EFFECTS OF VACCINATION SCHEDULE AND AMBIENT AMMONIA ON THE IMMUNE RESPONSE TO VACCINATION AND PROTECTION AGAINST HOMOLOGOUS CHALLENGE IN CHICKENS

by

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(Under the Direction of Mark W. Jackwood and Brian J. Jordan)

ABSTRACT

Over time, vaccination and management practices have adjusted to meet the demands imposed by the changing structure of the poultry industry. Vaccination programs typically involve serial administration of multiple vaccines, including infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and infectious laryngotracheitis virus (ILTV). Often the interval between immunizations is short, sometimes a week or less, and it is unknown whether the short interval between immunizations interferes with the development of immunity and protection against challenge. Additionally, birds are commonly exposed to ambient ammonia (NH_3), which has been linked to disease susceptibility. Our research aimed to determine the effects of vaccination schedule and ambient NH_3 on the immune response to vaccination and protection from homologous challenge in chickens. Specific-pathogen-free white leghorns were administered multiple live attenuated vaccines against IBV, NDV, and ILTV until 16 weeks-of-age (WOA), after which certain groups were challenged with IBV, NDV, or ILTV at 20, 24, 28, 32, and 36 WOA. Five days post-challenge, viral load, clinical signs, ciliostasis, tracheal histopathology, and antibody titers in serum and tears were evaluated. One-day-old broiler chicks

were administered IBV vaccine and exposed to NH_3 until challenge with a homologous strain at 28 DOA. Protection was measured by viral detection, clinical signs, ciliostasis, presence of airsacculitis, IBV-specific serum IgG and lacrimal fluid IgA titers, and Harderian gland immune cell phenotypes. Leghorns serially administered live attenuated vaccines against IBV, NDV, and ILTV were protected against homologous challenge with IBV, NDV, or ILTV until they were at least 36 WOA. Additionally, the timing of these vaccines and intervals between each vaccine did not interfere with the development of immunity to each virus and consequently protection against homologous challenge. Our data also indicated that ambient NH_3 exposure had no clear impact on the broiler immune response to IBV vaccination and also did not impact vaccine or challenge virus replication and clearance, ciliostasis, or tracheal histopathology scores. Collectively, our data promote poultry health and performance by contributing valuable information to the development of poultry vaccination and management programs.

INDEX WORDS: Ammonia, Airsacculitis, Broiler chickens, Ciliostasis, Immunity, Infectious bronchitis virus, Infectious laryngotracheitis virus, Leghorn chickens, Newcastle disease virus, Respiratory disease, Vaccination program

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DEDICATION

To my family and Annie

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CHAPTER 1

INTRODUCTION

The scope of poultry production in the U.S. has changed dramatically in the past century. In the early 1920's, chicken meat was generally reserved for special occasions, but a combination of improved technology, production practices leading to better poultry health and nutrition, and product marketing transformed the poultry sector from traditionally dual-purpose (eggs and meat) backyard flocks to highly specialized, vertically integrated agribusiness firms that dominate the industry today. The vast majority of poultry producers now operate under a contract system, which distributes some of the risk from the typically family-owned farms to large integrators in a market prone to wide fluctuations in poultry and egg prices.

The result of these advances is that poultry are often reared in high densities to achieve economies of scale, and consequently the maintenance of good air quality warrants special consideration. Determinants of air quality in a poultry house include gaseous products of animal respiration, byproducts of decomposing manure, and airborne dust carrying disease-causing agents (95). In particular, NH_3, CO_2, H_2S, and particulate matter (dust) negatively affect air quality (139), and other contributors such as humidity and temperature play an indirect role in air quality by exerting influence on gaseous products, especially NH_3.

One contributor to poor air quality is NH_3, which has received considerable attention due to its adverse effects on poultry health and performance. NH_3 is naturally emitted from feces in poultry litter, in which uric acid is converted to gaseous NH_3 by bacterial activity. The recommended maximum level of NH_3 exposure is 25 parts per million (ppm) (114), but

commercial poultry are often exposed to higher levels of NH_3, particularly during cold weather as ventilation rates are reduced to conserve heat in the winter months (28, 113, 133, 201). In addition, NH_3 levels are influenced by litter conditions such as moisture content, temperature and pH, and environmental conditions such as relative humidity, temperature and air movement (28, 113, 123).

NH_3 exposure has a number of negative effects on chicken health, especially in relation to ocular and respiratory tissues. Lesions affecting the eye generally include inflammatory infiltrates in the ocular tissues, conjunctivitis, and in severe cases, birds can develop corneal ulcerations. Tracheal lesions may involve deciliation, excessive mucus production, and inflammatory cell infiltrates (203), and ultrastructural analysis of lungs has revealed abnormalities affecting blood-gas exchange (3). NH_3 can also influence the immune response by increasing oxidative damage and downregulating immune response proteins responsible for antigen recognition and presentation, and it is also capable of increasing the production of proteins involved in muscle contraction, which may explain the cough and rales often observed among ammonia-exposed birds (203). Not all studies have identified ammonia-induced pathology, and some authors contend that the harmful effects of NH_3 may not always be apparent until the bird experiences additional environmental or microbial challenges, such as dust and *Escherischia coli* exposure (151). Notably, NH_3 exposure has been associated with increased susceptibility of chickens to either primary disease such as Newcastle disease (ND) (8) or secondary infections subsequent to primary infection, such as in IBV-vaccinated chickens that later developed airsacculitis (112, 159).

In addition to air quality, another important consideration when rearing birds in high densities is vaccination against disease. Some of the most economically important diseases of

poultry include the viral respiratory diseases, such as IB, ND, and infectious laryngotracheitis (ILT). Traditionally, vaccines used in poultry are live or inactivated, and vaccine selection depends on the type of pathogen targeted by the vaccine and the type of immune response required for protection. For example, cell-mediated immunity is generally an important component of immune responses to viruses, which are intracellular pathogens, and live vaccines are more successful at stimulating cell-mediated immunity because they can replicate in the host cell (87). Moreover, live vaccines are capable of stimulating local immunity (128), which is required for protection from many respiratory viral infections. As a result, vaccination regimes typically include several live vaccines to enable the birds to develop optimal protection against viral disease. Vaccination programs are designed to induce immunity in birds from an early age. This involves the administration of several vaccines against a variety of pathogens, and often the interval between immunizations is short, sometimes a week or less. In some cases, several different vaccines are administered together.

It is well known that some viruses can persist in a flock for weeks and even months, and it is routine for birds to be co-infected with multiple live vaccines. In addition, it has been established that certain viruses can block the growth of other viruses through a process called viral interference (48, 157). However, surprisingly few experimental studies of simultaneous virus infections have been published, and fewer yet have been considered in the context of poultry viral respiratory pathogens.

Economically important poultry viral respiratory tract diseases include IB, ND, and ILT. IBV infection is characterized by drops in egg production, reduced growth, and secondary bacterial infection leading to condemnations at the processing plant (32). IBV replicates in the respiratory epithelial cells, and some strains also replicate in the kidney, oviduct, or

gastrointestinal tract (104). As a result, clinical signs are varied and can include respiratory signs, depression, inappetence, decreased egg production and quality, and renal damage (44, 104, 161).

The first pathological signs of IBV infection are hyperplasia of goblet cells and alveolar mucous glands, which are responsible for nasal discharge, and virulent virus infection often leads to loss of cilia, epithelial degenerative changes, and the depletion of goblet cells and alveolar mucous glands (137). These changes stimulate the innate immune responses characterized by the induction of pattern recognition receptors (PRRs), recruitment of heterophils and macrophages to the site of infection, production of pro-inflammatory cytokines and chemokines, and activation of apoptosis (119, 121, 209). The adaptive immune response is an essential component of immunity to IBV, which mediates viral clearance and establishes immunological memory capable of generating an appropriate immune response to prevent re-infection. A cell-mediated immune response is key to developing host immunity to IBV infection with either an IBV vaccine or virulent strain, and cytotoxic $CD8^+$ T lymphocytes play an important role in mediating viral clearance (174). Humoral immunity is important for preventing IBV re-infection (88) and is also involved in disease resolution and virus clearance (39, 40).

Because IBV first enters the host via the respiratory tract, mucosal immunity to IBV infection is essential to inhibiting viral infection, replication, and reducing clinical disease. Tissues involved in the respiratory tract mucosal immunity include the paraocular lymphoid tissue, nasal-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT), and tracheal tissue (96). The paraocular immune system is a major system involved in IBV immunity and comprises the conjunctiva associated lymphoid tissue (CALT) and Harderian gland (HG) (72, 96). The HG contains IgA-, IgG-, and IgM-expressing plasma cells (13, 143), and constitutes the main source of antibodies in tears (172). The immunoglobulin (Ig)-secreting

populations in the respiratory immune system, in particular the HG, are varied and depend on anatomical location, bird age, and infection status. Consequently, there is no consensus regarding the frequency and proportion of IgA-, IgG-, and IgM-secreting cells in the HG and whether IgA or IgG, or a combination of both, are involved in protection from IBV infection (13, 88, 108, 150, 185, 186). However, as of yet, only IgA antibodies in tears have been associated with protection from IBV infection (185), although IgG has been speculated to also play a role (150).

Traditional vaccination protocols rely most commonly on initial mass vaccination of one to three serotypes of live, attenuated IBV at 1 day-of-age (DOA) to stimulate the mucosal and systemic immune responses. Live vaccines are generally administered to young birds to achieve early protection, and layers and breeders are also boosted with either live or inactivated vaccines (55). The vaccine strains used in poultry production vary based on their similarity to the circulating field viruses (55), however birds are usually poorly protected against heterologous challenge. One strategy employed to induce a larger spectrum of cross-protection against heterologous challenge is the "protectotype" vaccination strategy (41, 184). While this strategy is partially effective, vectored vaccines have been investigated as a potential alternative to traditional IBV vaccines and efforts to develop vaccines against conserved epitopes or vaccines expressing immunostimulatory cytokines have been ongoing and include the fowlpox virus (FPV), adenovirus (AdV), Marek's disease virus (MDV), and NDV vectors (190, 200, 204, 207). Although several of these vaccine candidates are promising, most of them do not completely protect against IBV infection.

A second poultry viral respiratory tract disease is ND, which affects both wild birds and domestic poultry and is an OIE designated disease (144) and a USDA select agent (24). Lentogenic strains are of low pathogenicity causing mild respiratory or enteric infections,

followed by mesogenic strains, while velogenic isolates are highly pathogenic often causing neurological signs and mortality (130). Birds infected with mild NDV strains may have no clinical signs or may exhibit depression, inappetence, respiratory signs such as nasal discharge and sneezing, and reduced egg production (130).

NDV infection first engages the innate immune response, which inhibits virus spread while stimulating the adaptive immune response to develop virus-specific responses to eliminate virus and establish immunological memory. The cell-mediated and humoral responses are important components of NDV immunity. The principal cells involved in the cell-mediated response are the cytotoxic $CD8^+$ and helper $CD4^+$ T lymphocytes (110). Mucosal humoral response appears to play a role in NDV immunity because IgM, IgG, and IgA antibodies were detected in respiratory tissues following NDV exposure (2). As with IBV, neutralizing antibodies are required for protection against NDV infection (2, 166).

ND vaccination regimes vary and may utilize a combination of live, inactivated, and virus-vectored vaccines (61). A common strategy is to vaccinate either one-day-old chicks or chicks at 2-4 WOA with a live vaccine, followed by a live booster at 2-4 weeks and again at 10 weeks (130). In the United States, the most widely used traditional vaccine strains comprise lentogenic B1 (or virus clones of the B1 strain) and LaSota strains (130). Inactivated vaccines are often used in layers and breeders.

Several viral-vectored vaccines are commercially available. Although they can be administered *in ovo* and induce both systemic and local immunity and strong cell-mediated responses, protective immunity requires 4-5 weeks to develop (61). Commercially available recombinant poultry vaccines for NDV include the FPV-vectored vaccine expressing the NDV fusion (F) or hemagglutinin (HN) protein and the herpesvirus of turkeys (HVT)-vectored vaccine

expressing the F protein (61, 135). One challenge to this vaccination strategy is that maternally derived antibodies (MDA) may interfere with the development of immunity (134).

ILT is a third viral respiratory tract disease of economic importance in the poultry industry and results in severe production losses due to increased mortality, reduced egg production, and decreased body weight gain. ILT is a herpesvirus that establishes latency in the host, and clinical signs include gasping, coughing, sneezing, depression, nasal discharge, expulsion of bloody mucus, and conjunctivitis (74).

Although ILTV infection leads to the production of virus-specific antibodies (99), antibody titers do not correlate with protection against ILTV infection (176). In fact, the cell-mediated response is the predominant arm responsible for the development of immunity to ILTV and is also capable of preventing re-infection in the absence of mucosal antibody (69).

Vaccination strategies often combine live and vectored vaccines and depend on the type of bird being vaccinated (79). Live vaccines against ILT may be of chicken embryo origin (CEO) or tissue culture origin (TCO). The CEO vaccine is the most widely used vaccine worldwide but has been shown to regain virulence following several passages in chickens (90), and ILTV outbreaks of vaccine origin are common (10). Although live, attenuated vaccines provide good protection when applied correctly and uniformly, protection in the field is diminished due to unrestricted vaccine use, poor flock vaccination coverage using mass application techniques, insufficient biosecurity measures, and comorbidity (79, 107).

Consequently, viral-vectored vaccines were developed as a safe alternative to live, attenuated ILTV vaccines. FPV- and HVT-vectored vaccines are commercially available and consist of the glycoprotein B (gB) gene and gD and gI genes, respectively (195). Although viral-vectored vaccines reduce clinical signs upon ILTV infection, they do not prevent viral shedding

and therefore confer only partial protection (79). In an effort to reduce the risk of outbreaks caused by revertant ILTV strains while achieving complete protection against viral replication, researchers are investigating the potential use of recombinant live attenuated ILTV vaccines with gene deletions.

The aforementioned vaccination strategies for IB, ND, and ILT have been designed to maximally protect chickens from infection, but until now they have not been considered in the context of a typical poultry vaccination regime in which multiple vaccines for a variety of diseases are administered. In addition, chickens are often exposed to suboptimal air quality, particularly NH_3, and very little is known about its effect on the development of immunity following vaccination and protection against challenge with a respiratory virus.

Specifically, this dissertation investigates the effects of vaccination schedule and ambient NH_3 on the immune response to vaccination and protection against homologous challenge in chickens. Specifically, this research aims to (1) determine the effect of pullet vaccination schedule on development and longevity of immunity, and (2) determine the effect of ambient NH_3 on the immune response to IBV vaccination and protection from homologous challenge in broiler chickens. Results of this research provide valuable insight that may aid in the design of poultry vaccination and management programs.

CHAPTER 2

LITERATURE REVIEW

U.S. Commercial Broiler and Layer Industries

The U.S. commercial broiler and layer industries are vertically integrated, which refers to a single firm owning and operating successive stages of poultry production. Within the broiler industry, eggs are sold from the breeder to an integrated broiler company (integrator), a firm that owns hatcheries, feed mills, and processing plants, and contracts farms to raise broilers in the grow-out stage. Eggs are hatched at an integrator-owned hatchery and delivered to a contract grower for the grow-out phase (125, 169). The layer industry is structured very similarly. Hatchery supply flocks lay fertile eggs that are hatched and sold to layer companies for grow-out into table egg layers (175).

The vast majority of the poultry and egg market operates under a contract system. Detailed information about the contract system between integrators and growers in the U.S. broiler industry is described in MacDonald and McBride (125). In a broiler contract system, integrators provide contract growers with chicks, feed, transportation services, veterinary and technical services, and may reimburse for some utilities. Growers are responsible for providing labor, housing, equipment, and most utilities, and receive a payment per pound of live broilers produced based on their performance relative to other growers. A layer contract system is very similar to a broiler contract system, and the grower is paid a fee based on the number of eggs produced and receives performance incentives.

An overview of contracts is reviewed in MacDonald et al. (124). Contracts reduce risk on the part of the farmers because wide price fluctuations are minimized, thus eliminating variable and uncertain income. A predictable income improves the ability of farmers to meet financial obligations and plan production and investment decisions. Broiler houses in particular have asset specificity, which refers to durable investments that are made in support of certain transactions and have little use in other markets. For example, broiler houses are outfitted with costly equipment designed specifically for broiler production that have little alternative uses. In addition, transactions are restricted to integrators within a certain proximity of the broiler house because of the high transportation costs of feed and birds and the indirect costs of stress-related weight loss or death during transport. Therefore, integrators contract with farms in close proximity to their feed mills, hatcheries, and processing plants.

Air Quality and Ammonia

Another feature of the poultry industry is that poultry are typically reared in high densities and managing air quality in a chicken house is important for the maintenance of optimal bird performance and health. Air quality is influenced by the gaseous products of animal respiration, the byproducts of decomposing manure, and airborne dust carrying disease-causing agents (95). The decomposition of livestock manure leads to the production of the following gases: methane, carbon dioxide (CO_2), oxygen, nitrogen, ammonia (NH_3), carbon monoxide, and hydrogen sulfide (H_2S) (141). In poultry houses, NH_3, CO_2, H_2S, and particulate matter (dust) have been identified as contributors to poor air quality (139). Dust irritates the respiratory tract upon inhalation, and it carries pathogenic microorganisms present in the house. Air quality may be indirectly affected by humidity, defined as the moisture content in the atmosphere in the form of water vapor (64). Humidity is often expressed as relative humidity (Rh) and indicates how

close the air is to becoming saturated with water vapor (64). Higher Rh promotes the settling of dust, thus reducing the amount of exposure to irritants and pathogens, whereas lower Rh results in increases in airborne dust and may predispose birds to respiratory disease (95). However, it is noteworthy that an increase in Rh is correlated with an elevation in NH_3 levels, which can be detrimental to bird performance and health (45). Finally, Rh is also inversely related to temperature, and therefore temperature fluctuations in the poultry house can also indirectly influence air quality (45). In summary, the determinants of air quality in a poultry house are complex, and it is important to understand the relationship between factors to more efficiently manage air quality.

Of the parameters affecting air quality in the poultry house, NH_3 is the most studied. The mechanisms of NH_3 emissions from manure have been summarized in Ni (138), in which uric acid in feces is converted to gaseous NH_3 by bacterial activity within poultry litter. The recommended maximum level of NH_3 exposure is 25 parts per million (ppm) (114), but commercial poultry are often exposed to higher levels of NH_3, particularly during cold weather as ventilation rates are reduced to conserve heat in the winter months and to reduce the cost of electricity required to power ventilation fans (28, 113, 133, 201). The higher NH_3 levels occur in part due to slower air turnover and consequently a buildup of ammonia in the house. NH_3 concentrations commonly average 39.9 ppm in the winter months and 6.3 ppm in the summer months (133), and broiler houses are prone to ammonia spikes leading to high levels of ammonia often during the nighttime when ventilation rates are reduced (45). A study by Davis and Morishita (51) measured NH_3 concentrations within 5 poultry houses using ammonia-diffusion indicator tubes and reported values between 2.64 ppm to 59.72 ppm. NH_3 concentrations in a poultry house are positively correlated with relative humidity, which increases the litter moisture

content and promotes the emission of NH_3 into the environment, and negatively correlated with temperature and ventilation rate (28, 113, 123). Although elevated NH_3 levels occur more commonly during cold weather, NH_3 in a broiler house may present a year-round challenge to bird health and production (133).

The effects of NH_3 exposure on the eye have been documented in numerous studies on chickens and vary depending on the level and duration of exposure. Broilers exposed to low levels of NH_3 (25 ppm) experienced diffuse lymphocytic and heterophilic infiltrates in the iris, and exposure to higher concentrations (50 and 75 ppm) resulted in corneal ulcerations and more numerous inflammatory infiltrates by 14 DOA (132). Notably, ocular lesions in this study quickly improved upon removal of ammonia. Barber (16) also observed rapid recovery from NH_3-induced ocular lesions, which occurred once chickens that had developed ulcerative keratitis and severe conjunctivitis during exposure to an unspecified high level of NH_3 had been placed in clean, well-ventilated rooms. Anderson, Beard and Hanson (8) also demonstrated an effect of graded levels of NH_3 exposure, which progressed in severity with longer durations of exposure. Keratitis, conjunctivitis, and corneal edema, ulceration, and neovascularization have also been reported in chickens treated with NH_3 (73). Beker, Vanhooser, Swartzlander and Teeter (19) reported increasing conjunctival lesions in broilers exposed to 30 and 60 ppm of ammonia for 21 days.

Several reports have investigated the effect of NH_3 on respiratory tissues. In general, those reports have described deciliation, goblet cell hypertrophy, epithelial hyperplasia, and inflammatory cell infiltrates in the upper-respiratory tract associated with NH_3 exposure (203). A report by Al-Mashhadani and Beck (3) detailed extensive mucus secretion and areas of cilia loss in tracheal epithelium after 7 days of exposure to 25 ppm, and NH_3 exposure to 50 ppm over 4

days preceded increased lung atrial wall thickness in chicken lungs, which was visualized by scanning electron microscopy (3). Ultrastructural analysis of turkey lungs revealed that prolonged exposure to ammonia levels as low as 10 ppm resulted in excessive mucus production, matted cilia, and areas of deciliation (136).

Despite NH_3-induced histological and ultrastructural abnormalities in respiratory tissues, it is uncertain whether NH_3 exposure alone increases inflammation in these tissues, but there is at least one report of NH_3 exposure leading to an increased percentage of circulating heterophils with a concomitant decrease in the percentage of lymphocytes (heterophil:lymphocyte ratio) (131). However, Miles (132) reported no increase in lymphocytic or heterophilic infiltrates in tissue from the trachea, lungs, and air sacs following a 28-day exposure period with up to 75 ppm of NH_3, and the authors speculated that the harmful effects of NH_3 may not always be apparent until the bird experiences additional environmental or microbial challenges. For example, a study by Oyetunde, Thomson and Carlson (151) demonstrated that the deleterious effects of NH_3 are compounded by dust and *Escherichia coli*, thus leading to increased trachea, lung, and air sac lesions. In another study, Anderson, Beard and Hanson (9) observed that natural exposure to NH_3, dust, and carbon dioxide for 6 days resulted in some deciliation of the upper portion of the trachea and turbinates, as well as an increase in mucus-secreting goblet cells.

NH_3 exposure has been associated with an increased susceptibility to disease. Anderson, Beard and Hanson (8) reported increased susceptibility to Newcastle disease infection after exposure to 20 ppm of NH_3 for 72 hours. In addition, some studies documented the occurrence of airsacculitis in NH_3-stressed birds vaccinated with live attenuated infectious bronchitis virus (IBV) (112, 159).

In contrast to reports demonstrating the increased susceptibility to disease among NH_3-exposed birds, some studies suggest no association between NH_3 exposure and infection with other diseases. For example, Barber (16) reported that chickens reared in an environment with ammonia produced naturally from feces was not associated with an increase in the incidence of leukosis. In a later study, Brewer and Koon (23) showed that atmospheric ammonia levels of approximately 200 ppm for two 24-hour periods had no significant effect on the incidence of Marek's disease (MD).

While the majority of studies have relied on morphometric and functional parameters to describe the effect of NH_3 on poultry health, very few have addressed the mechanisms by which NH_3 influences immunity. In a study by Xiong, Tang, Meng and Zhang (203), transcriptomic analysis of broiler tracheas following NH_3 exposure of 75 ppm for 20 days indicated that NH_3 increased the production of reactive oxygen species (ROS), downregulated immune response proteins responsible for antigen recognition and presentation, and upregulated proteins involved in muscle contraction and mucin production. The authors further discussed that the overproduction of mucins combined with tracheal muscle contraction may explain the clinical signs such as coughing and rales that may accompany NH_3 exposure.

Poultry Vaccination against Viral Disease

Vaccination against respiratory viral disease is standard practice in commercial poultry operations. Traditionally, vaccines administered to poultry are either live or inactivated, and differences are highlighted in Marangon and Busani (128). Briefly, live vaccines are preferred in many cases because they are relatively economical, stimulate local (or mucosal) immunity, confer a rapid onset of immunity, require a smaller quantity of antigen, and can be mass applied. However, they are easily killed by improper storage conditions, they may be more susceptible to

preexisting antibodies as in maternal immunity, booster vaccination often does not elicit a good immune response, there is a risk of vaccine contamination or tissue reactions, and viral interference may occur if combined with other live vaccines since the vaccine replication is required to stimulate robust immune responses. In addition, live vaccines in poultry production may confer added immune stress to the chickens, which leads to reduced growth performance (122). Inactivated vaccines may be a good alternative to live vaccines because they are easier to store, they are more capable of eliciting an immune response in the presence of maternal antibodies or in an immune host, there is a reduced risk of vaccine contamination, and multiple combinations are less likely to interfere with each other. On the other hand, inactivated vaccines require a large amount of antigen, are expensive to produce, require individual administration (as opposed to mass administration), and typically generate a slower onset of immunity.

The selection of live or inactivated vaccine is also dependent on the type of pathogen targeted by the vaccine, as well as the immune response required for protection. A particular challenge for vaccine development is successfully immunizing the host against intracellular pathogens such as viruses, and in general immunity against these pathogens is predominantly cell-mediated (87). Immunization of the host with a live vaccine often is more successful at stimulating robust immunity to an intracellular pathogen because, in addition to the advantages mentioned above, live vaccines have the potential to localize to a specific organ or cell type, replicate in the host, and most of the pathogen genome is expressed, which provides a wide range of potential epitopes (87).

The duration of immunity achieved following live vaccine administration can depend on the age and type of bird, levels of maternal immunity, disease targeted by the vaccine, immunogenicity of the vaccine, method of vaccine application, number of and interval between

boosters, virulence and similarity of the field strain challenge, interval between vaccination and challenge, and immunocompetency of the host (21, 55, 130).

Successful vaccine development has been a major factor enabling large-scale poultry production because commercial poultry are exposed daily to many environmental and pathogenic challenges, and the modern poultry industry is characterized by high stocking densities of birds combined with geographical clustering of poultry operations. Poultry must develop immunity against a variety of pathogens from an early age. Vaccination programs are designed to induce protection against common pathogens by serial or simultaneous administration of multiple vaccines. Sample vaccination regimes in different poultry sectors are reviewed in the Merck Veterinary Manual (www.merckvetmanual.com), in which the interval between vaccinations is often only a matter of weeks, and in many cases, vaccines are administered together. Baron, Lqbal and Nair (17) also provide a sample vaccination schedule for broiler breeders consisting of up to 15 different types of vaccines. Such programs require careful analysis to determine if the timing of vaccinations and intervals between vaccinations are sufficient for the birds to develop adequate immune protection against challenge for each virus.

The literature suggests that simultaneous viral infections may exhibit viral interference, in which one virus blocks the growth of another virus (48, 157). The mechanisms of interference have been proposed and involve immune response interactions (20, 62, 120), interference through viral proteins (177), and resource competition in which one virus blocks the other simply by being the first virus to infect the host cells (157). Relatively few experimental studies of simultaneous virus infections have been published, and fewer yet have been considered in the context of poultry viral respiratory pathogens. Costa-Hurtado et al. (43) demonstrated that co-

infection of chickens with a mesogenic strain of NDV and highly pathogenic avian influenza virus (HPAIV) affected HPAIV replication in chickens for 9 days but was dose-dependent.

Co-infections occur commonly in commercial poultry. It is feasible for a bird to be infected with multiple live vaccines despite intervals of weeks or even months between vaccinations, as live vaccine viruses have been known to persist in flocks. IBV vaccines have been detected in the respiratory tract up to 28 days post-vaccination (170), and IBV was isolated from tracheal and cloacal swabs collected at the point of lay and 19 WOA in hens that had been virus-negative for several weeks following recovery from inoculation at one day of age (32). In NDV B1-vaccinated chickens, vaccine virus was detected 14 days post-inoculation (71). Viral DNA from ILTV CEO-vaccinated chickens was detected in the conjunctiva and trachea at 10 and as late as 14 days post-vaccination (dpv) (168). In addition, intermittent shedding in the trachea from ILTV-immunized chickens occurred between 7 and 14 weeks post-vaccination (102). There is ample evidence of vaccine virus persistence in vaccinated flocks, yet it is unknown whether immunity to individual vaccine viruses is compromised when birds are serially or simultaneously administered multiple live vaccines targeting different viral respiratory tract pathogens.

Infectious Bronchitis Virus: Overview

Infectious bronchitis virus (IBV) is an upper respiratory tract viral pathogen of poultry that causes significant economic losses in poultry worldwide, leading to drops in egg production, stunted growth, and secondary bacterial infection resulting in condemnations at the processing plant (32).

IBV is spread by aerosol and mechanical vectors and has an incubation period of 18-36 h (32). IBV replicates in the respiratory epithelial cells, but certain strains also replicate in the

kidney, oviduct, and sometimes other organs including the gastrointestinal tract (104). Infected chickens develop respiratory signs (sneezing, rales, periorbital edema, and nasal discharge), depression, and inappetence. Some strains are also nephropathogenic, and renal damage often leads to mortality and severe dehydration (104, 161). Signs of IBV infection in layers also include decreased egg production and quality, characterized by soft-shelled, misshapen, or rough-shelled eggs (44). Respiratory signs in uncomplicated cases persist for 5-7 days (161). Young chicks tend to be most susceptible to severe respiratory signs and mortality (161), and mortality can range between 0% and 82% depending on the virus strain, birds' age, immune status and complicating secondary pathogens (104).

IBV is a gammacoronavirus belonging to the order Nidovirales and is an enveloped, positive sense single-stranded RNA genome of 27.6 kb that replicates in the cytoplasm (202). The 5' two-thirds of the viral genome contains open reading frame (ORF) 1ab encoding for nonstructural replicase proteins (nsp) 2-16, which are required for RNA replication and transcription (202). The 3' one-third of the genome encodes the structural proteins and accessory genes 3a, 3b, 4b/intergenic region, 5a, and 5b (202). The structural proteins include the spike (S) glycoprotein, membrane (M) glycoprotein, envelope (E) protein, and the nucleocapsid (N) protein (31). The spike protein comprises the N-terminal S1 subunit, which forms the globular head and is responsible for attachment of IBV to the host cell, and the C-terminal S2 subunit, which mediates fusion of the virion with the cell membrane (202). Fusion of the viral envelope with the cellular membrane occurs after endocytosis when the acidic environment of the endosome triggers conformational changes in the S protein (36). The spike protein is responsible for the development of neutralizing antibodies in the host (103). Meanwhile, the N protein as well as the spike protein have been implicated in the induction of cell-mediated immunity (173).

The spike protein is highly variable, evading immune recognition by mutation and genetic recombination, particularly in the hypervariable regions of the S gene (35).

Infectious Bronchitis Virus: Innate Immunity

The first changes to occur upon IBV infection are hyperplasia of goblet cells and alveolar mucous glands, which results in seromucous nasal discharge and catarrhal exudates in the trachea (137). Infection with virulent virus may cause loss of cilia, epithelial degenerative changes, and depletion of goblet cells and alveolar mucus glands, and the ensuing damage to the respiratory tract activates other immunological components (137).

Pattern recognition receptors (PRRs) are one of the first components of the immune system to be activated in the innate immune response, and they recognize conserved sequences on pathogens. PRRs are ubiquitous, located on dendritic cells, macrophages, lymphocytes, endothelial cells, mucosal cells, and fibroblasts and include the toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs). TLR3 is located in the endosome and recognizes intermediate double-stranded RNA (199). TLR3 mRNA expression was reported to increase 3 days post-infection in chickens infected with the IBV-M41 strain (199), and other reports documented the involvement of TLR3 in the immune response to IBV infection (88, 109). Elevated expression of TLR1 LA, TLR1 LB, TLR2, TLR3, and TLR7 in tracheal epithelial cells has been demonstrated in three-week-old chickens vaccinated with attenuated IBV-Mass by intranasal route (88). Treatment of 18-day-old embryos with deoxynucleotides containing CpG motifs (a potent stimulator of TLR21) prior to infection with IBV led to reduced viral load. Increased expression of melanoma differentiation associated protein 5 (MDA5) has been associated with IBV infection (38, 111).

Heterophils are one of the primary phagocytic cells recruited to the inflammation site during initial infection (137, 162). At least two studies reported a large increase in heterophil infiltration in respiratory lavage fluid during IBV infection from 24 to 72 hours post-infection (75, 88). However, heterophils are not believed to play an important role in innate immunity to IBV infection because heterophils did not reduce virus replication in the trachea (162). Whether the epithelial damage is exacerbated by heterophil migration remains to be clarified. Raj and Savage (162) reported that heterophils increased the severity of lesions and nasal discharge in IBV-infected birds, but Fulton, Thacker, Reed and DeNicola (76) indicated no correlation between heterophils and epithelial damage.

Macrophages are also important phagocytes involved in IBV infection but are present in much lower numbers than heterophils following infection (75). In contrast to mammals, fewer free-residing macrophages line the avian respiratory tract; consequently, phagocyte infiltration upon infection is believed to play an important role in the disease response (191). Following infection with IBV-M41 in chickens, macrophage numbers in the tracheal and bronchial lumen increased between 24 and 96 hours post-infection (75). IBV-Conn infection in chickens also led to macrophage infiltration 24 hours post-infection (109). One study documented an upregulation in gene expression of the following monocyte and macrophage signaling molecules in IBV-Mass-infected chicks: Spi-1/PU.1, GMF-B, M-CSFR (88). Spi-1 induces macrophage differentiation; GMF stimulates NF-kB, GM-CSF, and D4-/$CD8^+$ cell differentiation; and M-CSF is important for macrophage linage development (35).

In mammals, dendritic cells (DCs) are well-known to be the primary cell type responsible for priming naïve T cells to respond to a pathogen (14), but it is unclear which cell type is involved in naïve T cell priming in the chicken or whether these cells differ functionally in their

priming capacity (53). In contrast to macrophages, DCs have low levels of lysosomal proteases. Thus, antigen uptake results in decreased phagosomal acidification which does not fully destroy the antigenic peptides and enables the high antigen-presenting capacity of dendritic cells (163). de Geus and Vervelde (53) distinguished two cell types in the chicken lung based on the ability to decrease the pH of the endosomal compartment and anatomic location, which provides evidence that chicken DCs and macrophages are distinct but whether these two cells differ functionally remains to be explored.

Natural killer (NK) cells are cytotoxic lymphocytes involved in early defense against infection and are present in very low numbers (0.5% to 1.0%) in spleen and peripheral blood in birds compared to mammals (85). To date, few studies have examined the role of NK cells. Wakenell, Sharma and Slocombe (198) showed that the IBV-Mass Holland strain did not alter NK cell activity. On the other hand, Vervelde, Matthijs, van Haarlem, de Wit and Jansen (196) demonstrated that IBV-M41-infected chickens experienced rapid NK cell activation in lung and blood. These results indicate differential NK cell activation in response to a vaccine strain versus a virulent strain.

Cytokines are inflammatory mediators which regulate the immune system by binding to cell receptors and initiating downstream pathways that result in specific immune cell functions (35). IBV infection leads to interferon production in trachea, lung, plasma, kidney, liver, and spleen (35). Infection with attenuated IBV-Mass induced gene expression of IL-1B and common cytokine receptor g in tracheal tissue to bind IL-2, IL-3, IL-7, IL-9, and IL-21 (88). Distinct genetic lines have been implicated in the differential immune response to nephropathogenic IBV infection (12). While both genetic lines showed elevated IL-6 four days post-infection, IL-6 in the kidney and serum was twenty times higher and three times higher, respectively, in the

susceptible line compared to the resilient line. Jang, Koo, Jeon, Lee, Mo and Lee (106) demonstrated that infection with IBV strains of different genotypes (KIIa and ChVI) yielded differences in mRNA expression of pro-inflammatory cytokines (IL-6 and IL-1B) and lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor (LITAF), with the KIIa genotype associated with greater viral load and pro-inflammatory cytokine gene expression at 7 dpi in trachea and 9 dpi in kidney, contributing to enhanced disease. IL-6 and IL-1B upregulation associated with tracheal lesions and high viral loads has been further confirmed at 3 dpi in IBV-M41-infected chickens (145). In another study, relative expression of IFN-γ and IL-1β mRNA was downregulated during initial infection with IBV-Conn, but IL-1β expression increased dramatically throughout the infection (Kameka et al., 2014). Transcriptomic analysis of the chicken kidney post-infection revealed increased expression of IL-6, IL-18, IL-10RA, IL-17RA, CCL4, CCL20, CCL17, CCL19 and decreased expression of CXCL12 (38). Pei, Sekellick, Marcus, Choi and Collisson (156) demonstrated that chicken IFN type I inhibits IBV replication (Beaudette strain) *in vitro* and partially protects chicks from IBV infection. Finally, IFN-γ upregulation has been implicated in the immune response to IBV. IBV infection was shown to activate IFN-γ production in leukocytes, and stimulation was reduced when IBV was inactivated but still elevated compared with uninfected cells (11). This observation is another example of the differential immune response to inactivated virus compared to attenuated or virulent virus. Other reports indicate an increased expression of IFN-γ in lungs and PBMCS (196) and trachea (145) after infection of chickens with IBV-M41.

Chemokines mediate cell migration during the immune response. Vaccination with attenuated IBV-Mass resulted in increased expression of CXCR4, CCR6, and chemokine-like receptor 1/CHEMR23 in tracheas, including integrin β2 (CD18) and matrix metalloproteinase

(MMPs), which are involved in immune cell recruitment (88). Dar, Tikoo, Potter, Babiuk, Townsend, Gerdts and Mutwiri (49) demonstrated that pre-treating embryos with CpG ODN was associated with inhibition of IBV replication in the lung tissue and coincided with suppression of IL-6 and upregulation of IFN-γ, IL-8, and MIP-1β gene expression.

Apoptosis is another mechanism of the early immune response to IBV infection and reduces viral replication by inducing rapid cell death after a target cell is infected. Reports have shown that IBV Beaudette induces cell cycle arrest and apoptosis in infected Vero cells (119, 121). The specific mechanisms of IBV-induced apoptosis have been described and involve proapoptotic (e.g., Bax and Bak) and anti-apoptotic (e.g., Bcl-2 and Bcl-XL) proteins (119, 121, 209).

Infectious Bronchitis Virus: Adaptive Immunity

The adaptive immune response is an essential component of the host response to IBV infection and is responsible for eliminating the virus and establishing immunological memory capable of generating an appropriate immune response upon subsequent infection. The involvement of both humoral and cellular immunity in IBV infection in chickens has been investigated.

A cell-mediated immune response is key to developing host immunity to IBV infection with either an IBV vaccine or virulent strain. Cytotoxic T lymphocyte (CTL) responses in chickens following IBV infection have been correlated with early decreases in infection and clinical signs (156, 174). Further studies using adoptive transfer of IBV-primed lymphocytes indicated that the cell types responsible for early viral clearance were $CD8^+$ αβ T lymphocytes (174). Seo and Collisson (173) also discovered that chickens infected with the nephropathogenic IBV-Gray strain showed increased antigen-specific CTL activity at 3 dpi, which peaked at 10

dpi. Experiments conducted by Collisson, Pei, Dzielawa and Seo (37) demonstrated that major histocompatibility complex (MHC)-restricted CTLs are critical in controlling IBV infection in poultry.

Little research has focused on the possible role of $CD4^+$ T cells in mediating IBV clearance and resolution of clinical disease. While adoptively transferred $CD4^+$ T cells alone do not appear to be important in resolving IBV infection in chickens (Seo et al., 2000; Pei et al., 2003), it cannot be ruled out that $CD4^+$ T cells are not important to IBV immunity. $CD4^+$ T cells have been shown to be important in controlling virus infections (208). While $CD4^+$ T cells acting alone may not resolve viral infection, it is possible that these cells may act synergistically with $CD8^+$ T cells by secreting effector molecules that delay viral replication to allow sufficient activation and proliferation of effector $CD8^+$ T cells that ultimately clear virus (183, 208). Considering the role of memory $CD4^+$ T cells in the immune response to IBV infection may be of interest because memory $CD4^+$ T cells have advantages compared to memory $CD8^+$ T cells in that they are longer lived in some settings and are more polyclonal (182). Furthermore, $CD4^+$ T cell epitopes are less susceptible to immune escape than $CD8^+$ T cell epitopes, which may be explained in part by the indirect mechanisms of $CD4^+$ T cell function in targeting virally infected cells (94). An example of a SARS-CoV-specific $CD4^+$ T cell epitope conferring cross-protective immunity in SARS-CoV and MERS-CoV has been reported (208). Given these characteristics, investigating the potential role of $CD4^+$ T cells in IBV immunity may prove useful when designing immunization strategies to develop long-lasting, T cell-mediated immune responses to IBV.

T cell responses have been categorized into three stages: (1) activation and expansion, (2) death, and (3) memory (1), and initial studies investigating the CTL response in chickens

following IBV infection concentrated on effector T lymphocytes collected from inoculated chicks at 10 dpi during the peak of the activation and expansion stage following infection (37, 155, 156, 173, 174). Memory T cells, on the other hand, develop following resolution of infection and have been identified in chickens three weeks following infection (155). In mammals, memory T cells are present for many years and reach detectable levels by 4 weeks following infection with lymphocytic choriomeningitis virus (1). In chickens, however, it is unclear how long memory T cells are present in the absence of antigenic stimulation. Pei, Briles and Collisson (155) demonstrated that IBV-specific memory T cells could be detected in PBMCs beginning at 3 weeks following IBV infection and lasted for at least 10 weeks post-infection. The authors demonstrated that IBV-specific $CD8^+$ memory T cells generated at 3 to 6 weeks, but not 2 weeks, post-infection protected chicks from acute IBV infection. Annexin-V and propidium iodide staining revealed that a larger percentage of T cells generated at 2 weeks post-infection were apoptotic compared to T cells from uninfected controls or at 10 dpi, suggesting that by 2 weeks following infection they were losing their effector functions. It was further postulated that by 2 weeks memory T cells had not yet become established, and this notion has been supported in murine studies (1). The role of memory $CD4^+$ and $CD8^+$ T cells in protective immunity in mice infected with severe acute respiratory syndrome (SARS) coronavirus has been documented in other studies (34, 208).

To date, a majority of the research involving the immune response to infection with IBV vaccine strains and virulent strains has concentrated on humoral immunity. It is well known that IBV infection stimulates a humoral response in chickens (161). Although circulating antibody titers do not correlate with resistance to infection (160), the humoral response is important for clinical disease resolution and virus clearance because IBV-infected bursectomized chicks

experienced more severe, protracted disease than intact chicks, and virus titers were elevated and present longer in bursectomized chicks (39, 40). Chandra (33) further attributed the effect diminished antibody production via chemical bursectomy to more severe clinical signs and renal lesions coinciding with delayed IBV clearance. The effect of high antibody titer on virus clearance has been substantiated in studies reporting the absence of virus re-isolation from kidneys and genital tract (86) and protection against a reduced egg production (22).

IBV-specific neutralizing antibodies are important in preventing IBV re-infection (88, 100) and are discussed further in "Infectious Bronchitis Virus: Mucosal Immunity."

Infectious Bronchitis Virus: Mucosal Immunity

Since IBV first enters the host via the respiratory tract, mucosal immunity is paramount to inhibiting viral replication and reducing clinical disease. Mucosal vaccination can induce both systemic and local immunity, but systemic immunization generally is not sufficient to elicit strong mucosal immunity (35). It is believed that mucosal immunity is stimulated upon direct interaction of target cells with virus (29). Vaccine studies support this notion because live attenuated virus protected chickens from disease only when administered locally (30, 173). During primary exposure to IBV, the cells primarily responsible for IBV immunity are the local cytotoxic T lymphocytes (173), while the mucosal antibody response is believed to mediate IBV clearance upon subsequent exposure (88). Upon primary IBV immunization of three-week-old chickens with IBV-Mass, local innate immune factors activated in tracheal tissues included TLRs (i.e., TLR3, TLR7, and type 1 TLR2/6), RIG-I, type I interferons, complements, and IL-1β (88). These innate responses coincided with or immediately preceded a Th1-biased, cell-mediated adaptive immune response, characterized by the induction of T-cell signaling molecules, surface markers, and effector molecules. Following the secondary immunization, a

strong humoral immune response driven by upregulated immunoglobulin gamma chain occurred in the absence of innate, Th1 adaptive immunity, or IgA upregulation. These findings imply that the local memory response in the trachea is dominated by IgG. The results of this study agree with previous findings that IgG is a dominant antibody involved in the mucosal immune response to IBV.

It should be noted that the aforementioned study examined the local immune response in tracheal tissues. Another component involved in the mucosal immune response to IBV infection is the paraocular immune system, which is connected to the mouth by the nasolacrimal duct (15) and comprises the conjunctiva associated lymphoid tissue (CALT), nasal associated lymphoid tissue (NALT), and Harderian gland (HG) (72). The HG contains IgA-, IgG-, and IgM-expressing plasma cells (13, 143). IgA is locally synthesized in the HG, and IgG is both synthesized and transduced to the respiratory tract from systemic circulation (13, 50, 187). Immunoglobulin (Ig)-secreting plasma cells in the HG are the main source of antibodies in tears because removal of the HG eliminated IgM and IgA and reduced IgG titers in the tears (172). In addition to Ig-secreting plasma cells, the HG also contains macrophages, lymphocytes, dendritic cells, and heterophils (18, 147).

The type, proportion, and anatomical location of immune cells in the chicken is believed to orchestrate the type of predominating immune response (53, 142, 143). Several studies have examined the properties of Ig-secreting cells in the upper respiratory tract, but results have been varied and may have been affected by tissue type, bird age, genetic line, host immune status, and infection status. Nevertheless, it is worth considering the results of these studies to gain insight into the role of local antibody-secreting cells in the chicken.

Several studies report that IgG-containing cells in the upper respiratory tract were more frequent than IgM-or IgA-containing cells (142, 143). However, there is conflicting evidence concerning the proportion and immunological function of IgG- and IgA-expressing cells in the respiratory tract. One study documented that IgG- and IgA-positive cells formed the bulk of the lymphoid cell population in 4- to 9-week-old chickens, but as the birds aged, IgA emerged as the predominant class (4). Kuper et al. (115) demonstrated that IgA-expressing cells were rare in the nasal associated lymphoid tissue (NALT) but were not in bronchial associated lymphoid tissue (BALT), suggesting that immunoglobulin-expressing populations may vary based on anatomical location. A different study contradicts these findings, reporting many IgM- and IgA-positive plasma cells and very few IgG-positive cells in Harderian gland of 8- and 10-week-old chickens (148). In yet another study, large numbers of IgA-containing cells were present in the Harderian gland in chickens during the acute stage of mild Newcastle disease (153, 154). Supporting the notion that mucosal stimulation may initiate B-cell isotype switching and differentiation, Ohshima and Hiramatsu (142) discovered that intranasal immunization of mice with cholera toxin (CT) resulted in CT-specific IgA antibody-forming cells in the NALT.

It is worth considering that most of the previous studies did not assess the degree of activation of Ig-expressing cells and that the proportion and number of B lymphocytes in the mucosal tissues may be potentially less important than their degree of activation. One group addressed this possibility by comparing the immunoreactivity of these cells as characterized by periodic acid-Schiff (PAS)-positive Russell bodies (RB) identified in immunohistochemistry (143). The results showed that RB-containing cells positive for IgA were the most frequent, followed by those positive for IgM, and the rarest cell type were IgG-expressing cells. This

observation implies that IgA-containing cells may play an important role in the immune response in the HG even if they occur less frequently than IgG- and IgM-expressing cells (142, 143).

In addition to the studies on Ig-secreting cells in the HG, there have been several reports investigating the role of antibody in tears in the context of IBV immunity. An IBV challenge study performed by Toro and Fernandez (185) substantiated the role of IgA in the protection of mucosal surfaces by demonstrating that IBV-specific IgA titers in tears were associated with protection from IBV infection. At least two studies have shown that mucosal IgA levels are greater than mucosal IgG levels (13, 186). However, a lack of a significant increase in the IgA titers among vaccinated birds after challenge has been described (88, 108), which suggests that local antibody responses may be dominated by another antibody class. In fact, some researchers have proposed that IgG may play a role in mucosal immune responses to IBV, and Orr-Burks, Gulley, Gallardo, Toro and van Ginkel (150) demonstrated both IBV-specific IgG and IgA titers in tears have been detected following primary IBV exposure. Notably, after secondary exposure, significant IgA titers in tears were only detected at 1 day post-inoculation whereas significant IgG titers in tears were sustained for at least 14 days. In summary, IBV exposure stimulates both IgA and IgG antibody secretion into the tears, and although IgA antibodies in tears have been associated with protection against IBV, the role of IgG antibodies in IBV immunity has yet to be established.

Infectious Bronchitis Virus: Vaccination Strategies

Traditional vaccination protocols rely most commonly on initial mass vaccination of one to three serotypes of live, attenuated IBV at 1 DOA to stimulate the mucosal and systemic immune responses, leading to the development of neutralizing antibodies which protect against re-infection with IBV. Live vaccines are generally administered to young birds to achieve early

protection, and layers and breeders are also boosted with either live or inactivated vaccines (55). The vaccine strains used in poultry production vary based on their similarity to the circulating field viruses (55), but this strategy is generally effective only when chickens are exposed to homologous field challenge. Unfortunately, protection is generally poor when chickens are exposed to a heterologous strain, as serum antibodies do not cross-protect against different IBV serotypes (160). The threat of heterologous field outbreaks necessitates the development of vaccination strategies that confer cross-protective immunity.

The "protectotype" vaccination strategy is well known to induce a larger spectrum of cross-protection against heterologous IBV challenge (41, 184). Smialek, Tykalowski, Dziewulska, Stenzel and Koncicki (180) investigated the immunological aspects involved in the protectotype vaccination strategy and demonstrated that chickens vaccinated simultaneously with the Ma5 and 4/91 strains developed the greatest level of $CD8^+$ T cell stimulation in the spleen and HG, as well as a high level of IgA and IgG in upper respiratory tract washings and serum. Further studies mapping specific epitopes of these protectotype vaccine strains to B and T lymphocytes may prove useful in understanding the mechanisms by which particular vaccine strains used in protectotype vaccination strategies provide cross-protection from IBV infection.

Vectored vaccines have been investigated as a potential alternative to traditional IBV vaccines. Currently, there are no commercially available vectored vaccines to protect chickens against IBV infection. However, several studies have reported varying degrees of efficacy using recombinant fowlpox virus (FPV) (200, 204), adenovirus (AdV) (189, 205, 206), and NDV (190) vectors.

Yu et al. (204) evaluated protective immunity induced by the recombinant FPV expressing the carboxy (C)-terminal region of the nucleocapsid (N) protein, following reports of

CTL activity against the C-terminal region of the N protein (173, 181). The N protein of IBV strains is highly conserved among various IBV strains and has highly conserved regions (204). Vaccinated chickens were protected against some, but not all, heterologous IBV strains, and the authors speculated that there might have been amino acid differences between the C-terminal region of the N protein from heterologous strains, the S1 protein may have been additionally required for protection, or that the increased virulence of the H4 strain may have overcome the level of protection.

Wang et al. (200) assessed the protective efficacy of a recombinant FPV-vectored vaccine expressing IBV S1 compared to a rFPV vaccine co-expressing IBV S1 and chicken IFN-γ, important for activating macrophages, which are important to enhancing humoral and cellular immunity (140). 84-week-old SPF chickens were immunized via wing web injection with either rFPV/IBVS1 or rFPV/IBVS1-ChIFNγ. In terms of the duration of IBV shedding and pathological lesions, the greatest protection against IBV LX4 virus challenge occurred in the rFPV-IBVS1-IFNγ group, followed by the group vaccinated with live IBV vaccine, and finally by the rFPV/IBVS1 group. Chickens vaccinated with rFPV/IBVS1-IFNγ showed enhanced $CD8^+$ T lymphocyte proliferation in peripheral blood compare to the birds vaccinated with the rFPV/IBVS1. In addition, the group vaccinated with rFPV/IBVS1-IFNγ exhibited a shorter time period of pathological changes in the kidney. However, local challenge virus detection was not suppressed in either of the rFPV-vaccinated groups. One limitation to this study was that viral load was not quantified, and therefore it is unknown whether virus shedding was diminished. This study highlights the potential to enhance protection from clinical signs and pathological changes by co-expressing immunostimulatory molecules.

AdV-vectored vaccines have also been considered and reveal augmentation of humoral and cellular immune responses in chickens (189, 205, 206). Zeshan et al. (206) demonstrated that chickens vaccinated *in ovo* followed by an intramuscular inoculation at 15 DOA showed fewer nephropathogenic lesions and less severe clinical signs when challenged with a nephropathogenic IBV strain at 29 DOA. Chickens receiving the *in ovo* vaccination and boost were completely protected from mortality, while birds receiving the *in ovo* vaccination only suffered 15% mortality. rAdV/IBVS1 and rAdV/IBVS1-boost vaccinations conferred partial protection, as measured by viral detection in the kidney: 15% and 30%, respectively. A later study conducted by the same group (205) demonstrated an enhanced protective effect of immunizing chickens with a rFPV/IBVS1 co-expressing or co-administered with granulocyte-macrophage colony stimulating factor (GM-CSF). These two studies suggest that AdV may be a promising candidate as a vector expressing IBV antigens and immunostimulatory molecules.

Toro et al. (189) also used a recombinant AdV vector to evaluate the immune protection elicited by three distinct ArkDPI S1 proteins derived from subpopulations within an ArkDPI vaccine. SPF white leghorns were vaccinated intramuscularly at 7 DOA or vaccinated and boosted ocularly at 22 DOA. Virulent Ark challenge was administered ocularly and intranasally at 43 DOA. Surprisingly, ocular boosting did not improve protection against respiratory signs or reduce viral shedding in the tears compared to the vaccination only group. Another finding was that two rAdV/IBVS1 vaccines offered good immune protection, but the third conferred the least protection. Sequence comparisons reveal that all three S1 proteins had >95% homology, however the S1 protein from the poorly protecting vaccine differed only at amino acid position 56. This observation suggests that selection of an S1 sequence as a vaccine candidate should not be determined based only on percent sequence identity with field strains because even a change of

one amino acid might result in reduced protection. Consequently, the sequence variability of the S1 protein presents an ever-shifting challenge when designing S1-based vaccines against IBV challenge.

Toro et al. (190) also investigated the potential for immune protection using IBV S2 protein from ArkDPI expressed from recombinant NDV LaSota (LS) strain. Unlike the inherently variable S1 protein, the S2 protein is highly conserved in different IBV serotypes (116). NDV LaSota was selected based on its safety, worldwide use as a poultry vaccine, stability (rare recombination events), ease of mass administration, and induction of strong local and systemic immune responses. The study revealed significantly diminished clinical signs and viral load in chickens vaccinated with rLS/IBVS2 by eye drop at 4 DOA and boosted at 18 days with an attenuated Mass-type vaccine and challenged with a virulent Ark isolate. As previous studies using other vectors have implicated, recombinant NDV may also serve as a vector for IBV antigen delivery and confer good protection.

At least one study has evaluated the protection conferred by a recombinant MDV (CVI988/Rispens) expressing the IBV S1 protein (rMDV-S1) (207). One-day-old SPF white leghorns were vaccinated subcutaneously with rMDV-S1 were protected from challenge 4 weeks later with a QX-like IBV, as demonstrated by mild clinical signs, a reduced virus-shedding period, and low mortality.

The major challenges of successful viral-vectored vaccine development against IBV infection have yet to be overcome. The incomplete protection from IBV in these studies may explain why no commercially available viral-vectored vaccines against IBV exist for poultry. First, protection from heterologous challenge may be incomplete. Yu et al. (204) showed that the N protein induced a degree of cross-protection following heterologous challenge with one IBV

strain, but that it was insufficient to protect birds against challenge with a different heterologous strain. Second, studies involving the S1 protein (189) showed promise as potential vaccines, but the inherent variability of the S1 protein restricts the efficacy of recombinant vaccines expressing S1 protein to a very limited range of protection. Additionally, immune protection based on sequence identity has been proven to be unpredictable. Addressing the issue of sequence variability by using a highly conserved protein, such as S2, appears to also provide good protection against homologous IBV challenge, but heterologous protection has not yet been evaluated. In summary, recent efforts to develop an IBV vaccine that confers cross-protective immunity are encouraging but require further study.

Newcastle Diseases Virus: Overview

Newcastle disease (ND) is a highly infectious viral disease affecting both wild birds and domestic poultry. ND belongs to the genus *Avulavirus*, the subfamily *Paramyxovirinae*, and the family *Paramyxoviridae* (144), and the disease is caused by virulent strains of avian paramyxovirus type 1 (APMV-1), which has recently been reclassified as avian avulavirus 1 (AAvV-1) (7). NDV strains are characterized as lentogenic, mesogenic, and velogenic (viscerotropic or neurotropic), according to their mean death time in embryos (93). Lentogenic strains are of low pathogenicity causing mild respiratory or enteric infections, followed by mesogenic strains, while velogenic isolates are highly pathogenic often causing neurological signs and mortality (130). Virulent strains must demonstrate an intracerebral pathogenicity index score in day-old chicks of at least 0.7 or the virus must have multi-basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 (144). ND is an OIE (Office International des Epizootics) designated disease (144) and a USDA select agent (24), and

virulent strains of NDV are subject to international trade regulations, control measures, and policies.

NDV is transmitted primarily through aerosol or fecal-oral routes. The incubation period ranges from 2 to 15 days post-exposure, and virus can be shed for up to 2 weeks following infection in gallinaceous birds (chickens, turkeys, grouse, pheasants, and partridges) and several months to one year in psittacine birds (parrots, parakeets, and macaws) (65, 71). In birds infected with mild NDV strains, clinical signs may be absent or may involve depression, inappetence, respiratory signs (nasal discharge, sneezing, coughing), and reduced egg production (130). Infection with viscerotropic velogenic NDV often results in hemorrhagic intestinal lesions, whereas neurotropic velogenic NDV typically produces respiratory and neurological signs (tremors, ataxia, torticollis, paresis or paralysis of the wings or legs) (24).

NDV is a pleomorphic enveloped virus and has a non-segmented, negative sense, single-stranded RNA genome and is reviewed in Ganar, Das, Sinha and Kumar (78). The genome consists of a leader at the 3' end and trailer at the 5' end, which flank genes coding for nucleocapsid (N), matrix protein (M), phosphoprotein (P), fusion protein (F), haemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). The P protein encodes three proteins (P, V, and W) via RNA editing. The N protein interacts with genomic RNA, P and L proteins to form the ribonucleoprotein complex. F and HN surface glycoproteins contain epitopes for virus and neutralizing antibodies and form trimers and tetramers, respectively. The V and W proteins are accessory proteins, and the V protein has been implicated in NDV virulence through interferon (IFN) antagonism.

Newcastle Disease Virus: Immunity

The innate immune response is activated following NDV infection and quickly controls and inhibits virus spread while assisting the adaptive immune response in developing virus-specific responses that will eventually eliminate the virus and establish protection from re-infection. Pathogen-associated molecular patterns (PAMPs) on the virus are recognized by PRRs, which include toll-like receptors (TLR), nucleotide-binding oligomerization domain proteins (NOD), retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and C-type lectins. The binding of the virus by these host sentinel proteins leads to the production of interferon and cytokines, which limits virus replication via multiple mechanisms. Some key innate responses include NO induction in heterophils and peripheral blood mononuclear cells (PBMCs), IFN-α and IFN-β mRNA detection in macrophages, and IFN-y production in PBMCs. In addition, IRF-3 and IRF-7 have been implicated in resistance to NDV infection. Infection with velogenic NDV, compared to lentogenic LaSota, has been associated with an enhanced pro-inflammatory immune responses in the host via the production of INF-α, IFN-γ, IL-6, and IL-1β (110).

Infection with NDV also stimulates a humoral response. Both local and systemic antibodies are first detectable within 6-10 days of infection with NDV vaccine or pathogenic virus and peak at 21-28 days post-infection (2). Antibodies from all three classes (IgM, IgG, and IgA) are detected in respiratory tissues following NDV exposure and are involved in virus neutralization following re-exposure to NDV (2). Maternal antibodies interfere with live vaccine virus (82).

Cell-mediated immunity (CMI) is an important component of the host immune response to NDV (110). The principal cells involved in CMI are the cytotoxic $CD8^+$ and helper $CD4^+$ T

lymphocytes. Live vaccines stimulate both $CD8^+$ and $CD4^+$ T cell responses, whereas inactivated vaccines mainly stimulate $CD4^+$ cells through cytokine responses, and immune responses to inactivated vaccines take longer to develop and are not as robust (117). The virulence of the vaccine strain has also been implicated in the degree of stimulation of the cell-mediated immune response. Rauw et al. (164) showed that a more virulent vaccine strain induced a longer lasting and more robust CMI in chickens compared to a less virulent vaccine strain. The role of CMI in NDV immunity has been investigated, and a study by Reynolds and Maraqa (166) demonstrated that CMI specific for NDV, determined by blastogenesis microarray, is not enough to confer resistance to NDV infection and that neutralizing antibodies are required for protection.

Newcastle Disease Virus: Vaccination Strategies

ND vaccination regimes vary and may utilize a combination of live, inactivated, and virus-vectored vaccines (61). A common strategy is to vaccinate either one-day-old chicks or chicks at 2-4 WOA with a live vaccine, followed by a live booster at 2-4 weeks and again at 10 weeks (130). In the United States, the most widely used traditional vaccine strains comprise lentogenic B1 (or virus clones of the B1 strain) and LaSota strains (130), which have been used since the 1940s. In addition, other live attenuated NDV vaccines have been developed and are avirulent, lentogenic, or mesogenic (130). Live NDV vaccines are typically applied by mass vaccination techniques by coarse spray or drinking water. Although inactivated vaccines are expensive to produce and administer (they require individual administration), they are often used in layers and breeders because they stimulate high antibody titers that last longer, which is important for the passage of maternally derived antibodies to their offspring.

Advances in molecular biology and vaccine technology have led to a new era of NDV vaccine development. Multiple viral-vectored vaccines have been produced and are

commercially available for *in ovo* injection. Advantages of vectored vaccines are that they may not require adjuvants, can be administered via mass vaccination, induce both systemic and local immunity and strong cell-mediated responses (61). However, the onset of protection usually occurs later, requiring 4-5 weeks to achieve protective immunity (61).

One commercially available recombinant poultry vaccine is the fowlpox virus (FPV) vector-based vaccine expressing the NDV F or HN protein. However, the FPV-vectored vaccine is not widely used because it cannot be applied through mass application (61) and maternal antibodies interfere with vaccination (70).

Commercially available recombinant poultry vaccines include HVT-vectored vaccines expressing the F protein. The F protein is required for fusion of the viral envelope with plasma membrane of the host cells and is essential for cell-to-cell spread of infection (135). While HN protein, important for virus binding to host cell receptors (135), also has been expressed in HVT-vectored vaccines, F protein is the preferred immunogenic antigen as it has been shown to confer enhanced protective immunity compared to protection induced by HN protein expression (135), as measured by clinical signs and time of disease onset. Whether this discrepancy is explained by the differences in antigen expression *in vivo* or biologically relevant differences in neutralizing a protein required for virus binding (HN) versus cell-to-cell spread (F) remains to be understood, as both of these proteins have been shown to elicit neutralizing antibodies (135).

HVT/NDV-F-vectored vaccines establish both humoral and cell-mediated immunity (152). Cell-mediated immunity has been shown to be important in protection against ND (97). It is likely that rHVT-vectored vaccines also stimulate cell-mediated immunity, which has been demonstrated in HVT-induced immunity against Marek's disease (66). Heller and Schat (98)

hypothesized that the cell-mediated immune response following HVT exposure is explained by the tropism of HVT, which is highly cell-associated and replicates in lymphocytes.

Establishing an early onset of protection is critical for protection against NDV challenge. Immune protection provided by live ND vaccines has been demonstrated as early as five to seven dpv (134), but HVT/NDV vectored vaccines appear to induce protective immunity more slowly than that observed in conventionally vaccinated chickens (135). Morgan et al. (134) demonstrated that protection occurred between 14 and 21 dpv in rHVT/NDV-F-vaccinated chickens at one day of age, compared to birds vaccinated with Hitchner B1 (6 dpv) and inactivated NDV (14 dpv). While rHVT/NDV-F-vaccinated chickens were partially protected against challenge on 10 and 14 dpv, they were fully protected by 21 dpv. Two other studies report similar timing in the onset of protection, and reveal that protection is complete between three and four weeks post-vaccination in chickens vaccinated with rHVT/NDV-F *in ovo* or posthatch (66, 152). Heckert et al. (97) demonstrated earlier protection from NDV in SPF chickens vaccinated subcutaneously at one day of age with recombinant HVT expressing both F and HN proteins. Challenge administered at 4, 7, 10, and 14 dpv with lethal neurotropic GB Texas strain yielded protection levels of 0%, 35-75%, 85%, 94-100%, respectively. Possible explanations for the earlier protection as seen in this study include simultaneous expression of two immunogenic antigens, subcutaneous versus intra-abdominal immunization route, different vaccine dosages, and/or different protein expression levels in the constructs.

In young chicks vaccinated against NDV, possible interference of maternally derived antibodies (MDA) with the efficacy of the rHVT/NDV-F-vectored vaccine is a concern. A study by Morgan et al. (134) showed that although both rHVT/NDV-F and B1 vaccines protected broilers from neurotropic ND clinical signs, the birds immunized with rHVT/NDV-F were

poorly protected from viral replication in the trachea. rHVT/NDV-F-vaccinated broilers were only 20% protected (compared to 50% of leghorns), while B1-vaccinated broilers were 90% protected (compared to 100% of leghorns). In summary, MDA interference with local immune responses appears to be exacerbated following immunization with recombinant HVT-vectored vaccines.

Vaccines establishing a long duration of protection are of great value to the industry because they potentially require only a single immunization to establish lifelong immunity. *In ovo* vaccination of chickens with a recombinant HVT expressing F and HN glycoproteins from an NDV B1 strain, as well as glycoproteins A and B of a serotype 1 MDV, yielded persistent infection for at least 8 WOA (165). Chickens challenged with lethal GB Texas strain showed very good protection at 4, 6 and 8 WOA regardless of vaccination with recombinant versus live B1 vaccine. Viral detection in the spleen revealed vaccine virus in birds that received the rHVT-vectored vaccine for up to 8 weeks post-vaccination, however virus was only detected until one week post-vaccination in B1-vaccinated birds. Esaki et al. (66) further demonstrated long-lived protection until at least 19 WOA and anti-NDV antibodies maintained through 50 WOA. It has been hypothesized that this long duration of protection may be due to the long duration of viremia established by HVT, lasting at least 40 weeks (27). This persistent viremia may provide constant immune stimulation.

Infectious Laryngotracheitis Virus: Overview

Infectious laryngotracheitis (ILT) is a highly contagious, economically important viral respiratory tract infection of poultry that may result in severe production losses due to increased mortality, reduced egg production, and decreased body weight gain. ILT is caused by *gallid*

herpesvirus I (GaHV-1), which belongs to the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae* (52).

Transmission of ILTV occurs through the upper respiratory and ocular routes, and less commonly via mechanical transmission (91). The incubation period of ILTV depends on the inoculation route and varies from 6 to 12 days after natural infection and from 2 to 4 days following experimental infection, and ILTV establishes latency in the trigeminal ganglion (74). Clinical manifestations of ILT include gasping, coughing, sneezing, depression, nasal discharge, expulsion of bloody mucus, and conjunctivitis (74). The severity of clinical signs is associated with the virulence of the ILTV strain, and mortality rates range from 0 to 70% (74). Production losses are attributed to increased mortality, reduced egg production, decreased body weight gain, and susceptibility to other respiratory pathogens (79).

ILTV has a linear, double-stranded DNA genome containing long (U_L) and short (U_S) unique regions and inverted-repeat sequences (IR, TR), which flank the U_S region (74). ILTV consists of 76 ORFs that encode proteins, and 11 of these proteins are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM (74).

Infectious Laryngotracheitis Virus: Immunity

The innate immune response to ILTV is not well known, however there are a few studies of chemokine and cytokine secretion. Lee et al. (118) demonstrated that ILTV infection led to an increase in the expression of the following proinflammatory molecules: IL-6, IL-8, CXC K60, CCL17, CCL20, CCL4, chemokine ah221, CXCL14, and IL-15. Purcell (158) explained the epithelial hyperplasia following ILTV infection by demonstrating the upregulation of genes involved in cell growth and proliferation and further identified the trafficking of numerous macrophages and lymphocytes to the site of infection.

ILTV infection leads to the production of virus-specific antibodies (99), but antibody titers do not correlate with resistance to ILTV infection (176). Evidence of the dominant role of CMI in ILTV immunity was demonstrated in bursectomized chickens (69). In this study, bursectomized chickens cleared primary infections as effectively as intact chickens, which provides evidence that humoral responses are not required in primary exposure to ILTV. In addition, protection of chickens from re-infection was demonstrated in bursectomized chicks vaccinated against ILTV, which effectively prevented the replication of challenge virus in the absence of mucosal antibody. Finally, ILTV susceptibility has been found to decline with bird age (68, 178).

Infectious Laryngotracheitis Virus: Vaccination Strategies

Vaccination programs combining live vaccines and vectored vaccines are increasingly common and have been reviewed in Garcia (79). Vaccination practices vary with the type of bird. Most vaccination regimes for light-weight broilers apply vectored vaccines to avoid vaccine reactions from CEO vaccination. Heavy-weight broilers are more often vaccinated with CEO vaccine or a combination of vector and CEO vaccines. Broiler breeders are usually vaccinated via eyedrop with the safer TCO vaccine either once or, in endemic areas, they may be vaccinated at 4-5 WOA with TCO vaccine and again at 10-12 WOA with the CEO vaccine in the drinking water. Most commercial layer flocks in the United States are initially immunized with a viral-vectored vaccine at 1 DOA, followed by eye-drop vaccination with CEO or TCO, or CEO administered in the drinking water between 8 and 12 WOA.

Live vaccines against ILT may be of chicken embryo origin (CEO) or tissue culture origin (TCO), in which they are passaged multiple times in eggs or tissue culture, respectively. The CEO vaccine is the most widely used vaccine worldwide. Live, attenuated ILTV vaccines,

in particular CEO vaccines, have been shown to regain virulence following several passages in chickens (90), and it is well known that the live vaccines can be transmitted from vaccinated to unvaccinated birds and become latent in clinically normal chickens (107). Although the conventional vaccines provide good protection when applied correctly and uniformly, protection in the field is diminished due to unrestricted vaccine use, poor flock vaccination coverage using mass application (107), insufficient biosecurity measures, and comorbidity. These events explain why ILTV outbreaks of vaccine origin are common (10) and highlight the importance of administering a safe vaccine characterized by a lack of transmission, lack of latent infections, and no reversion to virulence.

Commercial vaccination against ILTV is widely practiced using viral-vectored vaccines as a safe alternative to live, attenuated chicken embryo origin (CEO) or tissue culture origin (TCO) vaccines. Although viral-vectored vaccines reduce clinical signs upon ILTV infection, they do not prevent viral shedding and therefore confer only partial protection (79). Vectored vaccines are administered *in ovo* and fractionating the vaccine dose especially hinders the protective efficacy of the vaccine, which leads to a poorly vaccinated flock (63).

FPV and HVT are used in viral-vectored vaccines for protection against ILTV. The commercially available rFPV-LT vaccine consists of the gB gene, and the rHVT-LT vaccine comprises the glycoprotein D (gD) and gI genes, which were licensed for subcutaneous vaccination of one-day-old chickens but are typically applied *in ovo* (195). gD is a highly conserved herpesvirus structural glycoprotein (74) and functions as a receptor for virus binding to susceptible cells (60, 101). gI is important in cell-to-cell spread of the virus (57). gB is involved in fusion and entry into the host cell (42).

Immunity to ILTV requires 4-6 weeks to develop (195). Vagnozzi et al. (195) compared protection in chickens vaccinated with one of the four commercially available types of vaccines: live, attenuated CEO or TCO vaccines administered as full doses by eye drop at 14 DOA; and recombinant HVT-LT or FPV-LT administered as half doses *in ovo*. Protection induced by each of these vaccines was compared as well as protection among birds challenged at 35 DOA compared to 57 DOA. Following challenge at 35 and 57 days, HVT-LT-vaccinated chickens exhibited reduced clinical signs that were not statistically significant from the CEO- and TCO-vaccinated groups. However, high levels of virus were detected from the trachea in birds challenged at 35 days. In contrast, HVT-LT-vaccinated chickens challenged at 57 days had reduced viral shedding compared to chickens challenged at 35 days, which suggests that immunity to ILTV infection, as measured by viral shedding, may take over one month to develop.

The lack of protection from viral shedding suggests that viral-vectored vaccines do not induce strong local respiratory immune responses. This hypothesis was investigated by Gimeno et al. (83), who reported that expression of ILTV and HVT genes were more highly expressed in spleens and feather follicles than in the lungs, which may explain the poor protection from viral replication in the respiratory tract.

In an effort to reduce the risk of outbreaks caused by revertant ILTV strains while achieving complete protection against viral replication, researchers have explored the potential use of recombinant live attenuated ILTV vaccines with gene deletions. In particular, the glycoprotein G gene deleted strain (ΔgG) and ORF C gene deleted strain (ΔORFC) emerged as vaccine candidates due to their ability to grow in cell culture (58, 80). ILTV gG is a virulence factor that modulates the host adaptive immune response via viral chemokine binding (59). The

gene-deleted strains were less pathogenic than the parental strains and may be an alternative to traditional live, attenuated vaccines.

CHAPTER 3

EFFECT OF PULLET VACCINATION SCHEDULE ON DEVELOPMENT AND LONGEVITY OF IMMUNITY [1]

[1] E. J. Aston, B. J. Jordan, S. M. Williams, M. Garcia, M. W. Jackwood. To be submitted to *Avian Diseases*.

Abstract

Avian respiratory disease causes significant economic losses in commercial poultry operations. Because of the need to protect poultry against a variety of viral pathogens from an early age, vaccination programs typically involve serial administration of multiple vaccines, including infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and infectious laryngotracheitis virus (ILTV). Often the interval between vaccinations is only a matter of weeks, yet it is unknown whether the short interval between immunizations interferes with the development of immunity and protection against challenge. Our objective was to determine whether serially administered, live attenuated vaccines against IBV, NDV, and ILTV influence the development and longevity of immunity and protection against challenge in long-lived birds. Specific-pathogen-free white leghorns were administered multiple live attenuated vaccines against IBV, NDV, and ILTV until 16 WOA, after which certain groups were challenged with IBV, NDV, or ILTV at 20, 24, 28, 32, and 36 WOA. Five days post-challenge, viral load, clinical signs, ciliostasis, tracheal histopathology, and antibody titers in serum and tears were evaluated. We demonstrate that pullets serially administered live attenuated vaccines against IBV, NDV, and ILTV were protected against homologous challenge with IBV, NDV, or ILTV for at least 36 weeks. Additionally, our study indicates that immunity to individual vaccine viruses was not compromised despite serial administration of multiple live attenuated vaccines targeting different viral respiratory tract pathogens.

INDEX WORDS: Infectious bronchitis virus, Newcastle disease virus, Infectious laryngotracheitis virus, Vaccination program, Immunity, Respiratory disease, Ciliostasis, Antibody response

ABBREVIATIONS:

CEO = chicken embryo origin

CT = cycle threshold

DOA = days-of-age

dpc = days post-challenge

IBV = infectious bronchitis virus

HG = Harderian gland

ILTV = infectious laryngotracheitis virus

NDV = Newcastle disease virus

SPF = specific-pathogen-free

TCO = tissue culture origin

WOA = weeks-of-age

Introduction

Vaccination against respiratory viral disease is standard practice in commercial poultry operations. Both live and killed vaccines are administered to poultry, and live vaccines are commonly used for a variety of pathogens because they are typically more effective when mass applied and are relatively economical (21). The duration of immunity achieved following live vaccine administration may depend on the age and type of bird, levels of maternal immunity, disease targeted by the vaccine, immunogenicity of the vaccine, method of vaccine application, number of and interval between boosters, virulence and similarity of the field strain challenge, interval between vaccination and challenge, and immunocompetency of the host (21, 55, 130).

Infectious bronchitis virus (IBV) is an upper respiratory tract viral pathogen of poultry and leads to reduced weight gain and feed efficiency, drops in egg production, stunted growth, and secondary bacterial infection resulting in airsacculitis (32). IBV initially replicates in the upper respiratory tract, followed by systemic replication in the reproductive tract and some strains can cause lesions in the kidney (32). Infected birds may exhibit nasal discharge, coughing, sneezing, and tracheal rales (32). IBV is prevented by vaccination, and live vaccines are commonly used to achieve protection. Live vaccines are generally administered to young birds to achieve early protection, and layers and breeders are also boosted with either live or inactivated vaccines (55). The vaccine strains used in poultry production vary based on their similarity to the circulating field viruses (55).

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirusvirus type 1 (APMV-1), which has recently been reclassified as avian avulavirus 1 (AAvV-1) (7). Depending on the strain of the virus, clinical signs of ND infection may be absent or may involve depression, inappetence, respiratory signs (nasal discharge, sneezing, coughing), reduced egg

production, and neurological signs (torticollis, circling, paralysis) (130). NDV strains are characterized as lentogenic, mesogenic, and velogenic, according to their mean death time in embryos (93). Lentogenic strains are of low pathogenicity causing mild respiratory or enteric infections, followed by mesogenic strains, while velogenic isolates are highly pathogenic often causing neurological signs and mortality (130). ND vaccination regimes vary and may utilize a combination of live, inactivated, and virus-vectored vaccines (61). A common strategy is to vaccinate either one-day-old chicks or chicks at 2-4 weeks-of-age (WOA) with a live vaccine, followed by a live booster at 2-4 weeks and again at 10 weeks (130). In the United States, the most widely used traditional vaccine strains comprise lentogenic B1 (or virus clones of the B1 strain) and LaSota strains (130).

Infectious laryngotracheitis (ILT) is a respiratory disease of poultry caused by *gallid herpesvirus I* (GaHV-1), and is economically important worldwide (79). Clinical manifestations of ILT include increased mortality, reduced egg production, decreased body weight gain, and susceptibility to other respiratory pathogens. Live vaccines against ILT may be of chicken embryo origin (CEO) or tissue culture origin (TCO), in which they are passaged multiple times in eggs or tissue culture, respectively. Although recombinant vaccines for ILT are commercially available, the CEO vaccine is the most widely used vaccine worldwide. Most commercial layer flocks in the United States are initially immunized with a viral-vectored vaccine at 1 day-of-age (DOA), followed by eye-drop vaccination with CEO or TCO, or CEO administered in the drinking water between 8 and 12 WOA (79).

Because of the need to protect chickens against a variety of viral pathogens from an early age, vaccination programs typically include multiple vaccines against a variety of pathogens. Sample vaccination regimes in different poultry sectors are reviewed in the Merck Veterinary

Manual (www.merckvetmanual.com), in which the interval between vaccinations is often only a matter of weeks, and in some cases, vaccines are administered together. Such programs require careful analysis to determine if the timing of vaccinations and intervals between vaccinations are sufficient for the birds to develop adequate immune protection against challenge for each virus. The literature suggests that simultaneous viral infections may exhibit viral interference, in which one virus blocks the growth of another virus (48, 157), but until now it is unknown whether this phenomenon results in reduced protection. In this study, we investigate how the combination of serially administered, live attenuated viral respiratory vaccines affect the development and longevity of immunity to vaccination and protection against homologous challenge.

Materials and Methods

Viruses

A commercial infectious bronchitis virus (IBV) MILDVAC-GA-98® vaccine (Merck Animal Health, Summit, NJ) was used in this study. The vaccine was diluted according to the manufacturer's recommendations. The challenge virus used was IBV GA98/CWL0474/98 and was prepared at a dose of $10^{3.19}$. Virus titers were calculated by Reed and Muench (197). A commercial Newcastle B1 vaccine (NEWHATCH-C2, Merck Animal Health, Summit, NJ) was used for both vaccination and challenge and was reconstituted following the manufacturer's instructions. A commercial CEO vaccine (Trachivax® Merck Animal Health, Summit, NJ) and pathogenic Georgia broiler strain 63140 (193) were used for infectious laryngotracheitis (ILT) vaccination and challenge, respectively. Strain 63140 was propagated in chicken kidney cells obtained from 3- to 4-week-old specific pathogen-free (SPF) chickens (167). The CEO vaccine was prepared following the manufacturer's recommendations. After inoculation, the median

tissue culture infective dose ($TCID_{50}$) was confirmed by titration of both viruses in chicken kidney cells as previously described (167).

Experimental design

Specific-pathogen-free eggs were obtained at 18 days of incubation and hatched at the Poultry Diagnostic & Research Center, Athens, GA. Chicks were placed on fresh pine shavings in colony houses and pens. Chicks were vaccinated via oculonasal route according to the following schedule; IBV at 1 DOA, NDV at 2 WOA, IBV at 4 WOA, ILTV at 8 WOA, NDV at 12 WOA, and ILTV at 16 WOA. In addition, a control group was not vaccinated. Homologous challenges were conducted at 20, 24, 28, 32, and 36 WOA, and necropsies were performed five days post-challenge (dpc). At challenge, birds received one of four treatments: IBV GA98, NDV B1, ILTV 63140, or no challenge. The treatment groups for each challenge virus per time point were as follows: nonvaccinated, nonchallenged (n=9-10); vaccinated, nonchallenged (n=9-10); vaccinated, challenged (n=17-19); nonvaccinated, challenged (n=9-10). All IBV-challenged birds received a median embryo infectious dose (EID_{50}) of $1 \times 10^{3.2}$ per bird. All NDV-challenged birds received the NDV B1 vaccine, reconstituted according to the manufacturer's protocol. All ILTV-challenged birds received the 63140 pathogenic strain at a dose of $1 \times 10^{3.5}$ $TCID_{50}$ per bird. For IBV and NDV challenges, birds were observed at 5 dpc for respiratory signs, as previously described (188): 0 = absent; 1 = mild; 2 = moderate; 3 = severe. For ILTV challenges, birds were observed at 3 and 5 dpc for dyspnea, conjunctivitis, depression, and mortality, as described previously (149). The choanal cleft (IBV- and NDV-challenged and control birds at 5 dpc) or trachea (ILTV-challenged and control birds at 3 and 5 dpc) was swabbed for virus detection, and swabs were stored in PBS at −80°C. At 28, 32, and 36 WOA, 50 µl of tears was collected by adding granulated NaCl to the eye. Blood was collected by wing

or cardiac puncture and added to a microcentrifuge tube to collect serum for antibody detection. Birds were sacrificed by cervical dislocation, and the eyelid, Harderian gland (HG), thymus, liver, spleen, cecal tonsils, and bursa were collected and stored at −80°C for virus detection and in 10% neutral buffered formalin. The trachea was removed, and one section was placed in 10% neutral buffered formalin, and the remaining portion of the trachea was submerged in tissue culture media for the ciliostasis test described below. The procedures were approved by the University of Georgia Institutional Animal Care and Use Committee.

Ciliostasis test

The ciliostasis test was performed on harvested tracheas collected in cell culture media (Dulbecco's Modified Eagle's Medium) at 37°C. For each trachea, five tracheal rings measuring approximately 1 mm thick were cut and represented the proximal, middle, and distal portions (41, 54). Cilia activity was observed using an inverted microscope (Olympus, Center Valley, PA). The scoring system follows: 0 = all cilia beating; 1 = 75% of cilia beating; 2 = 50% of cilia beating; 4 = no cilia beating. Each ring was scored by three individuals, and the average total score for each trachea was calculated. The ciliostasis protection score for each group was determined by the following formula: 100 − [(total of the individual scores for the group)/(the number of individuals in the group × 20) × 100], as previously described (41), and a score ≥50 was considered protected. The binomial protection score was calculated to assess the percentage of chickens that were protected in each group, as outlined in the European Pharmacopoeia (67). Briefly, a tracheal ring with ≥50% of the cilia showing vigorous activity was considered protected, and a chicken was considered protected when 90% of the rings were showing ≥50% of cilia beating. The vaccine was considered efficacious when 80% or more of the birds in a group were protected (67).

Tracheal histopathology

A section of each trachea was fixed in 10% neutral buffered formalin, processed into paraffin, and 5-µm sections were cut for hematoxylin and eosin staining. For IBV lesions, epithelial hyperplasia, lymphocyte infiltration, and epithelial deciliation were scored for each trachea. Scores were determined as follows: 1 = normal, 2 = focal, 3 = multifocal, and 4 = diffuse, as described previously (105). For NDV lesions, a descriptive analysis was performed. For ILTV lesions, microscopic lesions were scored on a scale of 0-5 (normal to very severe), as described previously (89).

RNA extraction and qRT-PCR

Viral RNA extraction from 50 µl of the PBS from each swab was conducted using a 5X MagMAX-96 Viral Isolation Kit (Thermo Fisher, Waltham, MA) on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Thermo Scientific, Waltham, MA), according to the manufacturer's instructions. The qRT-PCR was performed with the AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher, Waltham, MA), following the manufacturer's protocol. Each 25-µl reaction mixture contained 12.5 µl of 2× RT-PCR buffer, 10 µM of each primer, 4 µM of probe, 1 µl of 25× RT-PCR enzyme mix, and 5 µl of viral RNA. The qRT-PCR reactions were run on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 1 sec and 60°C for 60 sec. The primers and probe for the qRT-PCR were previously published (26), and are comprised of a forward primer IBV5′GU391 (5′-GCT TTT GAG CCT AGC GTT-3′), a reverse primer IBV5′GL533 (5′-GCC ATG TTG TCA CTG TCT ATT G-3′), and a Taqman® dual-labeled probe IBV5′G probe (5′-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′). The primers were obtained from Integrated DNA

Technologies (Coralville, IA), and Taqman probe was synthesized by BioSearch Technologies (Novato, CA). Real-time RT-PCR components and thermocycler parameters were previously described (26). The data are expressed as the average cycle threshold (CT) value for all samples in each group, with positive CT values based on the limit of detection for this test associated with virus detection in eggs (171). The data are expressed as the average cycle threshold (CT) value for all positive samples in the group, with positive CT values based on the limit of detection for this test associated with virus detection in eggs (19).

Each qRT-PCR reaction plate included a standard curve as an RNA extraction control and as a positive control. GA98 IBV isolated from allantoic fluid was used as the template for the standard curve. Negative controls were also included in each plate and consisted of PCR reagents with no RNA.

DNA extraction

For ILTV, total DNA was extracted from the tracheal swabs using the MegaZorb® DNA extraction miniprep 96-well kit (Promega, Madison, WI, USA), as described previously (192).

qPCR

Duplex real-time PCR assay that amplifies a fragment of the UL44 viral gene in ILTV and a fragment of the chicken a2-collagen gene was performed, as previously described (194). Briefly, each sample was normalized to the chicken a2-collagen gene and the relative amount of viral DNA in each samle was estimated as $\Delta\Delta Ct = \Delta Ct$ ILTV inoculated $- \Delta Ct$ mock inoculated expressed as $\log_{10}^{2-\Delta\Delta Ct}$.

Serum IBV-specific IgG antibody titers

IBV-specific IgG titers were detected using a commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME). Briefly, serum samples (stored at −20°C) were diluted 1:500, and the procedure was performed according to the manufacturer's protocol.

Tear secreted IBV-specific IgA antibody titers

Tear IBV-specific IgA was detected using the antigen from a commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME). Briefly, tears were serially diluted two-fold in PBS and incubated in duplicate in wells overnight at 4°C. All wash steps were performed using PBS-Tween 20 (0.05% Tween 20). Plates were incubated at 23°C for 2hr in monoclonal mouse anti-chicken IgA-BIOT (1:1000, clone A-1, Southern Biotech, Birmingham, AL), followed by 1hr in Streptavidin-HRP (1:4000, Southern Biotech, Birmingham, AL). Final antibody detection steps were completed according to the manufacturer's instructions. Endpoint titers were determined by reporting the lowest dilution at which the optical density (OD), recorded at 650 nm wavelength, was at least three standard deviations above the mean of 12 control wells incubated with no tear samples. Data from wells with a pinpoint color change due to residual substrate or air bubbles were excluded from analysis, and results were reported as $\log_2$ of the endpoint titer.

Statistical analysis

The data were analyzed using Prism v.6.0 software (GraphPad Software, Inc., La Jolla, CA; www.graphpad.com). For viral load and antibody data, a one-way ANOVA with Dunnet's posttest was used to compare treatment groups within each collection period. Clinical sign scores were analyzed using a Kruskal-Wallis test with Dunn's posttest to compare treatment groups within each collection period. Significant differences were determined at $p < 0.05$.

Results

Infectious Bronchitis Virus

At 5 days following challenge with IBV GA98, vaccinated/challenged birds had significantly lower RNA loads compared to positive controls at all collection times and in all tissue samples, with the exception of cecal tonsil at 24 WOA (Table 3.1). Compared to vaccinated controls, vaccinated/challenged birds had significantly higher viral loads at all collection times and tissues except in 24 WOA and 32 WOA cecal tonsil. Viral RNA loads were significantly lower in vaccinated/challenged birds, compared to positive controls, at all times and in all tissues except in 24 WOA choanal cleft and HG and 32 WOA cecal tonsil. IBV loads in negative controls and vaccinated controls in choanal cleft, HG, and conjunctiva were below the limit of detection, using the CT value of 36.17 reported in Jackwood, Jordan, Roh, Hilt and Williams (105), at all collection times except the 24 WOA HG negative controls. Viral RNA loads were detected in the cecal tonsil of vaccinated controls at 20 and 24 WOA, but at other times loads were below the limit of detection in negative controls and vaccinated controls.

Clinical signs of IBV infection measured at 5 days post-challenge were significantly reduced in vaccinated/challenged birds when compared to positive controls in all weeks except 28 WOA, but trends in clinical sign scores were numerically lower among vaccinated/challenged birds (Table 3.2). Clinical signs were absent in nonchallenged negative and vaccinated control birds, and signs in vaccinated/challenged birds were not different from nonchallenged controls.

Ciliostasis, defined as the cessation of tracheal ciliary movement, was measured at 5 dpc, and the proportion of birds positive for ciliostasis and ciliostasis protection scores were calculated for each group (Fig. 3.1). At all collection times, vaccinated/challenged birds were protected from ciliostasis (scores were >50), and positive controls were not protected (scores

were <50). The nonchallenged negative controls and vaccinated controls were protected (scores were >50) at all collection times.

IBV-specific IgG titers were measured in serum collected at 5 dpc. At all times except at 32 WOA, vaccinated birds from both nonchallenged and challenged groups exhibited significantly higher titers compared to nonvaccinated birds from both nonchallenged and challenged groups (Fig. 3.2). At 32 WOA, titers in vaccinated birds, regardless of challenge status, were significantly higher compared to positive controls. Titers in vaccinated/challenged birds did not significantly differ from titers in vaccinated controls until 36 WOA, when vaccinated/challenged birds had significantly higher titers. Compared to negative controls, vaccinated/challenged birds had significantly higher titers at all times.

IBV-specific IgA titers were measured in tears collected at 5 dpc at 28, 32, and 36 WOA. At 28 WOA, titers in vaccinated/challenged birds were significantly higher compared to titers in nonchallenged negative controls (Fig. 3.3). At 32 WOA, vaccinated/challenged birds showed significantly lower titers compared to positive controls. No other significant differences were detected.

Newcastle Disease Virus

At 5 dpc with NDV B1, vaccinated/challenged birds either had undetectable RNA loads or significantly lower loads compared to positive control titers at all collection times and in all tissues sampled (Table 3.3). Nonchallenged negative controls and vaccinated controls were negative for RNA virus.

Clinical signs measured at 5 dpc were significant at 20 WOA in positive controls, after which clinical signs in positive controls were no different from any of the other treatment groups

(Table 3.4). No significant differences in clinical signs existed between vaccinated/challenged birds and nonchallenged controls at any time.

Tracheas were observed for ciliostasis, and no groups exhibited ciliostasis (data not shown).

NDV-specific serum IgG titers in vaccinated/challenged birds were significantly higher than titers in both positive and negative controls at all collection times (Fig. 3.4). Vaccinated controls also exhibited significantly higher titers compared to positive and negative controls at all time points, except 36-WOA titers were only numerically higher compared to negative controls. There was no significant difference in titers between vaccinated/challenged birds and vaccinated controls at any time.

Infectious Laryngotracheitis Virus

At 5 dpc with ILTV 63140, vaccinated/challenged birds had significantly lower viral DNA loads than positive controls at all collection times and in all tissues (Table 3.5). Viral DNA loads in the trachea and HG were undetectable or low and did not differ significantly from viral DNA loads in nonchallenged negative and vaccinated controls, which were negative. In the conjunctiva, viral DNA loads in vaccinated/challenged birds were significantly higher than viral DNA loads from both nonchallenged negative and vaccinated controls, except at 28 WOA in which no significant difference was detected between vaccinated/challenged birds and vaccinated controls.

Clinical signs and viral DNA were detected in challenged birds at 3 dpc (data not shown), but signs in positive controls had become more severe by 5 dpc as tracheal viral load increased (Table 3.6). Clinical sign scores measured at 5 dpc were significantly reduced in vaccinated/challenged birds compared to positive controls, at all collection points except 36

WOA when scores were numerically reduced. All negative controls and vaccinated controls had no clinical signs, and clinical signs in vaccinated/challenged birds were not significantly different compared to these controls, except at 36 WOA when clinical sign scores were significantly higher.

No ciliostasis was observed in the tracheas of birds from any of the groups.

ILTV-specific IgG titers in serum collected 5 days post-challenge were significantly higher in vaccinated birds from both challenged and nonchallenged groups, compared to the positive and negative controls.

Discussion

In the present study, we demonstrated that pullets serially administered live attenuated vaccines against IBV, NDV, and ILTV were protected against homologous challenge with IBV, NDV, or ILTV for at least 36 weeks, as determined by viral load detection, clinical signs, and ciliostasis.

Additionally, our study showed that the timing of these vaccines and intervals between each vaccine did not interfere with the development of immunity to each virus and consequently protection against homologous challenge. We designed our vaccination protocol to exemplify a typical vaccination program for IBV, NDV, and ILTV in commercial layers, and oculonasal administration of vaccine virus was intended to mimic the infection route of vaccines administered by spray and water in the field while achieving uniform coverage among the birds. A particular challenge of poultry vaccination regimes is that live vaccine viruses have been known to persist in flocks, and until now, it has been unclear whether the immunity induced by a live vaccine could be compromised because of viral interference, a phenomenon in which one replicating virus blocks the replication of another virus (48, 157). Although the vaccines in the

present study were administered at intervals of 2 or 4 weeks, it is feasible that virus from a previous immunization was still present at the time of the next vaccination. IBV vaccines have been detected in the respiratory tract up to 28 days post-vaccination (170), and IBV was isolated from tracheal and cloacal swabs collected at the point of lay and 19 WOA in hens that had been virus-negative for several weeks following recovery from inoculation at one day of age (32). Fentie, Dadi, Kassa, Sahle and Cattoli (71) reported that chickens vaccinated with NDV B1 shed vaccine virus 14 days post-inoculation. Finally, a study by Hughes, Williams, Gaskell, Jordan, Bradbury, Bennett and Jones (102) demonstrated intermittent shedding in the trachea from ILTV-immunized chickens between 7 and 14 weeks post-vaccination.

Few experimental studies of simultaneous virus infections have been published, and fewer yet have been considered in the context of poultry viral respiratory pathogens. Costa-Hurtado et al. (43) demonstrated that co-infection of chickens with a mesogenic strain of NDV and highly pathogenic avian influenza virus (HPAIV) affected HPAIV replication in chickens for 9 days but was dose-dependent. Viral interference has been demonstrated *in vitro* by co-infection with two human respiratory tract viruses, influenza A virus (IAV) and respiratory syncytial virus (RSV), in which the virus that had infected the cells first suppressed replication by the other virus. We did not measure vaccine virus replication in the present study and therefore could not determine whether viral interference occurred following vaccination. However, chickens vaccinated with all three viruses were protected from viral replication and clinical signs following homologous challenge, which proves that immunity to individual vaccine viruses was not compromised despite serial administration of multiple live attenuated vaccines targeting different viral respiratory tract pathogens.

The detection of IBV RNA in the cecal tonsils of vaccinated/nonchallenged birds at 20 and 24 WOA but not at subsequent times indicates that residual vaccine virus RNA remained in the cecal tonsils until at least 24 WOA, following the second exposure to IBV vaccine. IBV is known to persist for several months in various internal organs and has been isolated from the cecal tonsils at 14 weeks post-infection (5, 32). Additionally, IBV RNA was detected in nonchallenged negative control HG at 24 WOA but was absent from all other tissues collected from negative controls during that collection time, and birds displayed no clinical signs of IBV infection. This observation likely represents contamination during HG processing.

As expected, IBV-vaccinated/challenged birds were protected from ciliostasis, and ciliostasis was observed among nonvaccinated positive controls. The European Pharmacopoeia states that ciliostasis can be evaluated to determine IBV vaccine efficacy, in which a lack of ciliostasis would indicate that the vaccine was efficacious (67). Therefore, these observations further confirm that IBV-vaccinated birds were protected from homologous challenge.

The observation of robust IBV-specific serum IgG titers in vaccinated birds reinforces previous knowledge that IBV infection stimulates a humoral response in chickens (161), but that circulating antibody titers do not correlate with resistance to infection (160). The lack of significant titers in nonvaccinated, challenged birds can be explained by the early time of collection post-challenge. The dynamics of circulating IgG antibody titers following primary exposure to IBV have been described in Orr-Burks, Gulley, Gallardo, Toro and van Ginkel (150), in which significant changes in IgG titer were not detected until 10 days post-inoculation.

IBV-specific IgA titers measured in tears at 5 dpc did not reveal consistent trends, and may be explained by the early time of collection post-infection. Orr-Burks, Gulley, Gallardo, Toro and van Ginkel (150) demonstrated a lack of significance in IBV-specific IgA titer in tears

5 days after both primary and secondary exposure to IBV, but IgA titer was significant between 6 and 16 days after primary exposure to IBV. We have confirmed these results in a separate experiment by demonstrating significant IBV-specific IgA titers in tears between 10 and 14 dpc in naïve chickens (unpublished data).

The decision to use the B1 vaccine for NDV challenge was based on biosecurity regulations and the lack of appropriate biosafety level 3 facilities that prohibited the use of mesogenic or velogenic strains of NDV. Notably, clinical signs of NDV infection in positive controls were significant only at 20 WOA. Only a few birds had mild clinical signs at 24 and 28 WOA, after which no challenged birds had clinical signs. This observation is consistent with existing knowledge of the pathogenicity of ND, which tends to be more severe in younger birds (6). However, vaccinated birds at each sampling time had significantly lower viral RNA loads after challenge compared to positive control groups, indicating that the vaccinated birds were indeed protected from infection whereas the nonvaccinated positive controls were not. Because lentogenic NDV only causes a mild respiratory or enteric infection (130), it is not surprising that respiratory signs were mild or absent despite the presence of viral RNA in the remaining collection times.

The lack of ciliostasis observed in NDV B1-infected positive controls contrasts with previous reports. Butler et al. (25) demonstrated that NDV caused ciliostasis within 2 to 6 days after infection of tracheal explants, and Malo, de Wit and Swart (126) reported that the peak of ciliostasis following vaccination of one-day-old chicks with lentogenic NDV occurred at 5 and 7 dpv and waned by 13 dpv. The discrepancy between our results and the previous studies may be explained by the age-related resistance of chickens to NDV described above or the different lentogenic NDV strains used. However, since we did not measure ciliostasis following

vaccination, we could not compare whether there was a difference in ciliostasis between young birds vaccinated with NDV B1 and naïve adult birds challenged with NDV B1. In addition, we used a B1 vaccine strain for both vaccination and challenge, in contrast with the LaSota strain used in Malo, de Wit and Swart (126). It is well established that the LaSota vaccine strain is more virulent than B1 (129), and also may explain the ciliostasis observed in LaSota-vaccinated chicks. Interestingly, Butler, Ellaway and Hall (25) found that B1 and LaSota strains caused similar levels of ciliostasis, but rather than infecting chickens and harvesting tracheas, the authors directly infected tracheal explants that might have eliminated host-dependent factors involved in age-related resistance. Taken together, we speculate that age-related resistance and the low virulence of the NDV B1 strain could account for the lack of ciliostasis observed in our study.

Robust NDV-specific circulating IgG antibody responses developed following NDV vaccination and stayed elevated, which was consistent with prior research indicating that antibodies may be detected for up to one year in birds immunized multiple times against NDV (6). With the exception of 28 WOA, the IgG titers in nonvaccinated/challenged birds were not significant, though titers at 32 and 36 WOA were also detectable. These low but detectable titers likely represented the initial humoral immune response. Previous literature indicates that antibodies are not detected in the serum until 6-10 days after exposure (6). Surprisingly, the nonvaccinated/nonchallenged birds at 36 WOA had positive titers, which may be attributed to contamination or laboratory error.

As expected, viral DNA loads in tracheal swabs, HG, and conjunctiva among vaccinated/challenged birds were significantly lower than positive control viral DNA loads and were accompanied by significantly reduced (20-32 WOA) or numerically reduced (36 WOA)

clinical sign scores. Notably, almost no viral DNA was detected in tracheal swabs and HG among vaccinated/challenged birds. In contrast, the conjunctiva had statistically significant viral DNA loads at most sampling times, though they were significantly lower than positive control loads. This observation indicates that vaccination prevented challenge virus replication in trachea and HG but did not completely block virus replication in the conjunctiva. Since the challenge virus was administered via eyedrop and intranasal route, the conjunctiva was likely one of the first tissues infected, and the virus load might have been higher in this tissue. The proximity of the conjunctiva to the inoculation site, combined with the pathogenic nature of the ILTV 63140 challenge strain, might explain why the local host immune response might not have been able to completely block viral replication in the conjunctiva but successfully cleared the virus before it could replicate in HG and trachea.

The absence of ciliostasis observed in ILTV-challenged positive controls at 5 dpc was not surprising given that Butler, Ellaway and Hall (25) found that only some strains of ILTV caused ciliostasis and did not correlate with virulence. Moreover, ciliostasis rarely occurred before 6 days, and sometimes even 9 days, after inoculation. In support of these data, Gerganov and Surtmadzhiev (81) also demonstrated ciliostasis in ILTV-infected tracheal organ cultures at 7-8 days post-infection. Therefore, our results combined with previous studies indicate that measuring ciliostasis may not be a reliable marker of protection from ILTV infection and that ciliostasis in ILTV infection studies may need to be evaluated at later times post-inoculation.

ILTV-specific IgG antibody titers were robust in vaccinated birds throughout the study and support previous data that antibodies may be detectable for at least a year (91). The lack of significant titers in nonvaccinated positive controls may be explained by the early time of collection post-challenge, as ILTV-specific antibodies do not become detectable until 5-7 days

post-infection and peak at 21 dpi (99). However, it is worth noting that antibody titers are not correlated to protection from ILTV infection (91).

Altogether, our data indicate that serially administered live attenuated vaccines against IBV, NDV, and ILTV in pullets do not interfere with immune responses to individual vaccines, and the birds are adequately protected against homologous challenge until they are at least 36 WOA.

Tables

Table 3.1 qRT-PCR average CT values for IBV RNA collected from choanal cleft, Harderian gland, conjunctiva, and cecal tonsil 5 days post-challenge with IBV GA98 at 20, 24, 28, 32, and 36 weeks-of-age. Letters (a-c) indicate significant differences at $p < 0.05$.

	Treatment		Mean ± SEM*			
Week	Vaccine	Challenge	Choanal Cleft	Harderian gland	Conjunctiva	Cecal Tonsil
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	36.5 ± 0.9^{b}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	24.9 ± 1.9^{b}
	+	+	27.9 ± 0.8^{c}	33.2 ± 0.6^{b}	31.9 ± 0.7^{b}	30.2 ± 1.2^{c}
20	-	+	17.5 ± 0.3^{d}	26.1 ± 1.3^{c}	22.7 ± 0.6^{c}	21.6 ± 1.1^{b}
	-	-	40.0 ± 0.0^{ab}	34.4 ± 1.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	41.1 ± 0.5^{a}	39.0 ± 0.7^{b}	40.0 ± 0.0^{a}	30.7 ± 3.3^{b}
	+	+	39.6 ± 0.4^{b}	33.8 ± 0.5^{a}	31.0 ± 0.7^{b}	30.5 ± 1.7^{b}
24	-	+	34.3 ± 0.5^{c}	21.0 ± 1.5^{c}	24.1 ± 0.8^{c}	26.1 ± 2.0^{b}
	-	-	40.0 ± 0.0^{a}	39.2 ± 0.6^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	39.6 ± 0.4^{a}
	+	+	30.4 ± 1.2^{b}	30.9 ± 1.7^{b}	31.5 ± 1.0^{b}	33.7 ± 1.6^{b}
28	-	+	22.3 ± 0.3^{c}	20.4 ± 1.3^{c}	22.8 ± 0.5^{c}	24.3 ± 1.8^{c}
	-	-	40.0 ± 0.0^{a}	38.9 ± 0.8^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	38.6 ± 0.7^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	32.3 ± 0.5^{b}	34.1 ± 0.4^{b}	34.5 ± 1.6^{b}	34.5 ± 2.0^{a}
32	-	+	20.6 ± 0.5^{c}	21.8 ± 1.3^{c}	24.2 ± 0.7^{c}	23.4 ± 1.3^{b}
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	30.0 ± 0.8^{b}	30.3 ± 0.9^{b}	28.3 ± 1.4^{b}	32.5 ± 1.0^{b}
36	-	+	22.2 ± 0.4^{c}	21.2 ± 1.4^{c}	25.0 ± 0.5^{c}	23.1 ± 1.2^{c}

*Standard error of the mean.

Table 3.2 Clinical signs measured 5 days following IBV GA98 challenge. Letters (a-b) indicate significant differences at $p < 0.05$.

	Treatment		Clinical Signs
Week	Vaccine	Challenge	Mean ± SEM*
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.0 ± 0.0^{a}
20	-	+	0.7 ± 0.2^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.1 ± 0.1^{a}
24	-	+	1.0 ± 0.3^{b}
	-	-	0.0 ± 0.0
	+	-	0.0 ± 0.0
	+	+	0.1 ± 0.1
28	-	+	0.5 ± 0.3
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.1 ± 0.1^{a}
32	-	+	1.2 ± 0.4^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.1 ± 0.1^{a}
36	-	+	0.4 ± 0.3^{b}

*Standard error of the mean.

Table 3.3 qRT-PCR average CT values for NDV RNA collected from choanal cleft, Harderian gland, and conjunctiva 5 days post-challenge with NDV B1 vaccine. Letters (a-b) indicate significant differences at $p < 0.05$.

	Treatment		Mean ± SEM*		
Week	Vaccine	Challenge	Choanal Cleft	Harderian gland	Conjunctiva
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	39.7 ± 0.4^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	38.2 ± 0.6^{a}
20	-	+	29.3 ± 1.1^{b}	28.6 ± 0.9^{b}	27.6 ± 1.9^{b}
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	39.5 ± 0.5^{a}
24	-	+	26.6 ± 1.0^{b}	34.2 ± 1.4^{b}	27.8 ± 1.0^{b}
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	37.0 ± 0.7^{b}
28	-	+	24.5 ± 0.7^{b}	33.0 ± 1.3^{b}	27.9 ± 0.8^{c}
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	39.2 ± 0.6^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
32	-	+	27.7 ± 2.2^{b}	31.5 ± 1.6^{b}	28.6 ± 1.2^{b}
	-	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	-	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}
	+	+	38.0 ± 1.1^{a}	39.1 ± 0.7^{a}	39.6 ± 0.4^{a}
36	-	+	24.9 ± 1.0^{b}	33.7 ± 1.8^{b}	30.3 ± 0.5^{b}

*Standard error of the mean.

Table 3.4 Clinical signs measured 5 days following NDV B1 challenge. Letters (a-b) indicate significant differences at $p < 0.05$.

	Treatment		Clinical Signs
Week	Vaccine	Challenge	Mean ± SEM*
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.4 ± 0.2^{a}
20	-	+	1.6 ± 0.2^{b}
	-	-	0.0 ± 0.0
	+	-	0.0 ± 0.0
	+	+	0.0 ± 0.0
24	-	+	0.2 ± 0.1
	-	-	0.0 ± 0.0
	+	-	0.0 ± 0.0
	+	+	0.0 ± 0.0
28	-	+	0.1 ± 0.1
	-	-	0.0 ± 0.0
	+	-	0.0 ± 0.0
	+	+	0.0 ± 0.0
32	-	+	0.0 ± 0.0
	-	-	0.0 ± 0.0
	+	-	0.0 ± 0.0
	+	+	0.0 ± 0.0
36	-	+	0.0 ± 0.0

*Standard error of the mean.

Table 3.5 qRT-PCR average CT values for ILTV DNA collected from choanal cleft, Harderian gland, and conjunctiva 5 days post-challenge with pathogenic ILTV strain 63140. Letters (a-c) indicate significant differences at $p < 0.05$.

	Treatment		Mean ± SEM*		
Week	Vaccine	Challenge	Trachea	Harderian gland	Conjunctiva
	-	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	+	40.0 ± 0.0[a]	39.7 ± 0.3[a]	35.4 ± 1.2[b]
20	-	+	24.2 ± 0.4[b]	22.7 ± 0.4[b]	18.2 ± 1.4[c]
	-	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	38.7 ± 0.9[a]
	+	+	40.0 ± 0.0[a]	40.0 ± 0.0[a]	34.5 ± 0.9[b]
24	-	+	25.2 ± 1.1[b]	26.0 ± 1.4[b]	17.3 ± 0.8[c]
	-	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	36.8 ± 1.3[b]
	+	+	39.5 ± 0.3[a]	40.0 ± 0.0[a]	38.5 ± 0.6[ab]
28	-	+	23.9 ± 0.9[b]	22.5 ± 1.4[b]	17.0 ± 0.5[c]
	-	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	-	40.0 ± 0.0[a]	39.7 ± 0.4[a]	40.0 ± 0.0[a]
	+	+	39.7 ± 0.3[a]	40.0 ± 0.0[a]	27.2 ± 0.6[b]
32	-	+	24.7 ± 1.0[b]	24.2 ± 1.1[b]	17.7 ± 0.6[c]
	-	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	40.0 ± 0.0[a]
	+	-	40.0 ± 0.0[a]	40.0 ± 0.0[a]	39.5 ± 0.5[a]
	+	+	39.8 ± 0.2[a]	38.3 ± 0.8[a]	32.0 ± 0.5[b]
36	-	+	26.4 ± 1.7[b]	34.9 ± 1.6[b]	18.8 ± 0.9[c]

*Standard error of the mean.

Table 3.6 Clinical signs measured 5 days following ILTV 63140 challenge. Letters (a-b) indicate significant differences at $p < 0.05$.

	Treatment		Clinical Signs
Week	Vaccine	Challenge	Mean ± SEM*
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.6 ± 0.2^{a}
20	-	+	4.7 ± 0.7^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.0 ± 0.0^{a}
24	-	+	2.5 ± 0.7^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.1 ± 0.1^{a}
28	-	+	4.9 ± 0.7^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	0.4 ± 0.1^{a}
32	-	+	4.0 ± 0.7^{b}
	-	-	0.0 ± 0.0^{a}
	+	-	0.0 ± 0.0^{a}
	+	+	1.1 ± 0.2^{b}
36	-	+	2.9 ± 0.4^{b}

*Standard error of the mean.

Figures

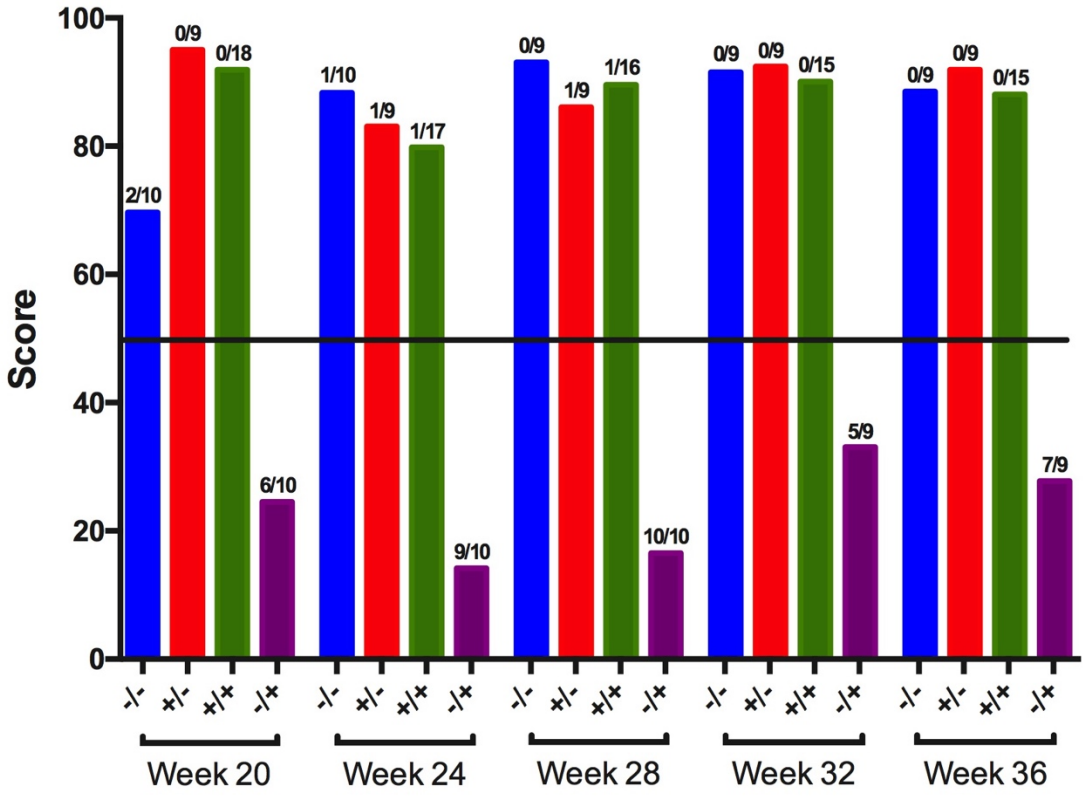

Fig. 3.1 Ciliostasis protection scores among tracheas collected 5 days post-challenge with IBV GA98 for different groups of birds challenged at 20, 24, 28, 32, and 36 weeks-of-age. Groups with a protection score >50 (horizontal line) are protected, and groups with a protection score <50 are not protected. Fractions above each bar represent the proportion of birds positive for ciliostasis for the respective group. –/– = nonvaccinated/nonchallenged, +/– = vaccinated/nonchallenged, +/+ = vaccinated/challenged, –/+ = nonvaccinated/challenged.

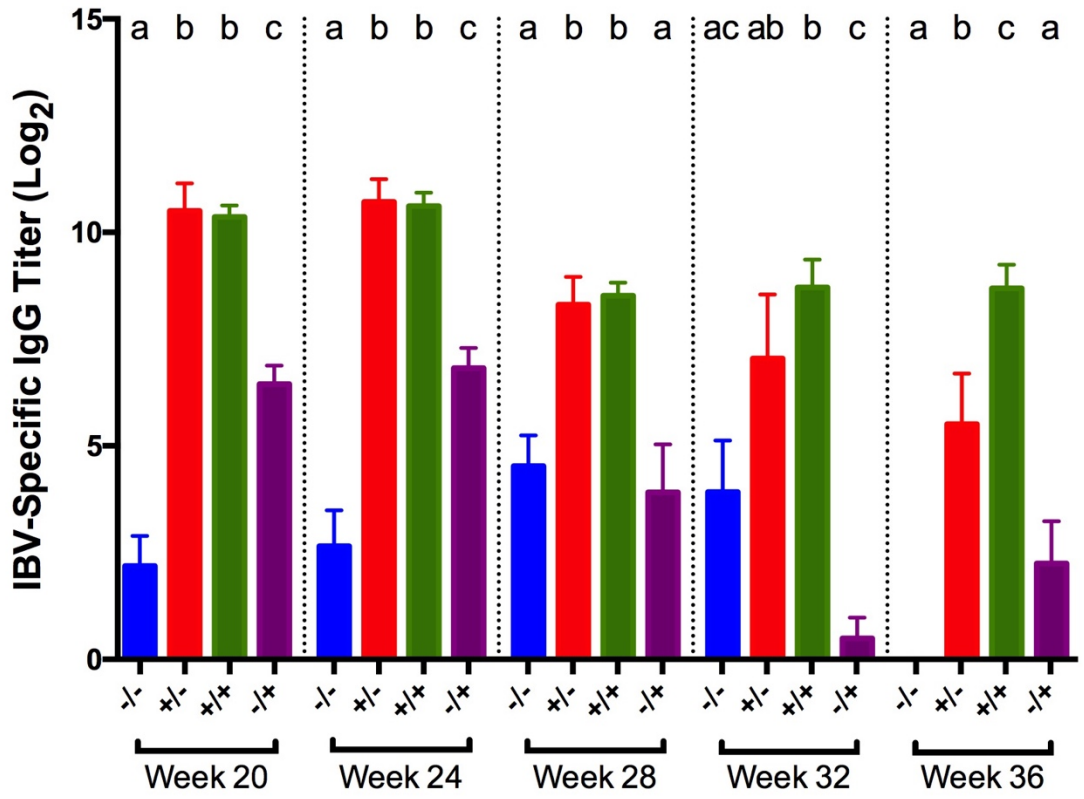

Fig. 3.2 IBV-specific IgG titers in serum collected 5 days post-challenge with IBV GA98 for different groups of birds challenged at 20, 24, 28, 32, and 36 weeks-of-age. Letters (a-c) indicate significant differences at $p < 0.05$. –/– = nonvaccinated/nonchallenged, +/– = vaccinated/nonchallenged, +/+ = vaccinated/challenged, –/+ = nonvaccinated/challenged.

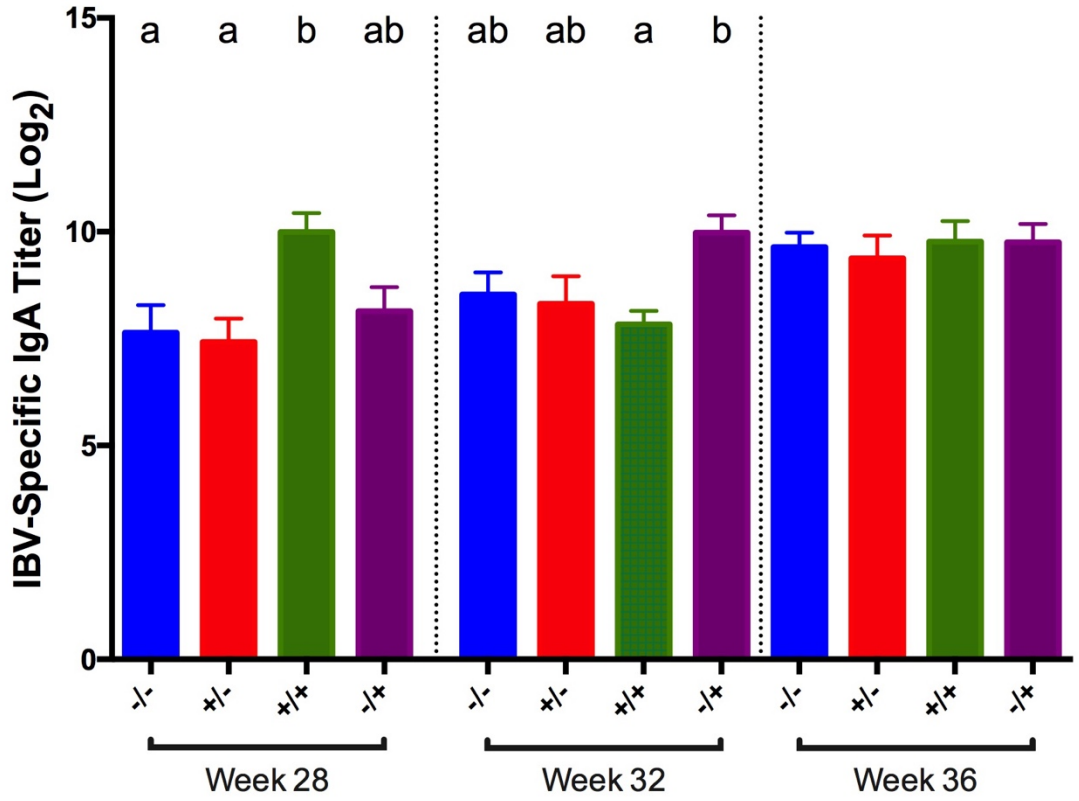

Fig. 3.3 Tear IBV-specific IgA titer in tears collected 5 days post-challenge with IBV GA98 for different groups of birds challenged at 20, 24, 28, 32, and 36 weeks-of-age. Letters (a-c) indicate significant differences at $p < 0.05$. –/– = nonvaccinated/nonchallenged, +/– = vaccinated/nonchallenged, +/+ = vaccinated/challenged, –/+ = nonvaccinated/challenged.

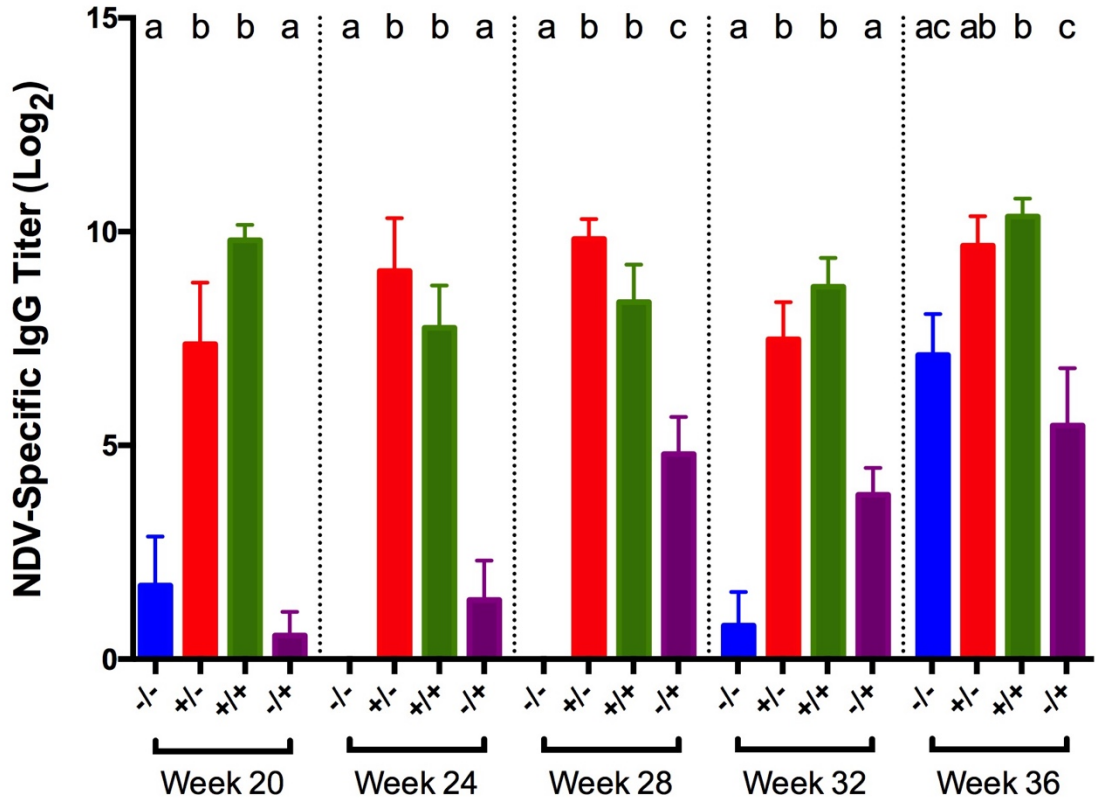

Fig. 3.4 NDV-specific IgG titers in serum collected 5 days post-challenge with NDV B1 vaccine for different groups of birds challenged at 20, 24, 28, 32, and 36 weeks-of-age. Letters (a-c) indicate significant differences at $p < 0.05$. –/– = nonvaccinated/nonchallenged, +/– = vaccinated/nonchallenged, +/+ = vaccinated/challenged, –/+ = nonvaccinated/challenged.

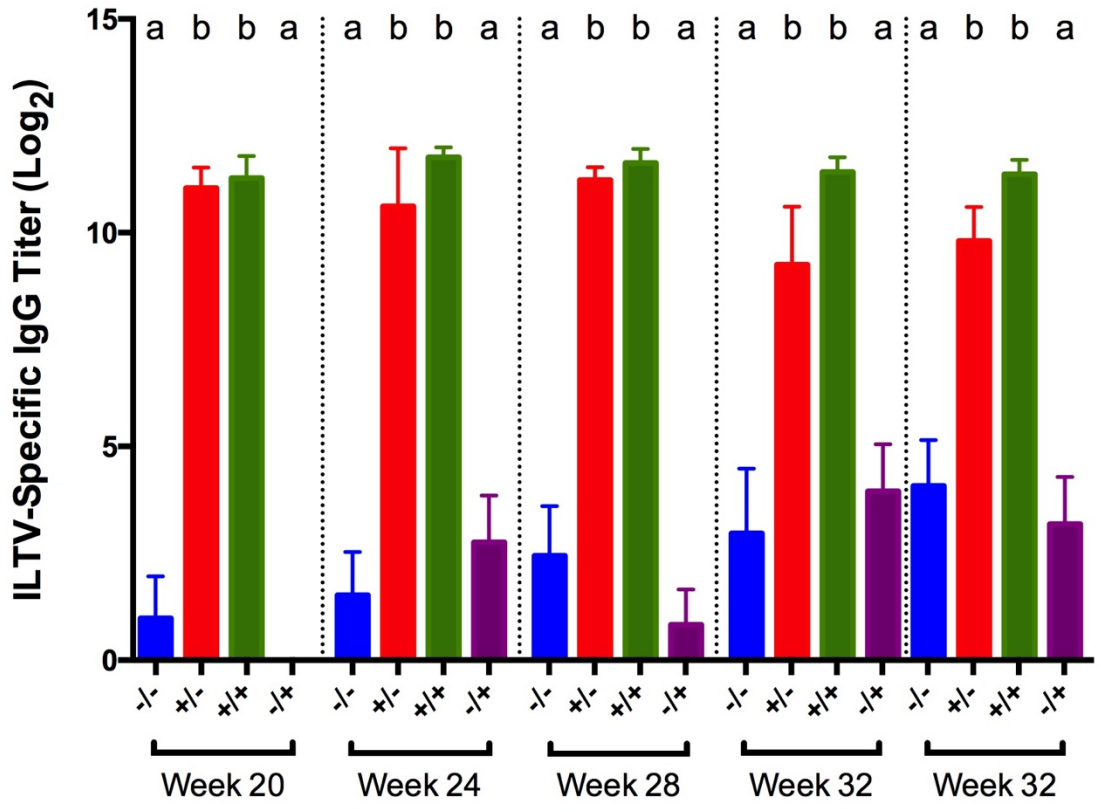

Fig. 3.5 ILTV-specific IgG titers in serum collected 5 days post-challenge with ILTV strain 63140 for different groups of birds challenged at 20, 24, 28, 32, and 36 weeks-of-age. Letters (a-b) indicate significant differences at $p < 0.05$. –/– = nonvaccinated/nonchallenged, +/– = vaccinated/nonchallenged, +/+ = vaccinated/challenged, –/+ = nonvaccinated/challenged.

CHAPTER 4

AMBIENT AMMONIA DOES NOT INHIBIT THE IMMUNE RESPONSE TO INFECTIOUS BRONCHITIS VIRUS VACCINATION AND PROTECTION FROM HOMOLOGOUS CHALLENGE IN BROILER CHICKENS [2]

[2] E. J. Aston, M. W. Jackwood, R. M. Gogal, Jr., D. J. Hurley, B. D. Fairchild, D. A. Hilt, S. Cheng, L. Tensa, M. Garcia, B. J. Jordan. To be submitted to *Veterinary Immunology and Immunopathology*.

Abstract

Commercial broilers during the grow-out period are commonly exposed to gaseous ammonia (NH_3) originating from degradation of nitrogen-containing excreta in the litter. NH_3 concentrations in the air are higher in poorly ventilated houses and appear to coincide with the elevated incidence of respiratory disease occurring during the winter months. This study examined the effect of NH_3 on the immune response to infectious bronchitis virus (IBV) vaccination and protection against homologous serotype challenge in commercial broiler chickens. One-day-old chicks were administered IBV vaccine and exposed to 30-60 ppm of NH_3. At 28 DOA, birds were challenged oculonasally with a pathogenic homologous IBV, and protection was measured by viral detection, clinical signs, ciliostasis, and presence of airsacculitis. IBV-specific serum IgG and lacrimal fluid IgA titers, as well as Harderian gland (HG) immune cell phenotypes, were evaluated. NH_3 exposure was associated with an increased incidence of airsacculitis among nonvaccinated, challenged birds. Vaccinated, NH_3-exposed birds were completely protected from IBV challenge. NH_3 had subtle effects on cilia morphology and function but did not affect vaccine or challenge virus replication and clearance, clinical signs, ciliostasis, tracheal histopathology scores, or immune responses. In the HG of vaccinated birds, the percent of leukocytes, MHC I^+/MHC II^{hi} expression, IgM^+ expression, and $CD8^+$ expression was increased, while mucosal IgA and serum IgG titers were nominal. Nonvaccinated, IBV-challenged birds exhibited an increased percent of leukocytes, MHC I^+/MHC II^{hi} expression, and IgM^+ expression in the HG at 5 dpc, followed by increased mucosal IgA and serum IgG titers and $CD8^+$ expression at 10-14 dpc. In contrast, vaccinated, IBV-challenged birds had a minimal increase in MHC I^+/MHC II^{hi} expression, and serum IgG antibody titers in vaccinated birds increased rapidly. The results indicate that commercial

broilers exposed to moderate levels of NH_3 are equally protected against IBV challenge if appropriately vaccinated, and the absence of robust immune activation in vaccinated, challenged birds suggests that the challenge virus was efficiently neutralized before establishing infection. In contrast, NH_3 exposure was associated with a higher incidence of airsacculitis in nonvaccinated, challenged birds, despite the apparent lack of differences in the immune response between birds in the NH_3-exposed and NH_3 control groups.

INDEX WORDS: Ammonia, Immune response, Infectious bronchitis virus, Broiler chickens, Respiratory disease, Airsacculitis

ABBREVIATIONS:

–/NAm/+ = nonvaccinated, no ammonia, challenged group

–/Am/+ = nonvaccinated, ammonia-exposed, challenged group

–/NAm/– = nonvaccinated, no ammonia, nonchallenged group

–/Am/– = nonvaccinated, ammonia-exposed, nonchallenged group

+/NAm/+ = vaccinated, no ammonia, challenged group

+/Am/+ = vaccinated, ammonia-exposed, challenged group

+/NAm/– = vaccinated, no ammonia, nonchallenged group

+/Am/– = vaccinated, ammonia-exposed, nonchallenged group

dpc = days post-challenge

dpv = days post-vaccination

HG = Harderian gland

hpc = hours post-challenge

IBV = infectious bronchitis virus

NH_3 = Ammonia

ppm = parts per million

Rh = relative humidity

Introduction

Commercial broiler chickens during the grow-out period are commonly exposed to ambient ammonia (NH_3), an irritant gas released from poultry manure. The mechanisms of NH_3 emissions from manure have been summarized in Ni (138), in which uric acid in feces is converted to gaseous NH_3 by bacterial activity within poultry litter. NH_3 concentrations in a poultry house are positively correlated with moisture content, temperature and pH, and NH_3 concentrations increase with reduced ventilation (28, 113, 123). Elevated NH_3 levels tend to occur during cold weather, but NH_3 in a broiler house may present a year-round challenge to bird health and production (133).

The effects of NH_3 exposure on the eye have been documented in numerous studies in chickens and vary depending on the level and duration of exposure. Broilers exposed to low levels of NH_3 (25 ppm) experienced diffuse lymphocytic and heterophilic infiltrates in the iris, and exposure to higher concentrations (50 and 75 ppm) resulted in corneal ulcerations and more numerous inflammatory infiltrates by 14 DOA (132). Keratitis, conjunctivitis, corneal edema, ulceration, and neovascularization have also been reported in chickens treated with NH_3 (73).

Surprisingly few reports have investigated the effect of NH_3 on respiratory tissues. In general, those reports have described deciliation, goblet cell hypertrophy, epithelial hyperplasia, and inflammatory cell infiltrates in the upper-respiratory tract associated with NH_3 exposure (9, 203). In an ultrastructural study of chicken lungs, NH_3 exposure to 50 ppm over three weeks preceded increased lung atrial wall thickness, leading to reduced gas exchange (3). Despite NH_3-induced histological and ultrastructural abnormalities in respiratory tissues, it is uncertain whether NH_3 exposure alone increases inflammation in these tissues. Miles (132) reported no increase in lymphocytic or heterophilic infiltrates in tissue from the trachea, lungs, and air sacs

following a 28-day exposure period with up to 75 ppm of NH_3, and the authors speculated that the harmful effects of NH_3 may not always be apparent until the bird experiences additional environmental or microbial challenges. Oyetunde, Thomson and Carlson (151) demonstrated that the adverse effects of NH_3 are compounded by dust and *Escherichia coli*, thus leading to increased trachea, lung, and air sac lesions. Anderson, Beard and Hanson (9) observed that natural exposure to NH_3, dust, and carbon dioxide for six days resulted in some deciliation of the upper portion of the trachea and turbinates, as well as an increase in mucus-secreting goblet cells. Anderson, Beard and Hanson (8) reported increased susceptibility to Newcastle disease infection after exposure to 20 ppm of NH_3 for 72 hours. Some studies documented the occurrence of airsacculitis in NH_3-stressed birds vaccinated with live attenuated infectious bronchitis virus (IBV) (112, 159).

While the majority of studies have relied on morphometric and functional parameters to describe the effect of NH_3 on poultry health, very few have addressed the mechanisms by which NH_3 influences immunity. In a study by Xiong, Tang, Meng and Zhang (203), transcriptomic analysis of broiler tracheas following NH_3 exposure of 75 ppm for 20 days indicated that NH_3 increased the production of reactive oxygen species (ROS), downregulated immune response proteins responsible for antigen recognition and presentation, and upregulated proteins involved in muscle contraction and mucin production. The authors further theorized that the overproduction of mucins combined with tracheal muscle contraction may explain the clinical signs such as coughing and rales that may accompany NH_3 exposure.

IBV is a common upper respiratory tract pathogen of economic significance in the commercial poultry industry and may lead to reduced weight gain and feed efficiency, drops in egg production, stunted growth, and secondary bacterial infection resulting in airsacculitis (32).

IBV initially replicates in the upper respiratory tract, including the Harderian gland (HG), nasal turbinates, and the trachea, followed by systemic replication in the reproductive tract and some strains can cause lesions in kidney (32). IBV has an incubation period of 18 to 36 hrs, and IBV-infected birds may exhibit nasal discharge, coughing, sneezing, and tracheal rales (32).

Mucosal respiratory responses are important in protection against IBV, and the HG is a major paraocular gland that contains IgA-, IgG-, and IgM-expressing plasma cells (13, 143). Previous studies have reported that IgA is locally synthesized in the HG, and IgG is both synthesized and transduced to the respiratory tract from systemic circulation (13, 50, 187). IgA is involved with protection of mucosal surfaces, and local IBV-specific IgA responses, in contrast to systemic IBV-specific IgG responses, have been associated with protection from IBV infection (185). In addition to immunoglobulin-secreting plasma cells, the HG also contains macrophages, lymphocytes, dendritic cells, and heterophils (18, 147).

Upon primary exposure to either an IBV vaccine or virulent strain, a cell-mediated immune response also develops. Cytotoxic T lymphocyte responses in chickens following IBV infection have been linked to an early decline in infection and clinical signs (156, 174), and the primary cell type responsible for early viral clearance is $CD8^+$ αβ T lymphocytes (174). The humoral response appears to be important in clinical disease resolution and viral clearance, since IBV-vaccinated, bursectomized chicks experienced more severe clinical disease coinciding with delayed viral clearance (39), and neutralizing antibodies are important in preventing IBV re-infection (88, 100).

The objective of this study was to analyze the effect of moderate levels of ambient NH_3 on the immune response to IBV vaccination and protection against homologous challenge in commercial broilers.

Materials and Methods

Viruses

A commercial Massachusetts (Mass) type vaccine (strain MILDVAC-Ma5^{TM}, Merck Animal Health, Summit, NJ) was used in this study. The vaccine was diluted in phosphate-buffered saline (PBS) according to the manufacturer's recommendations. The challenge virus used was Massachusetts Mass/Mass41/41 (Mass41). Virus titers were calculated by Reed and Muench (197).

Chickens

Maternal antibody-positive commercial broiler embryos from a commercial source were obtained at 18 days of incubation and hatched at the Poultry Diagnostic & Research Center, Athens, GA. Methods used in this study were approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA.

Experimental design

This study consisted of three *in vivo* experiments to investigate the effect of ambient NH_3 on the immune response to IBV vaccination and protection against homologous challenge in commercial broilers. Experiment 1 was a survey to gather preliminary data for the immune response post-vaccination (10-14 dpv), pre-challenge (24-28 dpv), and post-challenge (10-14 dpc). Experiment 2 included a negative control group post-challenge, as well as a –/Am/+ group to mimic incomplete or partial vaccination, a common result of mass vaccination in which some birds received a partial vaccine dose or no vaccine at all. Experiment 3 was designed like Experiment two but incorporated a larger sample size and additional collection times post-challenge.

Experiment 1

One-day of age commercial broilers (n=339) were arbitrarily divided into 3 groups: negative control/no NH_3 (–/NAm, n=113), vaccinated-only (+/NAm, n=113), and vaccinated, NH_3-exposed (+/Am, n=113) groups, and were placed in three climate-controlled rooms. Birds in groups receiving no NH_3 were placed on fresh shavings, while +/Am birds were placed on used litter, which served as a source of NH_3. To mimic field conditions, ventilation was controlled by three-speed 20-inch box fans, the stocking density was 0.6 ft^2/bird, and standard heat sources and feed were used. The one-day of age birds that were to be vaccinated were administered one dose each of IBV-Mass vaccine via oculonasal route (left eye/left nares), in a total volume of 50 µl. Temperature and relative humidity (Rh) were monitored in each room, and NH_3 levels were recorded in the +/Am room. Measuring NH_3 levels in all rooms was cost-prohibitive, so olfaction, with a mean odor detection threshold of 2.6 ppm (179), was used to confirm the absence of detectable levels of NH_3 in control rooms. Birds were observed daily for clinical signs. The immune response to vaccination was measured at 7, 10, 12, 14, 24, 26, and 28 DOA using two birds from each group per collection day. The choanal cleft was swabbed for vaccine virus detection, and swabs were stored in PBS at −80°C. Granulated NaCl was added to the left eye to collect 50 µl of tears, and samples were stored at −20°C. Blood was collected by cardiac puncture in a heparinized (1000 U/ml) syringe and added to both a microcentrifuge tube to collect plasma for antibody detection. Birds were humanely euthanized, and the HG was extracted and stored in PBS at room temperature for flow cytometry. The trachea was removed, and the distal portion was placed in 10% neutral buffered formalin. At 28 days, birds were transferred to Horsfall Bauer isolation units under positive pressure and provided with feed and water *ad libitum*. Birds were sub-divided into nonchallenge groups and challenge groups, and

challenges were administered with pathogenic Mass41 virus at a dose of 10^4 EID_{50}/bird in a volume of 100 µl, which was applied oculonasally (left eye/left nares). Five days post-challenge (dpc), necropsies were performed on the following groups: –/NAm/– (n=5), –/NAm/+ (n=10), +/NAm/– (n=5), +/NAm/+ (n=10), +/Am/– (n=5), +/Am/+ (n=10). Birds were observed for respiratory rales, as previously described (188): 0 = absent; 1 = mild; 2 = moderate; 3 = severe. Abdominal airsacs were assessed for airsacculitis, which was defined by the presence of opacity or foamy material or both in the airsacs at necropsy, as previously described (46). Tracheas were collected in formalin for histopathology and cell culture media (Dulbecco's Modified Eagle's Medium, DMEM) for the ciliostasis test described below. Post-challenge immune responses were measured at 10, 12, and 14 dpc using the same methods described above.

Experiment 2

One-day of age commercial broilers (n=452) were arbitrarily divided into negative control (–/NAm, n=113), NH_3-only (–/Am, n=113), vaccinated-only (+/NAm, n=113), and vaccinated, NH_3-treated (+/Am, n=113) groups, and were placed in four climate-controlled rooms. All environmental conditions and vaccine and challenge viruses and doses remained the same as in Experiment 1. Birds were monitored daily for clinical signs. At 7 dpv, 5 birds/group were swabbed via the choanal cleft, euthanized, and HG, blood, and trachea were collected as described above. Tears were collected from 5 separate birds/group. The immune response to vaccination was measured at 10, 12, 14, 24, 26, and 28 DOA using two birds from each group per collection day by applying the same methods described in Experiment 1. At 28 days, birds were transferred to isolation units and challenged with Mass41 as described above. Necropsies were performed at 5 dpc on the following groups: –/NAm/– (n=5), –/NAm/+ (n=10), –/Am/– (n=5), –/Am/+ (n=5), +/NAm/– (n=5), +/NAm/+ (n=10), +/Am/– (n=5), +/Am/+ (n=10). Birds

were observed for respiratory rales and lungs were assessed for airsacculitis. Tracheas were collected in formalin and DMEM. Post-challenge immune responses were measured at 4, 10, 12, and 14 dpc using the same methods described above.

Experiment 3

One-day of age commercial broilers (n=452) were divided into negative control (–/NAm, n=113), NH3-only (–/Am, n=88), vaccinated-only (+/NAm, n=113), and vaccinated, NH3-treated (+/Am, n=138) groups, and were placed in four climate-controlled rooms. Due to a number tracking error at placement, only 88 birds were placed in the –/Am room, and 138 birds were placed in the +/Am room. The population was later adjusted in the +/Am room to approximate 113, to maintain the same stocking density used in each experiment. All environmental conditions and vaccine and challenge viruses and doses were the same as in Experiment 1. Birds were monitored daily for clinical signs. In addition to swabbing birds via the choanal cleft at 5 dpv, the immune response to vaccination was measured at 7, 10, 12, and 14 dpv using five birds from each group per collection day, according to the methods outlined in Experiment 1. At 28 days, birds were transferred to isolation units and challenged with pathogenic Mass41 virus as described in Experiment 1. Necropsies were performed at 5 dpc on the following groups: –/NAm/– (n=5), –/NAm/+ (n=10), –/Am/– (n=5), –/Am/+ (n=10), +/NAm/– (n=5), +/NAm/+ (n=9), +/Am/– (n=5), +/Am/+ (n=10). Birds were observed for respiratory rales and lungs were assessed for airsacculitis. Tracheas were collected in formalin and DMEM. Post-challenge immune responses were measured at 18 and 48 hours post-challenge (hpc), and 3, 5, 10, 12, and 14 dpc using the same methods described in Experiment 1. At each time point, four samples per group were collected until 5 dpc, after which two to three samples per group were obtained.

Air quality measurements

The air quality parameters measured during this study included temperature, Rh, and NH_3. Temperature and Rh inside each room were measured and were recorded at 15-minute intervals using a Temperature/Relative Humidity Smart Sensor (Onset, Bourne, MA, S-THB-M002) connected to a HOBO® RX3000 Monitoring Station (Onset, Bourne, MA). NH_3 levels, measured in parts per million (ppm), were recorded at 15-minute intervals at the level of the drinkers in NH_3-treated rooms only by a Chillgard® RT Refrigerant Monitor (MSA, Cranberry Township, PA), which was connected to the same HOBO® monitoring station. Air quality parameters were measured from hatch until challenge at 28 DOA. A target range (30-60 ppm) for NH_3 was achieved by either adding water to the litter until it was damp and then turning the litter or adjusting the ventilation rate. Temperature and Rh were adjusted according to the temperature requirements of the birds.

Ciliostasis test

The ciliostasis test was performed on harvested tracheas collected in cell culture media (Dulbecco's Modified Eagle's Medium) at 37°C. For each trachea, five tracheal rings measuring approximately 1 mm thick were cut and represented the proximal, middle, and distal portions of the trachea (41, 54). Cilia activity was observed using an inverted microscope (Olympus, Center Valley, PA). The scoring system was as follows: 0 = all cilia beating; 1 = 75% of cilia beating; 2 = 50% of cilia beating; 4 = no cilia beating. Each ring was scored by three individuals, and the average total score for each trachea was calculated. The ciliostasis protection score for each group was determined by the following formula: 100 − [(total of the individual scores for the group)/(the number of individuals in the group × 20) × 100], as previously described (41), and a score ≥50 was considered protected. The binomial protection score was calculated to assess the

proportion of chickens that were protected in each group, as outlined in the European Pharmacopoeia (17). Briefly, a tracheal ring with ≥50% of the cilia showing vigorous activity was considered protected, and a chicken was considered protected when 90% of the rings were showing ≥50% of cilia beating.

Tracheal histopathology

A section of each trachea was fixed in 10% neutral buffered formalin, processed into paraffin, and 5-µm sections were cut for hematoxylin and eosin staining. Epithelial hyperplasia, lymphocyte infiltration, and epithelial deciliation were scored for each trachea on the basis of severity and distribution, severity and distribution scores for each parameter were averaged, and the three averaged scores were added to determine a total tracheal histopathology score for each trachea. Scores were determined as follows: 1 = normal, 2 = focal, 3 = multifocal, and 4 = diffuse, as described previously (105).

RNA extraction and qRT-PCR

Viral RNA extraction from 50 µl of the PBS from each swab was conducted using a 5X MagMAX-96 Viral Isolation Kit (Thermo Fisher, Waltham, MA) on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Thermo Scientific, Waltham, MA), according to the manufacturer's instructions. The qRT-PCR was performed with the AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher, Waltham, MA), following the manufacturer's protocol. Each 25-µl reaction mixture contained 12.5 µl of 2× RT-PCR buffer, 10 µM of each primer, 4 µM of probe, 1 µl of 25× RT-PCR enzyme mix, and 5 µl of viral RNA. The qRT-PCR reactions were run on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 1 sec and 60°C for 60 sec. The primers and probe for the qRT-

PCR were previously published (26), and are comprised of a forward primer IBV5′GU391 (5′-GCT TTT GAG CCT AGC GTT-3′), a reverse primer IBV5′GL533 (5′-GCC ATG TTG TCA CTG TCT ATT G-3′), and a Taqman® dual-labeled probe IBV5′G probe (5′-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′). The primers were obtained from Integrated DNA Technologies (Coralville, IA), and Taqman probe was synthesized by BioSearch Technologies (Novato, CA). Real-time RT-PCR components and thermocycler parameters were previously described (26). The data are expressed as the average cycle threshold (CT) value for all samples in each group, with positive CT values based on the limit of detection for this test associated with virus detection in eggs (171).

Each qRT-PCR reaction plate included a standard curve as an RNA extraction control and as a positive control. Mass41-type IBV isolated from allantoic fluid was used as the template for the standard curve. Negative controls were also included in each plate and consisted of PCR reagents with no RNA.

Serum and plasma IBV-specific IgG antibody titers

IBV-specific IgG titers were detected using a commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME). Briefly, serum or plasma samples (stored at −20°C) were diluted 1:500, and the procedure was performed according to the manufacturer's protocol.

Tear secreted IBV-specific IgA antibody titers

Tear IBV-specific IgA was detected using a commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME). Briefly, tears were serially diluted two-fold in PBS and 100 μl was added in duplicate to wells and incubated overnight at 4°C. All manual wash steps were performed using PBS-Tween 20 (0.05% Tween 20). Plates were washed, incubated at 23°C for 2hr with 100 μl of monoclonal mouse anti-chicken IgA-BIOT (1:1000, clone A-1, Southern

Biotech, Birmingham, AL) per well, washed a second time, incubated at 23°C for 1hr with 100 µl of Streptavidin-HRP (1:4000, Southern Biotech, Birmingham, AL) per well, and washed a final time. Final antibody detection steps were completed according to the manufacturer's instructions. Endpoint titers were determined by reporting the lowest dilution at which the optical density (OD), recorded at 650 nm wavelength, was at least three standard deviations above the mean of 12 control wells incubated with no tear samples. Data from wells with a pinpoint color change due to residual substrate or air bubbles were excluded from analysis, and results were reported as $\log_2$ of the endpoint titer.

Leukocyte enrichment and enumeration

Single-cell suspensions were obtained from the HG by mechanical disruption using size 60 mesh screens (Sigma-Aldrich, St. Louis, MO). Cell suspensions were centrifuged at 23°C for 7 min at 300 x g. Single cell suspensions of leukocytes from 10, 12, and 14 dpv were resuspended in PBS containing 1% BSA. Since the HG in older birds yielded greater cell counts, leukocytes collected at 24-28 dpv and 10-14 dpc were enriched by Histopaque density gradient (1.077 g/ml) (Sigma-Aldrich, St. Louis, MO) at 23°C for 30 min at 450 x g. Cells were collected from the interface, washed as described above, and resuspended in PBS containing 1% BSA. Cell were enumerated and viability was determined using trypan blue exclusion on a Cellometer Mini (Nexcelcom Bioscience, Lawrence, MA) with final cell dilutions set at 4×10^6 cells/mL.

Flow cytometry

The following antibodies were used to stain the enriched cells: mouse anti-chicken MHC Class I-FITC, mouse anti-chicken MHC Class II-PE, mouse anti-chicken CD4-PE, mouse anti-chicken CD8α-FITC, and mouse anti-chicken IgM-PE (Southern Biotechnology, Birmingham, AL) diluted in PBS, 1% bovine serum albumin, and 0.1% sodium azide at 4°C. Stained cells

were incubated on an orbital shaker for 30 min at 4°C, and 100 µl PBS, 1% bovine serum albumin, and 0.1% sodium azide was added to each well. The plate was then centrifuged at 7°C for 7 min at 400 x g, and fixed in 100 µl PBS, 1% bovine serum albumin, and 0.1% sodium azide, and 100 µl IC fixation buffer (Thermo Fisher, Waltham, MA) overnight at 4°C. Flow cytometric analysis was performed using a BD Accuri™ C6 (BD Biosciences, San Jose, CA) for Experiments 1 and 2, and a BD Accuri™ C6 Plus (BD Biosciences, San Jose, CA) for Experiment 3. Data were analyzed with BD Accuri C6 software. Leukocytes were identified by gating on singlets then discriminated by forward and side scatter gating. Avian lymphocytes were further discriminated by gating on a moderate forward and low side scatter distribution. Identification of leukocytes based on forward and side scatter was confirmed in a follow-up experiment by CD45 staining (not included in this study). All groups were represented in three experiments, except –/Am (Experiments 2 and 3). Each time point represented the average of three experiments, except 18 hpc, 48 hpc, and 5 dpc (Experiment 3 only).

Statistical analysis

Data were analyzed using Prism v.6.0 software (GraphPad Software, Inc., La Jolla, CA; www.graphpad.com). For data in which $n \geq 5$, a Kruskal-Wallis test with Dunn's multiple comparisons posttest was used to compare treatment groups. Significant differences were determined at $p < 0.05$. To standardize the identification of the percent of leukocytes in the HG, the data for leukocytes isolated over a Histopaque gradient were normalized to the data for cells collected by resuspension without Histopaque enrichment.

Results

Air quality measurements

Continuous measurements of NH_3, Rh, and temperature for each group were recorded each day. During the first 14 days, NH_3 concentrations for both +/Am and –/Am groups were generally within the target concentration of 30-60 ppm (Fig. 4.1). From 14 days until challenge, target NH_3 levels were maintained in Experiment 1 but declined to the low end of the range or below the target range in Experiments 2 and 3. No large differences between +/Am and –/Am groups were apparent, though NH_3 levels in the –/Am group tended to be lower than in the +/Am group in the final last six to ten days.

Rh trends in the NH_3-treated rooms in Experiment 3 were initially higher compared to Rh in the NH_3 control rooms, and differences were negligible after 12 days (Fig. 4.2). Rh trends in Experiments 1 and 2 and temperature in all three experiments, however, were comparable between NH_3-treated rooms.

Vaccine virus RNA loads post-vaccination

Intrachoanal swabs were collected from birds on various days following vaccination. Vaccine virus was first detected at 18 hpv in vaccinated birds (data not shown). Vaccine virus RNA load was highest at 5 and 7 dpv and declined by 10-14 dpv (Fig. 4.3). By 24-28 dpv, vaccine virus load was weakly positive (Experiments 1 and 2) or below the limit of detection (Experiment 3), determined at CT value 36.59 (171). No significant differences were observed between NH_3 treatment groups among both nonvaccinated and vaccinated birds. Nonvaccinated birds were negative for vaccine virus.

Post-challenge virus RNA loads

Intrachoanal swabs were collected from birds on various days following challenge. Post-challenge peak viral load in nonvaccinated, challenged birds occurred at 5 dpc (Fig. 4.4). By 10 dpc, virus loads had declined below the limit of detection in all challenge groups except –/Am/+ birds in Experiment 2, though this difference was not significantly different from –/Am/+ loads. All vaccinated birds were protected from IBV replication, and viral loads were below the limit of detection at all time points. Overall the trends in viral replication and clearance did not appear to be influenced by NH_3.

Clinical signs, airsacculitis, tracheal histopathology, and ciliostasis

Typical signs of IBV infection, which include watery eyes, tracheal rales, and mucus in the nares and trachea, were observed in the nonvaccinated, challenged birds at 5 dpc (Table 4.1). Mean scores in nonvaccinated, challenged birds were significantly higher than scores in vaccinated, challenged birds. NH_3 did not influence clinical sign scores. No clinical signs were observed in the negative controls. Trends in clinical sign scores were comparable among the three experiments.

Airsacculitis, denoted by the presence of opacity or foamy material or both in the airsacs at necropsy, was observed in several nonvaccinated, challenged birds, though only the nonvaccinated, challenged birds exposed to NH_3 had significantly higher scores compared to vaccinated, challenged birds (Table 4.1). Airsacculitis was absent in all vaccinated birds, regardless of NH_3 exposure, as well as in the negative controls. Airsacculitis trends were similar among all three experiments.

Post-challenge tracheal histopathology scores, scored on the basis of inflammation, epithelial hyperplasia, and deciliation, were higher in nonvaccinated birds compared to

vaccinated birds, and NH_3 had no impact on these scores (Table 4.1). Compared to scores from vaccinated, challenged birds and negative control birds, scores from nonvaccinated, challenged birds exposed to NH_3 were significantly higher in Experiments 2 and 3, and scores from nonvaccinated, challenged birds in the NH_3 control group were significantly higher in Experiments 1 and 3.

Tracheas were collected at 5 dpc and processed, and cilia activity was measured under a microscope. All nonvaccinated, challenged birds exhibited complete ciliostasis, and no effect on ciliostasis from NH_3 exposure could be observed (Table 4.1). However, cilia from the –/Am/+ birds were blunted and characterized by slower ciliary beating compared to the cilia from –/NAm/+ birds. All negative controls and all birds from vaccinated, challenged groups, independent of NH_3 exposure, passed the ciliostasis test. These observations were similar for all three trials.

IBV-specific IgG serum titers

Serum IBV-specific IgG maternal antibodies were present until at least 10-14 dpv in all experiments, and neither vaccine nor NH_3 status had an effect on titers (Table 4.2). Prior to challenge at 24-28 dpv, active IgG titers in vaccinated birds trended above baseline but were not significant, and titers were not influenced by NH_3. By 18 hpc, titers had increased dramatically among vaccinated, challenged birds and remained high for the duration of the study (data not shown for 18, n=4, and 48 hpc, n=4). Compared to negative control titers, significantly higher titers were observed in –/NAm/+ birds (Experiment 1) and –/Am/+ birds (Experiments 1 and 3). In contrast, titers in nonvaccinated, challenged birds did not increase until 10-14 dpc, when they were significantly higher than negative control titers. NH_3 had no effect on post-challenge IgG titers at any collection time.

IBV-specific IgA tear titers in tears

At all times post-vaccination, vaccinated bird IBV-specific IgA titers in tears were not significantly different from background titers, and titers were not influenced by NH_3 (Table 4.3). In the first week post-challenge, no significant differences in titers among bird groups were observed, regardless of vaccination or NH_3 status (data not shown for 18 hpc, 48 hpc, 3 dpc, and 7 dpc). By 10-14 dpc, titers in –/NAm/+ birds were significantly higher than titers in +/NAm/+ birds (Experiments 1 and 2). No other significant differences were found, though nonvaccinated, challenged birds in all experiments had upward trends in titers compared to vaccinated and negative control birds.

Percent of leukocytes and MHC I^+/II^{lo} and MHC I^+/II^{hi} cells

NH_3 had no impact on leukocyte percentages and leukocyte phenotypes in the HG, but vaccine and challenge virus-related differences among treatment groups were apparent (Table 4.4). At 10-14 dpv, the percent of leukocytes was significantly higher in +/NAm birds (Experiments 2 and 3) and +/Am birds (Experiments 1, 2 and 3). The percent of MHC I^+/II^{hi} cells was significantly higher in +/NAm birds (Experiments 1 and 3) and +/Am birds (Experiments 1, 2 and 3), while the percent of MHC I^+/II^{lo} cells was significantly lower in +/NAm birds (Experiment 3) and +/Am birds (Experiment 1 and 3). By 24-28 dpv, fewer significant differences were detected, though the overall trends still persisted, which consisted of an elevated percent of leukocytes and MHC I^+/II^{hi} cells accompanied by a lower percent of MHC I^+/II^{lo} cells.

At 18 and 48 hpc, vaccinated birds tended to have a higher percentage of leukocytes and MHC I^+/II^{hi} cells, and a lower percentage of MHC I^+/II^{lo} cells, compared to nonvaccinated birds (data not shown). In nonvaccinated birds, an upward trend in the percent of MHC I^+/II^{lo} cells was

observed relative to the pre-challenge levels. By comparison, there was no significant change in the percentage of MHC I^+/II^{lo} and MHC I^+/II^{hi} cells relative to the pre-challenge levels in vaccinated birds.

By 5 dpc, nonvaccinated birds experienced a strong peak in the percent of leukocytes and MHC I^+/II^{hi} cells, while the percent of MHC I^+/II^{lo} cells declined below the percent of MHC I^+/II^{lo} cells in negative controls. Vaccinated birds did not experience significant changes in the percentage of leukocytes or MHC I^+/II^{lo} cells, though a slight increase in the percent of MHC I^+/II^{hi} cells occurred. No statistical analysis could be performed due the low sample size (n=4) at this time.

At 10-14 dpc, the percent of leukocytes in nonvaccinated, challenged birds had declined, and though the data were not significant, the birds maintained higher trends in the percent of leukocytes compared to vaccinated, challenged birds and negative controls. Similarly, vaccinated birds displayed trends of a higher percent of leukocytes compared to negative controls. There were no significant differences among challenge and negative control groups in the percent of MHC I^+/II^{lo} cells. With respect to MHC I^+/II^{hi} cells, no significant differences were detected among challenge groups. Compared to negative controls, the only group to have a significantly higher percent of MHC I^+/II^{hi} cells was the –/Am/+ group in Experiment 3, but all challenge groups in Experiments 2 and 3 showed trends of a higher percent of MHC I^+/II^{hi} cells compared to negative controls.

Percent of IgM^+ cells

NH_3 exposure did not significantly affect the percent of IgM^+ cells across treatment groups and over time (Table 4.5). At 10-14 dpv, only the +/Am birds had a significantly elevated percent of IgM^+ cells (Experiments 2 and 3), but +/NAm birds also displayed higher trends in the

percent of IgM^+ cells (Experiments 2 and 3). By 24-28 dpv, the percent of IgM^+ cells was comparable across all treatment groups. At 18 hpc, the percent of IgM^+ cells remained comparable across all treatment groups, and at 48 hpc the percent of IgM^+ cells began to trend upward in nonvaccinated birds, but no differences were observed in the vaccinated birds (data not shown). By 5 dpc, nonvaccinated birds experienced a strong peak in the percent of IgM^+ cells relative to vaccinated birds and negative controls. In contrast, no clear changes were observed among vaccinated birds, though a higher trend was noted in +/Am/+ birds compared to +/NAm/+ birds and the controls. At 10-14 dpc, the percent of IgM^+ cells among all challenged birds, irrespective of vaccination or NH_3 treatment, were comparable to each other (all experiments) and negative controls (Experiments 2 and 3).

Percent of $CD4^+$ and $CD8^+$ cells

The HG was collected from 10-28 dpv and 18 hpc to 14 dpc and analyzed for the percent of T cell phenotypes via flow cytometry (Table 4.6). At 10-14 dpv, overall significant differences were not observed among treatment groups with respect to the percent of $CD4^+$ cells, though vaccinated birds showed upward trends in the percent of $CD4^+$ cells. The percent of $CD8^+$ cells was significantly increased ($p < 0.05$) in –/NAm birds (Experiment 3) and –/Am birds (Experiments 1, 2 and 3). Though no significant differences were detected in the CD4:CD8 ratio, vaccinated birds showed trends of a decreased CD4:CD8 ratio. NH_3 did not affect any of these parameters. Prior to challenge at 24-28 dpv, the percent of $CD4^+$ cells in vaccinated birds was significantly lower (Experiment 1), and no differences in the percent of $CD8^+$ cells were observed. Overall the CD4:CD8 ratio was not affected with the exception of the ratio in +/NAm birds in which the ratio was significantly lower ($p < 0.05$, Experiment 1) compared to the ratio in –/NAm birds. Although there were vaccine treatment-related trends in lower CD4:CD8 ratios

and shifts in the percent of CD4 and CD8 cells at 18 hpc, 48 hpc and 5 dpc, there were no significant differences in the T cell phenotypes across treatments (data not shown for 18 hpc and 48 hpc). By 10-14 dpc, no significant differences in the percent of $CD4^+$ cells were observed. Though significant differences were not detected in the percent of $CD8^+$ cells among challenge groups, nonvaccinated, challenged birds displayed trends in a higher percent of $CD8^+$ cells compared to vaccinated, challenged birds. This pattern was also observed in all challenge groups compared to negative controls, though only –/Am/+ birds had a significantly elevated percent of $CD8^+$ cells (Experiment 3). No significant differences in CD4:CD8 ratio were observed.

Discussion

In this study, we conducted three *in vivo* experiments to analyze the effect of ambient NH_3 on broiler immune response to IBV vaccination and protection against homologous IBV challenge. One challenge with NH_3 studies is maintaining exposure levels that are representative of NH_3 concentrations in commercial poultry operations. NH_3 levels vary considerably and may depend on season, time of day, geographic location, and individual poultry houses, and concentrations tend to be higher during cold weather as ventilation rates are reduced to conserve heat and reduce energy costs (28, 113, 133). NH_3 concentrations commonly average 39.9 ppm in the winter months, compared to 6.3 ppm in the summer months (133), and broiler houses are prone to NH_3 spikes leading to high levels of short duration (45). Because of the wide variation in NH_3 levels in the field and the difficulties with maintaining constant NH_3 concentrations using litter-sourced NH_3, we chose a target range (30-60 ppm) that encompassed average wintertime concentrations. While NH_3 concentrations in Experiment 1 were within the target range throughout the entire study, NH_3 levels in Experiments 2 and 3 stayed within the target range for only the two first weeks, after which they declined to the low end of or below the target range. In

spite of this observation, it is unlikely that maintaining target concentrations for the duration of the experiment would have led to different outcomes, as few clear differences were detected among the three experiments with regards to the parameters measured.

Based on the parameters measured in this study, NH_3 exposure had no clear impact on the immune response to IBV vaccination. Furthermore, +/Am/+ birds were protected from challenge virus replication and demonstrated an immune response comparable to the response in +/NAm/+ birds. Despite being protected from challenge, birds in the +/Am/+ group showed subtle effects of NH_3 on cilia morphology and function, characterized by blunted cilia and slower ciliary beating. In the event of higher NH_3 levels or acute exposure, we speculate that the morphological and functional effects of NH_3 might become more prominent, perhaps predisposing birds to respiratory disease. Nevertheless, in the present study, the deleterious effects of NH_3 became apparent only when nonvaccinated birds were challenged with IBV, which resulted in increased clinical sign scores in Experiment 3 and an elevated incidence of airsacculitis in both Experiments 2 and 3. Airsacculitis resulting from NH_3 exposure combined with live attenuated IBV vaccination has been reported previously (Kling and Quarles, 1974; Quarles and Caveny, 1979). However in the present study, vaccinated birds, irrespective of NH_3 exposure, were completely protected from challenge, and airsacculitis was not detected in either the vaccinated, challenged birds or the vaccinated, nonchallenged birds. Our data therefore suggest that the negative effects of NH_3 become apparent when nonvaccinated birds are exposed to a virulent IBV challenge virus, which lead to airsacculitis.

With regards to ciliostasis and tracheal histopathology scores, which were determined by evidence of inflammation, epithelial hyperplasia, and deciliation, exposure to NH_3 was not a factor as all of the non-vaccinated, challenged birds exhibited ciliostasis. Still, it is possible that

NH_3 might be associated with an increased incidence of ciliostasis should the birds be exposed to a less virulent IBV or smaller dose. Nevertheless, ciliostasis was not observed in the +/Am/+ birds, and scores were comparable to those for the +/NAm/+ birds. These results are in contrast with a report by Anderson, Beard and Hanson (9), which described some deciliation in the upper trachea and nasal turbinates in chickens, however those birds were housed in an environment consisting of poultry house dust, carbon dioxide, and NH_3 in which NH_3 concentrations were not recorded.

Except for some minor differences, NH_3 had no overall effect on vaccine and challenge virus replication and clearance by the host, nor did it seem to impact IBV-specific local and systemic serological responses measured in tears (IgA) and serum (IgG). Furthermore, it did not appear to impact IBV-specific local (IgA in tears) and systemic (IgG in serum) responses. Cell phenotypes of the local immune response measured in the HG, which included the percent of leukocytes, MHC I^+/II^{lo} and MHC I^+/II^{hi} cells, IgM^+ cells, and $CD4^+$ and $CD8^+$ T cells, also showed no NH_3-related differences. Although NH_3 exposure had no apparent effect on the immune response to IBV vaccination and challenge, vaccination status alone had a measurable impact.

Vaccination of maternal antibody-positive broiler chicks at hatch induced a weak-to-nonexistent serum IgG response and by 24-28 DOA, titers were only slightly higher than background titers in nonvaccinated controls. In contrast to IgG responses, post-vaccination IBV-specific IgA responses in tears were not detected. Nonvaccinated birds challenged at 28 DOA developed strong serum IgG responses by 10-14 dpc. As seen with serum IgG responses, nonvaccinated, challenged birds developed robust IgA titers in tears at 10-14 dpc. Orr-Burks et al. (2014) have reported similar timing in the development of plasma IgG and tear IgA titers in

chickens following ocular IBV vaccination at 28 DOA. Though we did not evaluate the effect of maternal antibodies on active antibody production in this study, the absence of robust titers in vaccinated birds in this study could be explained by maternal antibody interference which diminished the young birds' epitope-specific antibody response following IBV vaccination. Maternal antibody modulation of IBV epitope-specific antibody production in young chicks has been well documented and yet protection against homologous challenge does not appear to be compromised suggesting that other arms of the immune system are activated by the vaccine (160, 186).

Among the vaccinated birds in this study challenged with IBV, serum IgG titers increased rapidly, reaching high titers as early as 18 hpc. Titers were sustained for the duration of the study. In spite of these systemic IgG responses, it is worth noting that circulating antibody titers do not correlate with protection against IBV (160). By comparison, average post-challenge IgA titers in vaccinated birds did not differ from negative control titers at any time post-challenge. Though no increase in IgA was detected in tears, previous IBV challenge studies have suggested that local IgA responses may be a more reliable indicator of protection from active infection (185) and that mucosal IgA levels are greater than mucosal IgG levels (13, 186). Because we did not examine IgG titers in tears, we could not make direct comparisons between IBV-specific IgG and IgA levels in tears. The absence of a detectable IgA response in tears post-challenge may be explained by the presence of neutralizing antibodies in the tears, which would inhibit mucosal tissue access by IBV infection and subsequent stimulation of IgA responses, and therefore result in rapid viral clearance. The lack of a significant increase in the IgA titers among vaccinated birds after challenge has been described (88, 108). Alternatively, local antibody responses may be dominated by another antibody class that was not evaluated in this study. Orr-Burks, Gulley,

Gallardo, Toro and van Ginkel (150) demonstrated both IBV-specific IgG and IgA titers in tears following IBV vaccination. Nevertheless, our data provide novel insight into the role of tear IgA in IBV immunity.

The percent of leukocytes in the HG was higher in vaccinated birds post-vaccination and was sustained until challenge, which implies that the viral vaccine stimulated colonization and clonal expansion of leukocytes in the HG. It is believed that B cell colonization of the HG occurs prior to 2 WOA (77) and that the HG microenvironment orchestrates terminal B cell differentiation and immunoglobulin class switch (127). In the present study, the observation that MHC I^+/II^{lo} cells predominated in nonvaccinated birds whereas MHC I^+/II^{hi} cells were over-represented in vaccinated birds, would support that IBV vaccination induces an upregulation in MHC II expression as cells colonizing the HG become activated.

During the first 48 hr post-IBV challenge, there was no evidence of leukocyte migration or activation in the HG. By 5 dpc, nonvaccinated, IBV-challenged birds had a significant increase in the percent of leukocytes, whereas no change occurred in vaccinated birds. This observation coincided with a dramatic shift in MHC I^+/II^{lo} to MHC I^+/II^{hi} cells among nonvaccinated, challenged birds, and a slight shift from MHC I^+/II^{lo} to MHC I^+/II^{hi} cells among vaccinated, challenged birds. These observations are not surprising given that at 5 dpc virus replication peaked in nonvaccinated birds, but remained below the level of detection in vaccinated birds. By 10-14 dpc, the relative lack of differences in the percent of leukocytes and MHC I^+/II^{lo} and MHC I^+/II^{hi} cells between nonvaccinated and vaccinated birds indicated resolution of infection.

Cells that express MHC II were reviewed in Glimcher and Kara (84) and include B lymphocytes, macrophages, dendritic cells, thymic epithelium, and activated T cells, and the

level of MHC II expression is influenced by a large number of different stimuli. In addition, multiple cell types, including epithelial cells, may be induced to express MHC II by IFN-γ (84), a cytokine that is upregulated during IBV infection (146, 196). In a follow-up experiment using CD45 and CD115 markers that identify leukocytes and cells of the monocyte-macrophage lineage, respectively, we confirmed that the MHC I^+/II^{lo} population consisted mostly of macrophages and propose that macrophages in the HG in their resting state are MHC I^+/II^{lo}. We speculate that the macrophages become activated and upregulate MHC II expression post-infection, although further research is needed. Mature B lymphocytes are the only major population of cells that constitutively express large amounts of MHC II (84), and MHC II expression is largely lost during terminal differentiation into plasma cells (92). Literature citing constitutive expression of MHC II in high quantities on B cells, combined with our observations of IgM^+ B lymphocyte dynamics following IBV infection, suggest that a large proportion of the MHC I^+/II^{hi} cells in the HG consists of B lymphocytes. Therefore, it would follow that the shift in predominance from MHC I^+/II^{lo} cells to MHC I^+/II^{hi} cells following IBV infection represents, at least in part, macrophage activation and colonization and clonal expansion of mature B lymphocytes.

In Experiments 2 and 3, the percent of IgM^+ cells in the HG was initially greater among vaccinated birds at 10-14 dpv, and by 24-28 dpv the percent among nonvaccinated birds had increased to the level of vaccinated birds. The small sample size collected may account for the lack of consistency in the percent of IgM^+ cells between Experiment 1 and Experiments 2 and 3. Since IgM^+ cells measured in this study were not necessarily IBV-specific, we speculate that the increase in IgM^+ cells in nonvaccinated birds was a normal function of colonization and clonal expansion in the HG in response to other immunogens driving IgM responses. This is not

surprising, since the majority of lymphoid cells in the HG of young birds express IgM (4). Nonvaccinated birds challenged at 28 DOA experienced a peak percent of IgM^+ lymphocytes at 5 dpc, coinciding with the peak of virus load, and a decline to pre-challenge levels by 10-14 dpc, implying the cessation of IBV-induced IgM^+ B cell clonal expansion and the initiation of class switch and subsequent loss of IgM expression. These data suggest that IgM responses are rapid and transient, which complement previous work in IBV-specific IgM in sera demonstrating that IgM antibody responses are first detected five days after IBV exposure (56), reaching peak titers at 8 days and waning by 24 days (47). In contrast, vaccinated, challenged birds in our study showed no increases in the percent of IgM^+ cells in the HG, which would imply that vaccinated birds effectively neutralized the virus and that the IgM^+ cells were not activated.

The percent of $CD4^+$ and $CD8^+$ lymphocytes in the HG was initially greater among vaccinated birds at 10-14 dpv, and the lower CD4:CD8 ratio indicated that vaccinated birds experienced predominantly a $CD8^+$ T cell response following vaccination. These findings confirm previous research describing cytotoxic T cells as the major cells involved in primary exposure to IBV (173). These differences largely became nonsignificant by 24-28 dpv, and the CD4:CD8 ratio in vaccinated birds increased as the percent of $CD8^+$ cells decreased, which corresponded with virus clearance and the resolution of infection. The importance of the cytotoxic T cell response following primary IBV exposure was further confirmed when nonvaccinated birds challenged at 28 DOA exhibited a strong $CD8^+$ cell response, with a simultaneous decrease in CD4:CD8 ratio, at 10-14 dpc. Among vaccinated birds, no clear shift in the T cell populations post-challenge was detected, which implies that the HG T cell may have a minor role in the immune response to secondary IBV exposure or the level of humoral immunity

from vaccinated birds was robust enough to minimize mucosal penetration and mediate clearance.

Conclusions

Young commercial broilers exposed to NH_3 during a typical grow-out period responded well to both post-hatch IBV vaccine and a 28-day IBV challenge when compared to nonvaccinated birds in NH_3 control groups. Furthermore, NH_3 did not impact vaccine or challenge virus replication and clearance, ciliostasis, or tracheal histopathology scores. In addition, NH_3 also did not affect host immune response to the IBV vaccine or challenge. The only notable adverse observation was airsacculitis, which occurred in nonvaccinated, NH_3-exposed birds that were challenged with IBV, however nonvaccinated, IBV-challenged birds in the NH_3 control group also displayed airsacculitis, but at a lower incidence.

The IBV vaccine mobilized leukocytes with increased MHC I^+/II^{hi}, IgM^+, and $CD8^+$ expression in the HG, and serum IgG antibody titers remained elevated but comparable to nonvaccinated titers through 14 DOA. This observed IBV epitope-specific humoral immune profile pattern, measured in all bird groups, was likely due to a robust protective maternal immunity. Following IBV challenge, although all bird groups responded, nonvaccinated birds at 5 dpc had the greatest measurable increase in activated leukocytes denoted by increased MHC I^+/MHC II^{hi} and IgM^+ expression with mucosal and serum immunoglobulin levels and $CD8^+$ expression peaking at 10-14 dpc; the time period in which most of the birds had cleared the virus. It is also worth mentioning that the IBV challenge strain in the HG was cleared more quickly than the vaccine strain. Furthermore, the minimal immune activation in the HG of vaccinated, challenged birds indicates that the challenge virus was efficiently neutralized before mucosal penetration and viral replication.

Tables

Table 4.1 Clinical signs, airsacculitis, histopathology scores, and ciliostasis five days post-challenge.

a) Experiment 1

Treatment						Ciliostasis	
Vaccine	Ammonia	Challenge	Clinical Signs (Mean ± SEM*)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis Protection Score†	Binomial Protection Score‡
-	-	+	2.1 ± 0.4[a]	5/10[a]	9.8 ± 0.1[a]	0.0	10/10[a]
+	-	+	0.1 ± 0.1[b]	0/10[b]	6.4 ± 0.4[b]	86.2	1/10[b]
+	+	+	0.3 ± 0.2[b]	0/10[b]	5.1 ± 0.5[b]	87.3	0/10[b]
-	-	-	0.0 ± 0.0[b]	0/5[ab]	6.0 ± 0.4[b]	97.7	0/5[ab]
+	-	-	0.0 ± 0.0[b]	0/5[ab]	5.9 ± 0.4[b]	82.0	0/5[ab]
+	+	-	0.0 ± 0.0[b]	0/5[ab]	5.2 ± 0.4[b]	68.7	1/5[ab]

b) Experiment 2

Treatment						Ciliostasis	
Vaccine	Ammonia	Challenge	Clinical Signs (Mean ± SEM)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis Protection Score	Binomial Protection Score
-	-	+	3.0 ± 0.0[a]	2/5[ab]	9.5 ± 0.0[ab]	0	5/5[a]
-	+	+	3.0 ± 0.0[a]	9/10[a]	9.6 ± 0.1[a]	0	10/10[a]
+	-	+	0.0 ± 0.0[b]	0/10[b]	6.1 ± 0.1[bc]	90.7	0/10[b]
+	+	+	0.0 ± 0.0[b]	0/10[b]	6.2 ± 0.3[abc]	88.3	0/10[b]
-	-	-	0.0 ± 0.0[b]	0/5[b]	3.9 ± 0.4[c]	96.0	0/5[b]
-	+	-	0.0 ± 0.0[b]	0/5[b]	5.1 ± 0.6[bc]	91.0	0/5[b]
+	-	-	0.0 ± 0.0[b]	0/5[b]	4.8 ± 0.6[c]	92.0	0/5[b]
+	+	-	0.0 ± 0.0[b]	0/5[b]	5.5 ± 0.3[bc]	87.0	0/5[b]

c) Experiment 3

Treatment						Ciliostasis	
Vaccine	Ammonia	Challenge	Clinical Signs (Mean ± SEM)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis Protection Score	Binomial Protection Score
-	-	+	2.3 ± 0.3[a]	3/10[ab]	9.6 ± 0.2[a]	0	10/10[a]
-	+	+	2.7 ± 0.2[a]	7/10[a]	9.6 ± 0.1[a]	0	10/10[a]
+	-	+	0.2 ± 0.1[b]	0/9[b]	6.6 ± 0.2[b]	78.3	1/9[b]
+	+	+	0.0 ± 0.0[b]	0/10[b]	6.5 ± 0.6[b]	82.2	1/10[b]
-	-	-	0.0 ± 0.0[b]	0/5[b]	4.7 ± 0.5[b]	71.3	0/5[b]
-	+	-	0.4 ± 0.2[ab]	0/5[b]	5.6 ± 0.4[b]	90.0	0/5[b]
+	-	-	0.4 ± 0.2[ab]	0/5[b]	4.1 ± 1.1[b]	83.3	0/5[b]
+	+	-	0.4 ± 0.2[ab]	0/5[b]	6.0 ± 0.0[ab]	88.3	0/5[b]

*SEM = standard error of the mean.

†The protection score for each group was calculated using the following formula: 100 − [(total of the individual scores for the group)/(the number of individuals in the group × 20) × 100], groups with scores of >50 are considered protected.

‡The number of birds with ciliostasis per total in the group. Each ring was scored by three individuals. A tracheal ring with ≥50% of the cilia showing vigorous activity was considered protected, and a chicken was considered protected when 90% of the rings were showing ≥50% of cilia beating.

[a,b,c]Different letters are significant at $p < 0.05$.

Table 4.2 Serum IBV-specific IgG titer post-vaccination and post-challenge in maternal antibody-positive broilers.

a) Experiment 1

	Treatment			
Day	Vaccine	Ammonia	Challenge	IgG Titer (Mean ± SEM*)
	-	-	-	9.3 ± 0.1
	+	-	-	10.7 ± 0.3
7 dpv†	+	+	-	11.5 ± 0.5
	-	-	-	3.2 ± 0.8
	+	-	-	5.2 ± 1.2
10-14 dpv	+	+	-	6.5 ± 0.9
	-	-	-	0.4 ± 0.4[a]
	+	-	-	6.0 ± 1.4[ab]
24-28 dpv (Pre-Challenge)	+	+	-	7.9 ± 0.8[b]
	-	-	+	1.5 ± 0.6[ac]
	+	-	+	6.8 ± 0.6[b]
	+	+	+	8.0 ± 0.4[b]
5 dpc‡	-	-	-	0.0 ± 0.0[c]
	-	-	+	10.6 ± 0.6
	+	-	+	7.4 ± 1.2
10-14 dpc	+	+	+	8.2 ± 0.5

b) Experiment 2

	Treatment			
Day	Vaccine	Ammonia	Challenge	IgG Titer (Mean ± SEM)
	-	-	-	10.9 ± 0.5
	-	+	-	9.9 ± 0.5
	+	-	-	10.7 ± 0.2
7 dpv	+	+	-	10.8 ± 0.5
	-	-	-	7.8 ± 0.7
	-	+	-	6.5 ± 1.4
	+	-	-	5.1 ± 1.8
10-14 dpv	+	+	-	6.3 ± 1.0
	-	-	-	1.0 ± 1.0
	-	+	-	1.2 ± 1.2
	+	-	-	4.9 ± 0.9
24-28 dpv (Pre-Challenge)	+	+	-	4.1 ± 1.4
	-	-	+	4.1 ± 0.8[ab]
	-	+	+	3.6 ± 0.6[b]
	+	-	+	8.1 ± 0.6[ac]
	+	+	+	8.5 ± 0.4[c]
5 dpc	-	-	-	5.1 ± 0.8[abc]
	-	-	+	9.6 ± 0.4[a]
	-	+	+	9.0 ± 0.7[a]
	+	-	+	4.3 ± 1.7[ab]
	+	+	+	7.4 ± 1.3[ab]
10-14 dpc	-	-	-	1.2 ± 0.8[b]

c) Experiment 3

	Treatment			
Day	Vaccine	Ammonia	Challenge	IgG Titer (Mean ± SEM)
	-	-	-	12.4 ± 0.3
	-	+	-	12.3 ± 0.2
	+	-	-	11.5 ± 0.4
7 dpv	+	+	-	12.2 ± 0.1
	-	-	-	11.5 ± 0.3
	-	+	-	11.1 ± 0.4
	+	-	-	10.7 ± 0.5
10-14 dpv	+	+	-	10.8 ± 0.2
	-	-	+	7.0 ± 0.4[a]
	-	+	+	7.1 ± 0.6[a]
	+	-	+	9.6 ± 0.3[ab]
	+	+	+	10.6 ± 0.3[b]
5 dpc	-	-	-	4.1 ± 1.1[a]
	-	-	+	13.1 ± 0.5[ab]
	-	+	+	13.5 ± 0.2[a]
	+	-	+	10.3 ± 0.4[bc]
	+	+	+	11.8 ± 0.5[ab]
10-14 dpc	-	-	-	6.6 ± 0.4[c]

*SEM = standard error of the mean.

†dpv = days post-vaccination.

‡dpc = days post-challenge.

[a,b,c]Different letters are significant at $p < 0.05$.

Table 4.3 Tear IBV-specific IgA titer post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			IgA Titer (Mean ± SEM*)
	Vaccine	Ammonia	Challenge	
10-14 dpv†	-	-	-	7.3 ± 0.3
	+	-	-	8.3 ± 0.8
	+	+	-	8.0 ± 0.4
24-28 dpv (Pre-Challenge)	-	-	-	8.5 ± 0.7
	+	-	-	8.3 ± 0.8
	+	+	-	10.5 ± 0.8
10-14 dpc‡	-	-	+	13.3 ± 0.5[a]
	+	-	+	10.8 ± 0.4[b]
	+	+	+	10.5 ± 0.8[b]

b) Experiment 2

	Treatment			
Day	Vaccine	Ammonia	Challenge	IgA Titer (Mean ± SEM)
	-	-	-	5.8 ± 0.5
	-	+	-	4.8 ± 0.2
	+	-	-	6.6 ± 0.4
7 dpv	+	+	-	5.0 ± 0.4
	-	-	-	6.5 ± 0.5
	-	+	-	7.3 ± 0.3
	+	-	-	9.3 ± 1.1
10-14 dpv	+	+	-	8.0 ± 1.1
	-	-	-	8.8 ± 0.7
	-	+	-	9.1 ± 0.3
	+	-	-	10.1 ± 0.4
24-28 dpv (Pre-Challenge)	+	+	-	11.3 ± 0.7
	-	-	+	9.1 ± 0.5
	-	+	+	10.6 ± 1.1
	+	-	+	8.9 ± 1.1
	+	+	+	11.1 ± 0.3
5 dpc	-	-	-	11.4 ± 0.5
	-	-	+	12.6 ± 0.4[a]
	-	+	+	12.8 ± 0.6[a]
	+	-	+	9.0 ± 0.7[b]
	+	+	+	10.1 ± 0.8[ab]
10-14 dpc	-	-	-	10.1 ± 0.4[ab]

c) Experiment 3

	Treatment			
Day	Vaccine	Ammonia	Challenge	IgA Titer (Mean ± SEM)
	-	-	-	9.4 ± 0.6
	-	+	-	10.2 ± 0.2
	+	-	-	11.0 ± 0.4
7 dpv	+	+	-	9.8 ± 0.6
	-	-	-	10.4 ± 0.2
	-	+	-	10.1 ± 0.2
	+	-	-	10.7 ± 0.3
10-14 dpv	+	+	-	10.8 ± 0.3
	-	-	-	9.6 ± 0.3
	-	+	-	10.4 ± 0.3
	+	-	-	10.2 ± 0.4
24-28 dpv (Pre-Challenge)	+	+	-	9.8 ± 0.5
	-	-	+	8.6 ± 0.6
	-	+	+	9.2 ± 0.8
	+	-	+	9.8 ± 0.6
	+	+	+	9.8 ± 0.7
5 dpc	-	-	-	9.2 ± 0.7
	-	-	+	11.5 ± 0.5[ab]
	-	+	+	12.2 ± 0.6[a]
	+	-	+	9.8 ± 0.4[b]
	+	+	+	10.1 ± 0.6[ab]
10-14 dpc	-	-	-	10.2 ± 0.4[ab]

*SEM = standard error of the mean.

†dpv = days post-vaccination.

‡dpc = days post-challenge.

[a,b]Different letters are significant at $p < 0.05$.

Table 4.4 Percent leukocytes and MHC I^+/II^{lo} and MHC I^+/II^{hi} cells in Harderian gland post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			% Leukocytes (Mean ± SEM*)	% MHC I^+/II^{lo} of leukocytes (Mean ± SEM)	% MHC I^+/II^{hi} of leukocytes (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	1.3 ± 0.2[a]	26.2 ± 3.9[a]	10.8 ± 1.9[a]
	+	-	-	5.9 ± 1.8[ab]	19.3 ± 2.4[ab]	29.9 ± 2.1[b]
10-14 dpv†	+	+	-	6.2 ± 1.2[b]	16.0 ± 2.9[b]	36.4 ± 3.6[b]
	-	-	-	1.6 ± 0.3[a]	18.7 ± 2.7	18.4 ± 2.3
24-28 dpv (Pre-Challenge)	+	-	-	4.7 ± 1.2[ab]	13.3 ± 3.0	30.0 ± 8.1
	+	+	-	5.3 ± 1.1[b]	11.2 ± 2.0	38.1 ± 6.8
	-	-	+	10.2 ± 1.0	10.6 ± 1.4	26.1 ± 2.8
	+	-	+	7.4 ± 0.9	14.5 ± 3.4	28.0 ± 4.9
10-14 dpc‡	+	+	+	8.2 ± 1.3	14.4 ± 3.2	33.9 ± 4.5

b) Experiment 2

Day	Treatment			% Leukocytes (Mean ± SEM)	% MHC I^+/II^{lo} of leukocytes (Mean ± SEM)	% MHC I^+/II^{hi} of leukocytes (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	1.3 ± 0.1[a]	14.1 ± 1.6	11.5 ± 1.5[ab]
	-	+	-	1.4 ± 0.2[ab]	12.1 ± 1.7	10.3 ± 2.1[a]
	+	-	-	7.6 ± 2.0[bc]	12.0 ± 1.8	28.0 ± 4.8[b]
10-14 dpv	+	+	-	11.6 ± 3.5[c]	9.2 ± 1.5	28.8 ± 3.4[b]
	-	-	-	3.2 ± 1.9[a]	22.6 ± 8.3	13.1 ± 3.7
	-	+	-	3.1 ± 0.3[ab]	20.6 ± 2.7	15.1 ± 1.8
24-28 dpv (Pre-Challenge)	+	-	-	16.2 ± 3.2[b]	13.2 ± 3.8	40.5 ± 7.4
	+	+	-	6.4 ± 2.4[ab]	17.6 ± 5.9	32.6 ± 8.0
	-	-	+	7.3 ± 2.2[ab]	14.1 ± 3.7	19.3 ± 3.8
	-	+	+	7.3 ± 1.3[a]	11.5 ± 2.0	23.2 ± 3.3
	+	-	+	4.1 ± 1.3[ab]	19.7 ± 4.8	20.8 ± 4.2
	+	+	+	4.2 ± 1.0[ab]	10.6 ± 3.9	19.6 ± 5.2
10-14 dpc	-	-	-	1.3 ± 0.4[b]	22.1 ± 3.5	13.3 ± 2.2

c) Experiment 3

Day	Treatment			% Leukocytes (Mean ± SEM)	% MHC I^+/II^{lo} of leukocytes (Mean ± SEM)	% MHC I^+/II^{hi} of leukocytes (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	1.2 ± 0.1[a]	26.1 ± 2.9[ac]	5.4 ± 0.5[a]
	-	+	-	1.3 ± 0.1[a]	26.7 ± 2.1[a]	5.8 ± 0.4[a]
	+	-	-	11.5 ± 1.1[b]	15.6 ± 1.6[b]	32.4 ± 1.8[b]
10-14 dpv	+	+	-	9.1 ± 1.0[b]	16.5 ± 1.7[bc]	29.6 ± 1.9[b]
	-	-	+	17.3 ± 3.0	7.2 ± 1.4	69.5 ± 6.0
	-	+	+	24.4 ± 4.9	5.3 ± 0.4	67.6 ± 6.0
	+	-	+	4.6 ± 1.0	10.7 ± 1.0	43.5 ± 5.5
	+	+	+	5.1 ± 0.9	7.7 ± 1.5	51.3 ± 4.6
5 dpc	-	-	-	1.3 ± 0.2	24.2 ± 2.8	12.1 ± 1.2
	-	-	+	7.2 ± 0.9	8.0 ± 0.7	28.2 ± 5.8[ab]
	-	+	+	8.8 ± 1.3	6.1 ± 0.8	35.4 ± 2.5[a]
	+	-	+	6.1 ± 1.2	6.2 ± 0.9	33.5 ± 3.4[ab]
	+	+	+	4.4 ± 1.4	5.9 ± 0.9	26.1 ± 3.3[ab]
10-14 dpc	-	-	-	1.5 ± 0.9	7.6 ± 1.3	17.3 ± 2.1[b]

*SEM = standard error of the mean.

†dpv = days post-vaccination.

‡dpc = days post-challenge.

[a,b,c]Different letters are significant at $p < 0.05$.

Table 4.5 Percent IgM^+ lymphocytes in Harderian gland post-vaccination and post-challenge.

a) Experiment 1

	Treatment			
Day	Vaccine	Ammonia	Challenge	% IgM^+ (Mean ± SEM*)
	-	-	-	22.6 ± 2.6
	+	-	-	20.7 ± 5.0
10-14 dpv†	+	+	-	23.2 ± 4.5
	-	-	-	19.6 ± 1.7
	+	-	-	20.1 ± 6.0
24-28 dpv (Pre-Challenge)	+	+	-	32.2 ± 4.5
	-	-	+	31.6 ± 4.4
	+	-	+	21.8 ± 1.4
10-14 dpc‡	+	+	+	29.3 ± 2.9

b) Experiment 2

	Treatment			
Day	Vaccine	Ammonia	Challenge	% IgM^+ (Mean ± SEM)
	-	-	-	10.9 ± 0.8^a
	-	+	-	8.7 ± 2.3^a
	+	-	-	25.2 ± 6.6^{ab}
10-14 dpv	+	+	-	27.3 ± 1.7^b
	-	-	-	22.7 ± 2.1
	-	+	-	24.1 ± 1.5
	+	-	-	18.5 ± 3.1
24-28 dpv (Pre-Challenge)	+	+	-	16.9 ± 3.4
	-	-	+	19.0 ± 2.8
	-	+	+	20.0 ± 4.8
	+	-	+	19.9 ± 3.9
	+	+	+	21.1 ± 3.5
10-14 dpc	-	-	-	20.0 ± 5.1

c) Experiment 3

	Treatment			
Day	Vaccine	Ammonia	Challenge	% IgM^+ (Mean ± SEM)
	-	-	-	11.5 ± 0.7[a]
	-	+	-	12.2 ± 0.6[a]
	+	-	-	22.1 ± 2.1[ab]
10-14 dpv	+	+	-	22.3 ± 1.2[b]
	-	-	+	54.8 ± 5.6
	-	+	+	53.0 ± 7.4
	+	-	+	18.9 ± 0.8
	+	+	+	28.7 ± 3.7
5 dpc	-	-	-	19.7 ± 1.1
	-	-	+	29.0 ± 4.7
	-	+	+	23.7 ± 1.7
	+	-	+	19.2 ± 2.2
	+	+	+	19.1 ± 1.1
10-14 dpc	-	-	-	24.8 ± 2.6

*SEM = standard error of the mean.

†dpv = days post-vaccination.

‡dpc = days post-challenge.

[a,b]Different letters are significant at $p < 0.05$.

Table 4.6 Percent $CD4^+$ and $CD8^+$ lymphocytes and CD4:CD8 ratio in Harderian gland post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			% $CD4^+$ (Mean ± SEM*)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio† (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	17.7 ± 1.7	14.6 ± 2.7[a]	1.5 ± 0.4
	+	-	-	17.3 ± 3.9	22.2 ± 1.2[ab]	0.8 ± 0.2
10-14 dpv‡	+	+	-	16.8 ± 2.9	24.6 ± 2.1[b]	0.7 ± 0.1
	-	-	-	24.9 ± 1.5[a]	11.8 ± 0.6	2.2 ± 0.2[a]
	+	-	-	16.9 ± 1.7[b]	20.4 ± 2.8	0.9 ± 0.1[b]
24-28 dpv (Pre-Challenge)	+	+	-	16.9 ± 1.3[b]	15.3 ± 2.7	1.3 ± 0.3[ab]
	-	-	+	20.5 ± 1.8	20.7 ± 2.9	1.1 ± 0.2
	+	-	+	16.5 ± 1.4	16.6 ± 1.4	1.0 ± 0.1
10-14 dpc§	+	+	+	17.1 ± 2.1	13.2 ± 1.7	1.5 ± 0.4

b) Experiment 2

Day	Treatment			% $CD4^+$ (Mean ± SEM)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	7.4 ± 0.7[a]	5.5 ± 0.4[a]	1.4 ± 0.1
	-	+	-	7.1 ± 1.9[a]	6.0 ± 1.0[a]	1.2 ± 0.3
	+	-	-	13.5 ± 2.2[ab]	19.5 ± 4.2[ab]	0.8 ± 0.1
10-14 dpv	+	+	-	18.9 ± 2.8[b]	23.1 ± 2.0[b]	0.8 ± 0.1
	-	-	-	16.0 ± 1.8	11.0 ± 1.7	1.5 ± 0.1
	-	+	-	20.3 ± 2.3	11.2 ± 1.8	2.0 ± 0.4
	+	-	-	14.9 ± 1.6	11.3 ± 2.7	1.6 ± 0.3
24-28 dpv (Pre-Challenge)	+	+	-	13.3 ± 2.5	11.5 ± 2.4	1.2 ± 0.1
	-	-	+	17.9 ± 2.4	19.1 ± 3.0	1.0 ± 0.2
	-	+	+	22.2 ± 2.3	21.5 ± 3.6	1.2 ± 0.2
	+	-	+	14.2 ± 0.8	15.1 ± 0.9	1.0 ± 0.1
	+	+	+	13.5 ± 1.4	11.4 ± 1.1	1.2 ± 0.1
10-14 dpc	-	-	-	14.5 ± 2.6	14.7 ± 2.3	1.1 ± 0.2

c) Experiment 3

Day	Treatment			% $CD4^+$ (Mean ± SEM)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
	-	-	-	12.9 ± 1.2	15.8 ± 1.5[a]	0.9 ± 0.1
	-	+	-	12.3 ± 1.1	15.3 ± 1.4[a]	0.9 ± 0.1
	+	-	-	16.1 ± 1.1	24.7 ± 1.6[b]	0.7 ± 0.1
10-14 dpv	+	+	-	14.8 ± 1.3	24.3 ± 1.1[b]	0.6 ± 0.1
	-	-	+	12.0 ± 4.7	7.7 ± 1.9	1.5 ± 0.4
	-	+	+	7.9 ± 0.8	13.0 ± 4.4	0.7 ± 0.2
	+	-	+	10.4 ± 0.8	16.7 ± 2.2	0.6 ± 0.1
	+	+	+	11.9 ± 1.2	10.0 ± 1.9	1.3 ± 0.3
5 dpc	-	-	-	19.3 ± 0.9	8.1 ± 0.4	2.4 ± 0.2
	-	-	+	19.3 ± 3.9	20.3 ± 1.7[ab]	0.8 ± 0.2
	-	+	+	21.9 ± 0.9	22.1 ± 2.0[a]	1.1 ± 0.1
	+	-	+	18.3 ± 1.5	16.9 ± 1.8[ab]	1.0 ± 0.1
	+	+	+	17.4 ± 0.9	16.9 ± 2.3[ab]	0.9 ± 0.1
10-14 dpc	-	-	-	17.8 ± 1.0	12.1 ± 1.6[b]	1.8 ± 0.2

*SEM = standard error of the mean.

†CD4:CD8 Ratio = ratio of the percent of $CD4^+$ cells to the percent of $CD8^+$ cells.

‡dpv = days post-vaccination.

§dpc = days post-challenge.

[a,b]Different letters are significant at $p < 0.05$.

Figures

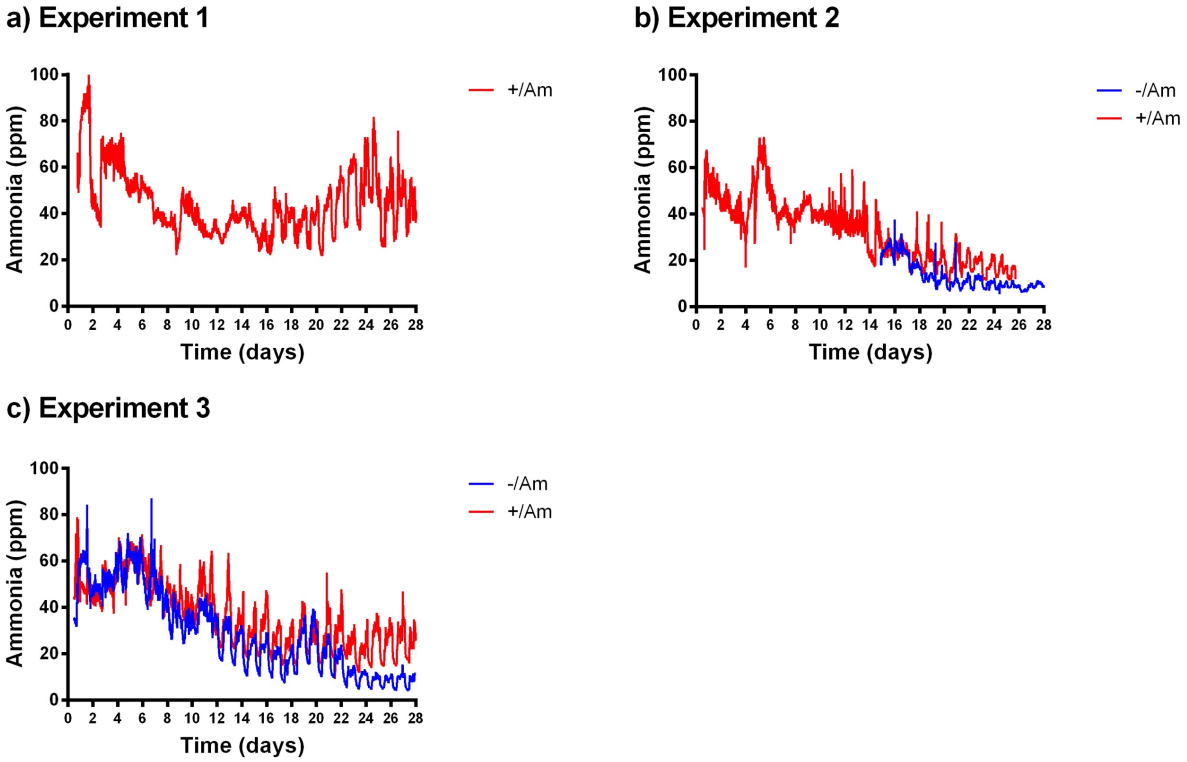

Fig. 4.1 NH_3 concentrations in NH_3-treated rooms. Due to technical difficulties, Experiment 2 NH_3 recordings in the –/Am room were not started until 14 dpv. –/NAm = nonvaccinated, no-NH_3; –/Am = nonvaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

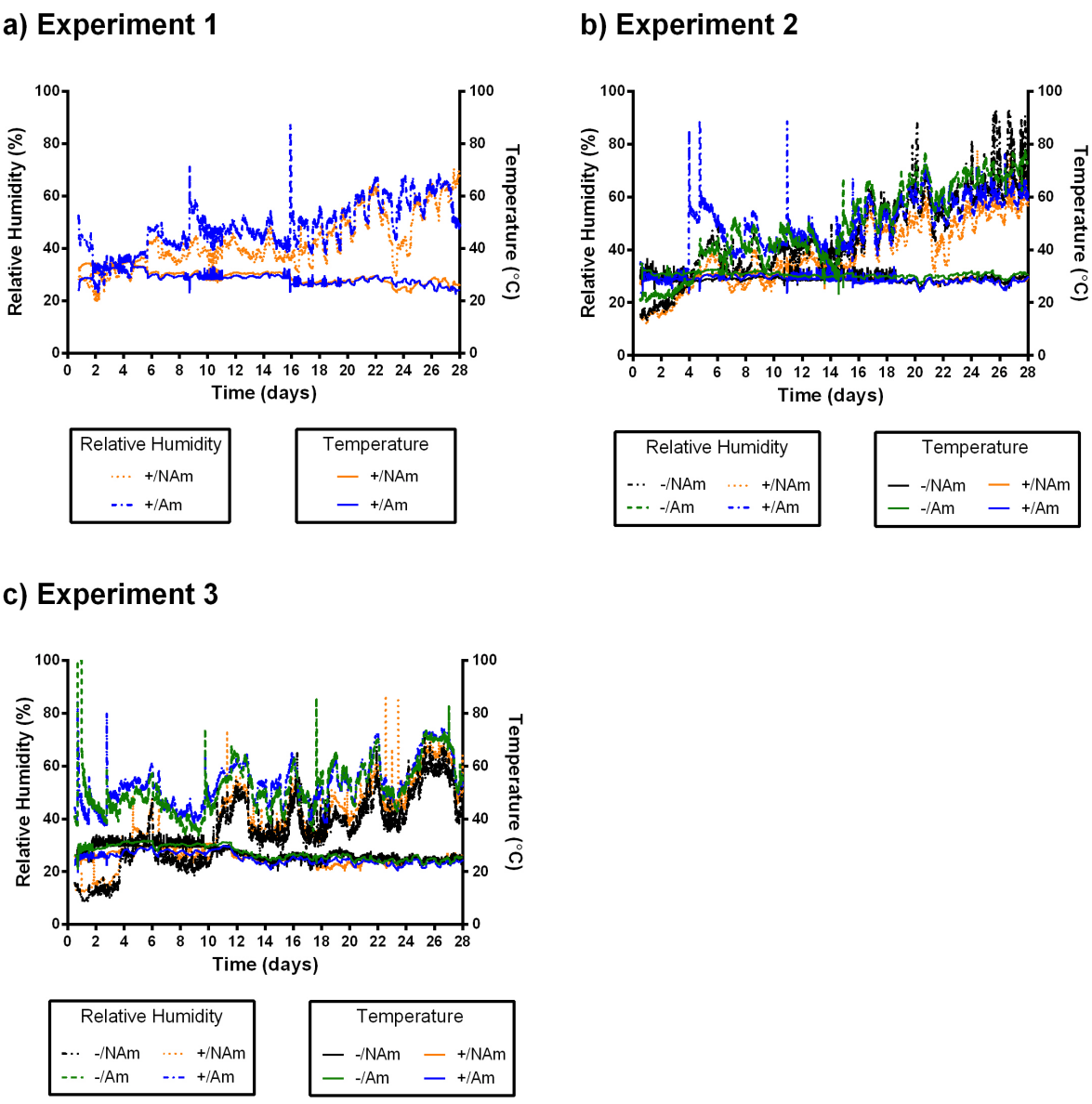

Fig. 4.2 Relative humidity (Rh, %) and temperature (°C). –/NAm = nonvaccinated, no-NH_3; –/Am = nonvaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

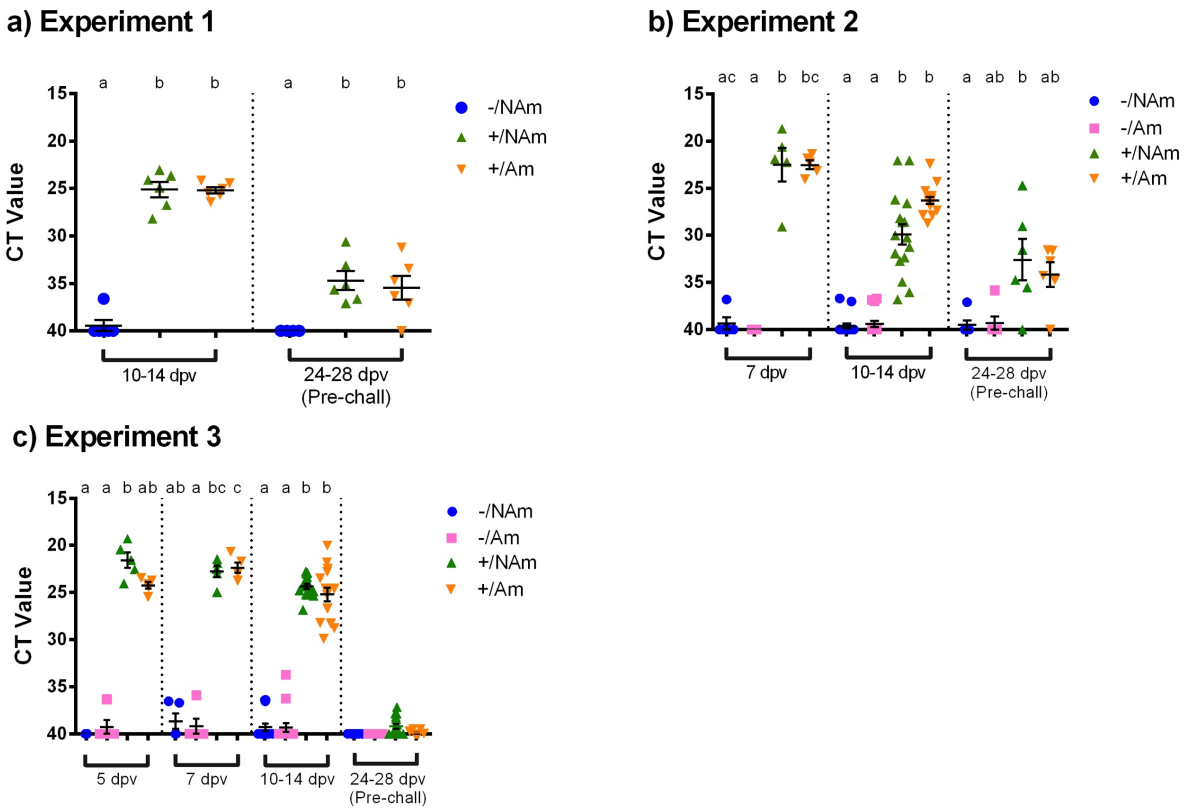

Fig. 4.3 qRT-PCR average CT values from choanal swabs collected post-vaccination. Error bars indicate standard error. Letters (a-c) are significant at $p < 0.05$. –/NAm = nonvaccinated, no-NH_3; –/Am = nonvaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

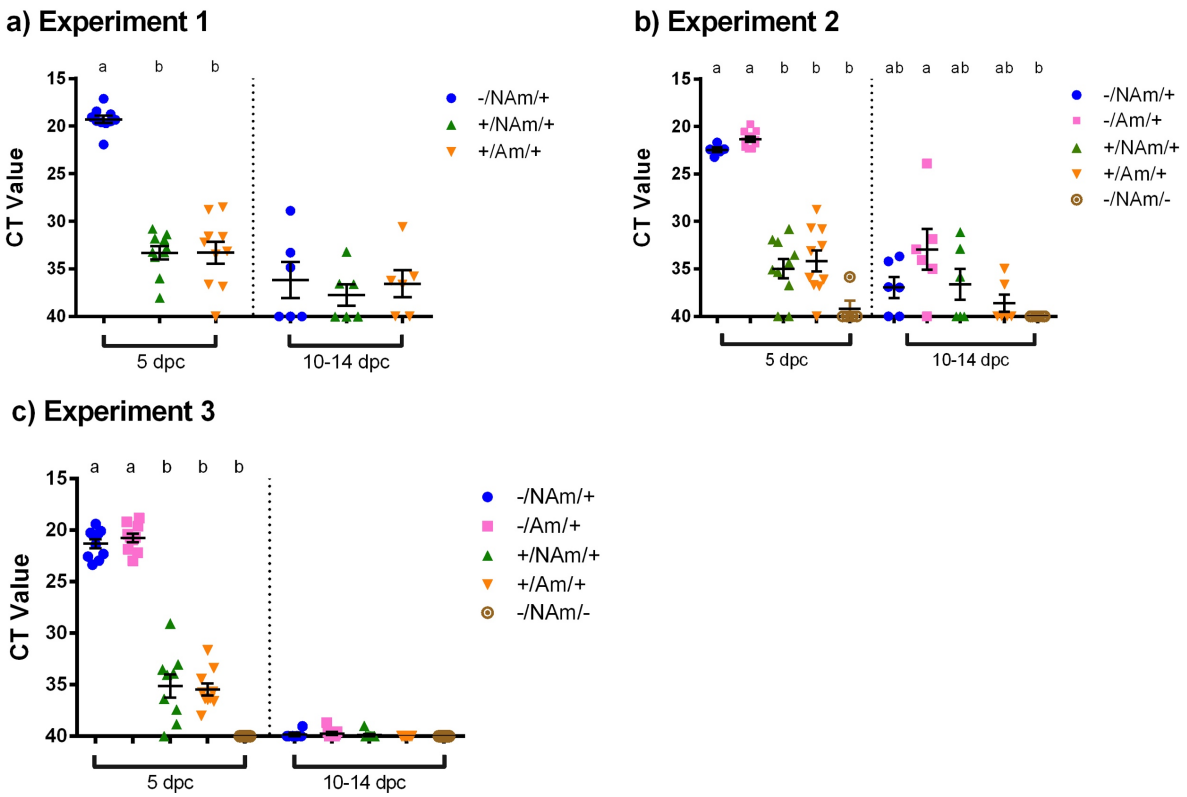

Fig. 4.4 qRT-PCR average CT values from choanal swabs collected post-challenge. Error bars indicate standard error. Letters (a, b) are significant at $p < 0.05$. –/NAm = nonvaccinated, no-NH_3; –/Am = nonvaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

CHAPTER 5

CONCLUSION

Over time, vaccination and management practices have adjusted to meet the demands imposed by the changing structure of the poultry industry. Therefore, careful consideration of these programs is important to achieving optimal health and performance of the birds.

The first phase of our research showed that pullets serially administered live attenuated vaccines against IBV, NDV, and ILTV were protected against homologous challenge with IBV, NDV, or ILTV until they were at least 36 WOA. Additionally, the timing of these vaccines and intervals between each vaccine did not interfere with the development of immunity to each virus and consequently protection against homologous challenge. We designed our vaccination protocol to exemplify a typical vaccination program for IBV, NDV, and ILTV in commercial layers. It is well known that live viral vaccines can persist in flocks, and until now, it has been unclear whether the immunity induced by a live vaccine could be compromised because of viral interference, a phenomenon in which one replicating virus blocks the replication of another virus (48, 157). We have demonstrated that chickens vaccinated for IBV, NDV, and ILTV were protected from viral replication and clinical signs following homologous challenge, which suggests that immunity to individual vaccine viruses was not compromised despite serial administration of multiple live attenuated vaccines targeting different viral respiratory tract pathogens. This observation supports industry practices that employ vaccination schedules similar to the one we used in our study, in which the interval between immunizations with live, attenuated IBV, NDV, or ILTV is a minimum of two weeks.

The second phase of our research revealed that ambient NH_3 exposure does not appear to impact bird immune response to IBV vaccination based on the parameters measured in our study. Furthermore, ambient NH_3 exposure did not alter vaccine or challenge virus replication and clearance, ciliostasis, or tracheal histopathology scores. In addition, +/Am/+ birds were protected from challenge virus replication and demonstrated an immune response comparable to the response in +/NAm/+ birds. Despite being protected from challenge, +/Am/+ bird mucosal tissues showed subtle effects of ammonia on cilia morphology and function, characterized by blunted cilia and slower ciliary beating. These observations support previous reports of ammonia-induced morphological changes in the respiratory tract (136). In the event of higher ammonia levels or additional environmental or pathogenic challenges, we predict that the morphological and functional effects of ammonia might become more prominent, perhaps predisposing birds to respiratory disease. Nevertheless, our results suggest that the deleterious effects of NH_3 became apparent only when nonvaccinated birds were challenged with IBV, which resulted in an elevated incidence of airsacculitis in both Experiments 2 and 3. Airsacculitis resulting from NH_3 exposure combined with live attenuated IBV vaccination has been reported previously (112, 159). However, in our study, vaccinated birds, irrespective of NH_3 exposure, were completely protected from challenge, and airsacculitis was not detected in either the vaccinated, challenged birds or the vaccinated, nonchallenged birds. Our data therefore suggest that the negative effects of NH_3 become apparent when nonvaccinated birds are exposed to a virulent IBV challenge virus, which lead to airsacculitis.

Based on our research, we additionally confirmed previous knowledge of IBV immunity and introduced new insight regarding IBV-induced mucosal immune responses in the HG. The IBV vaccine mobilized leukocytes with increased MHC I^+/II^{hi}, IgM^+, and $CD8^+$ expression in the

HG, and serum IgG antibody titers remained elevated but comparable to nonvaccinated titers through 14 DOA. This observed IBV epitope-specific humoral immune profile pattern, measured in all bird groups, was likely due to a robust protective maternal immunity. Following IBV challenge, although all bird groups responded, nonvaccinated birds at 5 dpc had the greatest measurable increase in activated leukocytes denoted by increased MHC I^+/MHC II^{hi} and IgM^+ expression. Mucosal and serum immunoglobulin levels and $CD8^+$ expression in nonvaccinated, challenged birds peaked at 10-14 dpc, by which time most of the birds had cleared the virus. It is also worth mentioning that the IBV challenge strain in the HG was cleared more quickly than the vaccine strain. Furthermore, the minimal immune activation in the HG of vaccinated, challenged birds indicates that the challenge virus was efficiently neutralized before mucosal penetration and viral replication. These observations highlight the importance of mucosal immunity in protection from IBV infection. Specifically, our data imply that the HG T cell may have a minor role in the immune response to secondary IBV exposure or the level of humoral immunity from vaccinated birds was robust enough to minimize mucosal penetration and mediate clearance though, however the specific antibody class responsible for virus clearance remains to be determined.

In conclusion, our data support the continued use of vaccination programs in which multiple live, attenuated vaccines for IBV, NDV, and ILTV are serially administered with intervals between immunizations of at least two weeks. In addition, we highlight that adverse health effects of ambient NH_3 in poultry houses may not be readily apparent until the birds are exposed to pathogenic challenges and further recommend that ambient NH_3 in poultry houses be minimized to reduce the incidence of secondary infections resulting from IBV infection.

Collectively, our data promote poultry health and performance by contributing valuable information to the development of poultry vaccination and management programs.

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