. The proventriculus had a thickened wall with loss of glandular integrity and lesions in mucosal lamina propria. Homogenized proventriculi were gavaged into SPF chickens and they became antibody positive to IBDV and remained antibody negative to CAV and reovirus. Exposed chickens had bursal atrophy, enlarged proventriculi, swollen kidneys and spleens, and lesions at the junction of the esophagus and proventriculus.

Detection of IBDV by ISH-staining using riboprobes specific for the VP2 gene of IBDV failed to detect that virus in the proventriculi of 3 week old chickens experimentally exposed to Delaware A, D78, or Bursavac®. Also, no histologicaly evident proventricular lesions were present after these exposures (205). Combined with previous findings, these results indicate that IBDV probably has no direct effect on the proventriculus.

Part 2: Infectious Bursal Disease Virus

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBD is a highly contagious viral disease of young chickens, characterized by destruction of the lymphocytes in the bursa of Fabricius, producing severe immunosuppression. IBDV is endemic in most poultry producing areas of the world. The virus is highly stable in the environment and has a tendency to persist in the environment despite

thorough cleaning and disinfection. There are two serotypes of IBDV: 1 and 2. All viruses capable of causing disease in chickens belong to serotype 1. Serotype 2 viruses may infect chickens and turkeys but are non-pathogenic for either species (109, 155). Chickens are the only avian species known to be susceptible to clinical disease and lesions produced by IBDV. Turkeys, ducks and ostriches are susceptible to infection with IBDV but are resistant to clinical disease (148, 156).

Despite widely used vaccination programs, IBD is one of the major economically important diseases of poultry worldwide. Most commercial chickens get exposed to IBDV early in life. In unprotected flocks, the virus causes mortality and immunosuppression. Although mortality can be quite significant, the major economic concern is the ability of IBDV to produce immunosuppression. Immunosuppressed flocks perform poorly and show reduced economic return (209).

History. The disease was first reported by Cosgrove in 1957. It was initially recognized as “avian nephrosis”, and the syndrome became known as “Gumboro disease” because it occurred in the Gumboro district of Delaware, USA. The clinical features of the syndrome included whitish or watery diarrhea, followed by anorexia, depression, trembling, severe prostration, and death. At necropsy, the birds exhibited dehydration, hemorrhages in the leg and thigh muscles, urate deposits in kidneys and enlargement of the bursa of Fabricius (37).

The early consensus was that avian nephrosis or Gumboro disease was caused by the Gray strain of infectious bronchitis virus (IBV) because of gross changes in the kidney and because IBDV and IBV were concurrent in many cases. This misconception also arose because the two infections were concurrent in many cases and IBDV was difficult to isolate with the available methods (135). After subsequent studies, Winterfield *et al*. (249), succeeded in

isolating the causative agent in embryonating eggs, and later Hitchner (93) proposed the term "infectious bursal disease" for the disease.

In 1972, Allan *et al*. (3) reported that IBDV infections at an early age were immunosuppressive. The recognition of the immunosuppressive capability of IBDV infections greatly increased the interest in the control of these infections. The existence of a second serotype was reported in 1980 (153).

In 1984 and 1985, the Delmarva peninsula broiler growing area experienced a significant increase in mortality. The clinical syndrome had significant variability, but often was respiratory in nature. Lesions ranged from moderate to severe, with death usually being attributed to *E. coli* infection (38). Using vaccinated sentinel birds, Rosenberger *et al*. (199) isolated four isolates designated as A, D, G, and E. These isolates differed from standard strains in that they produced a very rapid bursal atrophy associated with minimal inflammatory response. The available killed standard vaccines did not afford complete protection against these four new Delaware isolates. The Delaware isolates, A, D, G and E were designated as antigenic variants and killed vaccines were developed, tested and proven effective against them (199). Currently these and other similar variants are widely distributed in the United States (217, 218).

Since 1987, acute IBDV cases with up to 30% to 60% mortality in broiler and pullet flocks, respectively, became commonly reported in Europe. The first reports were made by Chettle *et al*. 1989 (30), and van den Berg *et al* in 1991 (242). Some of these acute outbreaks occurred in broiler flocks where appropriate hygienic and prophylactic measures had been taken. Although no antigenic drift was detected, these strains of increased virulence were identified as very virulent IBDV (vvIBDV) strains (242). The European situation has been dominated for a decade by the emergence of vvIBDV strains. These strains have now spread all over the world

(57). In the Americas, acute IBD outbreaks due to vvIBDV strains have already been reported in Brazil (41, 100), and the Dominican Republic (8).

Etiology. IBDV is a small, non-enveloped virus, belonging the *Birnaviridae* family, which is characterized by a bisegmented dsRNA genome (123). The *Birnaviridae* family includes three genera: Genus *Aquabirnavirus* (type species: infectious pancreatic necrosis virus or IPNV), Genus *Avibirnavirus* (type species: infectious bursal disease virus or IBDV), and Genus *Entomobirnavirus* (type species: *Drosophilla* X virus or DXV). (43). Other birnaviruses have been isolated from bivalve mollusks such as Tellina virus (236), and Oyster Virus (43, 129), and Japanese eels (139). To date, no Birnavirus capable of causing disease in mammals has been reported.

The virion has a single capsid shell of icosahedral symmetry composed of 32 capsomeres and a diameter of 60 to 70 nm (43, 81, 90, 174). By cryomicroscopy, the subunits forming the capsid are predominantly trimeric clusters. Due to the conformation of these subunits, the capsid acquires a nonspherical shape (20).

Viral genome structure and replication. The genome of IBDV is formed by two segments of double-stranded RNA (dsRNA) with the two segments detected by polyacrylamide gel electrophoresis (43, 113). Molecular weights of the two double stranded segments are 2.2 x 10^6 and 1.9 x 10^6 Da, respectively (162). The length of both segments is 3.2 kb and 2.8 kb respectively (98).

The larger segment A (approximately 3400 base pairs) contains two partially overlapping open reading frames. The first encodes a nonstructural polypeptide of 17 kDa known as VP5, which is dispensable for replication *in vitro* but important for virus-induced pathogenicity (165, 166). The second ORF encodes a 109-kDa polyprotein that is autoproteolytically cleaved into

three polypeptides, VPX, VP3 and VP4. VPX is further processed to produce a polypeptide known as VP2 (6, 98, 161). VPX, VP2, and VP3 are the major structural proteins that form the virus capsid (20), while VP4 appears to be responsible for the proteolytic maturation of the polyprotein (118, 126, 140).

Segment B encodes VP1, a 95-kDa protein which is the RNA-dependent RNA polymerase (RdRp) responsible for the replication of the genome and synthesis of mRNAs (44, 220). VP1 shares a number of primary sequence features with RNA polymerases from diverse origins (23).

At the 5' and 3' ends in both genome segments of IBDV, there are direct terminal and inverted repeats that are likely to contain important signals for replication, transcription and packaging. It is not known whether virulence variations are due to mutations in these regions (170). The inverted adjacent repeats at the 3' terminus on segments A and 5' terminus on segment B have the potential to form stem and loop secondary structures (124), which are involved in the processes of RNA replication, translation and encapsidation like other RNA viruses such as poliovirus (211).

The mechanism of synthesis of both virus-specific ssRNA and dsRNA during infection with IBDV has not been clearly determined. An RNA-dependent RNA polymerase has been demonstrated in IBDV (220). Genome-linked proteins have been demonstrated in three different Birnaviruses, (162, 186, 195, 220), indicating that they replicate their nucleic acid by a strand displacement (semiconservative) mechanism (17, 158, 220).

Viral Proteins. Four mature viral structural proteins designated VP1, VP2, VP3 and VP4 are detected in infected cells (13, 42, 43, 174). A non-structural protein designated VP5 has been

identified, the function of this protein is still unknown, but it is not essential for viral replication (165, 166).

During the processing of the polyprotein precursor into pVP2, VP3 and VP4, the existence of two sites, essential for the cleavage of the VPX-VP4 and VP4-VP3 precursors, respectively has been reported (202). These sequences are highly conserved among IBDV strains from both serotypes 1 and 2.

VP1, the RNA-dependent RNA polymerase of the virus, is present in small amounts in the virion, both as a free polypeptide and as a genome-linked protein (125, 163). It plays a key role in the encapsidation of the viral particles (146).

VP2 is the most abundant viral protein, accounting for 51% of the virus proteins of the serotype I IBDV's. This is the major protein component of the viral capsid, and is the host-protective antigen containing the antigenic region responsible for the induction of neutralizing antibodies and for serotype specificity (60). The transition from the precursor of VP2 (pVP2) to VP2 involves the cleavage of pVP2 near its C terminus (6). VP2 has also been identified as an inducer of apoptosis (62).

VP3 is also a structural protein, and accounts for 40% of the virion proteins (123). VP3 is found only on the inner surfaces of virus-like particles (150). This protein plays role in the assembly of viral particles, and packaging of the viral genome (146, 150, 225). VP3 is a group-specific antigen that is recognized by non-neutralizing antibodies, some of which cross-react with both serotypes 1 and 2 (14). It is likely that the outer subunits in the viral capsid consists of VP2, carrying the dominant neutralizing epitope, and that the inner trimers consist of protein VP3, (20).

VP4 is the viral protease involved in the processing of the precursor polyprotein(6). It is a proteolytic enzyme-like protein, which uses a Ser-Lys catalytic dyad to act on specific substrates and cleavage sites (18). The integrity of VP4 is essential for the proteolytic processing of the polyprotein (50, 118) and either itself, or through proteins under its control, plays a role in the activation of VP1 (18).

VP5 was the last IBDV protein identified (165). This protein is not essential for IBDV replication *in vitro* or *in vivo*, however, it plays an important role in viral pathogenesis (253). It has cytotoxic properties and it may play a role in the release of the IBDV progeny (147).

Host susceptibility. Domestic fowl are the natural host of IBDV (86). All breeds are affected. White Leghorns exhibit the most severe disease and have the highest mortality rate (148). Turkeys may be infected with serotypes 1 and 2 but do not show clinical signs of the disease (110, 156). There is, however, considerable potential for immunosuppression or interaction with other diseases under commercial conditions in turkeys (136). Serotype 2 was originally identified in clinically unaffected adult turkeys in Ireland (153). Ducks may develop IBDV infection and antibodies are detectable by serum virus neutralization, but neither gross nor microscopic lesions occur. Antibodies have been detected in wild birds. Five of 29 weavers (*Ploceus cucullatus*) and one of eight finches (*Uraeginthus bengalus*) (171) were seropositive. Surprisingly, seropositivity has also been detected in Antarctic adelie penguins, but the source of IBDV exposure has not been defined (66).

Transmission. IBDV is highly contagious and the disease may be spread by direct contact between infected and susceptible flocks. Infected chickens shed IBDV one day after infection and can transmit the disease for at least 14 days. There are neither experimental data

nor naturally-occurring observations to suggest that IBDV is transmitted vertically by the transovarian route (148).

Indirect transmission of virus most probably occurs on fomites (feed, clothing and litter) or through airborne dissemination of virus-laden feathers and poultry house dust (15). IBDV is very persistent in the environment of a poultry house. Houses from which infected birds were removed, still had virus infective for other birds 54 and 122 days later (16). The lesser mealworms, *Alphitobius diaperinus* may be reservoir hosts (152, 214). IBDV has also been isolated from *Aedes vexans* mosquitoes (96), and antibodies against IBDV have been detected in rats found on poultry farms (180). No further evidence supports the conclusion that either mosquitoes or rats act as vectors or reservoirs of the virus.

Clinical forms of IBDV. The classical form, as described since the early 1960s, is caused by the classic moderately virulent strains of IBDV. The incubation period of IBD ranges from 2 to 4 days after exposure. One of the earliest signs of the classical infection in a flock is the tendency for some birds to pick at their own vents. The disease also produces acute onset of depression, reluctance to move, ruffled feathers, white or watery diarrhea, pericloacal staining of feathers with urates, trembling, and prostration. The feed intake is depressed but water consumption may be elevated. Severely affected birds become dehydrated and die (37).

The immunosuppressive form, principally described in the United States, is caused by low-pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variants or GLS strains, which partially resist neutralization by antibodies against the so-called “classic” or standard strains (217).

The acute and very virulent form, described initially in Europe, and then spread to Asia, Africa and some countries in Latin America, is caused by hypervirulent strains of IBDV, and it is

characterized by an acute progressive clinical disease, leading to high mortality rates on affected farms. The initial outbreaks in Europe were characterized by high morbidity (80%) and mortality reaching 25% in broilers and 60% in pullets over a 7-day period (30, 177, 242).

Gross lesions. Chickens which die acutely of primary IBD infection show dehydration of the subcutaneous fascia and musculature of the thigh, inguinal and pectoral areas (37, 148). Hemorrhages occur in the mucosa of the proventriculus at its junction with the gizzard. Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules (37). The renal lesions were more prominent in early outbreaks in the United States, perhaps due to co-infection with nephropathogenic strains of avian infectious bronchitis (148).

The bursa of Fabricius is the main organ in which lesions develop following exposure to IBDV (31). Chickens that die or are sacrificed at early stages after the infection show a doubling in size of the bursa due to edema. The bursa is pale yellow and has striations. By the 5th day the bursa returns to normal weight, but it continues to atrophy, and from the 8th day forward it is approximately one-third its original weight (148). Variant strains have been reported that do not induce an acute inflammatory response (199, 208). However, at least one variant strain was reportedly able to induce such acute inflammatory lesions (83).

Splenic enlargement has been documented, with small gray foci uniformly dispersed through the parenchyma (148, 160). The vvIBDV strains are able to cause greater decrease in thymic weight index and more severe lesions in cecal tonsils, thymi, spleens, and bone marrow, but the bursal lesions are similar (148). IBDV has been suggested to be part of an etiologic complex causing proventriculitis in broilers (99).

Histopathologic lesions. Infection with standard or variant strains results in death of bursal B lymphocytes. Necrosis of lymphocytes in the medullas of bursal follicles can be

detected within one day of infection. By the third day an inflammatory response with edema, heterophil infiltration, congestion and hemorrhage is present in infections due to standard strains. At this time the follicles may be reduced to a necrotic center surrounded by heterophils. From the fourth day after infection the acute inflammatory reaction declines, and as necrotic debris are cleared by phagocytosis, cystic cavities are formed. Fibroplasia occurs in the surrounding connective tissue and the covering epithelium becomes infolded and irregular (31, 192). Sharma *et al*. (1989) observed that the infection with the variant A strain did not result in an acute inflammatory response, and follicular lymphoid necrosis was evident at three days after infection (208).

The development of lesions by IBDV in thymus depends on the pathotype of the virus (102, 226). IBDV induced cortical thymic lymphocyte depletion is caused by apoptosis (102). The highly pathogenic vvIBDV strains from Europe and Japan are associated with severe thymic lymphocyte loss when compared to less pathogenic strains (226). Although the thymus undergoes marked atrophy and extensive apoptosis of thymic cells during the acute phase of virus infection, there is no evidence that the virus actually replicates in T cells (228). Gross and microscopic lesions in the thymus are quickly overcome and the thymus returns to its normal state within a few days of virus infection (209).

The spleen may have hyperplasia of reticuloendothelial cells around the adenoid sheath arteries in early stages of the infection, and lymphoid necrosis in the germinal follicles and the periarteriolar lymphoid sheath by the third day (148). The Harderian gland may also be affected. Normally this gland is infiltrated and populated with plasma cells as the chicken ages. Infection with IBDV prevents this infiltration (223). In cecal tonsils, there may be acute heterophil inflammation, destruction of lymphocytes, and regeneration on the fifth day after infection (86).

Histologic lesions in the kidney are nonspecific and probably occur because of severe dehydration of affected chickens. Lesions observed consisted of large casts of homogeneous material infiltrated with heterophils, and also glomerular hypercellularity (86).

Pathogenesis and Immunosupression. The main target organ of IBDV is the mature bursa of Fabricius, which is the source for B lymphocytes in avian species. Bursectomized chickens did not develop clinical IBD despite the presence of infection (89). The severity of the disease is directly related to the number of susceptible cells present in the bursa of Fabricius; therefore the highest age susceptibility is between 3 and 6 weeks, when the bursa of Fabricius is at its maximum development. This age susceptibility is broader in the case of the vvIBDV strains (177).

After oral infection or inhalation, the virus replicates primarily in the lymphocytes and macrophages of the gut-associated tissues. From the gut, the virus is transported to other tissues by phagocytic cells, most likely resident macrophages (209, 240). By 13h post-inoculation (p.i.), most bursal follicles are positive for virus and by 16 h p.i. a second and pronounced viraemia occurs with secondary replication in other organs leading to disease and death (164). Similar kinetics are observed in vvIBDV but replication at each step is amplified (240).

Actively dividing, surface immunoglobulin M-bearing B-cells are lysed by infection (91, 92, 198), but cells of the monocyte-macrophage lineage can be infected in a persistent and productive manner, and play a crucial role in dissemination of the virus (25, 101) and in the onset of the disease (127, 133, 207). The exact cause of clinical disease and death is still unclear but does not seem to be related only to the severity of the lesions and the bursal damage. Prostration preceding death is very similar to what is observed in acute coccidiosis, and is reminiscent of a septic shock syndrome (240). The macrophage could play a specific role in this

pathology by exacerbated release of cytokines such as tumor necrosis factor of interleukin 6 (127). As macrophages are known to be activated by interferon, this role could occur through an increased secretion of interferon as has been described *in vitro* after infection of chicken embryo cultures or *in vivo* in chickens (67).

Clinical and subclinical infections with IBDV may cause suppression of both humoral and cellular immune responses (209). The first indication of damage in the immune system was reported by Helmboldt and Gardner in 1967 (86). In 1970, Cho demonstrated that white leghorn chickens exposed to IBDV at one day of age were consistently more likely to develop visceral tumors and nerve enlargement by Marek's disease virus (32). In 1972 Allan *et al*. reported that IBDV infection at an early age was immunosuppressive, and severely depressed the antibody response to Newcastle disease virus (3). IBDV replication in the bursa leads to extensive lymphoid cell destruction in the follicular medullas and cortices (227). The acute lytic phase of the virus is associated with a reduction in circulating IgM+ cells (92, 198). IBDV-exposed chickens produce suboptimal levels of antibodies against a number of infectious and noninfectious antigens (32, 61, 128, 250).

Only the primary antibody response is impaired, the secondary responses remain intact (68, 198, 208), and this humoral deficiency may be reversible (209). Although destruction of Ig-producing B cells may be one of the principal causes of humoral deficiency, other mechanisms are possible including the adverse effect of IBDV on antigen-presenting and helper T cell functions (208). A paradox associated with IBDV infections in chickens is that although there is immunosuppression against many antigens, the response against IBDV itself is normal, even in 1-day-old susceptible chickens (212). There appears to be a selective stimulation of the proliferation of B cells committed to anti-IBDV antibody production (148).

T-cells are resistant to infection with IBDV (91)(61), and there is no evidence that the virus actually replicates in thymic lymphocytes (208, 228). However, there is evidence that *in vitro* mitogenic proliferation from T cells of IBDV exposed birds is severely decreased. This mitogenic inhibition is likely mediated by macrophages, however how IBDV induces macrophages to exhibit this suppressor effect is not clear (209).

Sharma *et al*. (209) detected a dramatic infiltration of T-cells in the bursa during acute IBDV infection, accompanied by the precipitous drop in the number of IgM+ cells. By the seventh day of infection, the infiltrating cells were predominantly CD8+ lymphocytes. It was suggested that T-cells modulate the infection, limiting viral replication in the bursa in the early phase of the disease. They also promote bursal tissue damage and delay recovery, possibly through the release of cytokines and cytotoxic effects (135). Cytotoxic T cells may exascerbate virus-induced cellular destruction by lysing cells expressing viral antigens. T cells may also promote the production of pro-inflammatory factors, such as nitric oxide, increasing tissue destruction (209).

The effect of IBDV on innate immunity is centered in the modulatory effect of IBDV on macrophage functions. There is evidence that the *in vitro* phagocytic activity of these cells is compromised (209).

In addition to causing necrosis in the lymphoid cells of the bursa, IBDV also induces apoptosis (62, 132, 175, 228, 229, 245, 246). Apoptosis is characterized by cell shrinkage and chromatin condensation and does not generate a local inflammatory response. Induction of apoptosis in infected cells contributes to the pathogenesis of IBDV in the bursa (121, 179), chicken peripheral blood lymphocytes (245), and in the thymus (102, 228). Virally-induced apoptosis can occur in cells in the absence of detectable virus (121, 175, 228). A direct effect of

viral proteins like VP2 and VP5 has been implicated in the induction of apoptosis (62, 254). Apoptotic cells have also been observed in viral antigen-negative bursal cells, underlining the possible role of immunological mediators in this process (175, 228). And finally, apoptosis has also been observed in the proventriculus of IBDV challenged SPF leghorn chickens (173).

Diagnosis of IBDV. Several diagnostic procedures can be applied in the diagnosis of IBD. Diagnosis of the clinical forms of IBD is based on typical signs of the disease and on the lesions of the bursa of Fabricius. Differential diagnosis should include velogenic viscerotropic Newcastle disease, chicken infectious anemia, and mycotoxicosis. In subclinical and immunosuppressive forms of IBD, Marek's disease, chicken anemia and mycotoxicosis should be considered (136, 148).

Since the lesion caused by IBDV infection is well characterized (31, 192), diagnosis by histopathology is frequently used. This approach has the advantage of giving valuable information about the virulence of the IBDV strain involved and the possible time when the infection occurred.

Current serological tests include serum-virus neutralization and ELISA (200). ELISA is widely used because is a sensitive and rapid method. With ELISA is easy to handle large number of samples. Using serological techniques it is possible to detect the immunologic response in an outbreak or evaluate vaccination programs (178).

The virus can be isolated in embryonated eggs, cell cultures or by inoculation of susceptible birds. Inoculation in birds is the best method, because the other methods may modify the original characteristics of the naturally-occurring IBDVstrains (200).

Viral antigens may be detected by direct or indirect fluorescent antibody techniques, immunohistochemistry, agar gel immunodiffusion and antigen-capture ELISA (AC-ELISA). The

use of monoclonal antibodies in the capture detection allows for more precise antigenic characterization (216, 218).

The reverse transcription-polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (114, 138, 252). Differentiation of the strains is possible if the RT-PCR amplicons are further analyzed using restriction enzymes (113, 115, 117) or sequencing (7, 9). Other molecular techniques include the use of DNA probes (111, 112).

Immunity. Natural exposure to the virus, or vaccination with either live or killed vaccines, stimulates active immunity. Antibody levels are normally very high after field exposure or vaccination. Immunization of chickens is the principal method used for the control of IBD in chickens. The immunization of breeder flocks is especially important to confer passive immunity to their progeny (148). Antibody transmitted from the dam via the yolk of the egg can protect chicks against early infections with IBDV, with resultant protection against the immunosuppressive effect of the virus (148). Because maternal immunity interferes with vaccination, the major problem with active immunization of young maternally immune chicks is determining the proper time of vaccination. This determination is aided by monitoring antibody levels in a breeder flock or its progeny (241).

Satisfactory protection against IBDV can be achieved by immunization with live or inactivated vaccines. Classical live vaccines achieve lifelong and broad protection, but posses residual pathogenicity and a proportional risk of reversion to virulence (240). Many choices of live vaccines are available based on virulence and antigenic diversity. According to virulence, vaccines are classified as mild, mild intermediate, intermediate, intermediate plus, or hot. Vaccines that contain Delaware variants are also available (148). Killed vaccines in oil emulsions to stimulate high levels of maternal immunity are extensively used in the field (148).

Inactivated vaccines and live vaccines made from variant strains protect chickens from disease caused by either variant or standard strains, whereas inactivated vaccines made from standard strains do not protect, or only partially protect, against challenge with variant strains (105). Very virulent strains of IBDV can be controlled adequately under experimental conditions by vaccination with commercial vaccines prepared from classical attenuated strains (53, 182, 241).

In ovo vaccination may provide a way for vaccines to circumvent the effects of maternal antibody and initiate a primary immune response (65). Virus-antibody complex vaccines have also emerged and seem very promising (80). This new technology utilizes specific hyperimmune neutralizing antiserum with a vaccine virus under conditions that are not sufficient to neutralize the vaccine virus but which are sufficient for delaying the pathological effects of the vaccine alone. This allows chicks to be vaccinated more effectively in the presence of passive immunity even with a strain that would be to virulent for use *in ovo* or at hatching (80). IBDV proteins, expressed in yeast or via the baculovirus system, have been studied for the use as subunit vaccines (51, 149, 189, 239). An advantage of this technology is that a vaccine based on VP2 alone should allow monitoring of the field situation by the discrimination between antibody induced by vaccine (anti-VP2 only) and that induced by infection (anti-VP2 and VP3) (240). The use of a reverse genetics system could represent a basis for the genetic attenuation of strains and for the generation of new vaccines, although interference of passive immunity will still exist. Therefore, as they are less sensitive to neutralization by anti-IBDV maternally derived antibodies, recombinant viral vaccines expressing the VP2 protein, such as fowl pox virus (10), herpesvirus of turkey (HVT)(40, 234), or fowl adenovirus (210) might be able to prime an active immune response.

Antigenic variation. The high mutation rate due to the RNA polymerase of RNA viruses, generates a genetic diversification that could lead to natural emergence of viruses with new properties that allow them to persist in immune populations (240). In the case of IBDV, these mutations lead to antigenic variations and modifications in virulence *in vivo* and attenuation *in vitro* (240).

The capsid protein, VP2, is the major host protective immunogen. Immunization of susceptible chickens with purified VP2 elicits neutralizing antibodies and confers protection against homologous virulent virus challenge (14, 60). Monoclonal antibodies raised against VP2 have the ability to neutralize homologous virus (6, 14, 215, 218). Using one neutralizing monoclonal antibody, a specific antigenic region of VP2 between amino acids 206 and 350 was identified. Since this epitope was denaturated by SDS, it was determined that is a conformationaly-dependent epitope (6). Antigenic epitopes on VP3 protein have also been reported but these antibodies are not completely neutralizing (6, 59).

Antigenic diversity between IBDV serotypes has been recognized since 1980, when serotypes 1 and 2 were defined on the basis of their lack of *in vitro* cross neutralization (153). Based on studies with monoclonal antibodies, IBDV strains belonging to serotypes 1 and 2 have been found to not share major neutralizing epitopes (13, 203). Some researchers have developed polyvalent neutralizing antiserotype 1 monoclonal antibodies such as monoclonal antibodies 1, 6, 7 8 and 9 (55), monoclonal antibody 8 (218), and monoclonal antibodies 6F6 and 7C9 (243).

Antigenic differences have been demonstrated within serotype 1, and the study of different strains has led to dividing serotype 1 into six subtypes, differentiated by cross neutralization assays using polyclonal sera (108).

Studies with monoclonal antibodies demonstrated the presence of a number of modified neutralizing epitopes among antigenically variant strains detected in the United States. Based on this evidence, there may have been an antigenic shift in IBDV viruses in the US (216). There are a minimum of at least five neutralization epitopes on the standard IBDV strains as defined by the monoclonal antibodies 8, 179, R63, B69 and 10. Delaware viruses have lost the B69 site, GLS viruses lack the B69 and R63 sites, and the DS326 virus lacked the sites for monoclonal antibodies B69, R63 and 179 (216). Thus on the basis of the reactivities with various monoclonal antibodies, the IBDV viruses are antigenically grouped as classic or standard, GLS, DS326 and Delaware type variants (238).

In spite of their enhanced pathogenic properties, the vvIBDV strains were considered to be closely antigenically related to the standard strains such as the Faragher 52/70 strain, on the basis of high cross-neutralization indices (53). Using neutralizing monoclonal antibodies developed by Snyder to characterize US IBDV variants, van der Marel studied twelve European isolates of IBDV. He detected no important differences between the standard strain 52/70 and vvIBDV(244). Similar data was produced by Öppling *et al* (182). However, Etterradossi *et al*. (54) developed nine other monoclonal antibodies and using these he detected modified binding and neutralizing properties against French vvIBDV strains. All their monoclonal antibodies neutralized most mild or intermediate vaccines strains, whereas two monoclonal antibodies did not neutralize a French vvIBDV strain, as well as US variant A, and the European strain Faragher 52/70. Based on their results, they suggested a neutralizing epitope may be altered in the European vvIBDV strains, causing decreased antibody neutralization. This difference could be used to differentiate vvIBDV strains (54, 55).

Molecular basis of IBDV variability. Nucleic acid sequencing of genes coding for VP2 and subsequent deduction of their predicted amino acid sequences, lead to the identification of a hypervariable region. The amino acid changes between strains are not evenly distributed throughout the open reading frame but are clustered in certain regions. Most of the changes that occur in VP2 are located between amino acids 239 and 332 (9, 134, 238). This highly variable region falls entirely within those sequences of VP2 identified as the minimum region required for reaction with virus neutralization monoclonal antibody (6, 60, 251).

Hydrophilicity profiles of this region show that there are two hydrophilic peaks at either end of this region, the larger peak being from amino acids 212 to 224 and the other from 314 to 324. These hydrophilic regions have been shown to be important in binding of neutralizing antibodies and, hence, are presumed to be a main part of the neutralizing domain (85, 203). It is interesting that most of amino acid variations in this region fall within these two peaks (9, 134).

Variations in IBDV antigenicity depend on changes in hydrophilic peaks. The serotype 2 strain 23/82 (203), the North-American antigenic variants A, E, GLS and DS326 (85, 134, 238), and neutralization resistant escape mutants (203) all exhibit amino acid changes in these hydrophilic peaks. Only differences in the intervening hydrophobic domains are found between typical serotype 1 strains (238).

A nucleotide sequence comparison suggested that four amino acid alterations in the VP2 protein of the Delaware E strain allowed this variant to escape neutralizing antibodies. These amino acids were located at positions 213, 222, 318, and 323 (85). By restriction enzyme and amino acid sequence analysis, point mutations have been detected at residues 222, 254 and 323. Amino acid residues 222 and 254 are consistently mutated in the variant strains (47, 116).

Glycine is present in the standard strains, amino acid residue number 254, whereas the variants have serine at this position (47, 116).

Vakharia *et al*. (238) used monoclonal antibodies to correlate antigenic variations with amino acid sequence substitutions in the hypervariable region of VP2. They found that the amino acid residue glutamine at position 249 might be involved in the binding of neutralizing antibody B69, which recognizes epitopes in standard strains. All the variant viruses have lysine instead of glutamine at this position, and they escape binding with antibody B69.

Using a baculovirus expression system to synthesize all the structural proteins coded for in segment A of the IBDV genome, Vakharia *et al*. (237), produced virus like particles. They mapped the antigenic sites by producing chimeric cDNA clones of IBDV using the variant GLS plasmid as a backbone and inserting fragments from the D78 and Delaware strains. At least two antigenic sites are present on the surface of IBDV, one resides between amino acid residues 222 and 249, and the other between 269 and 323.

The role of VP1 in the virulence of IBDV is not yet established. It is likely that the viral polymerase would influence the replication rate and, thus the pathogenic potential of a virus. The VP1 sequences of very virulent IBDV strains are genetically distinct from those of classical virulent or attenuated strains thus, VP1 of vvIBDV constitutes a genetic lineage distinct from that of classical virulent or attenuated strains and serotype 2 strains as well (104).

In highly virulent strains three specific amino acid residues in VP2 have been reported at position 222 (Ala), 256 (Ile), 294 (Ile) and 299 serine which differ from classical strains (22). These substitutions are also present in other strains isolated from other countries such as Germany (256), Bangladesh (103) China (29), Israel (190), Japan (143, 252), Taiwan (144), Malaysia (33, 95), Nigeria (231, 256), Vietnam (231), and Brazil (41, 100). Positions 222 – 223

and 318 – 324 may be critical for the vvIBDV (56). These positions have been identified as "hot spots" for mutations in several escape mutants resistant to selected neutralizing monoclonal antibodies (203, 243).

Immunosuppression in chickens. In poultry production, immunosuppressive diseases have been and remain economically important. Vaccination failure, increased condemnation and mortality, poor feed conversion, and increased morbidity and medication costs commonly result from immunosuppression. Immunosuppression has been defined as "a state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease" (46). Numerous immunosuppressive agents affect avian and mammalian species (167) including viruses, prokaryotic and eukaryotic parasites, microbial toxins, chemicals, drugs, nutritional deficiencies (137) and various psychological or physical-environmental stressors (45). Infectious bursal disease virus (IBDV) is of major interest because of the widespread occurrence of the infection in commercial chickens. Infection with IBDV at an early age significantly compromises the humoral and local immune responses of the chickens (201). Chicken anemia virus is also an important pathogen in poultry and appears to target erythroid and lymphoid progenitor cells in the bone marrow and thymus respectively (1). Destruction of erythroid and myeloid progenitors in bone marrow results in severe anemia, and depletion of granulocytes and thrombocytes. Destruction of T cells result in depletion of mature cytotoxic and helper T cells with consequent immune suppression. In Marek's disease virus (MDV) infection, the degree of immunosuppression is determined by persistence of early cytolytic infection, atrophy of bursa of Fabricius and thymus, and histologic evidence of necrosis and atrophy in lymphoid organs (26, 28). Syndromes caused by dietary consumption of feed containing moderate to high levels of mycotoxins range from acute

mortality to slow growth and reduced reproductive efficiency (188). Consumption of lower levels of fungal toxic metabolites may result in impaired immunity and decreased resistance to infectious disease. Mycotoxin-induced immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement activity, or impaired macrophage-effector cell function (35).

Treatments of chickens with cyclophosphamide or cyclosporin have been used as a means of inhibiting the humoral or cell-mediated immune responses in order to determine the role of T and B cells in protective responses to infectious pathogens of chickens (36, 58, 63, 106, 196, 247).

Cyclophosphamide is an antineoplastic agent and immunomodulator used therapeutically in the treatment of tumors and autoimmune disorders. The parent compound, cyclophosphamide, *in vitro* is neither alkylating, cytotoxic, nor immunosuppressive (76). *In vivo*, cyclophosphamide is converted by hepatic microsomal enzymes to 4-hydroxycyclophosphamide (4-OHCP) that is reversibly altered to aldophosphamide (AP) (34). Then the 4-OHCP/AP compound is either enzymatically detoxified or undergoes spontaneous degradation to phospharamide mustard (PM) and acrolein within cells (34). This alkylating agent induces DNA cross-links – an important step in causing the development of point mutations and chromosome aberrations (34). Newly hatched chickens treated with cyclophosphamide are rendered irreversibly B cell deficient (142). Furthermore, selective B-lymphocyte cytotoxicity is most dramatically achieved when cyclophosphamide exposure occurs during embryogenesis (248). T cells can be killed or their proliferation slowed by single or multiple, high dose CP treatment in neonatal chicks, but the numbers of T cells in thymus can recover in two weeks (69). The selective toxicity of cyclophosphamide is primarily due to its differential lymphocyte sensitivity, and not due to

differential compound distribution, uptake by immune tissues, or to site-specific activation and detoxification (159). Structure-activity studies in the chick embryo revealed induction of selective B lymphocyte toxicity that was induced by cyclophosphamide analogs capable of forming DNA interstrand cross-links (248).

Cyclosporin, a selective T-cell immunosuppressant drug, depresses cell-mediated immunity in chickens, causing prolonged skin graft survival, depressed proliferative responses in mitogen-stimulated lymphocytes and decreased wattle T-lymphocyte responses to injected antigen (88). Cyclosporin prevents the synthesis of cytokines by T cells by blocking a late stage in the signaling pathway initiated by the T-cell receptor. This especially affects the production of interleukin-2 (IL-2), hence T cell proliferation is reduced. As a consequence, IL-2 dependent functions which include T-helper activities, cytotoxicity, natural killer cell activity and antibody dependent cell cytotoxicity would be depressed after cyclosporin treatment (88).

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CHAPTER 3

INFECTIOUS BURSAL DISEASE VIRUS AND PROVENTRICULITIS IN BROILER CHICKENS[1]

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SUMMARY.

Acute necrotic proventriculitis is a naturally occurring disease of broiler chickens that causes proventricular rupture during routine evisceration. Although infectious bursal disease virus (IBDV) has been implicated, it has not been proven to be a direct cause of this disease. To further study the role of IBDV in proventriculitis, proventriculi and bursas were collected during both acute and chronic phases of naturally occurring proventriculitis, and from chickens experimentally infected with seven different IBDV strains. All tissues were examined for IBDV using light microscopy, immunohistochemistry (IHC), real time reverse transcriptase polymerase chain reaction (RT-PCR), and for apoptosis by the TUNEL method. Tissues from naturally occurring proventriculitis had bursal and proventricular lesions. Two out of four bursas had no IHC-stainable IBDV antigen, or RT-PCR detectable IBDV sequences. No proventriculus had IBDV detectable by any of these methods. Bursas from chickens experimentally infected with IBDV had microscopically evident lesions, and IBDV was detectable by IHC and RT-PCR, and strong IHC staining for apoptosis was present. Proventriculi from these experimentally exposed chickens had no lesions, low levels of IBDV detectable by IHC or RT-PCR, and very little IHC-stainable apoptosis. We conclude that naturally occurring proventriculitis can occur in the absence of IBDV and that the IBDV strains tested do not directly produce proventriculitis or induce increased proventricular apoptosis.

Key words: proventriculitis, chickens, IBDV.

Abbreviations: DAB = diaminobenzidine; dpi = days postinoculation; H&E = hematoxylin and eosin; IBDV = infectious bursal disease virus; IHC = immunohistochemistry; LS = lesion score; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase polymerase chain reaction;

SPF = specific-pathogen free: TUNEL = terminal deoxynucleotidyl transferase-mediated dioxiuridine triphosphate nick end labeling.

INTRODUCTION

Proventriculitis is a naturally occurring disease of commercial chickens. Its economic impact occurs because of delayed transit of feed through an atonic dilated proventriculus, thinning of the proventricular wall, and resultant tearing of the proventriculus during routine mechanical evisceration. Spillage of the retained ingesta from the torn proventriculus into the body cavity causes contamination and carcass condemnation. Grossly, there is severe proventricular dilation, and histologically, there is glandular lumenal ectasia, acute glandular epithelial necrosis, fibroplasia, and interglandular interstitial lymphoid aggregate hyperplasia (3, 9). Some affected commercial flocks also have poor body weight uniformity (3).

Potential causes of proventriculitis include oral exposure to biogenic amines (2, 28), mycotoxins (6,27), lack of dietary fiber (29), and excessive copper sulfate (4,14). Infectious causes include adenovirus (20), reovirus (5,18,37), infectious bronchitis virus (40), and Megabacterium (30). However, none of these noninfectious or infectious agents has been found in a majority of cases. Electron microscopy has detected viral particles in acute lesions, but isolation of a virus from affected proventriculi has been unsuccessful (10,11,12).

Infectious bursal disease virus (IBDV) has also been implicated as the cause for this disease (3,12,24), and IBDV vaccination decreases its incidence (7,16). Proventriculitis can be reproduced by orally inoculating broilers with homogenized proventriculi collected from affected birds (3,17). A filterable agent found in these homogenates causes lesions similar to those found in field cases (3) and IBDV has been immunoprecipitated from these homogenates. Commercial

broilers exposed to this IBDV developed increased proventricular lesion scores but had no increase in proventricular size, a characteristic feature produced by exposure to infectious proventricular homogenates (12).

Infectious bursal disease is an acute, highly contagious viral disease of chickens that produces necrosis of lymphocytes in the bursa of Fabricius followed by immunosuppression (21,33,38). Classic strains produced lesions in the proventriculus of specific-pathogen-free (SPF) leghorns (34). Flattening of papillae, hemorrhage, exudate on the mucosal surface, and grossly visible prominences on the serosal surface were present, but not proventricular necrosis or enlargement. IBDV-induced proventricular lesions are transient and proventriculi returned to normal 11 days postinfection (34). However, by *in situ* hybridization staining with riboprobes specific for the VP2 gene of IBDV, no virus was detected in the proventriculi of 3 week-old chickens experimentally exposed to Delaware A, D78, or Bursavac® and no histologically evident proventricular lesions were produced (31).

In addition to causing necrosis in the lymphoid cells of the bursa, IBDV also induces apoptosis (8,19,25,26,35,36,39). Apoptosis is characterized by cell shrinkage and chromatin condensation and does not generate a local inflammatory response. Induction of apoptosis in infected cells contributes to the pathogenesis of IBDV in the bursa (15,26), chicken peripheral blood lymphocytes (39), and the thymus (13,35), and virally induced apoptosis can occur in cells in the absence of detectable virus (15,25,35). Apoptosis has also been observed in the proventriculus of IBDV challenged SPF leghorn chickens (24).

The purpose of our study was to determine if IBDV either directly, or indirectly through inducing apoptosis, causes proventriculitis in chickens. To address this we examined the proventriculi and bursas from chickens with naturally occurring proventriculitis as well as those

from SPF broilers experimentally infected with multiple strains of IBDV. The presence of IBDV in these tissues was determined by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) for viral gene sequences and viral antigen, respectively. The presence of apoptosis was examined by a modified TUNEL method, and lesions induced by the virus were examined by histopathology.

MATERIALS AND METHODS

Naturally occurring cases of Proventriculitis. Four diagnostic cases presented for naturally-occurring proventriculitis were selected for study. All cases were broiler chickens, 25 to 40 days of age that had grossly evident proventricular enlargement and thinning of the gastric isthmus wall. Proventriculi and bursas were collected at necropsy, fixed in 10% formalin (paired tissues from each case), examined for lesions by histopathology, for IBDV and apoptosis by IHC, and by RT-PCR as described below.

Experimentally infected chickens. Fertile white Plymouth Rock chicken eggs (Southeast Poultry Research Laboratory, USDA, Athens, GA) from a breeder flock maintained under SPF conditions were obtained, hatched, and maintained in positive-pressure Horsfal isolation units until they were 28 days of age. Specifically, the parent flock and all progeny used in these experiments were negative for IBDV antibody by commercially available assays (IDEXX, Westbrook, ME). Feed and water were provided *ad libitum*.

Viruses. The IBDV strains used represented classic, variant, and commercially available vaccine strains. Classic strains used were STC challenge strain 124-ADV (National Veterinary Services Laboratory, Ames, IA), and Lukert (Poultry Diagnostic and Research Center, Athens, GA). Variant strains used were Variant E (Intervet, Inc., Millsboro, DE), Variant A (Poultry

Diagnostic and Research Center, Athens, GA), and a GLS strain (Intervet, Inc.). Vaccine strains used were Bursine 2 (Solvay Animal Health, Inc., Mendota Heights, MN) and D78 (Intervet, Inc.).

Experimental Design. When chickens were 28 days old, they were separated into 8 groups of 4 birds. All chickens in 7 groups were inoculated with one of the IBDV strains listed above. Chickens in the eighth group were not exposed to IBDV and served as negative controls. Each group was exposed and housed in a separate isolation unit. Inoculations were given once *per os* and consisted of 100 µl total of infected fibroblast cell culture fluid or reconstituted vaccine containing at least 10^3 mean chicken infective dose or 10^3 mean tissue culture infective dose of IBDV, diluted in phosphate-buffered saline (PBS). At 4 days post-inoculation (dpi) 2 chickens from each group were killed by cervical dislocation and necropsied. The remaining 2 chickens in each group were killed and necropsied at day 6 postinoculation.

Sample collection and processing. At necropsy, experimentally exposed chickens were examined, and bursa, proventriculus, and thymus were collected from each and fixed immediately by immersion in 10% neutral buffered formalin for 24 hr. Tissues from naturally occurring and experimentally exposed chickens were then processed by routine histologic techniques and embedded individually in paraffin blocks.

RNA extraction. Sections totaling fifty µm in thickness were cut from each formalin-fixed paraffin-embedded tissue block with a microtome and a new blade for each block. Sections were then deparaffinized (HemoDe and 100% ethanol; Fisher Scientific, Pittsburgh, PA) and digested with 10% proteinase K (Sigma Chemical Co., St. Louis, MO) for 1 hr at 50 C. RNA was extracted with Trizol (Life Technologies, Inc. Gaithersburg, MD) according to the

manufacturer's recommendations, diluted in 90% dimethyl sulfoxide (DMSO), and frozen at -80 C until assayed.

Real time RT-PCR. Extracted RNA was denatured at 95 C for 5 min and put on ice. A reverse transcriptase polymerase chain reaction (RT-PCR) was performed with reagents from the Light Cycler-RNA Amplification SYBR® Green I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used were designed to amplify a 400 bp segment of the IBDV genome shared by all known IBDV strains that flanks a hypervariable region of the VP2 gene. Primer sequences were B4 5' TCTTGGGTATGTGAGGCTTG and B4 3' GGATGTGATTGGCTGGGTTA. Amplification and detection of specific products was also performed with a Light Cycler according to the manufacturer's recommendations (ROCHE Light Cycler version 3.0; ROCHE Molecular Biochemicals). Briefly, reverse transcription was done at 55 C for 10 minutes, followed by denaturation at 95 C for 30 sec. Forty PCR cycles were done consisting of denaturation (95 C for 1 sec), hybridization (55 C for 10 sec), and extension (72 C for 13 sec). A melting curve analysis was done after an initial denaturation at 95 C. DNA melting was accomplished with an initial temperature of 65 C for 10 sec and a gradual temperature increase of 0.1 C per second until reaching 95 C. The melting temperature of the expected 400 bp amplicon was between 82 and 84 C. This estimated melting temperature was used to confirm the identity of IBDV specific products obtained using real time RT-PCR. Additional confirmation of specific amplification was done by gel electrophoresis of the PCR products on 2% agarose (Sigma Chemical Co., St. Louis, MO) followed by ethidium bromide staining. RNA from samples was also amplified by a one-step RT-PCR in a normal thermocycler as described by Banda *et al*. (1).

Restriction endonuclease digestion. To confirm infection and positively identify the IBDV strain present in each experimental group, and to detect if any cross contamination occurred between groups, real time RT-PCR products from all infected birds and those of IBDV strains prior to inoculation were examined by enzymatic restriction analysis. This procedure confirmed chickens were infected with only the intended strain of IBDV. With *Sac*I, *Sty*I and *Nar*I (New England BioLabs Inc., Beverly, MA), digestion of real time RT-PCR products produced above was performed according to the manufacturer's recommendations. One microliter of RT-PCR product was used in each of three separate restriction reactions, each with a different enzyme. Products were then subjected to a melting curve analysis to determine if enzymatic digestion had occurred with that specific enzyme. Restriction results for this three-enzyme panel were then compared with similar restriction results obtained for the seven different IBDV strains prior to their inoculation into chickens. This comparison served to individually identify each of the seven IBDV isolates and detect any potential cross contamination between groups.

Histopathology. Paraffin-embedded tissues were sectioned, mounted, stained with hematoxylin and eosin (H&E), and examined for lesions using light microscopy. All sections were assigned a lesion severity score. For all tissues, a lesion score of 1 represented no lesions. For bursal sections, 2 was defined as mild variation in follicle size, 3 as moderate variation in size of follicles, and 4 as either necrosis or follicle atrophy. For thymic sections, 2 was defined as mild cortical thinning, 3 as moderate cortical thinning, and 4 as absence of cortical lymphocytes. For proventricular sections, 2 was defined as mild glandular lumenal ectasia, 3 as ectasia plus lymphoid infiltrates in the interglandular interstitium and 4 as either acute glandular necrosis or severe fibrosis with lymphoid infiltrates.

Immunohistochemistry. All procedures were done at room temperature. Tissue sections were cut (4 μm) from paraffin-embedded samples and mounted on charged glass slides (Superfrost/Plus; Fisher Scientific). Paraffin was melted from slides (10 min at 65 C) and removed by immersion in Hemo-De three times (5 min each). Slides were then air dried and digested with 10% proteinase K (DAKO, Carpinteria, CA) for 5 min to expose antigenic target sites. IHC staining was performed with an automated stainer (Leica ST 5050, Nussloch, Germany) with a non-biotin peroxidase kit (DAKO Envision System; DAKO) according to the manufacturer's recommendations. The primary antibody used was a monoclonal antibody specific to and cross reactive for all IBDVs (ATCC No.HB9490). After IHC staining, sections were counterstained with hematoxylin, air dried, coverslipped, and examined by light microscopy. Intensity of IBDV staining in each section was scored as follows: - = no staining, + = minimal staining, ++= moderate staining, and +++= intense staining.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. For the detection of apoptotic cells, the *in situ* cell death detection kit (DeadEnd Colorimetric TUNEL System; Promega Corp., Madison, WI) was used according to the manufacturer's instructions. Briefly, tissue sections were cut (4 μm) from paraffin-embedded samples (bursa, thymus, and proventriculus) and mounted on charged glass slides (Superfrost/Plus, Fisher Scientific). Paraffin was melted from slides (10 min at 65 C) and removed by immersion in Hemo-De three times (5 min each). Slides were washed in 100% ethanol for 3 min and rehydrated by sequentially immersing the slides through graded ethanol washes (95%, 85%, 70%, and 50%) for 3 min each. Slides were washed in 0.85% NaCl and PBS for 5 min each, and fixed by immersion in 10% buffered formalin for 15 min. After two washes in PBS for 5 min each, the slides were set on a flat surface and 100 μl of a solution of 20 μg/ml

proteinase K was added to each one. The slides were incubated for 10 min at room temperature and then washed twice for 5 min with PBS. Tissue sections were refixed by immersing the slides in 10% buffered formalin for 5 min. Slides were washed again twice in PBS and excess liquid was removed. The tissues were covered with 100 µl of equilibration buffer for 10 min. The equilibration buffer was then blotted off and the tissues were covered with 100 µl of TdT reaction mix, containing a biotinylated nucleotide mix and TdT enzyme. Tissue sections were covered with plastic coverslips to ensure even distribution and incubated at 37 C for 60 min inside a humidified chamber to allow the end-labeling reaction to occur. Then the coverslips were removed and the reaction was terminated by immersing the slides in 2X SSC (standard sodium citrate) for 15 min. Slides were washed twice in PBS for 5 min to remove unincorporated biotinylated nucleotides. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 5 min. Slides were washed again, 100 µl of the streptavidin horseradish peroxidase solution diluted 1:500 in PBS was added to each one, and the slides incubated for 30 min at room temperature. After the slides were washed twice in PBS, 100 µl of a solution containing the DAB (diamino-benzidine) substrate, the DAB chromogen, and hydrogen peroxide was added to each one and developed until there was a light brown background (approximately 10 min.). After the slides were rinsed several times in deionized water, they were mounted and coverslipped, and staining was observed under a light microscope.

RESULTS.

Naturally occurring cases. As expected, **s**evere proventriculitis was present microscopically in all field cases examined (Table 3.1; Fig. 3.1). Bursal necrosis or lymphoid depletion was mild to moderate. By real time RT-PCR and IHC for detection of IBDV on bursas,

two cases were positive and two were negative. All four proventriculi were negative for IBDV by the same methods. Apoptosis staining was present to varyious degrees in proventriculus and bursas from all four cases. Bursas that were positive for IBDV had stronger staining for apoptosis. Apoptosis in proventriculi was minimal and occurred mainly in the necrotic glands.

Experimental birds.

Controls. Real time RT-PCR and IHC successfully detected IBDV in formalin fixed paraffin-embedded tissues of all birds challenged with IBDV except those exposed to Lukert strain (Tables 3.2, 3.3; Fig. 3.2). Enzyme restriction of the PCR products followed by a melting curve analysis confirmed that all birds were infected with only the strain to which they were intentionally exposed and that non-inoculated birds were not infected. Real time RT-PCR was more sensitive than the one-step RT-PCR procedure for IBDV diagnosis, detecting IBDV nucleic acid in 12 out of 12 samples from inoculated chickens compared with 8 out of 12 detected by the routine one-step RT-PCR. Apoptosis staining was strongly positive in all bursas of birds challenged with IBDV (Fig. 3.2), compared with weak staining in tissues from noninoculated chickens and chickens exposed to Lukert strain.

Antemortem clinical signs and lesions. Birds challenged with the STC, GLS, Variant E, and Variant A strains of IBDV were depressed, whereas birds challenged with D78, Bursine 2®, and Lukert strains were clinically normal antemortem. No grossly evident proventriculitis or proventricular dilation was present at necropsy.

Proventriculus. Histologic sections of proventriculi from birds experimentally infected with different strains of IBDV had no lesions at 4 dpi and either no or mild lesions at 6 dpi (Tables 3.2, 3.3). Lesions present consisted of multifocal small lymphoid infiltrates in the glandular interstitium. Real time RT-PCRs for IBDV on these proventricular samples were

positive for 3 out of 14 at 4 dpi, and 6 out of 14 at 6 dpi (Tables 3.2 and 3.3). In samples positive by real time RT-PCR, the IBDV strain identified was identical to that used to inoculate that group.

IBDV antigen was detected by IHC in 1 of 14 of these proventricular samples at 4 dpi, and 3 out of 14 at 6 dpi. This staining was very mild and confined to lymphocyte infiltrates in the proventriculus. Apoptosis was observed in 10 proventricular samples and was present in the cells of the lymphocyte infiltrates. A very low number of epithelial cells were stained in proventricular glands.

Bursa. Histologic lesions of IBDV infection were present in all bursas of chickens exposed to all IBDV strains, except those given the Lukert strain. The damaged bursas also contained IBDV as measured by IHC (Fig. 3.2) and real time RT-PCR (Tables 3.2, 3.3). Chickens challenged with the Lukert strain had no lesions, no staining by IHC, and were real time RT-PCR negative.

Apoptosis was present in all bursas. Strong staining was present in bursas from chickens inoculated with IBDV (Fig. 3.2). Mild staining was present in the negative control chickens.

Thymus. Thymic lesions were not present in most challenged birds. IBDV was stained by IHC in only one sample at 4 dpi and in two at 6 dpi and was very faint (Tables 3.2, 3.3). Virus was detected by real time RT-PCR in thymuses from 5 groups on day 4, and from the same 5 groups on day 6. Chickens inoculated with the Lukert strain were consistently negative by IHC and RT-PCR.

Mild apoptosis was present in the thymuses of the IBDV-challenged birds, except those challenged with Lukert. The staining was stronger than that present in thymuses from unexposed chickens.

DISCUSSION.

In the past several years, IBDV has been implicated as the cause of proventriculitis in broiler flocks from northern Alabama (7) and Arkansas (16). The disease resulted clinically in poor feed conversion, body weight reduction, and mortality. Dormitorio *et al*. (7) reported that vaccination of SPF leghorns with a commercial vaccine containing both antigenic standard and variant IBDVs reduced the incidence and severity of proventriculitis in birds that were challenged with proventricular homogenates from proventriculitis cases. Huff *et al.* (12) reported the successful segregation, isolation, and propagation in SPF embryos of an IBDV from a proventricular homogenate. A bacterial isolate (*Clostridia* sp.) from this same proventricular tissue homogenate exacerbated experimental proventriculitis when reinoculated orally (12). Further studies showed that only the combination of this virus and bacteria reproduced proventriculitis similar to the proventricular homogenate. However, the severity of lesions and the effects on growth were more severe in birds exposed to the intact homogenate, suggesting the presence of additional agents or increased titers present in the homogenate. Huff *et al.* (12) concluded that a currently undetermined viral infection, as well as dietary factors, may facilitate bacterial invasion of the proventriculus and that more than one type of virus may act as the initiator for this disease syndrome.

In our study, broilers with naturally occurring proventriculitis did not have detectable IBDV in their proventriculus, even when lesions and IBDV in the bursas were present. Proventriculitis was not produced at 4 dpi in any of the birds exposed to IBDV and in only 1 of the birds at 6 dpi. Very low levels of IBDV were detected by RT-PCR in 9 of 28 proventriculi, suggesting that we were detecting either viremia or replication of IBDV in recruited inflammatory B lymphocytes. IBDV antigen staining was present only in lymphocytes within

proventriculi, supporting the latter speculation. The mild lesions in the proventriculi observed at day 6 with some of the IBDV strains (STC, GLS and Variant A) could be due to a generalized effect of the more pathogenic viruses (38).

The minimal apoptotic staining in the bursas of noninfected birds represents normal cellular turnover during maturation of B lymphocytes (22,23). Apoptosis and histologic lesions in the thymus of chickens infected with IBDV without correlating *in situ* replication has been reported (13,32) and indirect mechanisms of induction of apoptosis have been suggested (15). Apoptosis observed in individual cells of proventricular glands may be due to release of similar apoptosis-inducing factors by distant cells infected with IBDV (25,35). However, the apoptosis observed in the proventriculus of some IBDV-infected birds was so mild it is unlikely that it plays a significant role in the induction of proventriculitis.

In summary, SPF broilers experimentally infected with different strains of IBDV did not develop proventriculitis, and chickens with naturally occurring cases of proventriculitis did not have IBDV in their proventriculi. This shows that the strains of IBDV examined do not localize or cause direct damage in the proventriculus. Although the strains chosen for this study belong to five of the six molecular groups used to classify IBDV strains, it is possible that other untested strains may produce proventriculitis directly, or that proventriculitis is due to an undetermined cause and is exacerbated by immunosuppression produced by IBDV infection.

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Figure legends

Fig. 3.1. Photomicrographs of proventriculi from a normal broiler chicken (A and C), and from a broiler chicken with naturally occurring proventriculitis (B and D). H&E, 25X and 40X.

Fig. 3.2. Photomicrographs of bursas from broiler chickens, control and challenged with IBDV (STC strain). (A) IBDV antigen staining by IHC, negative control. (B) IBDV antigen staining by IHC, challenged. (C) Apoptosis staining by TUNEL method, negative control. (D) Apoptosis staining by TUNEL method, challenged. 100X.

Fig. 3.1.

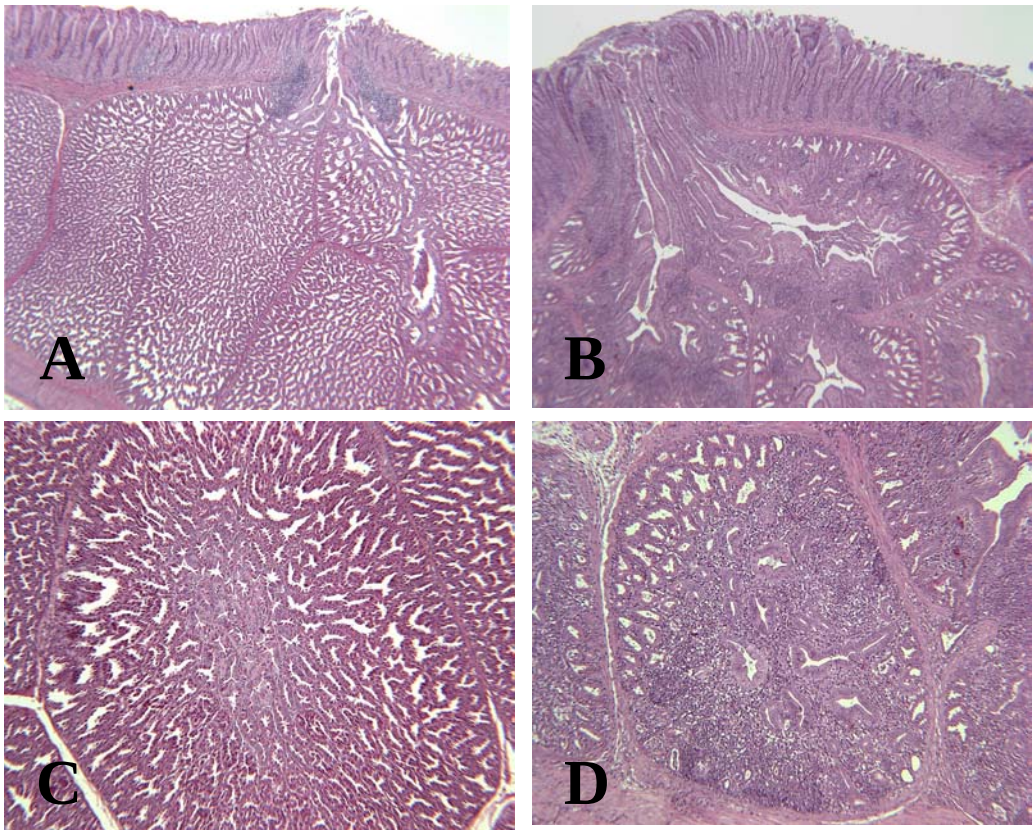

Fig. 3.2.

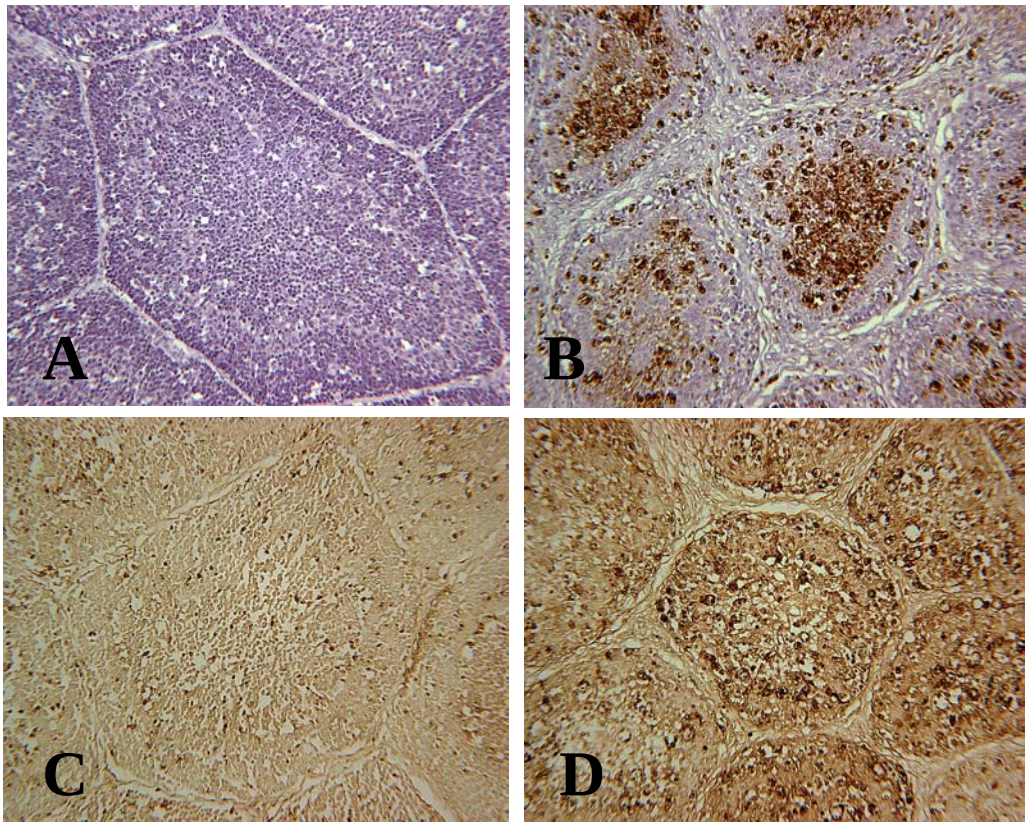

Table 3.1. Naturally occurring cases of proventriculitis. Histopathology , RT-PCR for IBDV, IHC for IBDV, and apoptosis staining (TUNEL) on formalin-fixed, paraffin-embedded tissue sections of bursas and proventriculi from broiler chickens with proventriculitis.

	Bursa				Proventriculus			
Bird	LS[A]	RT-PCR	IHC[C]	TUNEL[D]	LS	RT-PCR	IHC	TUNEL
1	2	-	-	+	4	-	-	+
2	3	+	+	++	4	-	-	+
3	2	-	-	+	4	-	-	+
4	3	+	+	++	4	-	-	+

[A]LS = lesion score. 1 = no lesions. For bursal sections, 2 = mild variation in follicle size; 3 = moderate variation in size of follicles; 4 = either necrosis or follicle atrophy. For proventricular sections, 2 = mild glandular lumenal ectasia; 3 = ectasia plus lymphoid infiltrates in the interglandular interstitium; 4 = either acute glandular necrosis or severe fibrosis with lymphoid infiltrates.

[B]Reverse transcriptase polymerase chain reaction; - = negative; + = positive.

[C]Immunohistochemistry; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

[D]TUNEL= terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

Table 3.2. Experimental chickens. 4 dpi. Histopathology, RT-PCR for IBDV, IHC for IBDV, and apoptosis staining (TUNEL) on formalin-fixed, paraffin-embedded tissue sections of proventriculi, bursas, and thymuses from broiler chickens challenged with different strains of IBDV. Data for individual birds.

	Proventriculus				Bursa				Thymus			
Strain[A]	LS[B]	RT-PCR[C]	IHC[D]	TUNEL[E]	LS	RT-PCR	IHC	TUNEL	LS	RT-PCR	IHC	TUNEL
Control	1	-	-	-	1	-	-	+	1	-	-	+
	1	-	-	-	1	-	-	+	1	-	-	+
STC	1	-	-	+	4	+	+++	+++	4	+	-	++
	1	+	+	+	4	+	+++	+++	3	+	-	++
GLS	1	-	-	-	2	+	+	++	1	-	-	++
	1	+	-	-	4	+	+++	+++	1	+	-	++
Var. E	1	+	-	+	4	+	++	+++	1	+	+	++
	1	-	-	-	4	+	++	+++	1	+	-	++
Var. A	1	-	-	-	4	+	++	+++	1	+	-	++
	1	-	-	-	4	+	+++	+++	1	-	-	++
D78	1	-	-	-	2	+	++	++	1	-	-	++
	1	-	-	-	2	+	+	++	2	+	-	++
Bursine	1	-	-	-	4	+	+	++	1	-	-	++
	1	-	-	-	4	+	++	++	1	-	-	++
Lukert	1	-	-	-	1	-	-	+	1	-	-	+
	1	-	-	-	1	-	-	+	1	-	-	+

[A]IBDV strain used for challenging chickens

[B]LS = lesion score. 1 = no lesions. For bursal sections, 2 = mild variation in follicle size; 3 =

moderate variation in size of follicles; 4 = either necrosis or follicle atrophy. For proventricular sections, 2 = mild glandular lumenal ectasia; 3 = ectasia plus lymphoid infiltrates in the interglandular

interstitium; 4, either acute glandular necrosis or severe fibrosis with lymphoid infiltrates.

[C]Reverse transcriptase polymerase chain reaction; - = negative; + = positive.

[D]Immunohistochemistry; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

[E]TUNEL= terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

Table 3.3. Experimental chickens 6 dpi. Histopathology, RT-PCR for IBDV, IHC for IBDV, and apoptosis staining (TUNEL) on formalin-fixed, paraffin-embedded tissue sections of proventriculi, bursas, and thymuses from broiler chickens challenged with different strains of IBDV. Data for individual birds.

	Proventriculus				Bursa				Thymus			
Strain[A]	LS[B]	RT-PCR[C]	IHC[D]	TUNEL[E]	LS	RT-PCR	IHC	TUNEL	LS	RT-PCR	IHC	TUNEL
Control	1	-	-	-	1	-	-	+	1	-	-	+
	1	-	-	-	1	-	-	+	1	-	-	+
STC	1	-	+	+	4	+	+++	+++	4	+	+	++
	3	+	+	+	4	+	+++	+++	3	+	-	++
GLS	1	-	-	+	4	+	+	+++	1	-	-	++
	2	+	+	+	4	+	+	+++	1	+	-	++
Var. E	1	-	-	-	4	+	++	+++	1	+	++	++
	1	+	-	+	4	+	++	+++	1	+	+	++
Var. A	1	-	-	-	4	+	++	+++	1	+	-	++
	2	+	-	+	4	+	++	+++	1	-	-	++
D78	1	-	-	-	4	+	+++	++	1	-	-	++
	1	+	-	+	4	+	+++	++	2	+	-	++
Bursine	1	-	-	-	4	+	+	++	1	-	-	++
	1	+	-	-	4	+	++	++	1	-	-	++
Lukert	1	-	-	-	1	-	-	+	1	-	-	+
	1	-	-	-	1	-	-	+	1	-	-	+

[A]IBDV strain used for challenging chickens

[B]LS = lesion score. 1 = no lesions. For bursal sections, 2 = mild variation in follicle size; 3 = moderate variation in size of follicles; 4 = either necrosis or follicle atrophy. For proventricular sections, 2 = mild glandular lumenal ectasia; 3 = ectasia plus lymphoid infiltrates in the interglandular

interstitium; 4, either acute glandular necrosis or severe fibrosis with lymphoid infiltrates.

[C]Reverse transcriptase polymerase chain reaction; - = negative; + = positive.

[D]Immunohistochemistry; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

[E]TUNEL= terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; - = no staining; + = minimal staining; ++ = moderate staining; +++ = intense staining.

CHAPTER 4

REPRODUCTION OF PROVENTRICULITS IN COMMERCIAL AND SPF BROILER CHICKENS[1]

[1]Pantin-Jackwood, M. J., T.P. Brown, and G. R. Huff. To be submitted to *Veterinary Pathology*.

SUMMARY.

Proventriculitis was studied by experimentally reproducing the disease in broiler chickens. One-day-old commercial and SPF broilers were orally gavaged with a proventricular homogenate produced from the proventriculi of broilers with proventriculitis. At 7 and 14 days post-inoculation differences in weight gain, organ/body weight ratios, and the presence of macro and microscopic lesions between these birds and controls were assessed. Both, commercial and SPF broilers had enlargement of the proventriculus with necrosis of the glandular epithelium and lymphocytic infiltrates in the proventricular gland. SPF broilers exposed to the proventricular homogenates developed Infectious Bursal Disease, and infectious bursal disease virus (IBDV) was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) in bursal and proventricular tissues. They also were positive by RT-PCR to infectious bronchitis virus (IBV) and developed nephritis. Commercial broilers developed mild nephritis but not bursal disease, and were negative for IBDV and IBV by RT-PCR. Both, commercial and SPF chickens, were negative for reovirus, and Newcastle disease virus (NDV), and positive for chicken anemia virus (CAV) and adenovirus by molecular techniques. Bacteria were not identified in histological sections nor were they isolated from affected proventriculi. Filtrates from the proventricular homogenates passed in embryos for virus isolation caused stunting but identification of the cause by electron microscopy (EM) was unsuccessful. However, allantoic fluid from the eggs was positive for IBV by RT-PCR. Thin sectioning EM on proventriculi from affected birds failed to identify a causative agent. In conclusion, the original proventricular homogenates had IBDV, IBV, adenovirus and CAV, but their role in producing proventriculitis was not proven.

Keywords: Chicken; Proventriculitis.

Abbreviations: CAV = chicken anemia virus; Dpi = days post-inoculation; EM = electron microscopy; H&E =hematoxylin and eosin; IBDV = infectious bursal disease virus; IBV = infectious bronchitis virus; IHC = immunohistochemistry; NDV = Newcastle disease virus; PBS = phosphate buffer saline; RT = reverse transcripatase; PCR = polymerase chain reaction; SPF = specific-pathogen free.

INTRODUCTION.

Proventriculitis is an infectious disease of chickens of unknown etiology (7). It is characterized by an enlarged, atonic proventriculus that is filled with fluid and feed (2, 8, 9, 13, 17, 28). The gastric isthmus connecting the proventriculus and gizzard is enlarged, with dilation of the constriction present at this juncture.

The economic impact of proventriculitis is mainly due to condemnation of contaminated carcasses subsequent to the rupture of the proventriculus during routine evisceration (2, 13). Proventriculitis is more severe in younger birds (4-5 wks of age) and has been associated with impaired growth, poor feed conversion, intestinal fragility, stunting syndrome and passage of undigested feed (1, 3, 13, 19, 24, 28, 30). The poultry industry reports sporadic, thought economically important, outbreaks of proventriculitis in broilers (13). Although broiler chickens throughout the world are commonly plagued by outbreaks of disease characterized at least in part by proventricular enlargement, lesions consistent with transmissible proventriculitis have been described in detail only in the United States (8, 9, 13), Holland (19), and Australia (28).

Routes of natural infection of proventriculitis are not known; however, chickens can be infected experimentally by oral inoculation with a homogenate prepared from proventriculi of chickens with proventriculitis (2, 9, 13, 28). Because the disease is reproduced with

proventricular homogenate filtrates (0.2 μm), a virus is suspected as the etiologic agent (9, 13, 28). Consequently, the disease is also termed transmissible viral proventriculitis (TVP)(8, 9). However, the severity of lesions and the effects on production are more severe in birds treated with unfiltered homogenates, suggesting an additive effect of other infectious agents (13).

Potential infectious causes of proventriculitis include adenovirus (19, 21), reovirus (20, 21, 24), infectious bronchitis virus (IBV)(35), infectious bursal disease virus (IBDV) (2, 13, 14, 17, 23, 31) and megabacterium (11, 12, 22, 26). However, none of these agents have been found in a majority of cases. Electron microscopy has detected adenovirus-like viral particles in acute lesions but isolation of this virus from affected proventriculi has been unsuccessful (8, 9, 13).

Our objective in this study was to reproduce proventriculitis in broiler chickens, characterize the changes present in the proventriculus and other organs, and examine the affected proventriculus for the presence of virus or bacteria by histological, bacteriological, virological, and molecular methods.

MATERIALS AND METHODS.

Chickens. One-day-old unvaccinated broiler chicks were obtained from a commercial hatchery. Also, Fertile White Plymouth Rock chicken eggs (SEPRL, USDA, Athens, GA, USA) were obtained from a breeder flock maintained under SPF conditions and hatched, the parent flock and all progeny were free of common poultry diseases, specifically IBDV, MDV, IBV, reovirus and CAV. All chicks were wing-banded, weighed, separated into groups and maintained in positive pressure Horsfal isolation units. Feed and water were provided *ad libitum*.

Proventricular homogenates. Two different proventricular homogenates were used. Homogenate 1 (Hom.1) was prepared from proventriculi from 4-wk old chickens with

proventriculitis, obtained from a commercial Cornish hen processing plant in northwest Alabama (2). Homogenate 2 (Hom.2) was prepared from proventriculi of broiler chickens that presented proventriculitis after being challenged at day of age with Hom.1 (13).

Experimental design. 18 one-day-old commercial broilers, and 18 one-day-old SPF broilers were divided into 3 groups each. The first group was inoculated by oral gavage with 1ml of sterile saline solution (negative control). The second group received 1 ml of proventricular homogenate 1 (Hom. 1). The third group received 1 ml of proventricular homogenate 2 (Hom. 2).

Sample collection and processing. At 7 and 14 days of age, 3 birds from each group were examined, weighed, bled, killed by cervical dislocation, and necropsied. Bursa, proventriculus, spleen, and right side of thymus were weighed and sections of these organs and of liver, kidney, duodenum, pancreas, heart, gizzard and bone were collected from each bird and fixed immediately by immersion in 10% neutral buffered formalin for 24 hours. Tissues were then processed and embedded in paraffin using routine histologic techniques. A section of proventriculi was also collected in a solution of 2% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, and 0.1M cacodylate buffer at pH 7.2- 7.3 for thin sectioning and electron microscopic examination. The remaining proventriculi were pooled per group and collected in sterile plastic tubes over ice, homogenates prepared (2, 13). Briefly, proventriculi were washed in sterile phosphate buffer saline (PBS) three times on a magnetic stirrer to remove feed residues and toxins. Washed proventriculi were then diluted 1:1 wt/vol in sterile PBS and homogenized with a commercial blender (Waring, New Hartford, Connecticut). The homogenates were then frozen at –80 C. Relative organ weights were obtained using the formula [Relative organ weight = (organ weight / body weight) x 100].

Histopathology. Paraffin-embedded tissues were sectioned, mounted, stained using hematoxylin and eosin (H&E), and examined blinded as to treatment for lesions using light microscopy. Tissue sections from proventriculus, bursa, thymus and spleen were assigned a lesion severity score. A lesion score of 1 represented no lesions. For bursal sections, 2 was defined as mild variation in follicle size, 3 as moderate variation in size of follicles, and 4 as either necrosis or follicle atrophy. For proventricular sections, 2 was defined as mild glandular lumenal ectasia, 3 as ectasia, mild glandular necrosis, plus lymphoid infiltrates in the interglandular interstitium, and 4 as either acute glandular necrosis or severe fibrosis with lymphoid infiltrates. For thymus sections, 2 was defined as mild cortical thinning, 3 as moderate cortical thinning, and 4 as absence of cortical lymphocytes. For spleen sections 2 was defined as mild lymphocyte depletion, 3 as moderate lymphocyte depletion, and 4 as severe lymphocyte depletion.

For identification of bacteria by light microscopy, tissue sections of proventriculi were stained by the Warthin-Starry technique (4), and a modified *Helicobacter pylori* and gastric stain (6).

Serology. Serum samples obtained at 7 and 14 days of age, from both commercial and SPF broilers, were examined for antibody to IBDV, IBV, NDV, CAV, reovirus, MS and MG, using commercially available ELISA tests (IDEXX Laboratories, Inc. Westbrook, Maine).

Bacteriology. Pooled proventricular homogenates from experimentally infected birds from each group were diluted in sterile saline and plated on Campy blood agar (Remel, Lenexa, TX). Inoculated plates were placed into GasPak pouches (BD Diagnostic Systems; Sparks, MD) and incubated at 42C for 48 hrs. To check for anaerobic growth, blood agar and PEA (phenylethyl alcohol) agar plates were streaked and incubated overnight at 37C in a GasPak

pouch. The proventricular homogenates were also plated on Sabouraud dextrose agar plates and incubated at 37C overnight, and then maintained at room temperature and examined daily for mold growth. Isolation of Salmonella sp. was attempted by standard protocol using tetrathionate enrichment broth (10).

Virus isolation. A portion of proventricular homogenate 2 (Hom.2), proventricular homogenate made from pooled proventriculi obtained from commercial chickens challenged with Hom. 2 (Hom.2 com.) at 7 dpi, and negative proventricular homogenate from control group (-PV), was frozen and thawed three times. Sediment was removed from the homogenates by centrifugation at 2,500 X g for 30 min at 4 C. The supernatants were forced through a series of glass fiber filters with a final membrane pore size of 0.2 μm. Four groups of five SPF leghorn chicken embryos were inoculated at 9 days of age via chorioallantoic membrane and allantoic cavity routes (29), with 0.2 ml of one of the following: Hom.2 filtrate, Hom.2 com. filtrate, –PV filtrate, and sterile saline. Eggs were examined daily for embryo death. At 7 days post inoculation chorioallantoic membranes (CAMs) and allantoic fluid were aseptically collected and placed in sterile microfuge tubes and frozen at –80C. A portion of the CAMs was collected in 10% buffered formalin and processed for histopathology. Five blind passes were done and at each, allantoic fluid and CAM's were diluted 1:10 in antibiotic diluent prior to reinoculation.

RNA extraction. RNA was extracted from formalin fixed paraffin-embedded bursas and proventriculus and from Hom.1, Hom. 2, pooled proventricular homogenates from experimental groups at 7 dpi, and from allantoic fluid from eggs inoculated with homogenate filtrates (fifth passage). Sections totaling fifty μm in thickness were cut from each formalin-fixed paraffin-embedded tissue block with a microtome and a new blade for each block. Sections were then deparaffinized (HemoDe and 100% ethanol; Fisher Scientific, Pittsburgh, PA). All tissues were

digested with 10% proteinase K (Sigma Chemical Co., St. Louis, MO) for 3 hr at 50 C. RNA was extracted with Trizol (Life Technologies, Inc. Gaithersburg, MD) according to the manufacturer's recommendations, diluted in 90% dimethyl sulfoxide (DMSO), and frozen at -80 C until assayed.

DNA extraction. DNA was extracted from Hom.1, Hom.2, pooled proventricular homogenates from experimental groups at 7 dpi, and from allantoic fluid from eggs inoculated with homogenate filtrates (fifth passage) using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturers recommendations. Extracted DNA was frozen at –80C until assayed.

Real time reverse transcriptase-polymerase reaction (RT-PCR). For detection of IBDV, IBV, NDV, and reovirus this protocol was followed: Extracted RNA was denatured at 95 C for 5 min and put on ice (only for IBDV and reovirus reactions). Real time RT-PCR was performed separately for each sample with reagents from the Light Cycler-RNA Amplification SYBR® Green I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used were specific for amplification of each of the viruses (Table 4.1). Amplification and detection of specific products was performed with a Light Cycler according to the manufacturer's recommendations (ROCHE Light Cycler version 3.0; ROCHE Molecular Biochemicals). Briefly, reverse transcription was done at 55 C for 10 minutes, followed by denaturation at 95 C for 30 sec. Forty PCR cycles were done consisting of denaturation (95 C for 1 sec), hybridization (55 C for 10 sec), and extension (72 C for 13 sec). A melting curve analysis was done after an initial denaturation at 95 C. The melting curve was established with an initial temperature of 65 C for 10 sec and then gradual temperature increase of 0.1 C per second until reaching 95 C. The melting temperature was used to confirm the identity of viral specific products obtained using

real time RT-PCR. Additional confirmation of specific amplification was done by gel electrophoresis of 8 µl of the PCR products on 2% agarose (Sigma Chemical Co., St. Louis, MO) followed by ethidium bromide staining. PCR products from IBDV positive samples were purified using the QIAgen purification kit and sequenced (Molecular Genetics Instrumentation Facility; University of Georgia, GA). Sequence data was then analyzed by DNASTAR and sequences compared to that of known IBDV. Samples positive for IBV were analyzed by RT-PCR RFLP for molecular grouping (15).

PCR. Detection of adenovirus and CAV was done as follows. Primers used for these reactions are specified in Table 4.1. PCR for adenovirus was performed using 'Ready to go' PCR Beads (Pharmacia Biotech) and following the protocol from Raue *et al.* (27). PCR for chicken anemia virus was performed following the same protocol used by Todd *et al*. (33). A 8 µl aliquot of each reaction was separated by electrophoresis in an 2% agarose gel (Sigma Chemical Co.) followed by ethidium bromide staining and examination with a U.V. transiluminator.

IBDV Immunohistochemistry (IHC) and Immunofluorecence Assay (IFA). All procedures were done at room temperature. Tissue sections were cut (4 µm) from paraffin-embedded bursas and proventriculi of inoculated chickens and mounted on positively charged glass slides (Superfrost/Plus; Fisher Scientific). Paraffin was melted from the slides (10 min at 65 C) and removed by immersion in Hemo-De three times (5 min each). Slides were then air dried and digested with 10% proteinase K (DAKO, Carpinteria, CA) for 5 min to expose antigenic target sites. Staining for IHC was performed on bursas and proventriculi with an automated stainer (Leica ST 5050, Nussloch, Germany) with a non-biotin peroxidase kit (DAKO Envision System; DAKO) according to the manufacturer's recommendations. The primary antibody used was a monoclonal antibody specific to and cross reactive for all IBDVs (ATCC

No.HB9490). After IHC staining, sections were counterstained with hematoxylin, air dried, coverslipped, and examined by light microscopy. Staining for IBDV was recorded as positive or negative staining. IFA was performed on proventriculus sections using as primary antibody a convalescent sera obtained from SPF chickens at 14 dpi and diluted 1:100 in sterile PBS. Slides were incubated for 20 min followed by three washes with PBS of 5 min each. For secondary antibody FITC monoclonal anti-chicken IgG (Accu-Specs) was used at a 1:40 dilution in PBS. Slides were incubated for 20 min then washed three times with PBS. Slide coverslips were mounted using 1:1 glycerol/PBS and the sections were examined using a fluorescent microscope (Leitz).

Transmission electron microscopy. Sections of proventriculus collected from inoculated chickens that presented proventriculitis, and allantoic fluid collected from the fifth pass in eggs of Hom. 2, Hom. 2 com. and -PV were sent for direct examination with a JEOL JEM 1210 transmission electron microscope.

Statistical analysis. The body weight gain, relative organ weight, and lesion scores were analyzed using ANOVA and means comparisons for all pairs using Tukey-Kramer HSD (JMP) Significance was assumed at the 0.05 level of probability.

RESULTS

Clinical signs and macroscopic lesions. No clinical signs were observed in the saline control groups or the commercial chickens that received the proventricular homogenates. SPF broilers that received the homogenates had mild depression. Gross lesions were observed in all proventriculi from homogenate-inoculated commercial and SPF chickens. At both, 7 and 14 days post-inoculation, the proventriculi were enlarged, mottled, and had a distended gastric isthmus

(Fig 4.1, 4.2). The proventricular wall was thickened, with a white lobular pattern observed when sectioned. These lesions were more evident in the commercial broilers than in SPF broilers, and at 14 days post inoculation for both groups. No macroscopic lesions were observed in any other organ of experimentally infected birds.

Body weight gain. Commercial broilers inoculated with proventricular homogenate had no significant suppression of weight gain compared to age matched control birds. Weight gain in SPF broilers was affected by both proventricular homogenate treatments (Table 4.2 and 4.3).

Organ weights and microscopic lesions. Commercial and SPF chickens that received the positive proventricular homogenates had increased proventricular organ/weight ratio, and microscopic lesions in the proventriculus at 7 and 14 dpi. Bursa and thymus organ/weight ratio was not affected in commercial broilers, but their spleen organ weight increased with the homogenate treatment (Tables 4.2 and 4.3). SPF broilers that received proventricular homogenate had smaller bursas, thymuses and spleens compared with controls at 14 dpi (Table 4.3). Microscopically, at 7 dpi, acute necrosis of the proventricular glandular epithelium was present in both, commercial and SPF chickens (Fig. 4.3, 4.4, 4.5). Collecting sinuses of the glands were dilated and contained desquamated epithelium. Severely affected glands coalesced. Nuclei of the glandular epithelial cells were enlarged and pale, with marginated chromatin. Lymphocytic infiltration was present in the lamina propria of the mucosa and in the glandular interstitium in areas containing affected glandular epithelial cells. At 14 dpi, proliferating hyperplastic and hypertrophic columnar cells lined primary, secondary, and tertiary gland ducts. Cuboidal to low columnar, pale, basophilic, and distinctly vacuolated ductlike epithelium replaced the destroyed alveolar secretory cells. Germinal center formation was present in the glands and mucosa. No difference in lesion scores were present in bursa, thymus and spleen

between commercial chickens and controls. Bursa and thymus of SPF chickens that received the homogenates had increased lesions scores when compared to controls (Table 4.2 and 4.3).

Mild lymphocytic infiltration was present in the intestine, pancreas and liver of commercial and SPF chickens inoculated with the proventricular homogenates, at both 7 and 14 dpi (Tables 4.4. and 4.5). All homogenate-inoculated SPF broilers also had moderate to severe lymphocytic infiltration in the kidneys at both 7 and 14 dpi. No other lesions were present in these or the remaining organs examined from both commercial an SPF chickens.

Serology. Commercial broilers that received the proventricular homogenate were negative for reovirus, NDV, MG, and MS at 7 and 14 dpi. These birds were positive for IBDV and IBV at both time points and also for CAV at 14 dpi. (Table 4.6). SPF broilers that received the proventricular homogenates were negative for IBDV, IBV, reovirus, NDV, MG, MS, and CAV at 7 dpi, but at 14 dpi seroconverted to IBDV, IBV, and CAV (Table 4.7).

Bacteriology. No bacteria were isolated from proventricular homogenates from birds experimentally infected by the methods described above. No bacteria were observed by direct light microscopy in routine or special stained sections.

Virus isolation. Embryo inoculated with proventricular homogenate 2 (Hom. 2) and proventricular homogenate from commercial chickens inoculated with Hom. 2 (Hom. 2 com.) were stunted from the second passage on. Chorrioallantoic membranes (CAMs) harvested from these eggs did not have plaque formation and no lesions were observed histopathologically.

RT-PCR and PCR results.

IBDV RT-PCR on paraffin-embedded tissues. Bursas and proventriculi of commercial broilers were all negative for IBDV (Tables 4.6). All bursas and some of the proventriculi of SPF

broilers that received either proventricular homogenate were positive for IBDV (Table 4.7). Amplicons were sent for sequencing and were most similar to variant A IBDV (data not shown).

RT-PCRs and PCRs on proventricular homogenates and allantoic fluids. All samples were negative for reovirus and NDV (Table 4.8). Hom. 1 was positive for IBDV, IBV, adenovirus and CAV. Hom. 2 was positive for IBDV, IBV, and CAV and negative for adenovirus. Proventricular homogenates from commercial broilers inoculated with the Hom. 1, and collected at 7 dpi, were negative for all virus examined except adenovirus. Commercial broilers inoculated with Hom. 2 were negative for all viruses examined. SPF broilers inoculated with Hom.1 were positive for IBDV, IBV, and adenovirus and negative for the rest. SPF broilers inoculated with Hom. 2 were positive for IBDV and IBV and negative for the rest. Allantoic fluids from embryos inoculated with Hom. 2 or Hom. 2 com. were only positive for IBV.

Molecular characterization of detected IBDV and IBV. Analysis of the sequence data obtained from the amplified IBDV revealed that this virus is a IBDV variant strain and is most similar to Variant A. RFLP analysis of the amplified IBV determined that this virus strain was Connecticut (data not shown).

IBDV Immunohistochemistry. Positive staining for viral antigen was detected in all bursas and some of the proventriculi of SPF chickens inoculated with the proventricular homogenates. None of the bursas or proventriculi of the commercial broilers were positive.

Immunoflourescence Assay (IFA). Positive fluorescent staining was present in glandular epithelial cells in the proventriculi from homogenate-inoculated chickens when examined at 7 dpi (Fig 4.6.). The specific reaction was seen localized within the cytoplasma of the glandular epithelial cells. Fluorescent staining was also present on the outer surface of what

seemed to be lymphocytes. No fluorescent staining was observed in proventriculi of saline-inoculated chickens.

Electron microscopy. No viruses were detected in the samples sent for examination.

DISCUSSION.

Proventriculitis was successfully reproduced by oral inoculation of commercial and SPF broilers with proventricular homogenates obtained from chickens with proventriculitis. Inoculated chickens had enlargement of the proventriculus and a distended gastric isthmus. The proventricular walls were thickened with a white lobular pattern observed when sectioned. Microscopic lesions consisted of degeneration and necrosis of the glandular epithelium, severe lymphocytic infiltration, and ductal epithelial hyperplasia. This loss of glandular tissue and ductal hyperplasia may result in loss of function of the proventriculus (10). This would explain the poor feed conversion and reduced growth rates reported in some naturally affected chickens with proventriculitis (28), and also the reduced weight gain observed in our homogenate-inoculated SPF chickens. However, the body weight gain in our commercial chickens was not affected. Bayyari *et al.* (2) found that proventriculitis was produced independently of an effect on growth, and a common field observation is that proventriculitis can occur in the best performing flocks when processed at 4-5 wk of age (13). This leads us to believe that proventriculitis may or may nor be associated with stunting in broilers, and that several agents or conditions most likely modify the severity of proventriculitis and its effect on weight gain. In fact, proventriculitis has been associated with infectious stunting or malabsorption syndrome in chickens (3), but cases of malabsorption syndrome may or may not include proventricular lesions (32). Filterable agents isolated in the Netherlands were originally linked to proventriculitis, causing runting syndrome

in broilers (19). These authors suggested the involment of both bacteria and viruses in the etiology of malabsorption syndrome (19, 20). A comparative study of the pathogenesis of five different malabsorption syndrome homogenates from the Netherlands and Germany distinguished the inoculated groups of chickens by their histopathologic lesions: proventriculitis, lesions in the intestine only, or combination of both (32). Lesions in the small intestine had more impact on weight gain depression than lesions in the proventriculus. In our study no intestinal lesions were observed in chickens inoculated with the proventricular homogenates.

Reoviruses have been implicated as a causative agent for concurrent proventricular lesions present in some flocks naturally affected with malabsorption syndrome (20), and proventriculitis was reproduced by inoculation of two reovirus isolates from the intestines of birds with malabsorption syndrome (24). In our study however, no reovirus were isolated from the homogenates, and no reovirus was detected by RT-PCR in any of the inoculated groups. Also none of the chickens seroconverted to this virus, which indicates that proventriculitis can occur in the absence of reovirus.

Mild proventriculitis has also been reproduced experimentally in chickens infected with some isolates of adenovirus (19, 21), though this virus hasn't been consistently isolated from diseased proventriculi. One of the proventricular homogenates used in our study was positive for adenovirus, and also the proventriculi of the chickens that were inoculated with this homogenate. However, the role of this virus in proventriculitis is not clear because the disease still occurred in its absence, and visualization of viral particles in affected proventricular glands by EM was unsuccessful. Goodwin *et al*. (8) reported the presence of intralesional virions in proventriculi from chicks that had proventriculitis, and suggested a causal relationship between the virus and the lesion in its host. Hexagonal intranuclear virus particles were described and resembled

adenovirus or poliomavirus. However, DNA *in situ* hybridization failed to detect adenovirus or poliomavirus nucleic acids. Huff *et al.* (13) also reported the presence of similar virus-like particles in the nuclei of many epithelial cells of the proventriculus of chickens experimentally inoculated with homogenate prepared from the proventriculi of chickens with proventriculitis. The particles, nonenveloped spheres of about 100-200nm in diameter, appeared hexagonal and were arranged in semiparacristalline arrays in the nuclei (13). These adenovirus-like particles have not been isolated so its role as causative agent in proventriculitis has not been corroborated.

IBDV has also been associated with proventriculitis (2, 13, 23) but its role in this disease is not clear. Both gross and microscopic lesions of the proventriculus have been produced by IBDV challenge in leghorn chickens (24) and vaccination against IBDV has been reported to decrease the incidence of proventriculitis (7,15). However, proventriculitis was not produced by inoculation of SPF broilers with different strains of IBDV (25). Both proventricular homogenates used in our study to induce proventriculitis were positive for IBDV by RT-PCR. Proventriculi of commercial broilers inoculated with these homogenates were negative for the virus by RT-PCR and IHC, and these birds did not present lesions or virus in the bursa. These chickens had antibody titers against IBDV at 7 and 14 dpi and were probably protected against the virus. On the other hand, SPF broilers had lesions in the bursa characteristic of IBDV infection, the virus was detected by RT-PCR and IHC in all bursas and some of the proventriculi, and some of the birds seroconverted at 14 dpi. Because proventriculitis was produced in commercial broilers independently of the presence of IBDV, this virus probably is not directly involved in the disease.

Both of the proventricular homogenates used in our study to induce proventriculitis were also positive for IBV by RT-PCR, and homogenates produced from the proventriculi of

inoculated SPF broilers were also positive by RT-PCR and seroconverted to IBV at 14 dpi. These birds also had moderate to severe nephritis, a lesion associated with infection with IBV (5). Commercial broilers inoculated with the proventricular homogenates were negative by RT-PCR for IBV but presented mild interstitial nephritis, and IBV was isolated in embryos when inoculated with a filtrate produced from the homogenate prepared from the pooled proventriculi of these birds. These commercial broilers had antibodies against IBV, most likely of maternal origin, detected at both 7 and 14 dpi, which probably offered some protection against the effect of the virus. Infectious bronchitis virus (IBV) isolates from naturally occurring cases in China have been reported to produce proventricular lesions in infected birds (35). The strain of IBV isolated in our study was determined to be Connecticut by RFLP, a strain that has been isolated also from cecal tonsils and intestine in chickens (16). The role of this strain of IBV in proventriculitis needs to be further explored.

Guy and Barnes (9) reproduced proventriculitis by administration of a filtrate (0.2-μm) from a homogenate produced from the proventriculi of chickens with proventriculitis. This inoculum was free of avian reovirus, avian group I adenovirus, infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV). Adenovirus-like particles, similar to those observed by Goodwin *et al* (8), were identified by thin-section electron microscopy in nuclei of affected glandular epithelium cells. These authors also detected intranuclear staining by IFA using as primary antibody hyperimmune sera from birds inoculated with infectious proventricular filtrates. The results of our immunofluorescent assays did not corroborate these findings. Although immunofluorescence was also observed in the affected glandular epithelial cells, it was localized in the cytoplasma, not the nucleous. Also staining of the surface of lymphocytes was observed, which was probably antigen attached to them.

Reece (28) reported that proventricular homogenates prepared from chickens with proventriculitis were highly infectious and transmissible for at least four passages in birds. Treatment of the inoculum with chloroform did not reduce infectivity supporting the hypothesis that the putative etiological agent of infectious proventriculitis was a non-enveloped virus. This virus did not grow in any of a wide variety of primary and established cell culture systems and viral isolation in embryos was unsuccessful. The original inoculum contained chicken anemia virus (CAV), fowl adenovirus type 8, avian nephritis virus and Marek's disease virus (MDV) but did not contain avian leucosis virus (ALV), infectious bronchitis virus (IBV), reovirus, Newcastle disease virus (NDV) or infectious bursal disease virus (IBDV). The proventricular homogenates used in our study were also positive for CAV and all birds treated with these homogenates seroconverted at 14 dpi. The role of this virus in proventriculitis also needs to be studied.

Huff *et al.* (13) reported the isolation of a unique bacterial agent (*Clostridia sp*.) from a proventriculus homogenate that caused proventriculitis, suggesting bacterial involvement in this syndrome. These authors conclude that a viral infection, as well as various dietary factors, may facilitate bacterial invasion of the proventriculus, and more than one type of virus may act as facilitator in this disease syndrome. In our study, no bacteria was isolated or identified by histopathology and special staining in the proventriculus of affected chickens, however the role of bacteria should be taken into consideration when studying proventriculitis.

In conclusion, proventriculitis can be transmitted by oral inoculation with homogenates produced from proventriculi of birds with proventriculitis. The causative agent(s) was not identified, although most likely is a virus. The severity of proventriculitis and its effect on weight gain is probably affected by other factors such as concomitant infection with other agents, viral

or bacterial, and nutritional factors. Viral candidates that seem to be involved in proventriculitis are IBV, IBDV, adenovirus and reovirus, however it has been demonstrated that none of them is found in every case of proventriculitis or can reproduce the disease when inoculated in chickens. This leads us to believe that another, non identified virus is the primary causative agent of proventriculitis.

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Figure legends.

Fig. 4.1. (A) Proventriculitis in a commercial chicken inoculated with an infectious proventricular homogenate at day of age (14 dpi). (B) Comparison between the proventricular wall of a normal chicken (upper section), and the proventricular wall of a chicken with proventriculitis (lower section) where the wall is thickened, with a white lobular pattern.

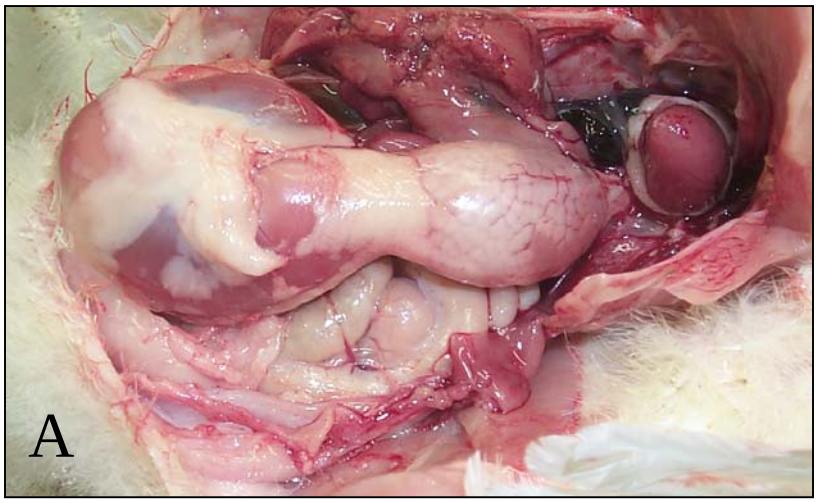
Fig. 4.2. (A and B) Proventriculi of a normal chicken (upper and on left) and a chicken with proventriculitis (lower and on right). The proventriculus is enlarged and the gastric isthmus distended in proventriculitis.

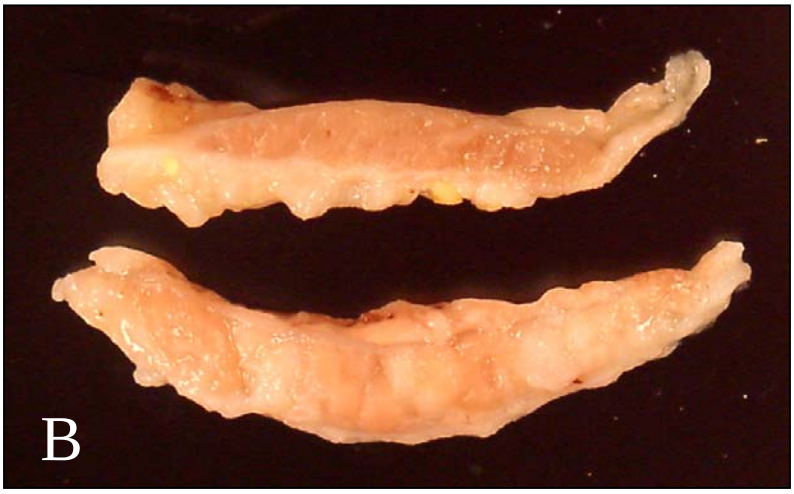
Fig. 4.3. Photomicrographs of proventriculi from a normal chicken (A) and from chicken with proventriculitis (B, C, and D). Degeneration and necrosis of glandular epithelium with coalescing of glands and lymphocytic infiltration in mucosa and glands (B). Dilation of glandular sinus with separation of epithelial cells from basement membrane (C). Lymphocytic infiltration in the glandular interstitium with ductal epithelial hyperplasia (D). H&E ,10 and 25X.

Fig. **4.4.** Photomicrographs of proventriculi from a normal chicken (A) and from chicken with proventriculitis (B, C, and D). Nuclei of affected glandular epithelial cells are enlarged and pale with marginated chromatin (B). Columnar ductal epithelium replacing secretory glandular epithelium (C). Hypertrophy and hyperplasia of ductal epithelium (D). H&E, 40X.

Fig. 4.5. Photomicrographs of proventriculi from a normal chicken (A) and from chicken with proventriculitis (B, C, and D) after immunofluorescent staining using as primary antibody convalescent sera from inoculated chickens. 25, 40X.

Fig. 4.1





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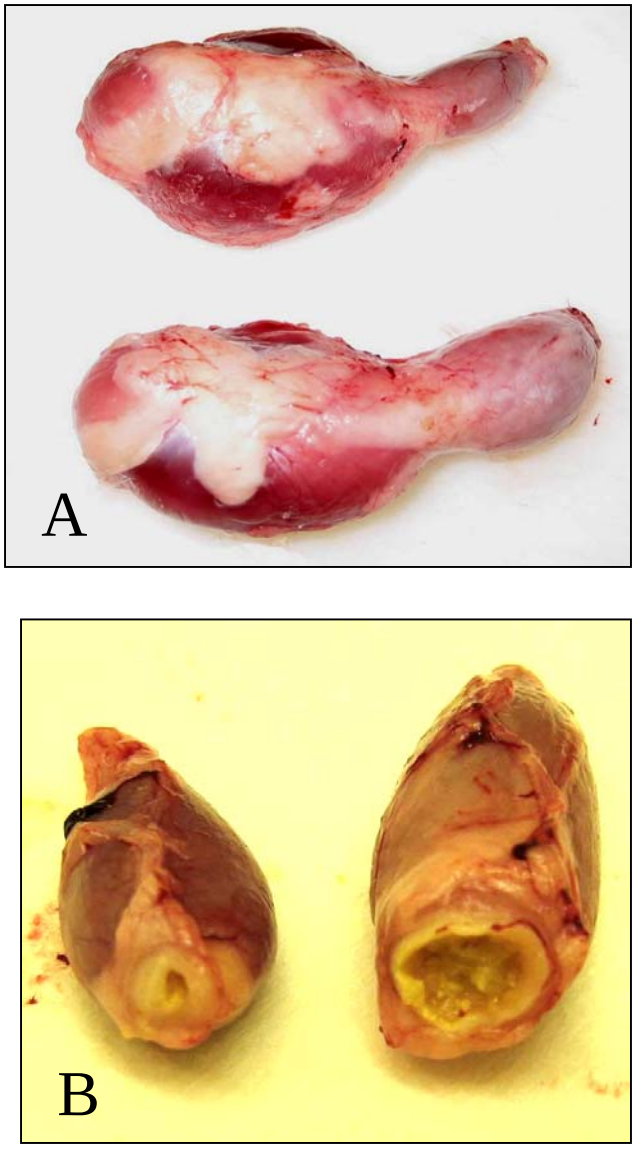

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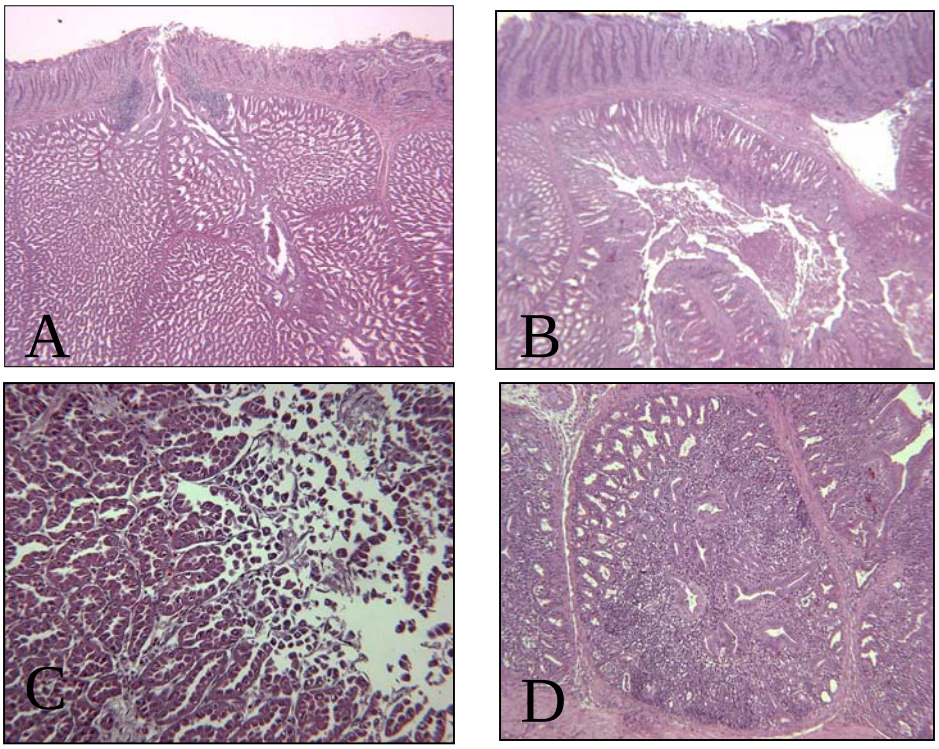

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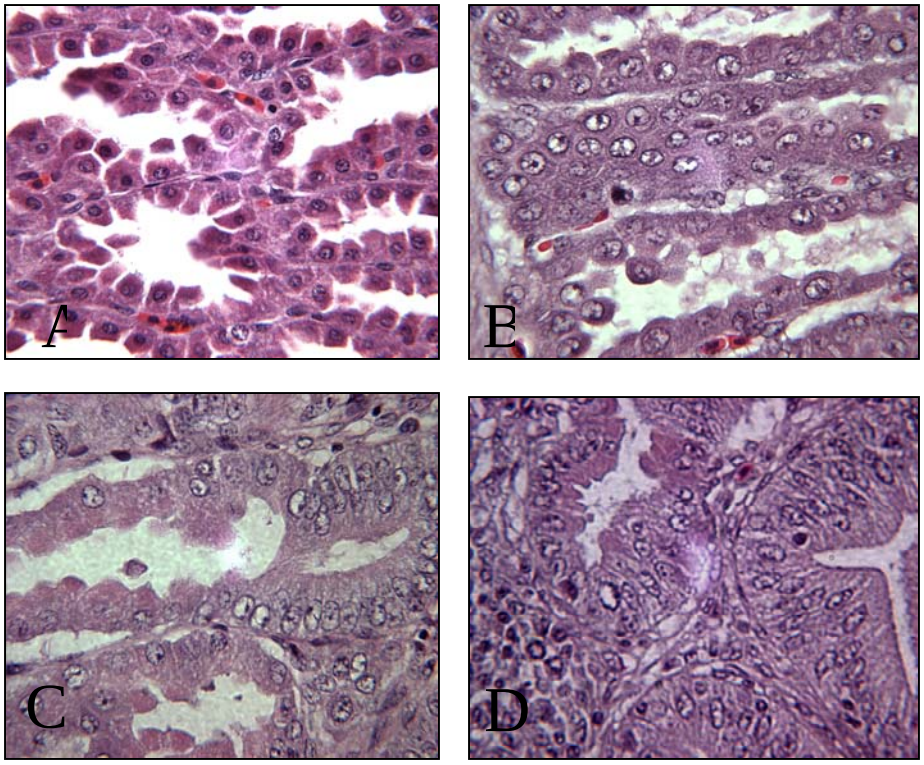

Fig. 4.5.

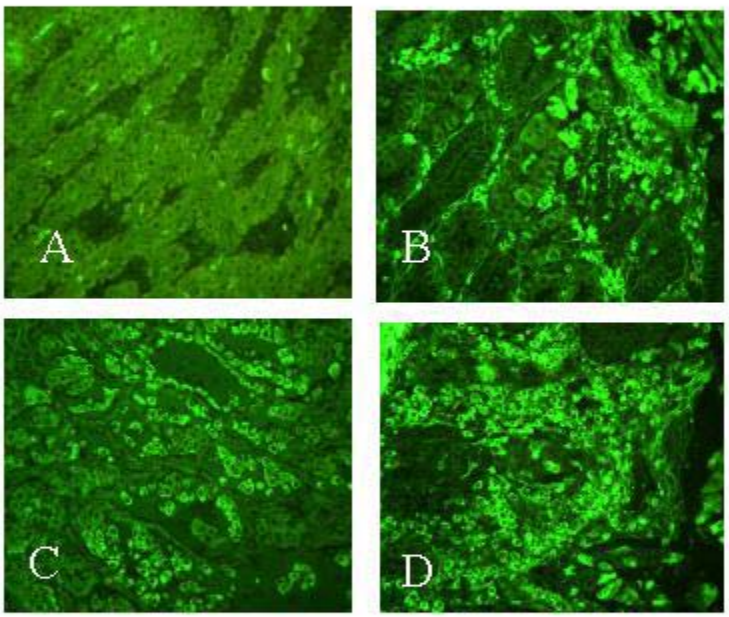

Table 4.1. Primers used for RT-PCR AND PCR analysis.

Virus	Primer Sequence	Product size	Reference
IBDV	B4 5'TCTTGGGTATGTGAGGCTTG B4 3'GGATGTGATTGGCTGGGTTA	400bp	Pantin *et al*(25)
Reovirus	MK87:GGTGCGACTGCTGTATTTGGTAAC MK88:AATGGAACGATAGCGTGTGGG	532bp	Xie *et al*. (33)
IBV	New S1 oligo 5':TGAAACTGGAACAAAAGAC S1 oligo 3': CATAACTAACATAAGGGCAA	1720bp	Jackwood *et al.* (15)
NDV	FOP1:TACACCTCATCCCAGACAGGGTC FOP2:AGGCAGGGGAAGTGATTTGTGGC	532bp	Kho *et al*. (18)
CAV	F:CTAAGATCTGCAACTGCGGA R:CCTTGGAAGCGGATAGTCAT	675bp	Todd. *et al.*(32)
Adenovirus	H1:TGGAC ATGGGGGCGACCTA H2:AGGG ATTGACGTTGTCCA	1219 bp	Raue *et al.* (27)

Table 4.2. Body weight gain (g), relative organ weights (% body weight) and organ lesions scores of commercial broilers orally challenged at day of age with sterile saline, proventricular homogenate 1 (Hom. 1) or proventricular homogente 2 (Hom. 2), and necropsied at 7 or 14 days postinoculation (dpi).

Dpi	Treatment	Body weight gain	PV relative weight	PV lesion score	Bursa relative weight	Bursa lesion score	Thymus relative weight	Thymus lesion score	Spleen relative weight	Spleen lesion score
7	Saline	120 ± 10[a]	.81 ± .09[a]	1.33±.57[a]	.10 ±.005[a]	2.00[a]	.10 ± .02[a]	1.00[a]	.02 ±.005[a]	2.00[a]
	Hom. 1	122 ± 6[a]	1.20±.01[ab]	3.00±1.0[b]	.20 ± .04[a]	3.00[a]	.23 ± .04[b]	1.00[a]	.12 ± .03[b]	2.00[a]
	Hom. 2	98 ± 16[a]	1.48± .33[b]	3.66±.57[b]	.17 ± .06[a]	3.0±1.0[a]	.16 ± .65[ab]	1.00[a]	.17 ± .02[b]	2.00[a]
14	Saline	399 ±50[a]	.53 ± .05[a]	1.33±.57[a]	.15 ± .03[a]	1.33±.5[ab]	.20 ± .03[a]	1.00[a]	.05 ±.005[a]	1.66±.57[a]
	Hom. 1	336 ± 2[a]	1.06 ±.37[b]	3.00±1[b]	.23 ± .03[a]	1.00[a]	.23 ± .03[a]	1.00[a]	.12 ± .03[b]	1.66±.57[a]
	Hom. 2	402 ±47[a]	.97 ± .17[b]	4.00[b]	.15 ± .03[a]	2.00[b]	.26 ± .07[a]	1.00[a]	.08 ±.08[ab]	1.00[a]

Table 4.3. Body weight gain (g), relative organ weights (% body weight) and organ lesions scores of SPF broilers orally challenged at day of age with sterile saline, proventricular homogenate 1 (Hom. 1) or proventricular homogente 2 (Hom. 2), and necropsied at 7 or 14 days postinoculation (dpi).

Dpi	Treatment	Body weight gain	PV relative weight	PV lesion score	Bursa relative weight	Bursa lesion score	Thymus relative weight	Thymus lesion score	Spleen relative weight	Spleen lesion score
7	Saline	41 ± 3 [a]	.93 ± .12 [a]	1.00 [a]	.22 ± .04 [a]	2.66±.57[a]	.20 ± .05 [a]	1.00 [a]	.55 ± .64 [a]	2.00 [a]
	Hom. 1	24 ± 7 [b]	1.29 ±.18[a]	1.33 ± .5 [a]	.08 ± .02 [b]	4.00 [b]	.13 ± .005 [a]	2.66 ± .57 [a]	.20 ± .06 [a]	2.00 [a]
	Hom. 2	22 ± 8 [b]	1.47 ±.46[a]	3.00±1.73[a]	.21 ± .02 [a]	3.33±.5 [ab]	.18 ± .007 [a]	2.00±1.73 [a]	.21 ± .06 [a]	2.00 [a]
14	Saline	126 ±19 [a]	.72 ± .06 [a]	1.00 [a]	.36 ± .12 [a]	1.33±.57[a]	.35 ± .19 [a]	1.00 [a]	.27 ± .08 [a]	1.66±.57[a]
	Hom. 1	88 ±18 [ab]	.98 ± .4 [ab]	2.00 ±1 [ab]	.11 ± .03 [b]	4.00 [b]	.17 ± .07 [b]	3.00±1.7 [ab]	.17 ± .06 [a]	2.00 [a]
	Hom. 2	53 ± 9 [b]	1.55±.49 [b]	3.33±1.1 [b]	.16 ±.04 [ab]	3.33±1.5[b]	.07 ± .03 [a]	4.00 [b]	.14 ± .08 [a]	2.00 [a]

Table 4.4. Lymphocytic infiltration in organs from commercial broilers inoculated with infectious proventricular homogenates (Hom.1 or 2) or saline, at 7 and 14 days post inoculation (dpi).

Dpi	Treatment	Intestine	Pancreas	Liver	Kidney
7	Saline	0/3[a]	0/3	0/3	0/3
	Hom 1	2/3	2/3	1/3	0/3
	Hom 2	3/3	2/3	0/3	0/3
14	Saline	0/3	0/3	0/3	0/3
	Hom. 1	2/3	1/3	2/3	1/3
	Hom.2	2/3	3/3	3/3	1/3

[a] = number of chickens positive/number of chickens inoculated

Table 4.5. Lymphocytic infiltration in organs from SPF broilers inoculated with infectious proventricular homogenates (Hom. 1 or 2) or saline, at 7 and 14 days post inoculation (dpi)

Dpi	Treatment	Intestine	Pancreas	Liver	Kidney
7	Saline	0/3[a]	0/3	0/3	0/3
	Hom 1	1/3	1/3	2/3	3/3
	Hom 2	1/3	2/3	2/3	3/3
14	Saline	0/3	0/3	0/3	0/3
	Hom. 1	3/3	2/3	3/3	3/3
	Hom.2	1/3	2/3	1/3	3/3

[a] = number of chickens positive/number of chickens inoculated

Table 4.6. Seropositivity by ELISA of commercial broilers inoculated with infectious proventricular homogenates (Hom.1 or 2) or saline, at 7 and 14 days post inoculation (dpi).

Dpi	Treatment	IBDV	Reovir.	IBV	NDV	CAV	MS	MG
7	Saline	3/3[a]	0/3	3/3	0/3	0/3	0/3	0/3
	Hom 1	3/3	0/3	3/3	0/3	0/3	0/3	0/3
	Hom 2	3/3	0/3	3/3	1/3	0/3	0/3	0/3
14	Saline	2/3	0/3	2/3	0/3	0/3	0/3	0/3
	Hom. 1	2/3	0/3	2/3	0/3	1/3	0/3	0/3
	Hom.2	1/3	0/3	0/3	0/3	1/3	0/3	0/3

[a] = number of chickens positive/number of chickens inoculated

Table 4.7. Seropositivity by ELISA of SPF broilers inoculated with infectious proventricular homogenates (Hom.1 or 2) or saline, at 7 and 14 days post inoculation (dpi).

Dpi	Treatment	IBDV	Reovir.	IBV	NDV	CAV	MS	MG
7	Saline	0/3[a]	0/3	0/3	0/3	0/3	0/3	0/3
	Hom 1	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	Hom 2	1/3	0/3	0/3	0/3	3/3	0/3	0/3
14	Saline	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	Hom. 1	1/3	0/3	2/3	0/3	1/3	0/3	0/3
	Hom.2	1/3	0/3	1/3	0/3	3/3	0/3	0/3

[a] = number of chickens positive/number of chickens inoculated.

Table 4.8. IBDV RT-PCR and IHC results from formalin fixed, paraffin embedded bursa and proventriculus tissues from commercial broilers inoculated with infectious proventricular homogenates (Hom.1 or 2) or saline, at 7 or 14 days post inoculation (dpi).

Dpi	Treatment	Bursa RT-PCR	Bursa IHC	PV RT-PCR	PV IHC
7	Saline	0/3[a]	0/3	0/3	0/3
	Hom 1	0/3	0/3	0/3	0/3
	Hom 2	0/3	0/3	0/3	0/3
14	Saline	0/3	0/3	0/3	0/3
	Hom. 1	0/3	0/3	0/3	0/3
	Hom.2	0/3	0/3	0/3	0/3

[a] = number of chickens positive/number of chickens inoculated

Table 4.9. IBDV RT-PCR and IHC results from formalin fixed, paraffin embedded bursa and proventriculus tissues from SPF broilers inoculated with infectious proventricular homogenates (Hom.1 or 2) or saline, at 7 or 14 days post inoculation (dpi).

Dpi	Treatment	Bursa RT-PCR	Bursa IHC	PV RT-PCR	PV IHC
7	Saline	0/3[a]	0/3	0/3	0/3
	Hom 1	3/3	3/3	2/3	0/3
	Hom 2	1/3	3/3	0/3	0/3
14	Saline	0/3	0/3	0/3	0/3
	Hom. 1	3/3	1/3	2/3	0/3
	Hom.2	1/3	0/3	1/3	0/3

[a] = number of chickens positive/number of chickens inoculated

Table 4.10. RT-PCR and PCR results from proventricular homogenate and allantoic fluid (AF) samples. Proventricular homogenates used for inoculation of chickens (Hom. 1 and 2), proventricular homogenates obtained from chickens inoculated with saline, Hom.1 or Hom.2; from commercial (Com.) and SPF broilers at 7dpi; allantoic fluid (AF) from embryos inoculated with Hom. 2. filtrate, proventricular homogenate filtrate obtained from the proventriculi of commercial broilers inoculated with Hom. 2, and from proventricular homogente filtrate made from the proventriculi of control chickens (-PV), harvested at the 5th pass.

Sample	IBDV	Reovirus	NDV	IBV	Adenovirus	CAV
Hom. 1	+	-	-	-	+	+
Hom. 2	-	-	-	+	-	+
Saline Com. 7dpi	-	-	-	-	-	ND[a]
Hom.1 Com 7dpi	-	-	-	-	+	ND
Hom.2 Com. 7dpi	-	-	-	-	-	ND
Saline SPF 7dpi	-	-	-	-	-	ND
Hom.1 SPF 7dpi	+	-	-	+	+	ND
Hom 2 SPF 7 dpi	+	-	-	+	-	ND
AF Hom.2 5th pass	-	-	-	+	-	ND
AF Hom.2 5th pass	-	-	-	+	-	ND
AF –PV 5th pass	-	-	-	-	-	ND

[a] = ND, not done

CHAPTER 5

PROVENTRICULITIS IN BROILER CHICKENS: EFFECTS OF IMMUNOSUPPRESSION[1]

[1]Pantin-Jackwood, M.J., T.P. Brown, Y. Kim and G. R. Huff. Submitted to *Avian Diseases,* 08/07/03.

SUMMARY.

Proventriculitis in broilers causes carcass condemnation when swollen proventriculi tear during evisceration. The cause of this proventriculitis is unknown but several infectious agents have been associated with it. One such agent, infectious bursal disease virus (IBDV), has been implicated as a cause of proventriculitis, but a direct effect of this virus on the proventriculus has not been proven. The role of IBDV in proventriculitis may be indirect due to its ability to cause immunosuppression. The objective of this study was to understand how immunosuppression affects the incidence of proventriculitis in broiler chickens. Immunosuppression was induced in commercial and SPF broiler chickens using chemicals (cyclophosphamide and cyclosporin) or virus (IBDV). All groups were then exposed to a proventricular homogenate produced from diseased birds. At 7 and 14 days post inoculation, the incidence of proventriculitis in these groups was compared to that produced by homogenate exposure in immunocompetent broilers. All birds exposed to the proventricular homogenate from diseased birds developed proventriculitis. Cyclophosphamide and IBDV, both B cell suppressors, did not significantly affect the incidence or characteristics of the proventriculitis observed, although they did have an effect on the size of the proventriculus at 7 days post inoculation. Chickens immunosuppressed with cyclosporin, a T cell suppressor, developed more severe lesions and had a higher incidence of proventriculitis. These findings indicate that both, B and T cells, are involved in the immune response against proventriculitis, but cell mediated immunity appears to have a more important role in controlling the disease. IBDV affects both humoral and cellular immunity in the chicken so although under experimental conditions it didn't have a major effect on proventriculitis, it may explain why control of IBDV in the field seems to reduce the incidence of proventriculitis.

Key words: Proventriculitis, immunosuppression, IBDV.

Abbreviations: CBH = cutaneous basophil hypersensitivity; CMI = cell-mediated immunity; CP = cyclophosphamide; CP = cyclosporin; IBDV = infectious bursal disease virus; RT-PCR = reverse transcriptase polymerase chain reaction; SPF = specific-pathogen free.

INTRODUCTION

Proventriculitis is a clinical condition that affects broiler chickens. It is characterized by enlargement of the proventriculus and weakness of the gastric isthmus. During routine evisceration at processing, affected proventriculi rupture causing spillage of the proventricular contents into the body cavity, which results in condemnation of affected carcasses for contamination. The disease has also been associated with impaired growth, and poor feed conversion (16, 13). Microscopically, degeneration and necrosis of proventricular glands is observed accompanied by marked intraglandular interstitial lymphocytic infiltration (4, 9,10).

Several agents have been implicated as potential causes of proventricular lesions. Noninfectious causes include oral exposure to biogenic amines (2,27), mycotoxins (26), lack of dietary fiber (29), and excessive copper sulfate (3,14,41). Infectious causes include adenovirus (19), reovirus (17,38), infectious bronchitis virus (39), and megabacterium (23,35). However, none of these noninfectious or infectious agents have been found consistently in a majority of cases. Electron microscopy has detected viral particles in acute lesions but isolation of a virus from affected proventriculi has been unsuccessful (9,10, 13).

Infectious Bursal Disease Virus (IBDV) has been implicated as the cause for this disease (4,13,24), and IBDV vaccination has been reported to decrease its incidence (7,15).

Proventriculitis can be reproduced by orally inoculating broilers with homogenized proventriculi collected from affected birds (16,4). A filterable agent found in these homogenates causes lesions similar to those found in field cases (4), and IBDV has been immunoprecipitated from these homogenates (13). Commercial broilers exposed to this IBDV developed increased proventricular lesion scores but had no increase in proventricular size, a characteristic feature produced by exposure to proventricular homogenates (13). These findings suggest other agents or conditions may be required to produce proventriculitis.

IBDV induces immunosuppression in chickens (21,34,40). Immunosuppressed flocks may have an increased incidence of secondary infections, poor feed conversion, and reduced protective response to commonly used vaccines (34). IBDV causes lytic destruction of IgM+ B lymphocytes that results in suboptimal levels of antibodies against a number of infectious and noninfectious antigens (8,30,34). Although the immunosuppression caused by IBDV is principally due to B lymphocyte damage, an effect on cell-mediated immunity (CMI) has also been demonstrated (5,18,33,34).

SPF broilers exposed to different strains of IBDV did not develop proventricular lesions or enlargement at 4 or 6 days post-inoculation (25). The virus was detected in large quantities in the bursa of these birds by RT-PCR and immunohistochemical techniques, but not in the proventriculus, indicating it is not a target organ for IBDV. However, the immunosuppressive effect of IBDV could explain its reported relationship to proventriculitis. The purpose of our study was to see if immunosuppression had any effect on the incidence, severity, or character of proventriculitis in broiler chickens. To address this, commercial and SPF one-day old broilers were immunosuppressed with cyclophosphamide (B cell suppressor), cyclosporin (T cell suppressor), or IBDV. Subsequently these chickens were exposed to a proventricular

homogenate from affected chickens, and the effect of immunosuppression on proventriculitis was determined.

MATERIALS AND METHODS

Animal housing. One-day-old chickens were divided into groups and housed in positive pressure Horsfal units. Unmedicated feed and water were provided *ad libidum.*

Experimental design. Trials 1 and 2. A total of 88 unvaccinated commercial broiler chicks, obtained from a local hatchery, were divided into 9 groups of 8 or 12 birds, and chicks in each group received either an immunosuppressive treatment or no treatment (Table 5.1). Chickens subsequently received as described below, either positive (+PV) or negative (-PV) proventricular homogenate, saline, or no homogenate. Group 1 had 12 birds, which were inoculated *per os* with 1 ml of sterile saline at 7 days of age. Group 2 had 8 birds, which were inoculated *per os* with 1 ml of -PV produced from normal commercial broilers at 7 days of age. Group 3 had 8 birds, which were inoculated *per os* with 1 ml of +PV produced from broilers that had proventriculitis at 7 days of age. Group 4 had 12 birds, which were immunosuppressed with IBDV administered at one day of age. Group 5 had 12 birds, which were immunosuppressed with cyclophosphamide (CP) starting at 1 day of age. Group 6 had 12 birds, which were immunosuppressed with cyclosporin (CS) starting at 1 day of age. Group 7 had 8 birds, which were immunosuppressed with IBDV administered at 1 day of age and treated with +PV at 7 days of age. Group 8 had 8 birds, which were immunosuppressed with CP starting at 1 day of age, and treated with +PV at 7 days of age. Group 9 had 8 birds, which were immunosuppressed with CS starting at 1 day of age, and treated with +PV at 7 days of age.

Trial 3. This trial was conducted as trials 1 and 2 with the following modifications.

Chickens. Fertile White Plymouth Rock chicken eggs (SEPRL, USDA, Athens, GA) from a breeder flock maintained under SPF conditions were obtained, hatched, and maintained in positive pressure Horsfal isolation units. The parent flock and all progeny used in this experiment were free of common poultry diseases, specifically IBDV, MDV, IBV, reovirus and CAV. The same experimental design and protocol as trials 1 and 2 was followed. Additional animals were included to allow a third sacrifice at 21 days post inoculation.

Immunosuppressive treatment groups. Chickens were immunosuppressed with either, IBDV, CP, or CS as described bellow.

IBDV Treatment. Birds in trial 1, (groups 4 and 7) were challenged with IBDV Variant E strain (Intervet, Inc. Millsboro, DE). In trials 2 and 3 chickens in groups 4 and 7 were treated with the STC challenge strain 124-ADV of IBDV (National Veterinary Services Laboratory, Ames, Iowa). Inoculations were given *per os* and by eye drop, and consisted of 100 µl containing at least 10^3 mean tissue culture infective dose of virus diluted in phosphate-buffered saline (PBS).

Cyclophosphamide (CP) treatment. B lymphocyte immunosuppression was induced using an established protocol (32). Briefly, groups 5 and 8 in all three trials received one intraperitoneal injection of 4 mg CP (Cyclophosphamide monohydrate; Sigma Chemical Co., St.Louis, MO) (0.1ml) daily for 4 days starting the first day after hatch. For injection, CP was obtained in a dry form, and an aqueous solution was prepared by reconstituting 1.6 g in 40 ml of calcium- and magnesium-free phosphate buffered sterile saline (CMF-PBS) and filtering this through a 0.22 µm syringe filter. The resulting solution contained 40 mg of CP/ml.

Cyclosporin (CS) treatment. T lymphocyte immunosuppression was induced using an established protocol (31). Briefly, chickens from groups 6 and 9 in all three trials received one intramuscular injection of CS, 100mg/kg body weight, every 3 days from the first day after hatch until the experiment ended. CS was prepared by diluting a stock solution (Sandimmune, 100mg/ml, Novartis Pharma AG, Basle, Switzerland) 1:1 in olive oil. Dilutions of the drug were adjusted as body weights increased.

Immunosuppression in IBDV, CP, and CS treated groups was assessed by histopathologic examination of immune organs (bursa, thymus and spleen), cutaneous hypersensitivity response testing (CBH), and humoral response to NDV vaccination.

Challenge with proventricular homogenates. At 7 days of age birds from groups 3, 7, 8, and 9 in trial 1 were inoculated by oral gavage with 1 ml of a positive proventricular homogenate (+PV) consisting of proventriculi obtained from commercial broilers with proventriculitis (13). Proventriculi from chickens in group 3 (+PV treated) of trial 1 were homogenized as previously described (4) and used to expose +PV groups in trial 2 and trial 3. Briefly, proventriculi collected from birds that developed proventriculitis were washed in sterile normal saline (PBS) three times on a magnetic stirrer to remove feed residues and toxins. Washed proventriculi were then diluted 1:1 wt/vol in PBS and homogenized with a commercial blender (Waring, New Hartford, Connecticut). The homogenates were frozen at –80 C and thawed at room temperature immediately before inoculation. The same procedure was used with proventriculi from normal broiler chickens without proventriculitis to produce a negative proventricular homogenate (-PV). This was used to inoculate birds from group 2 in all three trials. Birds of group 1 in all trials were sham inoculated with 1 ml of sterile saline.

Cutaneous basophil hypersensitivity (CBH) response. This test was performed to evaluate T-cell function in the immunosuppression treatment groups at 2 weeks of age as previously described (6). Four chickens from groups 1 (saline), 4 (IBDV), 5 (CP), and 6 (CS) were injected intradermally in the skin between the third and fourth digits of the left foot with 200 μg of Phytohemmagglutinin-P (PHA-P, Sigma, St. Louis, MO) in 100 μl of sterile physiological saline solution (PSS). The right foot of each chicken was similarly injected with 100 μl of PSS without PHA-P to serve as a control. The CBH response to PHA-P was evaluated by determining the thickness of the interdigital skin before injection, and at 12 and 24 hours after injection with a constant-tension, digital micrometer (Mitotuyo Co., Kanagawa, Japan). The CBH response was calculated by two methods: 1) CBH-1 or increased skin thickness = (post-injection skin thickness, left foot)-(pre-injection skin thickness, left foot); and 2) CBH-2 response = (PHA-P response, left foot)-(PSS response, right foot).

NDV vaccination. To asses humoral immune function 4 birds from groups 1 (saline), 4 (IBDV), 5 (CP), and 6 (CS) were vaccinated at 21 days old with killed Newcastle Disease vaccine (Vineland Laboratories, Vineland, NJ). Each bird was given one dose of 0.5 ml of vaccine intramuscularly as recommended by the manufacturer. Two weeks later birds were bled to obtain sera, and antibodies to NDV were quantified by ELISA (IDEXX Laboratories, Inc. Westbrook, Maine), and HI test using the diluted serum-constant virus procedure (37).

Sample collection and processing. All birds were wing banded and weighed at one day of age. At 14 and 21 days of age, 4 birds were randomly selected from each group and examined, weighed, bled, killed by cervical dislocation, and necropsied. Bursa, proventriculus, spleen, and the right half of the thymus were collected from each bird, weighed, and a portion of each fixed immediately by immersion in 10% neutral buffered formalin for 24 hours. Tissues were then

processed and embedded in paraffin using routine histologic techniques. The remaining proventriculi were collected in sterile plastic tubes over ice for subsequent preparation of homogenate as explained previously. Relative organ weights were obtained using the formula [Relative organ weight = (organ weight / body weight) x 100].

Histopathology. Paraffin-embedded tissues samples from bursa, proventriculus, spleen and thymus from each bird were sectioned, mounted, stained using hematoxylin and eosin (HE), and examined in a blinded fashion as to treatment for lesions using light microscopy. All sections of bursa and proventriculus were assigned a lesion severity score. A lesion score of 1 represented no lesions. For bursal sections, 2 was defined as mild variation in follicle size, 3 as moderate variation in size of follicles, and 4 as either necrosis or follicle atrophy. For proventricular sections, 2 was defined as mild glandular lumenal ectasia, 3 as ectasia plus lymphoid infiltrates in the interglandular interstitium and 4 as either acute glandular necrosis or severe fibrosis with lymphoid infiltrates. Also spleen and thymus were examined for the presence of lesions.

Serology. Serum samples obtained at days 14 and 21 of age were examined for antibody to IBDV, IBV, NDV, CAV, and reovirus using commercially available ELISA tests (IDEXX Laboratories, Inc. Westbrook, Maine).

Real time RT-PCR. RNA was extracted from formalin-fixed paraffin-embedded bursas and proventriculi and examined for IBDV nucleic acid by real time RT-PCR (25). Sections totaling fifty microns in thickness were cut from each formalin-fixed paraffin-embedded tissue block, deparaffinized in HemoDe (Fisher Scientific, Pittsburgh, PA), washed with 100% ethanol, and digested with 25µg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 1 hour at 50 C. RNA was extracted using Trizol (Life Technologies, Inc. Gaithersburg, MD) according to the manufacturer's recommendations, diluted in 25µl of 90% dimethyl sulfoxide (DMSO), and

frozen at –80 C until assayed. Extracted RNA was denatured at 95 C for 5 minutes and put on ice. A reverse transcriptase polymerase chain reaction (RT-PCR) was performed using reagents from the Light Cycler-RNA Amplification SYBR® Green I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used were designed to amplify a 400 bp segment of the IBDV genome shared by all strains, which flanks a hypervariable region of the VP2 gene. Primer sequences were B4 5' TCTTGGGTATGTGAGGCTTG and B4 3' GGATGTGATTGGCTGGGTTA. Amplification and detection of specific products was also performed using a Light Cycler (ROCHE Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's recommendations (ROCHE Light Cycler version 3.0, ROCHE Molecular Biochemicals, Indianapolis, IN). Briefly, reverse transcription was done at 55 C for 10 minutes, followed by denaturation at 95 C for 30 seconds. Forty PCR cycles were done consisting of denaturation (95 C for 1 second), hybridization (55 C for 10 sec), and extension (72 C for 13 sec). A melting curve analysis was done with an initial denaturation at 95 C. DNA melting was accomplished with an initial temperature of 65 C for 10 seconds and a gradual temperature increase of 0.1 degree C per second until reaching 95 C. The melting temperature of the expected 400 bp amplicon was between 82 C and 84 C. This estimated melting temperature was used to confirm the identity of IBDV specific products obtained using real time RT-PCR. Additional confirmation of specific amplification was done using routine gel electrophoretic techniques of the PCR products on 2% agarose (Sigma Chemical Co., St. Louis, MO) followed by ethidium bromide staining.

Statistical analysis. The body weight gain, relative bursal and proventricular weights, and bursal and proventricular lesion scores were analyzed using ANOVA and means

comparisons for all pairs using Tukey-Kramer HSD. Significance was assumed at the 0.05 level of probability.

RESULTS

Control Groups.

Proventricular homogenate controls. Chickens inoculated only with saline or negative proventricular homogenate (-PV) did not develop proventriculitis in any of the three trials. Macroscopic lesions were not observed when examined at necropsy (Fig. 5.1). Mean body weight gain and relative proventriculus weight for these two groups was very similar (Tables 5.2 and 5.3 respectively). Mild microscopic lesions consisting mainly of mild lumenal ectasia of the proventricular glands were present in some of these birds (Table 5.4). Chickens that were inoculated only with positive proventricular homogenate (+PV) had no significant suppression of weight gain compared to saline and –PV groups in all three trials (Table 5.2). There was a trend toward enlargement of the proventriculus in chickens that received the positive proventricular homogenate. Increased microscopic lesions were present in the proventriculus of chickens that received positive proventricular homogenate in trials 1 and 2 at 7 and 14 dpi, and in trial 3 at 14 dpi (Table 4). At necropsy, the proventriculus of these chickens were enlarged and swollen, with plaques or mottling visible on the serosal surface, dilation of the gastric isthmus, and mucosal lesions (flattened papillae, with secretion of white fluid) (Fig. 5.1). Microscopically, at 7 dpi, acute necrosis of the glandular epithelium was present. Collecting sinuses of the glands were dilated and contained desquamated epithelium. Nuclei of the glandular epithelial cells were enlarged and pale, with marginated chromatin. Lymphocytic infiltrates were present as sheets in the lamina propria of the mucosa and expanded the glandular epithelium between the epithelium

of the ducts and the glands (Fig. 5.2). At 14 dpi, glandular epithelium was replaced by ductal epithelium. Lymphocyte infiltrates and germinal center formation were present in the glands and mucosa (Fig. 5.2). In trial 3 chickens that were inoculated with +PV, showed similar mild to moderate lesions in the proventriculus at 21 dpi, but no significant increase in size of the proventriculus compared to saline or -PV controls. Small germinal centers were present in the glands (Fig. 5.2) of +PV-dosed chickens but not in those given saline or -PV.

No lesions or differences in relative organ weight of the bursa were observed between chickens that received saline, -PV, or +PV (Tables 5.5 and 5.6).

Immunosuppression controls. Commercial broilers (group 4) in trials 1 and 2, treated with IBDV strains Variant E and STC respectively, had no signs of IBDV infection at 7 and 14 dpi. Their bursas had no significant microscopic lesions, no difference in relative organ weight when compared to controls (Tables 5.5 and 5.6), and were negative for IBDV by RT-PCR. CBH response, and humoral response to NDV vaccination was similar to the saline control group (Tables 5.7 and 5.8), all indicating that challenge with IBDV in these birds did not produce IBDV infection. However, SPF broiler chickens in trial 3 exposed to IBDV strain STC did have signs of depression at 7 days post inoculation and their bursas were significantly smaller than saline control chickens at 7, 14 and 21 dpi (Table 5.5). Severe microscopic lesions were also observed (Table 5.6), and bursas were positive for IBDV by RT-PCR. Humoral immune response to NDV vaccination was significantly lower than saline controls (Table 5.8).

Cyclophosphamide (CP) control chickens (group 5) in all three trials tended to be smaller than chickens from the other groups, due to a reduction in their weight gain. This reduction was significant in the SPF broilers in trial 3 (Table 5.2). These chickens also had decreased feathering and appeared weak. The bursas of these chickens were significantly smaller in all three trials

(Table 5.5), and marked lymphocytic depletion and atrophy of the bursa was observed (Table 5.6). A small reduction of CBH response, was observed in these birds (Table 5.7), and humoral response to NDV vaccination was significantly reduced (Table 5.8).

Cyclosporin (CS) control chickens (group 6) in trials 1 and 2 appeared normal (similar to saline controls). Although their weight gain was reduced it was not significantly different from that of the saline controls (Table 5.2). Weight gain in chickens in trial 3 was reduced at 7 and 21 dpi. Bursas of birds treated with CS had no lesions and there was no difference in size compared to saline controls (Tables 5.5. and 5.6). Thymuses did not have any significant lesions, but the CMI immune capacity was significantly reduced (Table 5.7). The CBH-1 and CBH-2 responses were decreased ($p < 0.05$) compared to the saline control group. The humoral immune response, measured by antibody production after NDV vaccination, was not affected (Table 5.8).

The effect of the immunosuppressive treatments (IBDV, CP, CS) on the proventriculus relative weight or presence of lesions was very mild and not significantly different than that observed in saline or -PV controls (Tables 5.3 and 5.4).

Experimental groups.

Body weight gain. Chickens treated with CP and +PV had a significant reduction in body weight gain compared to the control groups (saline, -PV and +PV), including those given CP only, in trials 1 at 7 and 14 dpi and trial 2 at 7 dpi. (Table 5.2). The combination of CS and +PV had a detrimental effect on weight gain in trail 2 at 14 dpi and trial 3 at 7 and 21 dpi, but the difference from chickens given CS only was not significant in any instance.

Organ relative weights and lesions. No significant difference was observed between control and experimental groups for spleen and thymus in any of the trials. (Data not shown). The exceptions were the chickens treated with CP where at 7dpi their thymuses were smaller

than the rest of the groups, but by 14 dpi they were the same as controls. In all three trials, birds treated with CP and +PV had a significant decrease in bursal size and developed high lesion scores but these were no different than those in CP controls (Tables 5.5 and 5.6). In trial 3, lesions and a significant decrease in size of the bursa occurred in chickens that were challenged with IBDV and exposed to +PV, similar to that observed in the IBDV controls (Tables 5.5 and 5.6). These bursas were also IBDV positive by RT-PCR.

Relative proventricular weight of chickens that were immunosuppressed and treated with +PV was increased at 7 and 14 dpi when compared to the control chickens (saline and –PV), but in most cases there was no significant difference when compared to the +PV controls. Chickens in trial 1 and 2 at 7 dpi treated with the combination of CP/+PV, had a significant increase in relative proventricular weight relative to the +PV controls (Table 5.3). The lesion score of the proventriculi from all immunosuppressed birds treated with +PV was also similar to those observed in the +PV control groups at 7 and 14 dpi (Table 5.4), although there was an increase in the incidence of proventriculitis and a difference in the appearance and severity of the lesions observed in the birds treated with CS. This was more evident in the SPF broilers where all birds treated with the combination of CS and +PV had moderate to severe proventriculitis. CS/+PV scores were significantly higher than all other treatments at 21 dpi in trial 3. In all three trials, the incidence and severity of proventriculitis was highest at 14 dpi than 7dpi. In trial 3 at 21 dpi the relative weight and lesion score of the proventriculi of all birds that received +PV was similar to the negative controls, with the exception of the chickens treated with CP/+PV which scoring and weight remained significantly higher than birds in the other groups (Tables 5.3 and 5.4).

Chickens treated with +PV in all three trials, regardless of the immunosuppression treatment, had acute necrosis of the proventricular glands at 7 dpi with some lymphocyte

infiltrates, mostly in the mucosa. In some cases lymphocyte infiltrates also were present in the glands in the form of sheets. Hemorrhage and congestion were also sometimes present. Birds treated with CS had more severe lesions, with destruction and coalescence of the glands.

At 14 dpi, chickens treated with IBDV and +PV, or CP and +PV, had metaplastic replacement of proventricular glandular secretory epithelium by ductal epithelium, and lymphocyte infiltrates as observed in the +PV only-treated chickens. Proventricular lymphoid germinal centers were smaller, or not present, in birds treated with CP (in all three trials) or IBDV (in trial 3). Chickens treated with CS and +PV in trials 1 and 2 still had acute necrosis at 14 dpi, reduced lymphocyte infiltration and variable germinal center formation, and minimal metaplasia (Fig 5.3).

At 21 dpi, SPF broilers treated with IBD and +PV, or CP and +PV, had mild to moderate lesions, with very little lymphocyte infiltration. These were mostly in the form of small germinal centers. Chickens treated with CS and +PV had severe lesions consisting of acute necrosis of the glandular epithelium, coalescing of glands, and small and multiple germinal centers.

Serology. Chickens from all groups in trial 1 had ELISA titers against IBDV and IBV at 14 days of age (7 dpi), and had no titers against NDV, reovirus or CAV. These IBDV and IBV titers decreased but were still present at 21 days of age (14 dpi). Chickens in trial 2 had titers for IBDV, IBV, and NDV at 14 days of age (7 dpi), but not against reovirus. In both trials, chickens that received –PV or +PV (with the exception of birds treated with CP) developed titers against reovirus at 21 days of age (14 dpi).

SPF broiler chickens (trial 3) at 14 days of age (7 dpi) were seronegative for NDV, IBV, reovirus, and CAV. They also were negative for IBDV with the exception of those challenged with IBDV, which developed and had seroconversion at 14, 21 and 30 days of age (7, 14, and 21

dpi). At 21 and 30 days of age (14 and 21 dpi) birds that received +PV, but were not treated with CP, had titers against IBV, NDV, and reovirus. All birds were negative for CAV at all time points.

IBDV RT-PCR. IBDV was not detected in paraffin-embedded bursas or proventriculi from any of the birds in Trials 1 or 2. In Trial 3, IBDV was detected at 7, 14 and 21 dpi in paraffin-embedded bursas from all IBDV challenged birds. It was not detected in any of the proventriculi from these birds, or in bursas or proventriculi from chickens in the other groups in trial 3.

DISCUSSION

The relationship between IBDV and proventriculitis is not clear. Both gross and microscopic lesions of the proventriculus have been produced by IBDV challenge in leghorn chickens (24) and vaccination against IBDV has been reported to decrease the incidence of proventriculitis (7,15). However, proventriculitis was not produced by inoculation of SPF broilers with different strains of IBDV (25). Commercial chickens get exposed to IBDV early in life, and although mortality in unprotected flocks can be quite significant, the major concern for the poultry industry is IBDV's ability to cause immunosuppression. Immunosuppressed birds often fail to respond adequately to vaccination and are susceptible to secondary infections. The mechanisms of IBDV-induced immunosuppression are not fully understood. Both humoral and cellular immune responses are compromised (34). Inhibition of humoral immunity is more severe and is attributed to the destruction of immunoglobulin-producing B cells by the virus. IBDV-exposed chickens produce suboptimal levels of antibodies against a number of infectious and non-infectious antigens (34). Although T cells do not serve as targets for IBDV replication,

cell-mediated immune responses of virus-exposed chickens seem to be compromised (5,18,33,34).

Protection against IBDV is achieved by the induction of neutralizing antibodies, as well as by passive transfer of maternal antibodies to young chickens. These maternal antibodies may interfere with IBDV vaccination schedules. In our study, commercial broiler chickens (Trials 1 and 2) inoculated with an infecting dose of IBDV did not develop disease. No lesions were observed in their bursas, and RT-PCR did not detect any virus. Consequently, these birds were not immunosuppressed by IBDV as intended, and had a normal response to NDV vaccination. On the other hand, SPF broiler chickens were successfully infected with IBDV when intentionally challenged at one day of age. Their bursas were significantly smaller than controls, had lesions typical of IBDV infection, and were positive for the virus by RT-PCR. They also developed antibodies against IBDV, and were immunosuppressed as measured by their low seroconversion to NDV. However, infection with this particular strain of IBDV (STC) produced no proventriculitis.

CP treatment has been used to inhibit humoral immunity in order to determine its role in the pathogenesis of infectious pathogens of chickens (1,31). Chickens treated with CP had bursas that were significantly smaller and depleted of lymphocytes, and they did not develop specific antibody after NDV vaccination, demonstrating their humoral immunosuppression. Both CP and IBDV have minor effects on CMI (32,34). There was a mild depression of the CBH response in birds treated with IBDV (trial 3) or CP, but this was not significant when compared to controls.

As expected, chickens from all three trials treated with CS exhibited a significantly decreased CBH response (6). CS prevents cytokine synthesis in T cells by blocking a later stage of T cell receptor initiated signaling, reducing production of interleukin-2 (IL-2), and hence T

cell proliferation (12,28). As a consequence, IL-2 dependent functions, which include T-helper activities, cytotoxicity, natural killer cell activity, and antibody dependent cytotoxicity, are decreased (11). As expected, humoral immune response of birds treated with CS was not affected, and they developed anti-NDV antibodies following NDV vaccination.

The homogenate used to induce proventriculitis in trial 1 was known to contain IBDV (13). In an attempt to reproduce a proventriculitis as close to that observed in naturally occurring cases, commercial broilers with maternal antibodies to IBDV were used in trials 1 and 2. Inoculation of these chickens in trial 1 with the IBDV-bearing homogenate produced proventriculitis but no IBDV infection since their anti-IBDV antibody was protective. Since proventriculitis still occurred, this suggests that proventriculitis was not directly produced by infection with the IBDV present in that homogenate, but does not exclude IBDV as a potential contributing factor. In trials 2 and 3, proventriculitis was produced by inoculating birds with positive proventricular homogenate produced from birds with proventriculitis in trial 1. Excluding those challenged with IBDV intentionally, chickens given this homogenate in trials 2 and 3 developed proventriculitis but no IBDV infection. These data suggest our serial passage of the original proventricular homogenate through antibody positive broiler chickens cleared it of IBDV and propagated the causative agent responsible for proventriculitis.

The proventriculitis produced in trial 1 was more severe than that in trials 2 and 3. This may be due to reduction in titer of the causative pathogen by *in vivo* passage in the presence of antibody, or clearance of the IBDV as described above. Even so, the incidences of proventriculitis within groups and the effects of immunosuppression on proventriculitis were similar across all three trials.

Immunosuppression induced by cyclophosphamide (CP) in all three trials, and by IBDV in trial 3, did not affect the incidence or lesion severity of the proventriculitis observed. Proventricular lesions observed in chickens that received CP/+PV were similar to that observed in the +PV controls. There was acute glandular necrosis and some lymphocyte infiltrate at 7 dpi, and glandular metaplasia with severe lymphocyte infiltrates at 14 dpi. Both, sheets and follicles of lymphocytes, were present, representing T and B cells respectively (22,25), however in these birds less follicle formation was observed. Helper and cytotoxic T cells are both present in normal proventriculi (22) and their numbers increase dramatically in proventriculitis (25). Lower numbers of B cells are present in normal proventriculi, and in proventriculitis their numbers also increase. Although the lesions observed in the proventriculi of birds treated with CP/+PV were similar to that of the controls, at 7 dpi chickens from these groups in trials 1 and 2, had significantly higher proventriculus weights than the +PV controls. This suggests a role of B cells in the early stages of proventriculitis, where compromised production of antibodies could exacerbate the severity of the condition.

All chickens with T-cell suppression due to cyclosporin (CS) and treated with +PV had equal or higher incidence and lesion scores of proventriculitis than +PV controls. The proventricular relative weights also tended to be higher than +PV controls, being more evident in the SPF birds in Trial 3 where this difference was significant at 21 dpi. Cell mediated immune (CMI) responses have been suggested to play a key role in the elimination of avian enteric pathogens (1,20,36), and our data indicate that T cell functions play a role in controlling proventriculitis. The high incidence of lesions in the proventriculi of birds in Trail 3 at 21 dpi that were immunosuppressed with CP and treated with +PV indicates the importance of T lymphocytes in the clearing and resolution of proventriculitis. It is well known that IBDV can

affect the CMI response (5,18,33,34) and although we saw little effect of IBDV-induced immunosuppression on the severity of proventriculitis in this study, it is possible that preventing severe immunosuppression in the field through vaccination against IBDV, could diminish the severity of proventriculitis.

Serologic results infer that the original positive proventricular homogenate used in this study contained IBDV, IBV, NDV and reovirus because some dosed experimental chickens seroconverted to these agents. Passage of this homogenate in commercial broilers seemed to have eliminated IBDV because SPF's challenged with the subsequent proventricular homogenate did not seroconvert to this virus or develop bursal disease. The objectives and experimental design of the present study were not designed to determine the role(s) of these other agents in proventriculitis, so no conclusions should be drawn from their presence here.

In conclusion, B cell immunosuppression, by CP or IBDV, did not have an effect on the incidence of proventriculitis, and the lesions observed were similar to those produced by +PV alone. However, proventricular enlargement was more evident in these birds at 7dpi, indicating that humoral response might be important in the early stages of the disease probably by controlling the causative agent by production of antibodies. T cell suppression by CS, on the other hand, did have an effect on the incidence of proventriculitis, and the lesions observed were more severe and lasted longer than in +PV controls. T cells are more abundant in the proventriculus than B cells, which suggests their importance in immune responses to infectious agents in this organ. In this study, by affecting T cell function, the severity of proventriculitis was increased and resolution of the disease was prolonged.

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Figure legends.

Fig. 5.1. Photographs of proventriculi from broiler chickens (14 days of age): inoculated with saline (A, and C), or with infectious proventricular homogenate (B, and D). Increase in size of the proventriculus and gastric isthmus and a white lobular pattern in a thickened mucosa can be observed in chickens with induced proventriculitis.

Fig. 5.2. Photomicrographs of proventriculi: A, normal proventriculus of chickens inoculated with saline (negative control) (7 dpi). B, proventriculitis in chickens inoculated with positive proventricular homogenate (+PV) (7 dpi) with necrosis of the glandular epithelium, coalescing of glands, and diffuse lymphocytic infiltration in glands and mucosa. C, proventriculitis in chickens inoculated with +PV (14 dpi), with ductal epithelium replacing glandular epithelium. D, proventriculus in SPF broilers inoculated with +PV (21 dpi), with small germinal centers. HE, 10X.

Fig. 5. 3. Photomicrographs of proventriculi from broiler chickens inoculated with positive proventricular homogenate (+PV) (14 dpi). A and B, treated with CP and +PV, with metaplastic replacement of proventricular glandular epithelium by ductal epithelium with minimal necrosis. C and D, treated with CS and +PV, with acute necrosis of the epithelium with coalescing glands and variable germinal center formation. HE, 10, and 25X.

Fig. 5.1

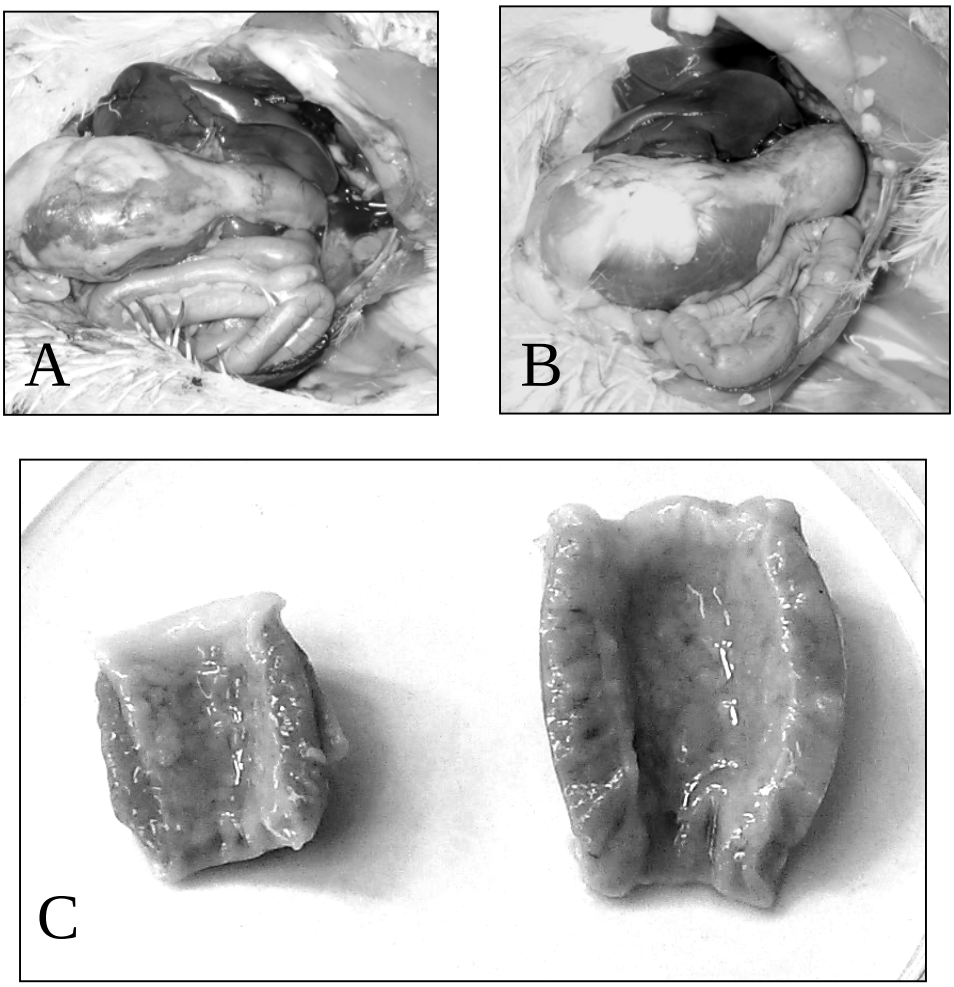

Fig. 5.2

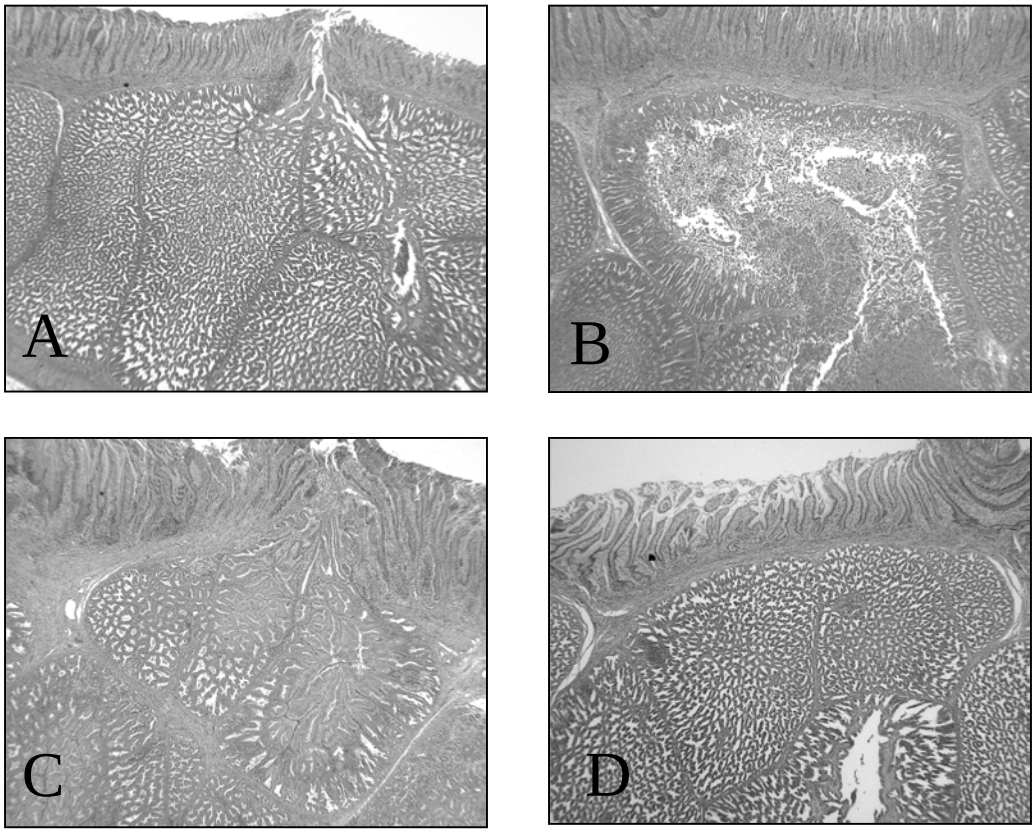

Fig. 5.3

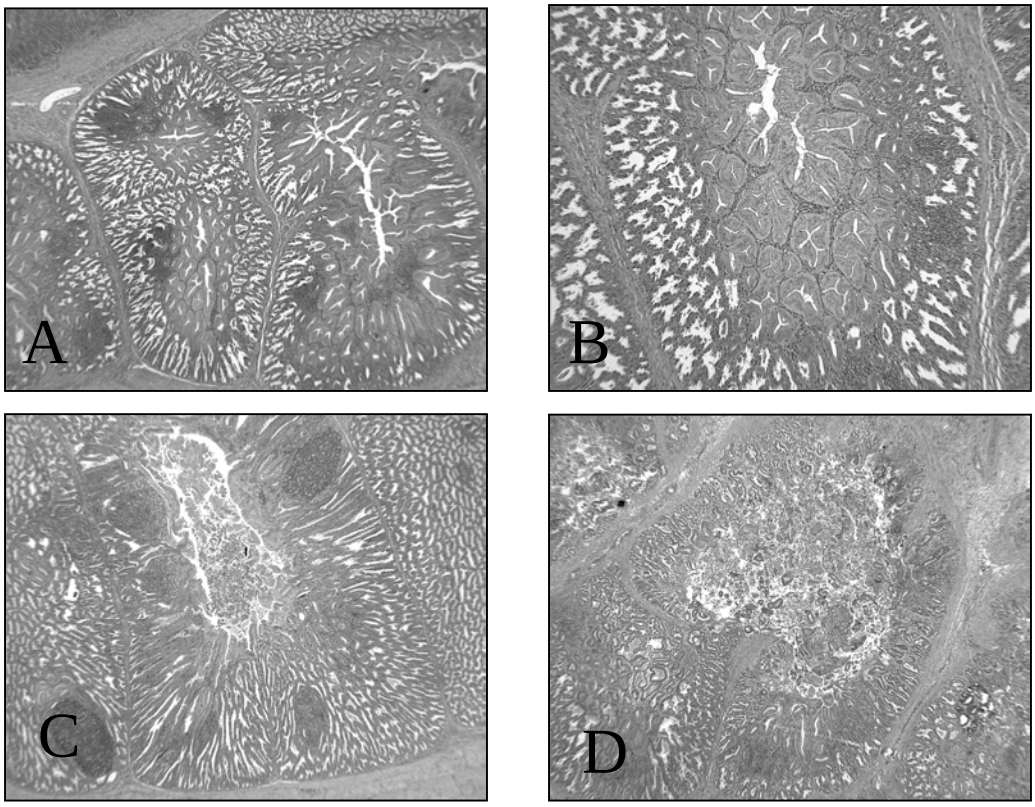

Table 5.1. Experimental protocol for trials 1 and 2 (commercial broilers), and trial 3 (SPF broilers). Four birds were necropsied per group on day 14 (7dpi) and 21 (14dpi) in all three trials, and also on day 28 (21dpi) in trial 3.

GROUPS	ONE DAY OF AGE IMMUNOSUPPRESSION TREATMENT[1]	SEVEN DAYS OF AGE HOMOGENATE TREATMENT[2]
1. Saline	-	Saline
2. –PV	-	-PV
3. +PV	-	+PV
4. IBDV	IBDV	-
5. CP	CP	-
6. CS	CS	-
7. IBDV/+PV	IBDV	+PV
8. CP/+PV	CP	+PV
9.CS/+PV	CS	+PV

[1] IBDV treatment: 10^3 CID_{50} *per os* strains Variant E (trial 1) or STC (trials 2 and 3). Cyclophosphamide (CP) treatment: 4 mg intraperitoneally for 4 days starting at one day of age. Cyclosporin (CS) treatment: intramuscular injection of 50 mg/Kg body weight every third day, starting on one day of age.

[2] Saline: 1ml sterile saline *per os*; –PV= proventricular homogenate from normal chickens, 1ml *per os*; +PV= proventricular homogenate from chickens with proventriculitis, 1ml *per os*.

Table 5.2. Body weight gain (g) of commercial broilers (trials 1 and 2), and SPF broilers (trial 3), orally challenged at 7 days of age with sterile saline, negative proventricular homogenate (-PV), or positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post inoculation (mean ± standard deviation)[1].

Groups	Trial 1	Trial 2	Trial 3
Day 14 (7dpi)			
1. Saline	360.5 ± 34.3 [a]	403.5 ± 14.5 [a]	146.2 ± 10.2 [a]
2. –PV	392.0 ± 7.16 [a]	411.2 ± 31.8 [a]	160.0 ± 16.7 [a]
3. +PV	349.0 ± 24.9 [a]	356.6 ± 45.0 [ab]	147.5 ± 10.1 [a]
4. IBDV	406.0 ± 42.6 [a]	400.8 ± 26.1 [a]	131.2 ± 13.2 [ab]
5. CP	329.2 ± 95.9 [a]	326.6 ± 50.8 [ab]	76.7 ± 21.6 [c]
6. CS	332.0 ± 83.7 [a]	360.7 ± 54.7 [ab]	128.7 ± 7.5 [ab]
7. IBDV/+PV	340.0 ± 25.9 [a]	361.8 ± 28.4 [ab]	130.7 ± 2.7 [ab]
8. CP/+PV	174.7 ± 40.5 [b]	220.1 ± 43.0 [b]	84.7 ± 8.1 [c]
9. CS/+PV	370.7 ± 29.9 [a]	290.3 ± 76.2 [ab]	114.7 ± 8.13 [b]
Day 21 (14 dpi)			
1. Saline	800.2 ± 26.1 [a]	807.7 ± 39.1 [a]	258.7 ± 18.7 [ab]
2. –PV	831.7 ± 67.5 [a]	714.4 ± 52.5 [a]	294.0 ± 19.3 [a]
3. +PV	807.7 ± 45.9 [a]	689.0 ± 24.3 [ab]	285.2 ± 24.2 [a]
4. IBDV	741.2 ± 104.5 [a]	773.4 ± 8.1 [a]	254.2 ± 32.8 [ab]
5. CP	733.2 ± 65.8 [a]	549.0 ± 80.0 [ab]	144.2 ± 39.1 [c]
6. CS	816.2 ± 43.8 [a]	506.2 ± 75.8 [ab]	229.5 ± 256 [ab]
7. IBDV/+PV	729.2 ± 123.9 [a]	712.5 ± 81.9 [ab]	249.5 ± 43.2 [ab]
8. CP/+PV	539.2 ± 77.5 [b]	392.2 ± 148.7 [b]	169.5 ± 36.5 [c]
9. CS/+PV	658.0 ± 72.0 [ab]	528.4 ± 157.6 [ab]	213.5 ±11.3 [b]
Day 28 (21 dpi)			
1. Saline			561.3 ± 73.0 [a]
2. –PV			561.0 ± 109.9 [a]
3. +PV			532.0 ± 97.5 [a]
4. IBDV			518.6 ± 92.6 [ab]
5. CP			316.0 ± 67.2 [b]
6. CS			484.0 ± 68.9 [ab]
7. IBDV/+PV			553.0 ± 92.9 [a]
8. CP/+PV			393.0 ± 95.3 [ab]
9. CS/+PV			422.0 ± 74.1 [ab]

[1] Means within a column and time point with no common lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group.

Table 5.3. Relative proventriculus weight (% body weight) of commercial broilers (trials 1 and 2), and SPF broilers (trial 3), orally challenged at 7 days of age with sterile saline, negative proventricular homogenate (-PV), or positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post inoculation (mean ± standard deviation) [1].

Groups	Trial 1	Trial 2	Trial 3
Day 14 (7dpi)			
1. Saline	0.602 ± .051 [a]	0.582 ± .047 [a]	0.677 ± .097 [a]
2. –PV	0.654 ± .042 [ab]	0.562 ± .040 [a]	0.707 ± .058 [a b]
3. +PV	0.932 ± .023 [ab]	0.812 ± .250 [a]	0.925 ± .750 [abc]
4. IBDV	0.550 ± .045 [a]	0.670 ± .083 [a]	0.685 ± .120 [a]
5. CP	0.754 ± .098 [ab]	0.685 ± .023 [a]	0.965 ± .054 [abc]
6. CS	0.670 ± .080 [ab]	0.696 ± .064 [a]	0.892 ± .180 [abc]
7. IBDV/+PV	0.962 ± .220 [b]	0.770 ± .153 [a]	1.010 ± .212 [c]
8. CP/+PV	1.406 ± .330 [c]	1.002 ± .208 [b]	0.985 ± .105 [bc]
9. CS/+PV	0.930 ± .095 [ab]	0.895 ± .175 [a]	1.020 ± .099 [c]
Day 21 (14 dpi)			
1. Saline	0.540 ± .075 [a]	0.473 ± .030 [a]	0.552 ± .061 [a]
2. –PV	0.510 ± .060 [a]	0.610 ± .140 [a]	0.582 ± .022 [a]
3. +PV	0.922 ± .194 [ab]	0.743 ± .089 [a]	0.745 ± .140 [abcd]
4. IBDV	0.532 ± .072 [a]	0.480 ± .036 [a]	0.650 ± .083 [abc]
5. CP	0.490 ± .057 [a]	0.540 ± .045 [a]	0.852 ± .140 [bcd]
6. CS	0.535 ± .050 [a]	0.580 ± .060 [a]	0.685 ± .100 [abc]
7. IBDV/+PV	0.900 ± .204 [ab]	0.723 ± .130 [a]	0.820 ± .110 [abcd]
8. CP/+PV	0.950 ± .154 [ab]	0.706 ± .210 [a]	1.020 ± .152 [d]
9. CS/+PV	1.202 ± .470 [b]	0.886 ± .370 [a]	0.927 ± .170 [bcd]
Day 28 (21 dpi)			
1. Saline			0.463 ± .083 [a]
2. –PV			0.436 ± .073 [a]
3. +PV			0.580 ± .111 [a]
4. IBDV			0.483 ± .149 [a]
5. CP			0.676 ± .005 [a]
6. CS			0.546 ± .096 [a]
7. IBDV/+PV			0.506 ± .046 [a]
8. CP/+PV			0.640 ± .103 [a]
9. CS/+PV			0.970 ± .261 [b]

[1] Means within a column and time point with no common lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group.

Table 5.4. Incidence and scoring of the severity of proventricular lesions in commercial broilers (trials 1 and 2), and SPF broilers (trial 3), orally challenged at 7 days of age with sterile saline, negative proventricular homogenate (-PV), or positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post inoculation [1].

Groups	Trial 1		Trial 2		Trial 3	
Day 14 (7dpi)						
1. Saline	1.00 [a2]	0/4[3]	1.00 [a]	0/4	1.50 [a]	2/4
2. –PV	1.00 [a]	0/4	1.00 [a]	0/4	1.75 [a]	2/4
3. +PV	3.00 [b]	3/4	2.50 [b]	2/4	2.50 [ab]	3/4
4. IBDV	1.00 [a]	0/4	1.25 [a]	1/4	1.50 [a]	2/4
5. CP	1.25 [a]	1/4	1.25 [a]	1/4	1.00 [a]	0/4
6. CS	2.00 [a]	2/4	1.00 [a]	0/4	1.50 [a]	2/4
7. IBDV/+PV	3.00 [b]	3/4	2.00 [ab]	2/4	2.50 [ab]	3/4
8. CP/+PV	2.50 [ab]	3/4	2.50 [b]	3/4	1.25 [a]	1/4
9. CS/+PV	3.50 [b]	4/4	2.75 [b]	3/4	3.25 [b]	4/4
Day 21 (14 dpi)						
1. Saline	1.25 [a]	1/4	1.00 [a]	0/4	1.25 [a]	2/4
2. –PV	1.00 [a]	0/4	1.50 [a]	2/4	1.50 [a]	2/4
3. +PV	3.75 [b]	4/4	3.50 [b]	4/4	3.25 [b]	4/4
4. IBDV	1.50 [a]	2/4	1.25 [a]	1/4	1.00 [a]	0/4
5. CP	1.25 [a]	1/4	1.25 [a]	1/4	1.25 [a]	1/4
6. CS	1.25 [a]	1/4	1.00 [a]	0/4	1.50 [a]	2/4
7. IBDV/+PV	3.25 [b]	4/4	3.50 [b]	4/4	2.50 [ab]	2/4
8. CP/+PV	3.00 [b]	4/4	2.50 [ab]	3/4	2.75 [ab]	3/4
9. CS/+PV	4.00 [b]	4/4	4.00 [b]	4/4	3.25 [b]	4/4
Day 28 (21 dpi)						
1. Saline					1.25 [a]	1/4
2. –PV					1.50 [a]	2/4
3. +PV					1.50 [a]	2/4
4. IBDV					1.25 [a]	1/4
5. CP					1.25 [a]	1/4
6. CS					1.50 [a]	2/4
7. IBDV/+PV					1.50 [a]	2/4
8. CP/+PV					1.50 [a]	2/4
9. CS/+PV					3.50 [b]	4/4

[1] Means within a column and trial with no common lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group

[2] Proventriculus score: 1: no lesions; 2: mild glandular lumenal ectasia; 3: ectasia plus lymphoid infiltrates in the interglandular interstitium; and 4: either acute glandular necrosis or severe fibrosis with lymphoid infiltrates.

[3] Number of birds with mild, moderate or severe lesions in the proventriculus/ number of birds necropsied.

Table 5.5. Bursa relative weights (% body weight) of commercial broilers (trials 1 and 2), and SPF broilers (trial 3), orally challenged at 7 days of age with sterile saline, negative proventricular homogenate (-PV), or positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post inoculation (mean ± standard deviation) [1].

Groups	Trial 1	Trial 2	Trial 3
Day 14 (7dpi)			
1. Saline	0.127 ± .075 [a]	0.220 ± .034 [a]	0.310 ± .045 [a]
2. –PV	0.170 ± .027 [a]	0.160 ± .039 [a]	0.320 ± .029 [a]
3. +PV	0.195 ± .020 [a]	0.205 ± .035 [a]	0.365 ± .090 [a]
4. IBDV	0.160 ± .010 [a]	0.165 ± .052 [a]	0.065 ± .010 [b]
5. CP	0.032 ± .009 [b]	0.060 ± .008 [b]	0.017 ± .015 [b]
6. CS	0.150 ± .017 [a]	0.213 ± .020 [a]	0.370 ± .067 [a]
7. IBDV/+PV	0.200 ± .026 [a]	0.152 ± .035 [a]	0.172 ± .009 [b]
8. CP/+PV	0.055 ± .012 [b]	0.060 ± .024 [b]	0.085 ± .005 [b]
9. CS/+PV	0.202 ± .022 [a]	0.182 ± .088 [a]	0.325 ± .098 [a]
Day 21 (14 dpi)			
1. Saline	0.250 ± .098 [a]	0.230 ± .081 [a]	0.340 ± .065 [a]
2. –PV	0.272 ± .090 [a]	0.230 ± .095 [a]	0.304 ± .045 [a]
3. +PV	0.225 ± .036 [a]	0.196 ± .075 [a]	0.305 ± .084 [a]
4. IBDV	0.190 ± .060 [a]	0.193 ± .068 [a]	0.112 ± .009 [b]
5. CP	0.032 ± .009 [b]	0.050 ± .017 [b]	0.055 ± .012 [b]
6. CS	0.232 ± .033 [a]	0.153 ± .055 [a]	0.387 ± .098 [a]
7. IBDV/+PV	0.225 ± .042 [a]	0.193 ± .064 [a]	0.080 ± .049 [b]
8. CP/+PV	0.070 ± .018 [b]	0.063 ± .020 [b]	0.070 ± .040 [b]
9. CS/+PV	0.282 ± .052 [a]	0.190 ± .066 [a]	0.377 ± .054 [a]
Day 28 (21 dpi)			
1. Saline			0.233 ± .023 [a]
2. –PV			0.243 ± .015 [a]
3. +PV			0.316 ± .047 [a]
4. IBDV			0.073 ± .036 [b]
5. CP			0.066 ± .005 [b]
6. CS			0.325 ± .051 [a]
7. IBDV/+PV			0.060 ± .010 [b]
8. CP/+PV			0.060 ± .036 [b]
9. CS/+PV			0.463 ± .027 [a]

[1] Means within a column and time point with no common lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group.

Table 5.6. Incidence and scoring of the severity in bursal lesions of commercial broilers (trials 1 and 2), and SPF broilers (trial 3), orally challenged at 7 days of age with sterile saline, negative proventricular homogenate (-PV), or positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post inoculation [1].

Groups	Trial 1		Trial 2		Trial 3	
Day 14 (7dpi)						
1. Saline	1.00 [a 2]	0/4[3]	1.50 [a]	2/4	1.25 [a]	1/4
2. –PV	1.00 [a]	0/4	2.00 [a]	4/4	1.25 [a]	1/4
3. +PV	1.75 [a]	2/4	2.25 [a]	4/4	2.50 [b]	4/4
4. IBDV	1.25 [a]	1/4	1.75 [a]	3/4	4.00 [c]	4/4
5. CP	4.00 [b]	4/4	4.00 [b]	4/4	4.00 [c]	4/4
6. CS	1.75 [a]	3/4	1.25 [a]	1/4	1.75 [a b]	3/4
7. IBDV/+PV	1.75 [a]	3/4	1.25 [a]	1/4	4.00 [c]	4/4
8. CP/+PV	4.00 [b]	4/4	4.00 [b]	4/4	4.00 [c]	4/4
9. CS/+PV	1.50[a]	2/4	1.50 [a]	3/4	2.00 [a b]	4/4
Day 21 (14 dpi)						
1. Saline	1.25 [a]	1/4	1.25 [a]	1/4	1.25 [a]	1/4
2. –PV	1.25 [a]	1/4	1.00 [a]	0/4	1.50 [a]	2/4
3. +PV	1.25 [a]	1/4	1.25 [a]	1/4	1.75 [a]	2/4
4. IBDV	1.00 [a]	0/4	1.25 [a]	1/4	4.00 [b]	4/4
5. CP	4.00 [b]	4/4	4.00 [b]	4/4	4.00 [b]	4/4
6. CS	1.50 [a]	2/4	1.25 [a]	1/4	1.50 [a]	2/4
7. IBDV/+PV	1.00 [a]	0/4	2.00 [a]	4/4	4.00 [b]	4/4
8. CP/+PV	4.00 [b]	4/4	4.00 [b]	4/4	4.00 [b]	4/4
9. CS/+PV	1.75 [a]	2/4	1.00 [a]	0/4	1.00 [a]	0/4
Day 28 (21 dpi)						
1. Saline					1.00 [a]	0/4
2. –PV					1.25 [a]	1/4
3. +PV					1.00 [a]	0/4
4. IBDV					4.00 [b]	4/4
5. CP					4.00 [b]	4/4
6. CS					1.00 [a]	0/4
7. IBDV/+PV					4.00 [b]	4/4
8. CP/+PV					4.00 [b]	4/4
9. CS/+PV					1.00 [a]	0/4

[1] Means within a column and trial with no common lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group.

[2] Bursa score: 1: no lesions; 2: mild variation in follicle size; 3: moderate variation in size of follicles; and 4: either necrosis or follicle atrophy.

[3] Number of birds with mild, moderate or severe lesions in the bursa/ number of birds necropsied

Table 5.7. Effect of immunosuppression treatments[1] on the cutaneous basophil hypersensitivity (CBH) response[2] induced by injection of phytohemagglutinin P (PHA-P) and physiological saline solution (PSS) in 2 week-old chickens.

Treatment	Trial 1		Trial 2		Trial 3	
	CBH-1	CBH-2	CBH-1	CBH-2	CBH-1	CBH-2
Saline	.74 ± .14	.87 ± .10	.72 ± .21	.74 ± .18	.58 ± .18	.54 ± .17
IBDV	.75 ± .25	.80± .24	.82 ± .27	.80± .24	.45 ± .22	.30 ± .08
CP	.67 ± .07	.73 ± .01	.69 ± .18	.69± .2	.34 ± .06	.31 ± .07
CS	.42 ± .14*	.49 ± .15*	.33 ± .21*	.27± .14*	.19 ± .03*	.14 ± .02*

[1] IBDV treatment: 10^3 CID_{50} *per os* strains Variant E (trial 1) or STC (trials 2 and 3). CP treatment: 4mg intraperitoneally for 4 days starting at one day of age. CS treatment: intramuscular injection of 50 mg/Kg body weight every third day, starting on one day of age.

[2] Data expressed as mean ± standard deviation; n= 4.

[3] CBH-1 = (skin thickness at 12 h post-injection, left foot) – (pre-injection skin thickness, left foot).

[4] CBH-2 = (skin thickness, PHA-P injected foot) – (skin thickness, PSS injected foot).

* Significantly different from groups in the same column ($P<0.05$).

Table 5.8. Immune responses to killed Newcastle Disease (ND) vaccine in chickens inoculated with either sterile saline, Infectious Bursal Disease Virus (IBDV), cyclophosphamide (CP), or cyclosporine (CS). 14 days postinoculation.[1]

Trial 1	Saline[2]	IBDV[3]	CP[4]	CS[5]
Mean ELISA titer	3,966[a]	3,433[a]	1.0[b]	3,034[a]
Mean HI titer	200[a]	160[a]	0[b]	160[a]

Trial 2	Saline[2]	IBDV[3]	CP[4]	CS[5]
Mean ELISA titer	5,277[a]	5,240[a]	36.0[b]	4,801[a]
Mean HI titer	200[a]	200[a]	0[b]	200[a]

Trial 3	Saline[2]	IBDV[3]	CP[4]	CS[5]
Mean ELISA titer	11,784[a]	4,202[bc]	1.0[c]	11,140[a]
Mean HI titer	480[a]	160[bc]	0[c]	360[ab]

[1] Means within a row with no common lowercase superscript are significantly different ($P < 0.05$). Means calculated from four birds.

[2] Saline: 1ml sterile saline *per os*

[3] IBDV treatment: 10^3 CID_{50} *per os* strains Variant E (trial 1) or STC (trials 2 and 3).

[4] CP treatment: 4mg intraperitoneal for 4 days starting at one day of age.

[5] CS treatment: intramuscular injection of 50 mg/Kg body weight every third day, starting on one day of age.

CHAPTER 6

PROVENTRICULITIS IN THE BROILER CHICKENS: CHARACTERIZATION OF THE LYMPHOCYTIC INFILTRATION IN THE PROVENTRICULAR GLANDS[1]

[1]Pantin-Jackwood, M. J., T. P. Brown, G. Kerce, and G. R. Huff. To be submitted to *Veterinary Pathology*.

SUMMARY.

Broiler chickens with transmissible proventriculitis have severe lymphocytic infiltration in the proventricular glands and the mucosa. The distribution of T cells (CD3+, CD4+, and CD8+) and B cells in the proventriculus of affected chicken was studied immunohistochemically and histopathologically. One-day-old commercial boilers were orally gavaged with a proventricular homogenate produced from broilers with proventriculitis to reproduce this disease. Resulting proventricular lesions were studied at 7, 14 and 21 days post-inoculation (dpi). Lymphocytic infiltrates in the proventricular glands and the lamina propria of the mucosa were observed at all time points, and were most prominent at 14 days post-inoculation with well-developed lymphoid aggregates present. Both T and B Lymphocytes were present during acute and chronic proventriculitis, but their distribution varied within the glands. Lymphocytic infiltrates in both the proventricular glands and in the lamina propria were mainly T cells (CD3+), and were predominantly CD8+ T lymphocytes. CD4+ T cells and B cells tended to form aggregates as the proventriculitis became chronic. These findings show that both cell mediated and humoral immune responses are induced during transmissible proventriculitis, and that the cell mediated immune response is morphologically greater.

Keywords: Chicken; Proventriculitis; T and B Lymphocytes; Immunohistochemistry.

Abbreviations: Dpi, days post-inoculation; IEL, intraepithelial lymphocytes; MALT, mucosa associated lymphoid tissue; -PV, negative proventricular homogenate; +PV, positive proventricular homogenate.

INTRODUCTION

Proventriculitis is a transmissible disease that occurs in commercial broiler chickens. It is characterized by enlargement of the proventriculus and weakness of the gastric isthmus. During routine evisceration at processing, affected proventriculi rupture causing spillage of the retained ingesta into the body cavity, which results in condemnation of affected carcasses for contamination. The disease has also been associated with impaired growth and poor feed conversion (10, 12). Microscopically, degeneration and necrosis of the proventricular glandular epithelium is accompanied by marked lymphocytic infiltration (4, 10, 11, 12).

The etiology of proventriculitis is not clear. Several agents have been implicated as potential causes of proventriculitis. Noninfectious causes include oral exposure to biogenic amines (3), mycotoxins (24), lack of dietary fiber (25), and excessive copper sulfate (5,13). Infectious causes include adenovirus (17), reovirus (16,29), infectious bronchitis virus (33), infectious bursal disease virus (4,10,12,20) and megabacterium (27). However, none of these noninfectious or infectious agents have been found consistently in a majority of cases. Electron microscopy has detected viral particles in acute lesions but isolation of a virus from affected proventriculi has been unsuccessful (10,11,12). Proventriculitis has been successfully reproduced by inoculation with proventricular homogenates produced from diseased chickens (10,11,12). Filtrates from these homogenates also produced lesions in the proventriculus suggesting that a virus is the cause of the disease (10,11,12). However, proventriculitis is more severe when birds are inoculated with the unfiltered homogenate suggesting that infectious proventriculitis has a complex etiology involving both viral and bacterial agents (12).

The main histologic finding in transmissible proventriculitis is a marked lymphocytic infiltration of the proventricular glands (22). The purpose of this study was to characterize this

lymphocytic infiltrate to gain insights into the identity of these cells and their functional role in generating a protective immune response in the proventriculus. To accomplish this we experimentally infected commercial broiler chickens with proventricular homogenates from affected broilers and studied the proventricular lesions using histopathology, staining for lymphocyte cell-surface markers, and by identifying the distribution of these different lymphocyte subsets.

MATERIALS AND METHODS.

Chickens. One-day-old unvaccinated broiler chicks were obtained from a commercial hatchery. All chicks were wing-banded, weighed, separated into groups and maintained in positive pressure Horsfal isolation units. Feed and water were provided *ad libitum*.

Proventricular homogenates. A proventricular homogenate (+PV) was prepared from proventriculi from 2 to 4-week old chickens with proventriculitis (12). A second proventricular homogenate (-PV) was similarly prepared from proventriculi of normal healthy broiler chickens without proventriculitis and was used as a control inoculum. Both +PV and –PV were prepared as previously described (4) and frozen at –70C, and thawed immediately prior to use.

Experimental design. We divided 24 one-day-old commercial broilers into 2 groups. The first group was inoculated by oral gavage with 1ml of -PV. The second group received 1 ml of the +PV. At 7, 14 and 21 days post inoculation (dpi) four birds from each group were weighed and killed by cervical dislocation. Bursa, proventriculus, and the right side of the thymus were weighed, and sections from these organs were collected from each bird and fixed immediately by immersion in 10% neutral buffered formalin for 24 hours for histopathology. Sections of proventriculus, bursa and thymus were also placed in Cryo-Gel embedding medium

(Instrumedics, Inc.,Hackensack, NJ) and immediately frozen in liquid nitrogen and kept at –70C until immunohistological studies were performed. Tissues in formalin were later processed using routine histologic techniques and embedded in paraffin. Also, a part of the proventriculus from each bird was washed several times in sterile saline, homogenized, and frozen at –70C.

Histopathology. Paraffin-embedded tissues were sectioned, mounted, stained using hematoxylin and eosin (HE), and examined, blinded as for treatment, for lesions using light microscopy. All sections were assigned a lesion severity score. For all tissues a lesion score of 1 represented no lesions. For bursal sections, 2 was defined as mild variation in follicle size, 3 as moderate variation in follicle size, and 4 as either necrosis or follicle atrophy. For thymic sections 2 was defined as mild cortical thinning, 3 as moderate cortical thinning, and 4 as absence of cortical lymphocytes. For proventricular sections, 2 was defined as mild glandular lumenal ectasia, 3 as ectasia, necrosis of the glandular epithelium, plus lymphoid infiltrates in the interglandular interstitium, and 4 as either acute glandular necrosis or severe fibrosis with lymphoid infiltrates. The wall thickness of the sections of proventriculi mounted on the slides was measured with a millimeter ruler on the thickest part.

Monoclonal antibodies. Monoclonal antibodies for T lymphocytes (Southern Biotechnology Associates Inc., Birmingham, Alabama) were: mouse anti-chicken CT-3 (anti-CD3), CT-4 (anti-CD-4), and CT-8 (anti-CD8). HisCl antibody (Cedi Diagnostics BV, Lelystad, The Netherlands) was used for B lymphocytes.

Immunohistochemistry. Optimal conditions for immunohistochemical staining with each monoclonal antibody were determined using bursa and thymus tissues from normal chickens. All monoclonals stained cell populations in positive-control tissues with equal intensity. These tissues were included as controls during the staining of each group of slides.

Frozen tissue blocks were cut in a cryostat into 5 μm sections, and placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). They were fixed immediately in acetone for 10 minutes and stored at –70 C until stained. Immunostaining was accomplished using a nonbiotin peroxidase kit (DAKO Envision System, DAKO, Carpinteria, CA) according to the manufacturer's recommendations. Briefly, the sections on slides were placed in a moist chamber and washed for 5 min. in 0.1 M phosphate buffered saline (PBS), followed by incubation for 5 min. in peroxidase blocking reagent (DAKO Envision System). Sections were then washed in PBS for 5 min. and incubated with monoclonal antibodies at 4C overnight (CD-3, CD-4, and CD-8 were used at a dilution of 1:100; His-C1 at a dilution of 1:50). Following primary antibody incubation, sections were washed in PBS for 5 min. and incubated with the secondary antibody (peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins, DAKO Envision System) at room temperature for 45 min. After washing in PBS for 5 min., bound antibody was detected by a 5-10 min. incubation with 3,3'-diaminobenzidine substrate- chromogen (DAB, DAKO Envision System). After IHC staining, sections were counter-stained with hematoxylin, air dried, cover slipped, and examined using light microscopy.

Statistical analysis. The relative organ weights and lesion scores were analyzed using ANOVA and means comparisons for all pairs using Tukey-Kramer HSD. Significance was set at a 0.05 level of probability.

RESULTS

Clinical signs and macroscopic lesions. No clinical signs were observed in any of the chickens in control or experimental groups. Gross lesions were observed in all proventriculi from +PV-inoculated chickens. At 7 and 14 days post inoculation (dpi) proventriculi were enlarged

with a mottled appearance and a distended gastric isthmus. At 21 dpi the enlargement still was present but was less severe. No enlargement of the proventriculi was observed in the chickens given -PV. The proventricular wall of chickens inoculated with the +PV was thickened, with a white lobular pattern observed when sectioned. No macroscopic lesions were observed in any other organ of experimentally infected birds.

Body weight gain. At 7 and 14 dpi, chickens inoculated with positive homogenate had no significant suppression of weight gain compared to age matched chickens given –PV. At 21 dpi there was a decrease in body weight gain in birds that received +PV (Table 6.1).

Organ weights. Chickens that received +PV had increased proventricular organ/weight ratio at 7 and 14 dpi, which was statistically significant when compared to chickens that received -PV (Table 6.1). There was no difference in the bursa and thymus organ/weight ratio between birds inoculated with +PV and those given -PV (data not shown).

Microscopic lesions. Proventricular lesion scores were significantly higher in birds that received +PV compared to compared to those given -PV. No lesions were present in the proventricular glands of the –PV treated birds at 7dpi. At 14 and 21 dpi, 2 of 4 birds in this group had mild lumenar ectasia of the glandular lumen. Lymphocytes were observed at all time points in the lamina propria of the mucosa especially in areas surrounding the orifices of the excretory ducts of the deep proventricular glands (Fig. 6.1-A). By 21 dpi these lymphocytes formed small aggregates in the proventricular glands (Fig. 6.1-B).

Proventriculi of chickens challenged with +PV presented necrosis of the glandular epithelium at 7 dpi (Fig. 6.1-C). Collecting sinuses of the glands were dilated and contained desquamated epithelium and debris. Nuclei of glandular epithelium were enlarged and pale, with marginated chromatin. At 7 and 14 dpi lymphocytic infiltrates were present in large numbers in

the lamina propria of the mucosa and also in affected glands expanding the glandular interstitium (Fig. 6.1-C and 6.1-D). At 14 and 21 dpi, the glandular epithelium in some of the glands was replaced by ductal epithelium (Fig. 6.1-E). At 21 dpi there was less necrosis of the glandular epithelium, but there was regeneration or metaplasia to ductal epithelium. At that time, lymphocytes were still present, mostly forming aggregates or germinal centers (Fig. 6.1-F).

Proventricular wall thickness. There was a significant difference in thickness of the proventricular wall between chickens that were inoculated with –PV and those inoculated with +PV at all time points (Table 6.1).

Localization of CD3+, CD4+, CD8+ and B cells. Both T and B cells were present in the lamina propria of the proventricular mucosa of chickens treated with –PV. Most lymphocytes in the proventricular glands were T cells, and were localized to the interstitium between the glands, and intraepithelially as individual lymphocytes (Fig. 6. 2-1 and 6.1-B). Small lymphoid aggregates were present in the glands at 14 and 21 dpi, and were mostly composed of B cells.

In chickens that received +PV, T cells predominated at all time points and were dispersed within the lamina propria of the mucosa and in deeper areas of proventricular glands. B cells were also present, but their distribution varied depending on the stage of the proventriculitis. Initially, B cells were localized similar to the T cells but in lower numbers (Fig. 6.2-C). As the proventriculitis progressed, B cells formed aggregates (germinal centers) in deeper portions of proventricular glands and less frequently in the lamina propria of the mucosa (Fig. 6.2-E and 6.2-G). T cells surrounded these germinal centers and infiltrated the proventricular glands and the mucosa (Fig 6.2-D, 6.2-F, and 6.2-H).

The two subsets of T lymphocytes studied (CD4+ and CD8+) were distributed differently in affected proventriculi (Fig 6.4. A to H). Both subsets were found at all time points in large quantities in the lamina propria of the mucosa, but CD4+'s predominated at 7 dpi. At 14 and 21 dpi, CD4+ positive cells were found mostly surrounding the B cell germinal centers and forming aggregates that by HE stain were germinal centers. Also, CD4+ cells infiltrated these B cell germinal centers. The CD8+ cells were more widely distributed, were surrounding the germinal centers, and also infiltrated the proventricular glands in the intraepithelial spaces. In glands with acute necrosis of the epithelium, CD8+ lymphocytes were the predominant cells infiltrating the gland. In the chronic lesions this subset was still observed in large numbers throughout the gland, while CD4+ and B cells formed aggregates located in deeper portions of proventricular glands (Fig 6.4-A and 6.4-B).

DISCUSSION

In the present study, lymphocyte subpopulation changes during proventriculitis were investigated. Proventriculitis was successfully reproduced by inoculation with a proventricular homogenate derived from proventriculi collected from broiler chickens affected with proventriculitis (+PV). Microscopic changes in these proventriculi included necrosis of the glandular epithelium and replacement of this epithelium with ductal epithelium. This loss of glandular tissue and ductal hyperplasia may result in loss of function of the proventriculus (10). This would explain the poor feed conversion and reduced growth rates observed in naturally affected chickens with proventriculitis, and the reduced body weight observed in our experimental chickens at 21 days post inoculation.

Severe lymphocytic infiltration was observed in all experimentally infected chickens. The distribution of these lymphocytes in the proventriculus varied. In the acute or early stages lymphocytes were present as sheets in large numbers in the lamina propria of the mucosa and infiltrating affected glands. In the later stages the lymphocytes formed aggregates in both the lamina propria of the mucosa and deep in the proventricular glands. These chronic changes were accompanied by less necrosis and ductal hyperplasia. Staining of these lymphocytes showed that both B and T cells are increased in number during proventriculitis but occupied different histologic locations within the proventriculus depending on the stage of the disease.

Lymphocytes are present in the mucosa of normal chicken organs as the mucosal associated lymphoid tissue (MALT). This complex immune apparatus has developed in the chicken in response to antigens entering the body through mucosal surfaces lining the respiratory, digestive and genitourinary tracts, and provides the first line of defense against these antigens (2). Matsumoto and Hashimoto (19) described the normal distribution and developmental changes of the lymphoid tissues in the chicken proventriculus. They observed the development of lymphoid masses in the proventricular lamina propria underneath the surface epithelium and near the duct orifice, which suggested that the local mucosal immune mechanism develops primarily with a dominant participation of T lymphocytes in the early post-hatching period. The development of B lymphocytes occurs following the invasion of the antigens associated with food intake, owing to immunological information from the prerequisite T lymphocytes. In our study, the response to a non-defined infectious agent present in the positive proventricular homogenate induced proliferation of the lymphoid tissue present in the proventriculus. This immune response was similar to that observed in the mucosa of other organs in response to different pathogens (2,6,8,11,18,21,26,28,31,32). Intrapithelial lymphocytes (IEL)

could be observed in the deep proventricular gland and Matsumoto and Hashimoto (19) identified them as γδ T lymphocytes, similar to those found in the chicken intestine. These authors could not demonstrate the presence of M cells in the proventriculus suggesting that there are alternative routes for uptake of intraluminal antigens.

The cause of proventriculitis is not known but it seems most likely that a virus is the primary agent involved (11,12). T cell mediated immune responses to viral pathogens are well established, and occur by a number of different mechanisms, including induction of cytotoxic activity, recognition of target antigens in conjunction with the major histocompatibility complex (MHC), and production of lymphokines such as interferon-γ, interleukin-2 and tumor necrosis factor-β. Cells mediating these different activities can be identified by cell surface antigens, CD4+ for helper T cells, CD8+ for cytotoxic and suppressor T cells, and CD3+ as a common T cell antigen (30). Most virus specific cytotoxic T lymphocyte (CTL) activity identified is MHC class I restricted and mediated by CD8+ T cells. The CD4+ subset has an important role in virus infections as it provides the helper T cell necessary to promote the clonal expansion and differentiation of virus-specific B cells (1). The activation of B cells and their differentiation into antibody secreting plasma cells is triggered by antigen and usually requires helper T cells. In our study, CD4+ T cells were the most abundant lymphocyte subset found in the lamina propria of the mucosa in the early stages of proventriculitis. These lymphocytes were later found surrounding what appeared to be B-cell germinal centers.

The CD8+ T cells found in the affected proventricular glands, formed sheets infiltrating the glandular epithelium. The influx of CD8+ T cells suggests cytotoxic activity associated with pathogen clearance. The CD8+ CTL response has been shown to be critical for the control of primary, persistent, and reactivated virus infections (7). The antiviral action of CTL is mediated

by direct lysis of infected cells (e.g. by perforin/ granzyme release), the induction of apoptosis (e.g. by Fas/ Fas ligand interaction) and the production of antiviral cytokines (7). Kotani *et al* (15) studied the lymphocytic subsets in the trachea of chickens inoculated with infectious bronchitis virus (IBV) and concluded that the chicken's immune system may utilize specific CTL to eliminate IBV at the early stage of infection, and in the later stage may depend on humoral immunity to control viral infection. In an earlier study it was found that cellular immunosuppression increased the severity and duration of proventriculitis, underlining the importance of T cells in the immune response against proventriculitis (23).

Songserm *el al* (28) also observed an increase of CD8+ cells in the intestine of chickens inoculated with malabsorption syndrome homogenate. They found that an increase of cytotoxic activity was associated with the intestinal lesions and weight gain depression. In our study the influx of CD8+ cells in the proventriculus appeared to occur after the onset of the necrosis of the glandular epithelium. However, the increase of lymphocytes in the glands exacerbated the lesions present in the proventriculus increasing the loss glandular epithelium. Lesions in the proventricular glands did not occur simultaneously. The necrosis and influx of T lymphocytes appeared to start in the area surrounding the mucosal papillae and spread to the glands that drained through these papillae. Microscopically, some glands present lesions and other appear normal, and depending on the severity of the proventriculitis, more glands would be affected.

In addition to CD4+ and CD8+ cells, natural killer cells (NK) may play a role in the defense against gut pathogens. NK cells are phenotypically defined as CD8+ lacking T (CD3+) or B lineage specific markers. Gobel *et al* (9) demonstrated by these criteria that approximately 30% of CD8+ intestinal intraepithelial lymphocytes (IEL) were NK cells. The physiological role of the intestinal NK cells is not known but might constitute the first line of defense once

epithelial cells get infected serving similar functions as T cells (9). In our study, because we didn't have a marker for NK cells, this specific subset was not analyzed and we cannot draw conclusions about the role of NK cells in proventriculitis. Most of the lymphocytes observed in the affected proventriculi that stained with the cytotoxic T cell marker (CD8+) also stained with the pan T cell marker (CD3+) with a low percentage not staining for the CD3+ marker. The presence of NK cells in the proventriculus, and their role in proventriculitis needs to be further investigated.

In conclusion, the influx of CD4+ cells during proventriculitis suggests that these cells are involved in the induction of the immune response, whereas the CD8+ cells most likely act as effector cells. The influx of B cells and formation of highly organized germinal centers, indicates that antibody-mediated mechanisms are also involved in the control of proventriculitis in chickens. Germinal center formation occurred in the later stages of the disease when the lesions in the proventriculus were less severe. In this study, staining of B cell immunoglobulins (IgG, IgM, and IgA) was not performed. IgM and IgA in the intestinal secretions prevent environmental antigen influx into internal body compartments, neutralization of viruses and microbial toxins, and prevention of adherence and colonization of mucosal surfaces by pathogens (18). The role of these immunoglobulins is not clear for some poultry infections and further study of the their importance in proventriculitis ought to be done.

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Figure legends.

Fig. 6.1. Photomicrographs of proventriculi: A. From chicken inoculated with –PV at 7 dpi. Lymphocytic infiltration in the lamina propria of the mucosa and surrounding the orifice of the secretory duct. B. From chicken inoculated with –PV at 21 dpi. Small lymphocyte aggregations are present in the proventricular gland. C. From chicken inoculated with positive +PV at 7 dpi. Severe necrosis of the glandular epithelium, dilation of sinus with desquamated epithelium and lymphocytic infiltration of the proventricular gland. D. and E. From chickens inoculated with +PV at 14 dpi. Diffuse lymphocytic infiltration in the proventricular gland and the lamina propria of the mucosa. Tubular epithelium replacing glandular epithelium. F. From chicken inoculated with +PV at 21 dpi. Lymphocyte aggregations present in the proventricular glands. HE, 25X.

Fig. 6.2. Immunohistochemistry (IHC) staining of proventricular lymphocytes: A. From chicken inoculated with -PV, B cell staining in the lamina propria of the mucosa, 7dpi. B. From chicken inoculated with -PV, CD3+ T cell staining in the lamina propria of the mucosa, interstitium between proventricular glands, and deep in the glands. 7dpi. C. From +PV-inoculated chicken, B cell staining in the proventricular gland, 7 dpi. D. From +PV-inoculated chicken, CD3+ T cell staining in the proventricular gland, 7 dpi. E. and G. From +PV-inoculated chicken, B cell staining of lymphocyte aggregations in the gland and mucosa, 14 dpi. F. and H. From +PV-inoculated chicken, CD3+ T cell staining in the glands and mucosa, 14 dpi. 25 and 50X

Fig. 6.3. Immunohistochemistry (IHC) staining of proventricular lymphocytes from +PV-inoculated chicken at 14 dpi. A. and E. B cell staining. B. and F. CD3+ T cell staining. C. and G. CD4+ T cell staining. D. and H. CD8+ T cell staining. 50 and 100X.

Fig. 6.4. Immunohistochemistry (IHC) staining of proventricular lymphocyte aggregations from +PV-inoculated chicken at 14 dpi. A. B cell stain. B. CD3+ T cell stain. 10X.

Fig. 6.1

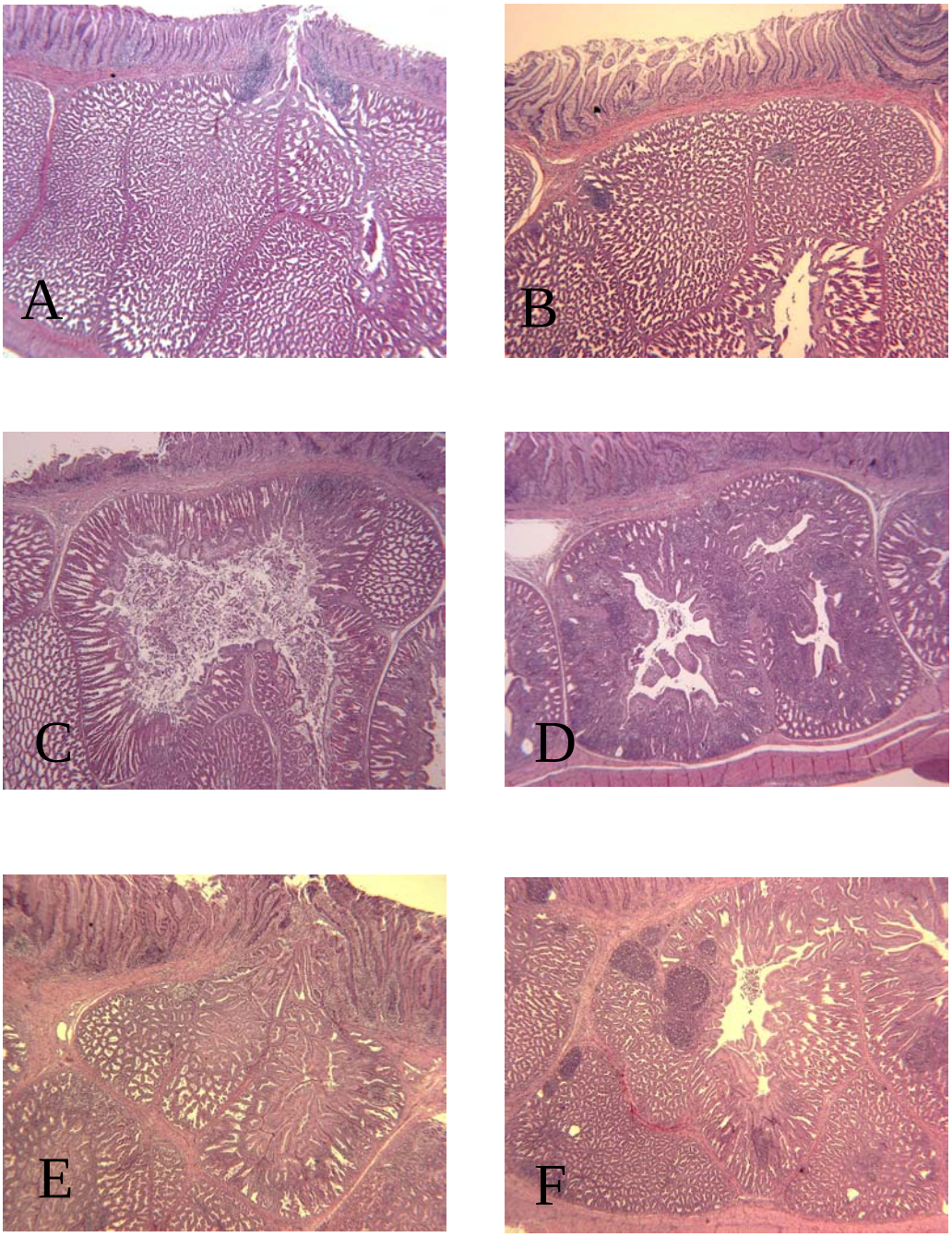

Fig. 6.2

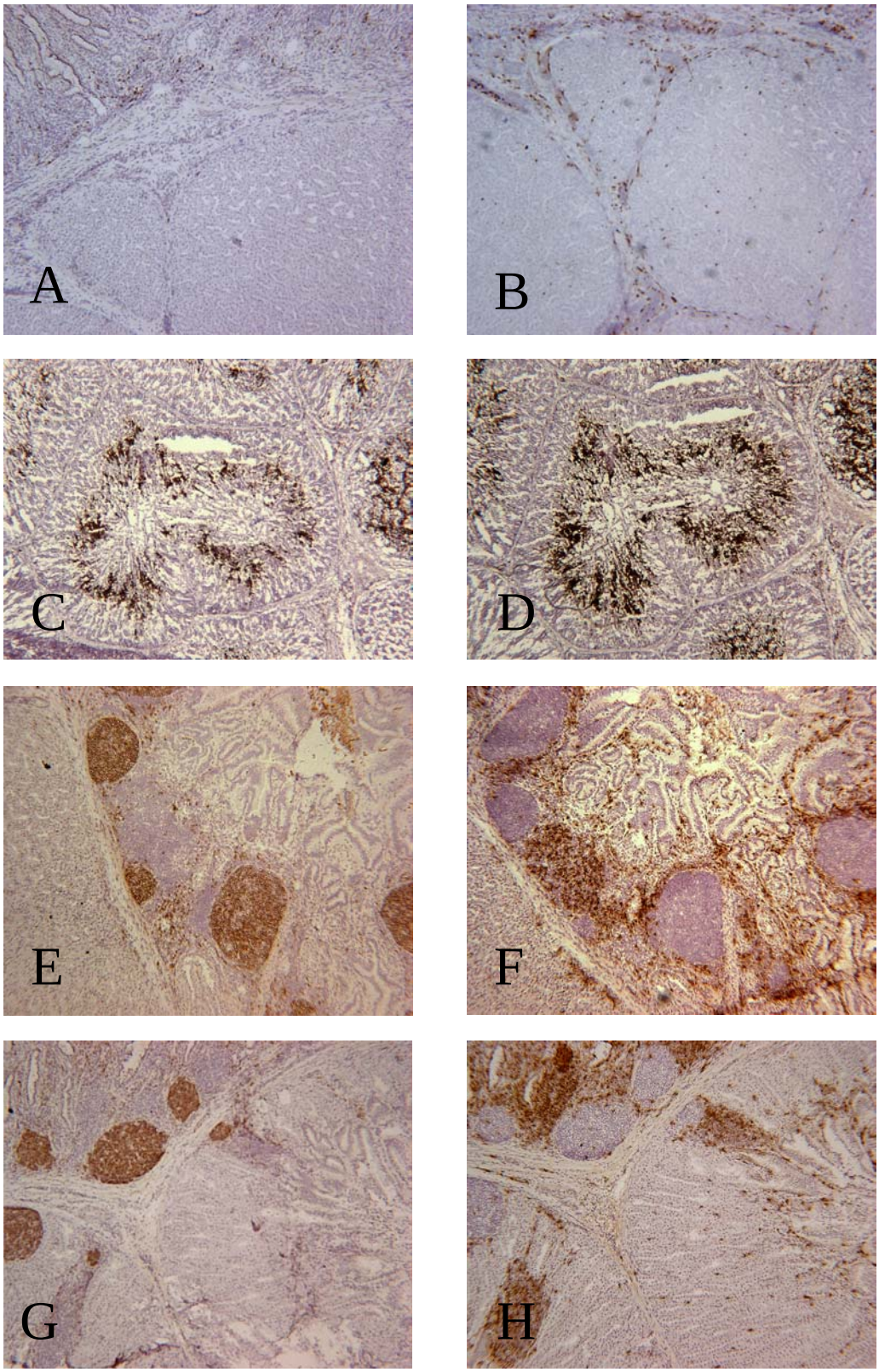

Fig. 6.3

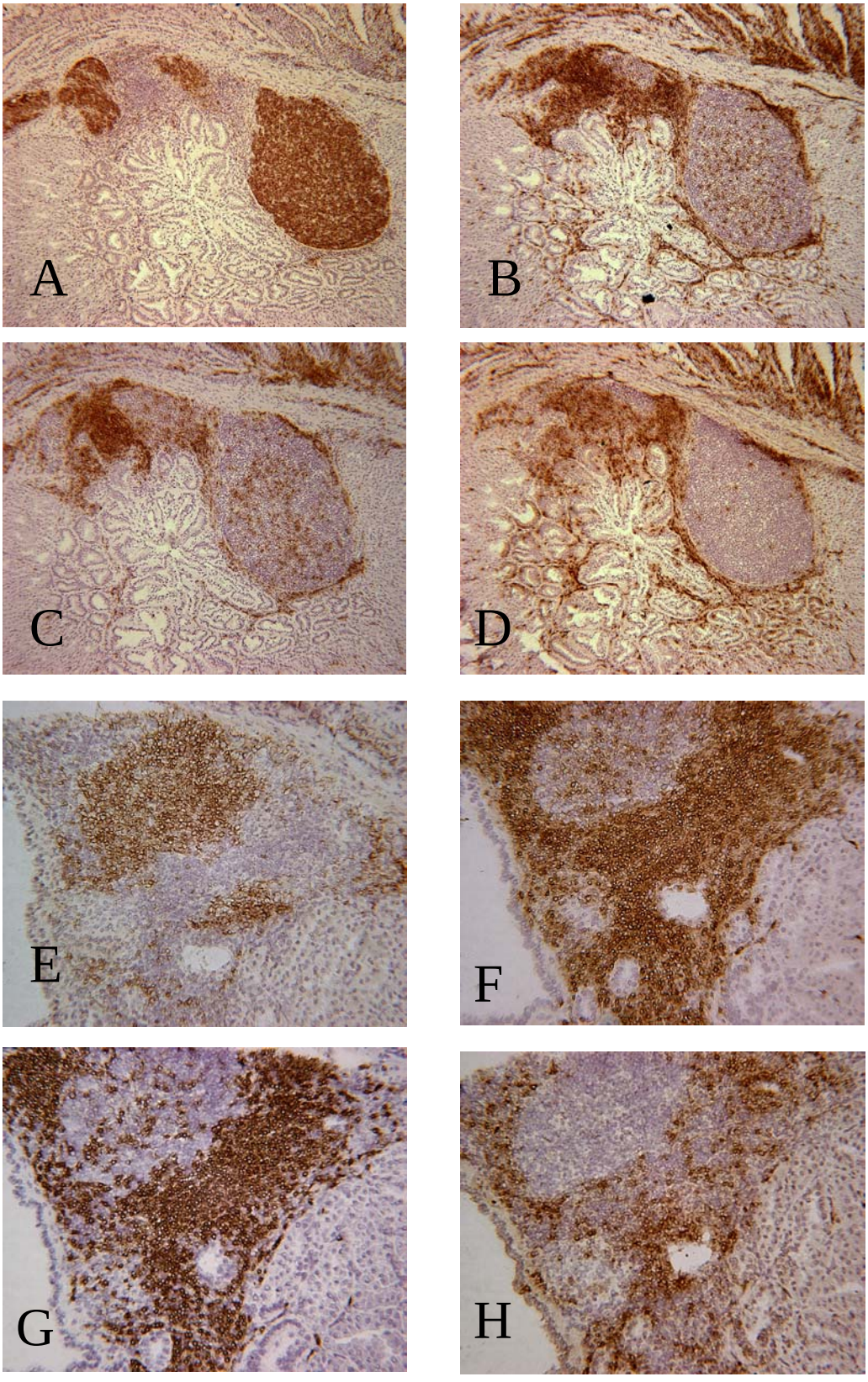

Fig. 6.4

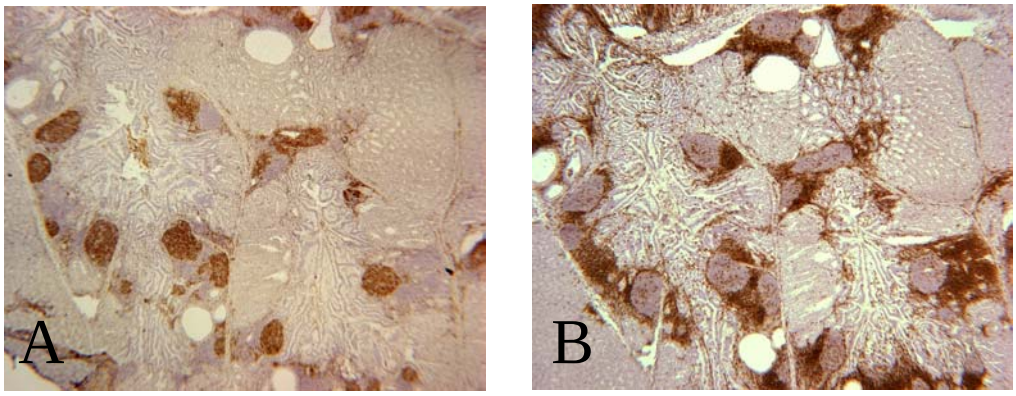

Table. 6.1. Body weight gain (g), relative proventriculus weight (% body weight), proventriculus lesion score and incidence of proventriculitis, and proventriculus wall thickness (mm) of commercial broilers orally challenged at day of age with a negative proventricular homogenate (-PV), or a positive proventricular homogenate (+PV), and necropsied at 7, 14, and 21 days post-inoculation (mean ± standard deviation).[1]

Days post-inoculation	*Treatment*	*Body weight gain*	*PV relative weight*	*PV lesion score and incidence*	*PV wall thickeness*
7	-PV	153.7 ± 29.6[a]	0.85 ± .11 [a]	1 [a] 0/4	3.62[a]
	+PV	144.0 ± 10.6 [a]	1.26 ± .22 [b]	3 [b] 3/4	4.62[b]
14	-PV	370.0 ± 44.2 [a]	0.69 ± 0.08 [a]	1.5 [a] 2/4	4.25 [a]
	+PV	331.0 ± 62.8 [a]	0.95 ± 0.09 [b]	4 [b] 4/4	5.10 [b]
21	-PV	882.0 ± 35.7 [a]	0.54 ± .005 [a]	1.5 [a] 2/4	5.00 [a]
	+PV	749.0 ± 20.8 [b]	0.78 ± 0.15 [a]	3 [b] 4/4	7.75 [b]

[1]Means within a column and time point with different lowercase superscript are significantly different ($P<0.05$). Means calculated from four birds in each group.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

SPF broilers experimentally infected with different strains of IBDV did not develop proventriculitis, and chickens with naturally occurring cases of proventriculitis did not have IBDV in their proventriculi. Although the strains chosen for this study belong to five of the six molecular groups used to classify IBDV strains, it is possible that other untested strains may produce proventriculitis directly, or that proventriculitis is due to an undetermined cause and could be exacerbated by immunosuppression produced by IBDV infection.

Proventriculitis was studied by experimentally reproducing the disease in broiler chickens. One-day-old commercial and SPF broilers were orally gavaged with a proventricular homogenate produced from the proventriculi of broilers with proventriculitis. Both, commercial and SPF broilers presented enlargement of the proventriculus with necrosis of the glandular epithelium and lymphocytic infiltrates in the proventricular gland. SPF broilers exposed to the proventricular homogenates developed Infectious Bursal Disease, and infectious bursal disease virus (IBDV) was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) in bursal and proventricular tissues. They also were positive by RT-PCR to infectious bronchitis virus (IBV) and developed nephritis. Commercial broilers developed mild nephritis but not bursal disease, and were negative for IBDV and IBV by RT-PCR. Both, commercial and SPF chickens, were negative for reovirus, and Newcastle disease virus (NDV), and positive for chicken anemia virus (CAV) and adenovirus by molecular

techniques. Bacteria were not identified in histological sections nor were they isolated from affected proventriculi. Filtrates from the proventricular homogenates passed in embryos for virus isolation caused stunting but identification of the cause by electron microscopy was unsuccessful. However, allantoic fluid from the eggs was positive for IBV by RT-PCR. Thin sectioning EM on proventriculi from affected birds failed to identify a causative agent. In conclusion, the original proventricular homogenates had IBDV, IBV, adenovirus and CAV, but the role of each in producing proventriculitis was not proven.

B cell immunosuppression, by CP or IBDV, did not have an effect on the incidence of proventriculitis, and the lesions observed were similar to those produced by positive proventricular homogenate (+PV) alone. However, proventricular enlargement was more evident in birds immunosuppressed with these agents at 7dpi, indicating that a humoral response might play a role in the early stages of the disease probably by controlling the causative agent by production of antibodies. T cell suppression by CS, on the other hand, did have an effect on the incidence of proventriculitis, and the lesions observed were more severe and lasted longer than in +PV controls. T cells are more abundant in the proventriculus than B cells, underlining their importance in immune responses to infectious agents in this organ. In this study, by affecting T cell function, the severity of proventriculitis was increased and resolution of the disease was prolonged.

The lymphocytic infiltrates present during proventriculitis in both the proventricular gland and the lamina propria, were mainly T cells. The influx of CD4+ cells suggests that these cells are involved in the induction of the immune response, whereas the CD8+ cells most likely act as effector cells. The influx of B cells and formation of highly organized germinal centers,

indicates that antibody-mediated mechanisms are also involved in the control of proventriculitis in chickens.

In conclusion, proventriculitis can be reproduced by oral inoculation of chickens with homogenates produced from proventriculi of birds with proventriculitis. The causative agent(s) was not identified, although most likely it is a virus. The severity of proventriculitis and its effect on weight gain is probably affected by other factors such as concomitant infection with more than one agent, viral or bacterial, and nutritional factors. Proventriculitis was reproduced in the absence of IBDV and IBDV did not cause proventriculitis when susceptible chickens were inoculated with the virus. IBDV affects both humoral and cellular immunity in the chicken, so although under experimental conditions it didn't have a major effect on proventriculitis, it may explain why control of IBDV under commercial conditions reduces the incidence of proventriculitis.