INFLUENCE OF THE CHICKEN MAJOR HISTOCOMPATIBILITY COMPLEX ON THE PATHOGENESIS OF BACTERIAL SKELETAL DISEASE, CHICKEN INFECTIOUS ANEMIA AND INFECTIOUS BRONCHITIS

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Kellye Sue (Bessinger) Joiner, daughter of Delbert Leo and Edith Sue (Felts) Bessinger, was born March 19, 1973, in Bowling Green, Kentucky. She graduated from Greenwood High School in 1991. She attended Western Kentucky University and completed the pre-veterinary medicine curriculum in May 1994. She began veterinary studies at Auburn University in September 1994 and graduated with a Doctor of Veterinary Medicine (cum laude) in June 1998. Following graduation, she entered a combined residency program in Anatomic Pathology and Master of Science graduate degree program in Pathobiology at Auburn University's College of Veterinary Medicine. In the fall of 2003, she was admitted to the Doctor of Philosophy degree program in Biomedical Sciences at Auburn University. She married Jesse Mack Joiner, III, son of Jesse M., Jr. and Elizabeth Ann (Fikes) Joiner, on July 5, 2003. They have one daughter, Lindsey Elizabeth, born June 3, 2005.

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Genetic resistance to several poultry pathogens has been unequivocally linked to the major histocompatibility *B* complex (MHC). Relatively few MHC association studies have been conducted during natural disease outbreaks, or in commercial chickens. Specific objectives during this three-part investigation were to identify potential MHC influence on the development of naturally occurring bacterial skeletal disease in a commercial flock of broiler breeders, characterize the pathogenesis of experimental chicken infectious anemia in commercial broiler breeder chickens selected for specific MHC genotypes, and evaluate potential MHC association with response to infectious bronchitis virus (IBV) in commercial leghorn chickens possessing select MHC genotypes.

Bacterial osteomyelitis and tenosynovitis, primarily caused by *Staphylococcus* spp., were observed with increased frequency in a line of commercial broilers expressing *B* A4/A4 (equivalent to standard B21) and *B* A12/A12 MHC genotypes by comparison

with 14 other genotypes in the flock. Previous studies have shown that the *B* A4 haplotype is relatively susceptible to *Escherichia coli* induced cellulitis. These findings are suggestive that the *B* A4 haplotype may be linked to increased susceptibility to bacterial disease.

The pathogenesis of chicken infectious anemia virus (CAV) was examined in the same line of commercial broilers using chickens with select MHC genotypes. Differences in clinical and pathologic lesions were not observed in 2 week old, orally inoculated chickens or between MHC genotypes. However, differences in seroconversion and CAV genomes per cell were detected in birds expressing *B* A9/A9 and *B* A9/A4 MHC genotypes. These findings suggest that certain MHC genotypes may influence CAV infection; however additional studies are needed to better define the interaction of CAV with the broiler MHC.

Infectious bronchitis disease progression was evaluated following challenge in vaccinated commercial white leghorn chickens with the *B* 2/15 and *B* 2/21 MHC genotypes. The incidence of clinical disease and the frequency of IBV-specific IgA detection in tears were higher in *B* 2/21 genotype birds. Differences between genotypes were discrete, suggesting that IB susceptibility might be associated with MHC genotype.

Collectively, the findings presented in this dissertation indicate that identifying candidate genes and characterizing MHC associations with other diseases may elucidate specific immune mechanisms involved in responses to certain pathogens. Further insight may be gained into the immunopathogenesis of several human diseases caused by related pathogens.

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I. INTRODUCTION

Poultry is the largest agricultural commodity in Alabama, which is currently ranked third in the nation for broiler production, behind Georgia and Arkansas (124). An Auburn University report showed that in 1993 Alabama produced 2.5 billion table and hatching eggs, ranking Alabama 11th nationally in egg production (124). In 1999, Alabama poultry growers produced 971.2 million broilers valued at $1.9 billion, and 2.5 billion eggs with a farm value of $281.8 million (public domain, http://www.alfafarmers.org/commodities/poultry.phtml). Multimillion dollar losses occur in the United States each year due to mortality and diminished production associated with poultry diseases. Therefore, reducing disease-associated production losses could have a substantial economic impact on this important Alabama industry.

Disease resistance is regulated by a series of complex interactions between a network of immune mediator proteins with various physiologic and environmental factors. These proteins enable specific recognition of antigens, interactions between antigen presenting cells with B and T lymphocyte subsets, and are crucial for appropriate immunoresponsiveness. Diversity among immune response elements is mainly due to intrinsic genetic polymorphism, and is a major source for differences in immunocompetence traits and disease resistance. Several genes with a proven or potential role in disease resistance have been identified; however, the best-characterized genetic

control of disease resistance in the chicken is that associated with the major histocompatibility *B* complex (MHC) (16, 29, 156).

The avian MHC contains two closely linked class I loci, *B-F1* and *B-F2*, and class II loci designated *B-Lβ1* and *B-Lβ2*, which code for proteins necessary for antigen presentation. As such, the MHC genes are a crucial component for antigen recognition and presentation, which are central to cell mediated immune responses.

The chicken MHC strongly influences resistance to several infectious diseases including Marek's disease (MD) (29), Rous sarcoma (18), avian leukosis (309), fowl cholera (158), salmonella (58) and cecal coccidiosis (134, 165). MHC class I cell-surface expression and cytotoxic T lymphocyte (CTL) responses (85, 244) may play a role in MHC-based genetic resistance to MD and potentially to other viral pathogens (144). The majority of MHC association studies have been conducted in experimentally inoculated, inbred populations of white leghorn chickens. Relatively few MHC association studies have focused on bacterial skeletal disease (57), chicken infectious anemia (39) or infectious bronchitis (17, 34), all of which are common diseases in the Alabama poultry industry and throughout the Southeastern United States.

The research described in this dissertation examines the pathogenesis of and potential MHC associations with 1) naturally occurring bacterial skeletal disease in a line of broiler breeders located at a commercial facility in the Southeastern United States, 2) experimental CAV infection in broiler breeder chickens, and 3) experimental IB in a commercial line of white leghorn chickens.

II. LITERATURE REVIEW

Major histocompatibility complex

Responses to antigenic stimuli including infectious pathogens are associated, at least in part, with the major histocompatibility complex (MHC) (86). The chicken MHC, or *B* complex, was the second MHC recognized, preceded only by the murine *H-2* histocompatibility complex and followed by the human *HLA* complex. Briles *et al*. (31, 32) initially identified the *B* complex as a highly polymorphic blood group antigen system that was associated with selective advantages for fitness and survival. Erythroid alloantigens were linked to major transplantation antigens by acute allograft rejection (245), graft-versus-host (GVH) splenomegaly (128) and chorioallantoic membrane (CAM) pock formation by peripheral blood leukocytes (PBLs) (246). In addition, PBLs from birds of different haplotypes were used to associate the MHC or closely linked gene(s) with levels of GVH competence (169) and the mixed lymphocyte reaction (186). In MHC-recombinant chicken lines, gene products controlled by MHC class II loci were shown to restrict antigen presenting cell / T cell interactions (285), and serve as restriction elements in B cell / T cell cooperation (282). Collectively, these immune responses were similar to the mouse *H-2* and human *HLA* counterparts (147), establishing the *B* complex as a component of the chicken MHC.

The chicken MHC consists of two major regions, the *B* complex (99, 221) and the *Rfp-Y* (restriction fragment polymorphism-Y) region (190, 193) which are located near to and separated by the nucleolar organizer region (*NOR*) (101) on chromosome 16 (22, 81).

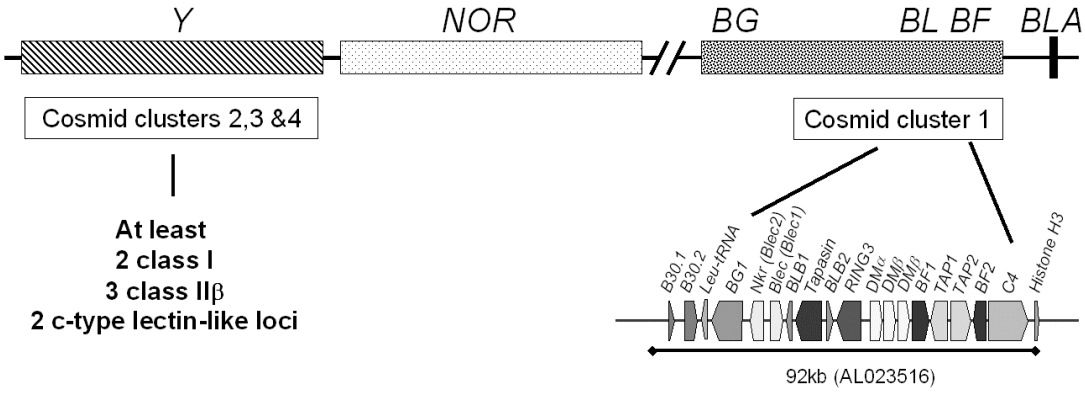

Fig. 1. Map of the major histocompatibility complex gene region on chicken chromosome 16 (187).

Cosmid mapping and segregation analysis indicate that *Rfp-Y* and *NOR* are linked; however, the *B* complex does not appear to be linked to the *Rfp-Y* region and segregates independently due to the presence of the *NOR* between them (99, 193). Major regions were originally cloned as four large cosmid contigs (CC1, CC2, CC3 and CC4) using a DNA library from the CB chicken strain (*B12* haplotype) (101). Molecular maps localized CC1 to portions of the *B* complex, and CC2, CC3 and CC4 to the *Rfp-Y* region, which will be described in greater detail below.

The *B* complex is subdivided into three tightly linked polymorphic regions (221), designated *B-F*, *B-Lβ* and *B-G*, that code for the three classes of MHC molecules. *B-F* and *B-Lβ* regions correspond to classical MHC class I and class II molecules, respectively (22, 80, 101, 221). Two class I (*B-F1* and *B-F2*) and two class II (*B-Lβ1* and

B-Lβ2) loci amid 15 other genes, including a single *B-G* locus, have been mapped to a 92 kb portion of CC1 (144). Other *B-G* genes are located approximately 100 kb outside of CC1, and constitute a large family of polymorphic immunoglobulin (Ig)-superfamily genes. Mammalian MHC class III molecules are complement components; however, most of the genes responsible for complement production in birds are not associated with the *B* complex and a distinct avian MHC class III associated region has not been described (43). Recombination between the *B-F*, *B-Lβ* and *B-G* regions is rarely observed so that *B* haplotypes encompassing alleles in all three loci are the units of inheritance most often considered in relating the chicken MHC to immunity and disease responses (25-29, 105, 108, 109, 114, 152).

B-F antigens (MHC class I molecules) are constitutively expressed on almost all chicken cells, including nucleated erythrocytes (314). *B-F* antigen protein structure was deduced by papain cleavage (208), and partial sequence analysis of the class I antigen amino terminus revealed sequence homology with mammalian class I molecules (125, 293). The *B-F* region gene products consist of a membrane bound polymorphic glycoprotein consisting of a single α heavy chain (40 to 43 kDa) (154, 217, 314). The *B-F* protein is non-covalently associated with β_2-microglobulin, a conformationally conserved protein involved in membrane transport that is essential for the expression of class I antigens on the cell membrane (153, 252). The α heavy chain is subdivided into α_1, α_2, and α_3 domains forming a cleft or groove for peptide antigens to bind to the MHC molecule for subsequent processing and cell surface expression (156). Variation in the molecular weight of the α heavy chain is a reflection of the polymorphism of different *B-F* alleles.

Guillemot *et al*. (101) identified six class I genes (*B-FI* through *B-FVI*) by southern blot analysis. However, further analysis showed that there are only four class I genes (144), and that the *B-FII* and *B-FIII* loci were artifacts of complex hybridization data. Two loci encoding class I heavy chains (*B-FI* and *B-FIV* genes) have been identified within a 44 kb segment of CC1 (144), whereas the other two class I loci (*Y-FV* and *B-FVI*) were localized to the *Rfp-Y* region (144, 190). The *B-FIV* locus, now called BF2, is predominantly expressed and produces the majority of the class Iα MHC molecules; hence, this locus is often referred to as *B-F* major, while the less well-expressed *B-FI* locus, now called BF1, is designated as *B-F* minor (144, 154, 214). In a comparison of eleven *B-FIV* alleles with mammalian class I alleles, similar nucleotide substitutions with high variability were found in exons 2 and 3, which encode the α_1 and α_2 domains (123). However, the region of exon 3 encoding the α2 helix has a much lower nonsynonymous to synonymous nucleotide substitution ratio, suggesting that the α2 helix in *B-FIV* is more conserved than the similar *HLA* region (123).

B-FI and *B-FIV* proteins are classified as MHC class Iα molecules due to associations with acute skin graft rejection (108), graft-versus-host reaction (221), mixed lymphocyte reaction (61, 292), and their ability to present antigen to CTLs (84, 148). Recently, Henry Hunt and colleagues have shown that only *B-FIV* proteins present antigen (unpublished data). Antigen restricted CTLs have been identified as the effector cells associated with MHC restricted cell-mediated cytotoxicity (300). In addition, it has been shown that the MHC restricts CTL reactions with some virus-infected or transformed cells (242). For example, when CTLs are combined with reticuloendotheliosis virus (REV) infected cells, significant cytotoxicity occurs only with

syngeneic effector and target cell combinations (172), suggesting that MHC and virus-restricted CTLs play an important immune surveillance mechanisms (84).

The two class II loci (*B-LβI* and *B-LβII*) contain genes that are related to the class II β-chain genes identified within the *D* region of the human *HLA* system (http://www.ebi.ac.uk/ipd/mhc/index.html), the murine *H-2 Ab1* and *H-2 Eb1* loci (http://www.informatics.jax.org) and in the *DB* loci of quail (155), pheasant (303), and passerine species including house sparrows (23), house finch (115), and two species of warbler (233). *B-L* antigens (MHC class II molecules) are homologous to mouse *Ia* molecules, and are expressed on a limited repertoire of immune cells including: B-cells, macrophages, dendritic cells, endothelial cells and a few additional cell types in specialized lymphoid tissues, *e.g.*, lymphoid aggregates and thymus (221). *B-L* antigens are expressed on most bursal and peripheral B-cells and a subpopulation of macrophages; however, few unstimulated peripheral T-cells express class II antigens (62, 77). Conversely, 50–80% of mitogen stimulated T-cells (62, 77) and 95-100% of antigen specific T-lymphoblasts express class II antigens (283).

Cross reactivity against *B* antigens with Prague CC line alloantiserum was used to initially identify a 30 kDa protein encoded by the *B-L* region (108, 221). Further analysis of *B-L* antigens characterized a heterodimeric protein composed of a highly polymorphic 34 kDa α and two 30 to 35 kDa β polypeptide chains (62, 220). Variations in molecular weight of the β polypeptide chains were shown to be haplotype specific (62). The *B-L* α and β chains are covalently-linked, forming a class II MHC restricted antigen binding cleft similar to that of the *B-F* region α heavy chain (62, 156, 220). However, in contrast to *B-F* antigens, *B-L* molecules are not expressed on erythrocytes, and *B-L* α and β chains

are not associated with a β_2 microglobulin molecule (235). Studies have also shown that *B-L* antigens are lectin-binding glycoproteins (33, 220), and have 55% sequence homology with *DQ*, *DR*, *DP*, *I-A* and *I-E* mammalian class IIβ genes (102).

Six class II genes (*B-LBI* through *B-LBVI*) have been identified (315). Zoorob *et al*. (315) classified the sequences from the six loci into three families: *B-LBII* (containing *B-LBI* and *B-LBII* genes), *B-LBIII* (containing *B-LBIII*, *B-LBIV* and *B-LBV* genes that now map to *Rfp-Y*) and *B-LBVI*. Recently, Jacob *et al.* (127) showed that the predominantly expressed *B-LB* gene (designated *B-LB*2) is located between *Tapasin* and *RING3*, and belongs to the *B-LBII* family of class IIβ chain genes. Likewise, the poorly expressed *B-LB* gene (designated *B-LB1*) is located between *Tapasin* and *Blec1*, and belongs to either the *B-LBII* or *B-LBVI* class IIβ gene family (127). These findings suggest that *B-LBII* and *B-LBVI* genes are two lineages of *B-LB* genes, and either of these gene families is interchangeable for the same locus within the *B* complex (127, 303). Based on high levels of polymorphism, sequence divergence and RNA expression, the *B-LBII* and *B-LBVI* locus is comprised of classical MHC class IIβ genes (215), whereas, the *B-LBIII* locus consists of non-classical class IIβ genes (315). *B-LBIII* genes map to the *Rfp-Y* region, and have been renamed the *Y-L* genes (193).

Class II molecules are recognized by T lymphocytes and serve as restriction molecules for B / T cell cooperation (282). Toivanen *et al.* demonstrated that *B-L* protein compatibility alone was sufficient for successful B / T cell cooperation, suggesting that the *B-L* region encodes MHC class II molecules (271).

The *B-G* antigens are polymorphic Ig-superfamily membrane glycoproteins that are present primarily on erythrocytes and erythroid precursors (241). *B-G* antigens are

also present on thrombocytes and B and T lymphocytes (241), and are generally used for serological haplotyping. *B-G* polypeptide structure was deduced by using *B-G* specific antiserum to precipitate *B-G* antigens from radiolabelled cell membranes (108, 221). *B-G* antigens consist of highly polymorphic, glycosylated polymers composed of covalently linked monomers, and two-dimensional gel electrophoresis revealed that *B-G* antigen structure is unique in individual haplotypes (189, 191). Several groups have analyzed *B-G* extracts from erythrocyte membranes using monoclonal anti-*B-G* antibodies or *B-G* alloantisera. Miller *et al.* (192) extracted a single 48 kDa polypeptide chain under reducing conditions; however, others observed two distinct 47 kDa and 42 kDa polypeptide chains under reducing conditions and a single 105 kDa polypeptide under non-reducing conditions (304).

The *B-G* region is unique to chickens, but sequence homologues have been recognized in the mammalian MHC. For example, physical and phylogenetic mapping of the human B30 and B7 multigenic families showed that the N-terminal domain of butyrophilin is similar to rat myelin/oligodendrocyte glycoprotein and *B-G* proteins (112, 291). Natural *B-G* alloantibodies are detectable in rats, mice, humans, alligators and allogenic chickens, further suggesting that *B-G* gene products are not unique to the chicken MHC (168). *B-G* antigens are not associated with differential immunoresponsiveness or disease resistance (29, 106, 212, 222). However, in studies with *B* recombinant chickens, production of *B-F* antibodies required the co-presence of heterologous *B-G* antigens, whereas *B-G* antibodies formed regardless of the presence or absence of the heterologous *B-F* antigen (106). Therefore, *B-G* antigens may serve as

adjuvants for the production of anti-*B-F* antibodies, and may play an integral role in general immune responses.

The *Rfp-Y* region was originally identified as a second genetically independent, polymorphic cluster of MHC-like class I and class II genes (30). At least one *Rfp-Y* class I locus (*Y-F*V) is widely transcribed *in vivo* (8); however, the precise number of class I and class II loci within the *Rfp-Y* region is not known. Some *Rfp-Y* haplotypes are more complex than others. Miller and collegues have identified 16 class I loci thus far in the *Rfp-Y* of the Red Jungle Fowl (unpublished data), but only two *Rfp-Y* class I loci have been found in line CB chickens (8). *Rfp-Y* alleles show divergent specialization. Alloantigens can be detected in skin graft assays (216, 265), and on erythrocytes and PBLs by antisera raised against *Rfp-Y* class I molecules expressed as transgenes (194). In addition, c-type lectin-like loci have been identified in the *Rfp-Y* region, suggesting that this region could play a role in innate immunity (193, 236).

The chicken MHC also contains non-polymorphic genes that do not belong to any of the traditional MHC gene families (144). The 92 kb region of CC1 containing *B-L* and *B-F* genes, was sequenced and shown to contain a total of 19 genes (144), including an avian homologue of the human G-protein β subunit (12.3 gene), *histone H3*, *C4*, *TAP1*, *TAP2*, two *DMB* loci, *DMA*, *RING3*, *Tapasin*, *Leu-tRNA* and two *B30.2* gene sequences (144). Other biological functions that have been correlated to MHC genotype include expression of cytokines and specific complement proteins (223, 258). Functional studies of monocytes and macrophages have found a correlation between the MHC haplotype and level of response (72, 227, 228). For example, the 12.3 gene expresses a GTP binding protein β subunit in leukocytes (100), and influences monocyte response to mitogen

(lipopolysaccharide [LPS]) or chemoattractants (N-formyl-methionyl-leucinyl-phenylalanine) (227). Additional chicken MHC *B* genes mapping to chromosome 16 include the class II α-chain gene *BLA* and a homologue of the mammalian class II G9a gene (259).

Cell mediated immunity

MHC associated antigen recognition, processing and presentation are fundamental for the initiation of adaptive immune responses, including cell mediated and humoral immunity (1). T lymphocytes are the central mediator of cell mediated immune (CMI) responses (44). T lymphocytes originate from hematopoietic stem cells, migrate to, differentiate and mature in the thymus, and enter the systemic circulation as effector cells for antigen-specific interactions. T lymphocytes express a diverse repertoire of T-cell receptors (TCR) capable of recognizing a great number of different antigens, as well as numerous cell surface markers such as cluster of differentiation (CD) antigens, Th-1 and Ly-4 alloantigens, which play an integral role in self-recognition and costimulation (83, 88). In general, mature T lymphocytes can be divided into two main subclasses according to their surface antigen repertoire: T helper (Th) cells, which are CD antigen 4 positive ($CD4^+$), and cytotoxic T cells (CTL), which express CD antigen 8 ($CD8^+$) (66). Expression of either CD4 or CD8 is related to the cells' ability to recognize antigen in association with MHC class II or class I, respectively, and will be discussed in greater detail below. Additionally, CD3 antigen is expressed by all T lymphocytes in conjunction with the TCR, regardless of the type of TCR expressed or TCR antigen specificity.

TCRs are expressed during differentiation and maturation in the thymus. Monoclonal antibodies that specifically recognize mammalian TCR homologues were used to identify and initially characterize the chicken TCR (47, 256). The three types of avian TCRs: TCR1 (γδTCR), TCR2 (αβ1TCR) and TCR3 (αβ2TCR) characterize the three different sublineages of T lymphocytes (44, 45, 256). The functional definition of these different T cell sublineages is still limited, but it has since been shown that TCRα and β gene sequences share homology with their mammalian counterparts.

The first TCR isotype to appear in development is the TCR1. Approximately 5-15% of thymocytes and 15-25% of PBLs and splenocytes express TCR1. Splenic and intestinal TCR1 expressing cells are $CD8^+$ (261), and may be capable of cytotoxicity or suppressor activity (44). The majority of chicken T cells in peripheral tissues are TCR2 positive and express either CD4 or CD8 molecules in peripheral tissues. These cells respond to T cell mitogens, produce cytokines and are capable of killing allogeneic target cells (47). TCR3 is expressed in 5-15% of thymocytes and 10-15% of PBLs and splenocytes (44). TCR3 cells in the thymic cortex express both CD4 and CD8 molecules. The majority of peripheral TCR3+ cells express CD4 and are localized in the periarteriolar sheathes of the spleen.

Adaptive immunity is dependent on T-lymphocyte regulation (14), particularly the functional capabilities of $CD4^+$ Th cells (14, 44). While the dichotomy of the adaptive immune responses (i.e. preferential development of either cell mediated or humoral immunity) has been associated with certain types of antigens or antigenic modifications (290), the initiation of those responses is critically dependent on regulation by two distinct types of Th cells (14). Following antigenic recognition, Th cells secrete various

cytokines, providing activation signals for the ensuing immune response. Type 1 Th (Th1) cells are effective in directing the adaptive immune response toward a cell mediated response, whereas type 2 Th (Th2) cells favor the development of a humoral response (1).

Th cell TCR interacts with antigenic peptides expressed on the surface of an antigen-presenting cell (APC) in association with an MHC class II (*B-L*) molecule (176). Genetic diversity of the MHC is the essential feature, as the alleles present determine the response to a particular antigen (72). However, for different cell types of the immune system to communicate effectively, they must share at least one MHC haplotype. TCR recognition of antigen-peptide complexes in association with particular MHC alleles is known as MHC restriction (283, 284). This defines T-cell specificity both in terms of the antigen recognized and in terms of the MHC molecule that binds the peptide fragment. $CD4^+$ Th cells are MHC class II restricted. Therefore, to elicit an immune response, the APC must possess the same MHC class II molecule recognized by the Th cell (1). Hence, cells that function as APCs for Th cells are limited to MHC class II expressing cells, and include: dendritic cells, macrophages and B lymphocytes. Th CD4 molecules engage class II molecules on the APC by binding to conserved regions. Additional signals such as cytokines or other direct molecular interactions between the Th cell and APC via adhesion molecules (LFA-1 to ICAM-1) or costimulatory molecules (CD2 to LFA-3, CD40L to CD40 or CD28 to B7) complete the activation of Th cells (1).

Activation by Th cell results in proliferation and differentiation of naïve T cells into effector or memory cells. Memory T cells are inherent to appropriate immunity, which involves differentiation into antigen-specific effector cells and rapid elimination of

the inciting antigen. Effector functions of Th cells primarily involve the production of lymphokines / cytokines and expression of membrane bound cell-surface molecules that help in the activation of other cells in the immune system. Effector Th1 cells produce cytokines such as interferon-γ, tumor necrosis factor α and interleukin 2 (IL-2) which play a role in the activation of CMI, whereas Th2 cells produce cytokines such as IL-4, IL-5, transforming growth factor β and IL-10, which are involved in humoral immune responses and antibody isotype switching (1, 130).

CMI is largely dependent on effector functions of CTLs which target and destroy virally infected and neoplastic cells by direct cell-cell contact or through soluble factors (1). CTLs express antigen specific TCR-CD3 complexes and cell surface CD8 molecule αβ heterodimers or αα homodimers. $CD8^+$ T cells are MHC restricted; however, this restriction is limited to interactions with antigenic peptides associated with class I (*B*-F) MHC molecules (176). Since all nucleated cells express class I MHC molecules, specialized APCs are not needed for CTL recognition.

In poultry, target cell recognition and killing by $CD8^+$ T cells in the context of MHC class I restriction has been well established (84, 172, 301). Although $CD8^+$ T cell mediated cytotoxicity has not been fully elucidated in birds, the process appears to be similar to that described for mammals and includes the induction of apoptosis in the target cell (68, 297, 307). Recent reports examined the role of natural or experimental CAV infection on the generation of antigen-specific CTL (175). Investigators demonstrated that reticuloendotheliosis virus (REV) specific CTL responses developed in chickens with maternal antibodies to CAV; however REV-specific CTL failed to develop when chickens negative for CAV specific maternal antibodies were infected with CAV,

thus providing evidence that CAV impairs CTL response. Collisson *et al*. (52) have shown that CTLs are important for controlling IB infection. Adoptive transfer of IBV primed $CD8^+$, αβ T lymphocytes protected naïve chicks from IB infection. Moreover, adoptive T cell transfer was used to provide evidence that IBV specific $CD8^+$ memory T cells protect MHC-matched recipient chicks from acute IBV infection (209). This study also revealed that avian T cell responses involve stages similar to those of murine T-cell responses, including activation and expansion, effector cell death, and development and expansion of memory cells. In other IBV studies, antigen-specific CTL activity could be detected in IBV-infected chickens as early as 3 days post infection (DPI), reaching maximum levels by 10 DPI (248). During the acute phase of IBV infection, CTL activity was directly associated with resolution of IBV infection and viral clearance, and a decline in CTL activity corresponded to an increase in apoptotic T cells.

Humoral immunity

Humoral immune responses are mediated by B-lymphocytes. Multipotent hematopoietic stem cells migrate from the bone marrow and colonize the bursa of Fabricus, where they subsequently differentiate into B-lymphocytes (121). During the embryonic phase of bursal development, bursal follicles are colonized and expanded by proliferating B-cell clones bearing immunoglobulin (Ig) M surface molecules beginning around the 12^{th} day of embryonic development (299). Mature B-lymphocytes emigrate into the peripheral circulation by the 18^{th} day of embryonic development. Continuous expansion of the bursal follicles and seeding of B-cells into the peripheral lymphoid organs occurs for several weeks following hatch. By four weeks of age, a sufficient number of stem cells have migrated out of the bursa, thus installing the mature chicken

B-lymphocyte immune repertoire (269). The bursa of Fabricus is unique to birds, and the local bursal environment is critical for stem cell maturation and B-lymphocyte development (270). For example, bursectomy prior to the 17^{th} day of embryonic development induces severe agammaglobulinemia and prevents the bird from mounting a humoral immune response to any immunizing antigen (56, 89).

In traditional mammalian models, the sequence of B-cell development progresses from pro-B cell to pre-B cell to immature B cell to mature B cell (129). During these developmental stages immunoglobulin gene segments rearrange to form one functional heavy chain gene and one functional light chain gene capable of recognizing specific antigen. However, in birds there is no apparent pre-B cell stage and antibody diversity relies on limited gene rearrangement of pre-bursal cells (299). Committed precursor B cells enter the bursa of Fabricus with fully rearranged Ig genes and rely on upstream pseudogenes to create diversity for both light and heavy Ig chains (174, 232). The numbers of gene segments for variable (V) domains of both light and heavy chains of birds are limited to a small subset of gene segments. The limited repertoire includes individual V and joining (J) light chain genes (V_L1 and J_L1), individual V and J heavy chain genes (V_H1 and J_H1), and 16 diversity segment heavy chain genes (D_H) that rearrange prior to cellular entry into the bursa. Following entry into the bursa of Fabricus, recombination can occur in V domain gene segments between approximately 25 pseudo V_L and 80 pseudo V_H upstream genes. However, VDJ diversification is not an obligatory component of B-cell development.

Approximately 5% of the developing B cells will mature and emigrate from the bursa of Fabricus to the peripheral tissues to participate in active humoral immune

responses. A variety of regulatory elements influence B cell development. Masteller and colleagues (177) showed that the cell surface marker sialyl Lewis^X was important for migration and movement of progenitor B cells into the bursa of Fabricus. In addition, Notch1, a transmembrane component of the B cell surface, was shown to play a role in regulating B cell differentiation and selection in the bursa (196). Notch 1 activation leads to apoptosis and arrest of B cells in the G_1 phase of the cell cycle, suggesting that Notch1 may contribute to the selection of functional B cells and serve to promote B cell maturation prior to bursal emigration. Investigators have also shown that various cytokines play a role in B-cell differentiation (251). For example, interleukin (IL)-6 promotes B-cell transition towards antibody secreting plasma cells. Other cytokines such as IL-4, IL-5 and IL-7 are produced by T_H2 cells and are crucial for B cell activation, proliferation and differentiation in mammals.

In adult chickens, B-lymphocytes are found in the bursa, blood, peripheral lymphoid organs, such as lymph nodules and spleen, and other lymphoid tissues. Mature B-lymphocytes express several cell surface antigens, particularly Ig molecule isotypes, including IgM, IgA and IgG. These Ig molecules are structurally and functionally similar to their mammalian counterparts, and particular isotypes are associated with particular immune responses (*e.g*. mucosal immunity is associated with IgA secretion) (306).

Antibodies act to directly neutralize specific pathogens, opsonize pathogens to promote phagocytosis, or activate complement, enhancing opsonization and promoting microbial killing. Antibody production and immune responses to a variety of synthetic polypeptides including (T,G)-A-L, GAT and GT (20, 103, 213); soluble antigens such as

bovine serum albumin; and cellular antigens e.g., *Salmonella pullorum* bacterin, and sheep red blood cells (SRBC) have been linked to the chicken MHC (170, 211).

MHC associated disease resistance

Disease resistance is a multigenic trait governed by a diverse repertoire of immunological factors. Phenotypic variation in disease resistance is associated with intrinsic polymorphism of immune intermediary proteins such as MHC molecules, TCRs, immunoglobulins, and secreted proteins such as cytokines and antibodies (73, 110). The association between MHC polymorphism and resistance or susceptibility differences to several pathogens has long been recognized, and is the best-characterized example of genetic-mediated disease resistance (37, 157). The MHC mediates interactions between various cellular components of the immune system, and as such, has a profound effect on immune response to specific antigens. Genetic enhancement of natural immunity may be a plausible mechanism for increasing resistance to whole classes of pathogenic organisms, thus protecting birds in production under a wide variety of environmental conditions.

Direct selection for desired immunocompetence traits has been used to enhance disease resistance. In long-term selection experiments for antibody response to sheep red blood cells (SRBC), correlated changes in MHC allelic frequencies have been detected (74, 98, 219). Egg-type chickens producing higher levels of antibodies to SRBC were more resistant to several parasites and viruses than low-antibody producing chickens. For example, studies by Pinard and coworkers found that mortality following challenge with a virulent Marek's disease virus strain was lower in high-antibody producing chickens expressing select MHC erythrocyte antigens (218). Leitner and colleagues

(162, 163) have shown that lines selected for rapid early antibody response to *E. coli* were more resistant to *E. coli* challenge. Differences in the frequency of MHC class IV restriction fragment length polymorphisms have also been found in meat-type chicken lines divergently selected for early antibody response to *E. coli* vaccination (281). More recent studies have shown that MHC associated gene regions *Tap2* and *B-F* are associated with *E. coli* antibody production (36).

Cell mediated immune responses have also been associated with the MHC. The delayed wattle reaction (DWR) is a standard measure of cell mediated immunity (CMI), and has been used to assess immunoresponsiveness (149). DWR following administration of phytohemagglutinin (PHA), a polyclonal T-lymphocyte activator, was significantly lower in B^2/B^2 leghorns than B^5/B^5 leghorns at 72 and 96 hours post-injection (264). This study also demonstrated sex-related differences among leghorns where PHA-induced DWR was less in females than males at 24 and 48 hours post-injection. These results suggest that while sex-related differences play a role in early responses, MHC genotype influences late DWR response and resolution. MHC may also influence T-lymphocyte and macrophage activation. For example, CB and CC MHC congenic chickens have increased percentages of CD4 and CD8 lymphocytes (107). Chemotactic activity of peripheral blood leukocytes and recruitment and activation of peritoneal macrophages differ among MHC congenic lines (229). While these studies have focused on general immune response mechanisms, MHC disease association studies have been used to elucidate specific responses against certain pathogens.

One of the most interesting features of the chicken MHC is the strong influence it exerts on resistance to several infectious diseases, most notably Marek's disease (MD), a

lymphoproliferative disease caused by a herpesvirus (16, 29, 113, 302). *B-F* and *B-L* region recombinants have been used to map MHC associated resistance to MD (16, 72, 113). Among the standard MHC haplotypes that have been studied, *B*21 is considered the prototypic haplotype for strong MD resistance in unvaccinated birds, whereas, *B*13, *B*15 and *B*19 are relatively susceptible to disease (16). Levels of MHC class I molecule expression differ among chicken lines and the expression correlates with MD susceptibility (64). For example, resistant *B*21 haplotype chickens have significantly lower MHC expression than the susceptible *B*19 haplotype birds. However, variation in response to MDV by sublines that are identical at the *B* locus, illustrates the significance of non-MHC gene effects on MD response (16, 247). Gene(s) responsible for controlling resistance to MD have not been identified, but MHC class I and II genes are strong candidates due to their immune response functions.

The MHC also influences response to other virally induced diseases, including Rous sarcoma virus (RSV) and laryngotracheitis virus (LTV). Similar to MD, RSV is a lymphoproliferative virus associated with tumor formation. In first backcross generation (BC_1) progeny expressing standard MHC *B* complex haplotypes *B*13 and *B*6, RSV induced tumors spontaneously regressed in 92% of *B* 6/13 genotype chickens, while only 4% of the tumors regressed in *B* 13/13 birds (247). In a similar study conducted in cross inbred lines expressing *B*2 and *B*5 haplotypes, RSV induced tumors spontaneously regressed in 72%, 35% and 0% of *B* 2/2, *B* 2/5 and *B* 5/5 genotype chickens, respectively (50). LTV is an alpha herpesvirus that causes a highly contagious respiratory disease characterized by generalized respiratory distress and depression. Loudovaris *et al*. (171) observed that inbred lines of White leghorn chickens homozygous for the *B*113 haplotype

were significantly more resistant to LTV than *B*114 or *B*15 homozygous birds. In a study comparing LTV susceptibility and resistance in three lines of White leghorn chickens, the inbred SC line (*B* 2/2) was shown to be significantly less susceptible to LTV than two other lines when experimentally infected (224). Resistance to other diseases such as fowl cholera (158), obese strain spontaneous thyroiditis (237) and coccidiosis (35) has also been linked to the MHC *B* complex. Resistance to various poultry pathogens is summarized in Table 1. Most MHC association studies have used in inbred or MHC congenic chicken lines. Few field studies during natural disease outbreaks or experimental studies using commercial broiler and leghorn lines have been conducted.

Table 1. Disease associations with standard MHC *B* complex haplotypes.

	Pathogen							
standard haplotype	MDV	RSV	LTV	IBV	CAV	*P. multocidia*	*S. aureus*	Coccidiosis
*B*1	-	-	-	-	-	R	S	-
*B*2	R	R	I	S	-	-	R	S
*B*5	-	S	-	-	-	-	-	-
*B*6	I	R	-	-	-	-	S	-
*B*13	S	S	R	-	R	-	-	S
*B*15	S	I	S	R	-	-	-	S
*B*19	S	S	-	-	S	S	S	-
*B*21	R	-	-	S	S	-	S	R

R = resistant, S = susceptible, I = intermediate
Adapted from (17, 29, 34, 39, 57, 134, 158, 171)

The range and variety of diseases influenced by the MHC *B* complex is extensive; however, there is not a single haplotype that responds optimally in all genetic backgrounds to all disease challenges. This is not surprising given the many different immune mechanisms involved in resistance to each specific disease and the levels at which genetic control is exerted by the MHC. The repeated associations with the MHC

suggest that there are genes affecting disease response within the MHC, but the lack of consistent association with specific haplotypes suggest that MHC linked genes other than the classical MHC class I and II genes may have a major influence.

Bacterial skeletal disease complex

Generalized leg weakness and lameness are predominant economic and welfare concerns among poultry producers (181). According to a 1994 review, annual losses in the United States from skeletal problems were estimated at $80 million to $120 million in broilers, and $32 million to $40 million in turkeys (262). Various non-infectious etiologies such as long bone growth disturbances involving the growth plate (225), tibial dyschondroplasia (161), and angular limb deformities (182, 234) have long been recognized as predominant causes of lameness. However, infectious etiologies have been increasingly recognized as important causes of lameness in commercial chickens.

Localized bacterial infections, primarily chondronecrosis and osteomyelitis are the most common cause of lameness in broilers between 25 and 45 days of age (182). Thorp & Waddington found bacterial chondronecrosis of the proximal femur and/or tibiotarsus in 17.3% of lame commercial broilers in Great Britain, the Netherlands and Northern Ireland (266). Likewise, in a longitudinal study of two commercial broiler flocks, typical bacterial skeletal lesions were observed in 20.4% of the lame birds, further exemplifying the significance of bacterial induced lameness (182). In these commercial flocks, *Staphylococcus aureus* was recovered from 63.1% and 62.5% of the bone lesions in the lame birds. Various other pathogenic and opportunistic bacteria including *Staphylococcus hyicus*, coagulase-negative staphylococci, *Escherichia coli*, *Mycobacterium avium*, *Salmonella* spp., and *Enterococcus* spp. have been recovered

from bone and joint lesions; however, *Staphylococcus aureus* remains the most commonly isolated pathogen (182, 267).

Staphylococcosis is a common systemic disease of poultry that frequently manifests as fulminating tenosynovitis, arthritis and osteomyelitis. Staphylococcal arthritis was initially described in pheasants and turkeys during the 1930s (119, 138), and was first reported as a cause of lameness in commercial broiler chickens in Australia in 1972 (200). Today, staphylococcal induced skeletal disease complex is considered one of the most important causes of lameness, and is commonly associated with large economic losses due to decreased weight gain, decreased egg production and carcass condemnations (19, 198).

Staphylococcus spp. are normal inhabitants of skin and mucous membranes of poultry, and are ubiquitous in environments where poultry are hatched, reared or processed (12). Commensal staphylococcal species suppress other potential pathogens through interference or competitive exclusion. Cutaneous damage or disruption of colonized mucous membranes provides a portal of entry, and intrinsic characteristics of *Staphylococcus aureus* may enhance bacterial virulence leading to disease. For example, surface protein A increases the severity of *Staphylococcus aureus* infections by inactivating complement, blocking the Fc portion of immunoglobulin involved in opsonization and preventing the accumulation of neutrophils around bacteria (49, 151).

Much of the information regarding the pathogenesis of staphylococcal induced skeletal disease has been obtained through experimental models that employ direct intracardiac or intravenous inoculation to induce disease development (12, 116, 181). In 1973, Nairn (199) found that intravenous injection of turkeys with *Staphylococcus aureus*

produced osteomyelitis indistinguishable from that seen in spontaneous disease. Subsequently, intravenous inoculation in broilers was used as a model for acute hematogenous staphylococcal osteomyelitis in infants (76). Jensen *et al*. (132) suggested that the respiratory tract serves as the most likely portal of entry for *Staphylococcus aureus* due to the preferential adherence of the bacteria to respiratory cells. However, experimental models for aerosol-mediated staphylococcal osteomyelitis required repeated high-dose challenge with inconsistent results (69, 198). More recently, a successful experimental model was developed (181). In this model, day-old broilers exposed to *Staphylococcus aureus* by aerosol inoculation, and subsequently inoculated with chicken infectious anemia virus (CAV) and infectious bursal disease virus (IBDV) at 21 days of age, developed osteomyelitis with greater frequency than birds receiving aerosolized *Staphylococcus aureus* alone. These findings support the hypothesis that immunosuppressive viruses may play a role in osteomyelitis development (267). Bacterial virulence factors, particle size of the aerosolized inoculum, broiler genotype, and environment may also contribute to the development of a successful model.

The proximal femur and tibiotarsus are the sites most commonly affected by osteomyelitis (182). Lesions also occur with less frequency in the proximal tarsometatarsus, distal femur, distal tibiotarsus, proximal humerus and vertebrae. Osteomyelitis develops following bacterial colonization of the terminal physeal capillary network in the zone of hypertrophy. Capillaries are lined by fenestrated endothelium facilitating bacterial extravasation and adherence to the physeal and metaphyseal cartilage (76). Ultrastructural studies revealed that an extensive staphylococcal glycocalyx anchors the bacterium to the type II collagen fibers of the cartilaginous matrix

(257). In addition, decreased circulation associated with low blood velocity and positional blood flow restriction in sitting birds may contribute to accumulation of bacteria in physeal capillaries (267). Septic arthritis and tenosynovitis are frequently observed in conjunction with osteomyelitis. Transphyseal blood vessels connect the metaphyseal and physeal capillaries to the epiphyseal vessels, potentially providing an extension to the adjacent joints and tendon sheathes (9).

Staphylococcal induced skeletal disease complex typically occurs in four to eight week old birds (200). When *Staphylococcus aureus* was administered by aerosol, the majority of lesions developed in 31 to 40 day old birds (181). This peak incidence coincides with the maximum stocking density of the birds, and is at a time when the growth plate is rapidly proliferating.

The onset of clinical signs may be insidious, and is frequently associated with a rising mortality in the flock (200). Affected birds often have ruffled feathers, closed eyes and weak response to external stimuli (197). Characteristic gait deficits range from weight bearing with a slight limp to non-ambulatory and sternal recumbancy (146). Birds are often reluctant to walk and may use one or both wing tips for support during locomotion, and vocalize loudly when pressure is applied to the affected region (267). Advanced lameness is frequently associated with decreased feed and water consumption, leading to weight loss and decreased growth rate (75, 97).

Grossly within affected bones, focal to locally extensive accumulations of fibrinonecrotic exudate are observed adjacent to or extending from the physis into the medullary cavity, adjacent joint and associated tendon sheathes (12). There is variable bony and cartilaginous lysis. Microscopically, lesions consist of coccoid bacterial

colonies embedded in necrotic debris, fibrin and a predominantly heterophilic inflammatory infiltrate. Lesions are typically located within vessels in the physeal zone of hypertrophy or have a perivascular distribution. Bacterial toxins promote degradation of the surrounding cartilaginous matrix and bacterial colonies are frequently observed in chondrocyte lacunae (75). These histologic changes can be observed as early as 24 hours after infection.

Despite the prevalence of staphylococcal induced skeletal disease, intervention strategies such as vaccination are often ineffective. This implies that humoral immune responses do not play an important role in preventing *Staphylococcus aureus* infections. In fact, specific antibodies to *Staphylococcus aureus* may promote the development of *Staphylococcus*-related infections in chickens (82). In contrast, cell mediated responses have been used to assess immune status to bacterial pathogens (298). The MHC is a central mediator for CMI; however, relatively few studies have described MHC associations with bacterial pathogens, including staphylococcosis.

Cotter and coworkers demonstrated MHC *B* complex associated differences in DWR following sensitization and subcutaneous inoculation with staphylococcal antigen (59). DWR was significantly greater and subsequent recovery was quicker in *B* 2/5 heterozygous males than either the *B* 2/2 or *B* 5/5 homozygous White Leghorn chickens (59). Subsequent studies found that congenic leghorn lines expressing *B* 2/2 and *B* Q/Q were more resistant to challenge with moderately and highly pathogenic *Staphylococcus aureus* strains at three days of age than *B*19 and *B*21 homozygotes (57). Differences were not observed among haplotypes challenged with moderate or highly pathogenic staphylococcal strains at six weeks of age. Collectively, these findings provide evidence

for MHC mediated staphylococcal resistance; however, MHC associations have not been demonstrated in broiler chickens.

Chicken infectious anemia

Chicken anemia virus (CAV) is a globally distributed immunosuppressive pathogen that causes considerable economic loss in the commercial poultry industry. CAV was first recognized in Japan in 1979 (313) as a novel agent associated with transient aplastic anemia in young chicks, and was subsequently isolated in 1989 from commercial broilers in the United States by Rosenberger and Cloud (239). However, a recent retrospective serological survey identified CAV-specific antibodies in sera as early as 1959, providing circumstantial evidence that CAV was present in the United States long before its first isolation (272). The disease is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression, leading to increased susceptibility to secondary infections and decreased responsiveness to vaccination. Disease mainly occurs in chickens less than 3 weeks of age; however, immunosuppression in older birds greatly impacts the poultry industry. McIlroy *et al.* (180) reported an approximately 18.5% net income loss due to decreased weight at processing and increased mortality around three weeks of age in fifteen clinically affected broiler flocks. Decreased weight gain and suboptimal feed conversion ratios resulting in a 13% lower net income were also observed in subclinically infected flocks in Northern Ireland (185). Indeed, the presence of CAV has been associated with an increased incidence of coccidiosis, gangrenous dermatitis and respiratory disease, which have contributed to significant production losses due to decreased livability and increased condemnations in commercial broilers in Alabama (104).

CAV is a small (23 -25 nm), nonenveloped, negative sense, DNA virus belonging to the *Gyrovirus* genus in the Circoviridae family (226). The 2.3 kb single-stranded, circular DNA genome consists of a 5'nontranscribed promoter/enhancer region, and three partially overlapping functional open reading frames that are transcribed as a major 2.0 kb mRNA transcript and two smaller 1.3 kb and 1.2 kb transcripts (139, 243). The unspliced 2.0 kb mRNA clearly encodes all of the viral proteins and is the major transcript throughout the infectious cycle (139). Three viral proteins, designated VP1, VP2 and VP3, are transcribed using alternate protein transcription start sites regulated by the promoter/enhancer region containing four or five 21 bp direct repeats resembling hormone response elements (HRE) (204, 243). VP1 is a 50 kDa structural protein found in the viral capsid, and has limited similarity to histone proteins. VP2 is an enzyme (210) that may direct VP1 folding during virion assembly. Together, VP1 and VP2 are necessary for the formation of CAV-specific neutralizing antibodies (206). VP3, also called apoptin, associates with the nuclei in infected cells and is a strong inducer of apoptosis in chicken thymocytes and lymphoblastoid cell lines (205). VP3 and perhaps VP2 are important for the pathogenesis of infection and subsequent immunosuppression (4).

Chickens are considered the only host for CAV, although antibodies to CAV have been reported in Japanese quail (79). All ages of chickens are susceptible to infection, but clinical disease is generally only seen in chicks less than 3 weeks of age without maternal antibodies. CAV is ubiquitous in commercial production flocks, and is spread either vertically into eggs from the hen or horizontally by the oral route. CAV infects and depletes hemocytoblasts in the bone marrow and lymphoblasts in the thymic cortex.

While T-cell lines may differ in their susceptibility to infection, differences are unrelated to cellular phenotype based on the CD4/CD8, TCRαβ1 and TCRαβ2 surface markers on the cell lines (38). Investigators found differences in infection and viral replication of Cux-1 and CIA-1 CAV isolates in two sublines of MDCC-MSB1 cells, and suggested that these differences may be due to an interaction between a putative receptor and the hypervariable region in VP1 (231). Infection and destruction of these cells, along with the direct and indirect virus-induced modulation of cytokine networks, results in profound suppression of immune functions (3).

Within 3 to 4 days postinfection (DPI) viral antigens can be detected in hemocytoblasts (3, 254). Hemocytoblast destruction and depletion ultimately leads to anemia, the characteristic feature of the disease. Hemocytoblasts also give rise to thrombocytes, and reduced numbers are probably related to an increase in hemorrhages. In addition, platelet alpha granules contain several CC and CXC chemokines that are essential to initiating immune responses, especially responses directed toward bacterial pathogens (150). Granulocytes are also derived from hemocytoblasts and reduced numbers of circulating heterophils may be found in the peripheral blood (263).

T lymphocyte progenitor cells in the thymus are particularly susceptible to CAV infection and appear to be a major target for the virus (3). Double labeling studies on thymic T cell populations containing CAV antigen revealed that cortical lymphocyte precursors expressing cytoplasmic CD3 are initially infected (5). This study also revealed that a higher percentage of infected cells were $CD8^+$. In addition, *in vivo* studies have demonstrated that a majority of infected lymphocytes in the spleen are $CD8^+$ cells (5, 48).

Viral replication induces cell death by apoptosis, and clinical signs such as marked thymic atrophy, pale bone marrow, anemia, hemorrhages and secondary infections typically appear by 8 to 14 DPI (133, 243). Clinical syndromes vary depending on the age of exposure, presence of protective antibody and presence of secondary infections. In chicks infected prior to 2 weeks of age, hematocrits range from 6 to 27% and affected birds are often depressed with diminished weight gain. Mortality may be as high as 20%, but usually ranges from 1 to 5% and surviving chicks recover by 20 to 28 DPI. In heavily infected flocks, horizontal infection may be associated with a second spike in mortality between 30 to 34 days (137). Chickens older than 2 weeks do not usually exhibit clinical signs, due to increased maturity of the immune system and rapid production of virus neutralizing (VN) antibodies. However, morbidity and mortality are considerably enhanced if chicks are concurrently infected with other immunosuppressive pathogens such as MDV or infectious bursal disease virus (IBDV) (48, 67). Despite the lack of overt disease in immunologically competent older chickens, immunosuppression is substantial and severe effects on the immune system have been demonstrated.

Lymphocyte function studies were used to evaluate splenic lymphocyte responses to the T-cell mitogen Concanavalin A in chicks inoculated with CAV at one day of age (6). While lymphocyte transformation and T cell growth factor were decreased at 8 and 15 days post CAV inoculation, responses subsequently increased and returned to control levels by 43 days post inoculation . In addition, IL-2 production declines, suggesting that severe defects in T-cell mediated functions occur following CAV infection. Both splenic macrophage concentrations and functions including IL-1 production, Fc receptor expression, phagocytosis and microbicidal activity are also reduced following CAV

infection (48, 178, 179). In addition to direct damage to T-cell, granulocyte and macrophage progenitors, cytokines produced by those are depleted, indirectly affecting other cell populations such as B lymphocytes. For example, destruction of Th subsets reduces IL-4, IL-6 and IL-8 cytokine concentrations, effectively reducing antibody responses (243). Disruption of the cytokine networks enhances the immunosuppressive effects of the virus, increasing susceptibility to a number of opportunistic pathogens including colibacillosis and pulmonary aspergillosis (91). Dual infections with several viruses including adenoviruses, retroviruses, Newcastle disease virus (NDV), MDV and IBDV are often synergistic exacerbating the effects of both viruses (243).

Gross pathologic findings in CAV infected chickens include diffuse thymic and bone marrow atrophy (243). In some instances, thymic lobes are difficult to find. In depleted, aplastic bone marrow only fat is visible. Hemorrhages in the proventricular mucosa and subcutaneous and muscular hemorrhages are associated with severe anemia and thrombocytopenia. Histologic changes are characterized by panmyelophthisis and generalized lymphoid atrophy. Thymic depletion is initially seen in the cortex and frequently there is hydropic degeneration of residual cells and focal necrosis. In chicks that recover, thymic lymphocytes begin to proliferate by 20 to 24 DPI, and thymic repopulation is complete by 32 to 36 days. Eosinophilic nuclear inclusions are rarely observed in infected cells, and have been detected most frequently at 5 to 7 days following experimental infection.

There is little information on differences in resistance to CAV infection. Cardona *et al.* observed that CAV seroconversion rates were significantly lower in experimental line S13 chickens expressing MHC genotype *B* 13/13 than P2a (*B* 19/19) and N2a (*B*

21/21) line chickens, even after vaccination (39). In contrast to these experimental lines, genetic resistance to CAV infection has not been demonstrated in either commercial layer or broiler chickens.

Infectious bronchitis

Infectious bronchitis (IB) was originally described in 1931 (78) and is currently recognized as an acute, highly contagious respiratory disease of chickens worldwide (42). Virtually all broiler, broiler breeder and layer breeder flocks are routinely vaccinated against IB; however, outbreaks continue to occur throughout the world. These outbreaks are frequently associated with major production losses due to decreased weight gain and egg production. Mortality is typically low, but some nephropathogenic strains are associated with mortality rates of up to 30%. While the economic significance of IB has not been precisely determined, it has been estimated that multimillion dollar losses may occur in the United States each year due to IB associated mortality and diminished production.

The disease is caused by infectious bronchitis virus (IBV), a Group 3 *Coronavirus* and prototype for the Coronaviridae family. IBV is a round to pleomorphic, enveloped positive-strand RNA virus with characteristic club-shaped surface projections (spikes). The virion contains four major structural proteins: the internal nucleocapsid protein (N), which coils around the 27.6 kb RNA genome to form the ribonucleoprotein; the integral membrane protein (M); the small envelope protein (E), which is essential for virus particle formation; and the spike protein (S) (42). The S protein is post-translationaly cleaved into the outer S1 and membrane-bound S2 glycopolypeptides, and mediates virion/host cell recognition for subsequent attachment and membrane coalescence (21,

51). Hemagglutination-inhibiting and most virus-neutralizing (VN) antibodies are induced by S1 (41).

IBV serotype or genotype classification is based on features of the S protein, particularly the S1 subunit, which exhibits sequence variation among several IBV strains (Beaudette strain sequence are reviewed by Wang *et al*. (296) and accessible in GenBank nucleotide sequence database [GenBank accession M95169]). Massachusetts M41 is the prototype strain; however, new antigenically distinct serotypes and strain variants have been increasingly recognized. These new serotypes can emerge as a result of only a few changes in the amino acid sequence of the S1 gene (40). VN antibodies, induced by S1, have been traditionally used to define different IBV serotypes (41). However, VN-distinct IBV isolates can still induce partial or complete cross immunity, suggesting that protective immunologic relationships exists between IBV strains, e.g. protectotypes (71). More recently, monoclonal antibodies (Mabs) directed toward antigenic sites on the S1 glycopolypeptide have been used to select neutralization resistant variants of North American, European and Australian IBV serotypes (126, 140, 142). For example, Massachusetts, Connecticut 46 and Arkansas 99 strain-specific Mabs selectively react with IBV serotypes with homologous S1 subunits (142). Currently, Mabs-based indirect or antigen capture enzyme-linked immunosorbent assays (ELISA) are commonly used for IBV detection and serotype determination, and are less labor intensive as compared to conventional virus-neutralization tests (126).

The chicken is the only natural host for IBV, and susceptibility to disease varies among breeds or strains of chickens. The disease affects all ages; however, young chicks are highly susceptible, and mortality can be as high as 25% in chickens less than 6 weeks

of age (12). IBV is an epitheliotrophic virus that primarily replicates in ciliated epithelium and mucous-secreting cells of the upper respiratory tract (11), causing generalized respiratory distress characterized by wheezing, coughing, tracheal rales, nasal discharge and labored breathing. During the clinical phase of the disease, maximum virus titers are typically detected in the tracheal epithelium between 5 and 10 DPI (11, 207). This peak clinical phase is followed by viremia with widespread dissemination. The virus also replicates in the lungs and airsacs, with maximum titers observed between 4 and 11 DPI (202).

At necropsy, the tracheal mucosa of IBV-infected chickens is thickened and serous or catarrhal exudate fills the nasal passages, sinuses and trachea. Bronchopneumonia is often observed and the air sacs may appear cloudy or contain a yellow caseous exudate. IBV causes ciliostasis, which has been used as a criterion for assessing tracheal damage caused by different IBV isolates; however, little or no difference in effects on ciliary activity has been observed among IBV strains (70).Tracheal lesions can be divided into three stages, which correspond to disease progression: degenerative, hyperplastic and recovery (201). Deciliation and desquamation of epithelial and mucous-secreting cells occur during the degenerative phase. There is mild infiltration of heterophils and lymphocytes in the tracheal mucosa, and heterophils are often seen infiltrating between ciliated epithelial cells and occasionally in the lumen of the trachea. During the hyperplastic stage, newly-formed epithelial cells lacking cilia begin to proliferate, covering and repairing mucosal defects. By 4 to 6 DPI, the reparative processes begin with complete recovery by 10 to 20 days.

In addition, some IBV strains exhibit urinary, reproductive and intestinal tropism, and have been isolated from the kidneys, ovaries and cecal tonsils (10, 60). IBV has also been isolated from the Harderian gland. Following ocular IBV vaccination, Toro and colleagues detected IBV antigen in the Harderian gland by both immunofluorescence using a monoclonal antibody and virus reisolation in embryonated chickens eggs through day 14 postvaccination (274).

Several intrinsic and extrinsic factors such as age, environmental conditions and concurrent infections with other pathogens influence the pathogenesis of IBV infections. In a study comparing IBV susceptibility in 2- and 6-week-old chickens, clinical signs were more severe in the 2-week-old birds; however, virus recovery from the trachea was similar in both age groups (13). Neutralizing antibody was detected earlier and in slightly higher concentrations in 6-week-old birds, suggesting that susceptibility may be related to the ability to produce neutralizing antibody. Ambient temperature has a dramatic effect on IBV-induced morbidity and mortality; a reduction in temperature from 20 ° C to 16 ° C increased mortality from 8 to 50% (63). Relative humidity and cold air temperatures influenced the development of *Mycoplasma synoviae*-induced airsacculitis in 3-week-old broilers previously infected with IBV (308). Airsacculitis was most extensive at low temperatures, regardless of humidity. Others have also found that dual infections with IBV and *Mycoplasma* spp. were synergistic, increasing the incidence of coryza, tracheitis and airsacculitis (7).

Concurrent infections with immunosuppressive viruses enhance IBV pathogenicity by impairing humoral and cell mediated immune response directed toward IBV. For example, IBV-specific antibody titers were lower and airsac lesions were

greater in chicks infected with IBDV at 1 day of age and IBV at 14 days of age than in chicks receiving IBV alone (87). Rosenberger and Gelb also showed that infectious bursal disease virus (IBDV) decreased immunoresponsiveness to IB vaccines, thus lowering resistance to IBV challenge (240). There are few reports of dual infections with IBV and CAV. Toro *et al.* recently evaluated the effects of immunodeficiency caused by co-infection with CAV and IBDV on the outcome of IB (280). Prolonged IB respiratory disease, and lower levels and delayed production of local IBV-specific IgA were detected in immunodeficient chickens. Moreover, van Santen *et al*. (289) demonstrated that either CAV or IBDV infection alone delays local antibody response to IBV, delays clearance of IBV, and prolongs respiratory signs. The delay of development of local antibody in CAV-infected chickens could be due to effects of CAV on helper T cells or cytokine expression.

Both humoral and cell mediated immune responses play a fundamental role in protection against IBV. Cook *et al*. (53) demonstrated that in ovo bursectomy increased both the severity and duration of IB in white leghorn line C chicks, exemplifying the protective role of humoral immune responses against IBV. Immunoglobulin G (IgG) is the major circulating antibody and anti-IBV IgG can be detected in the serum by HI and ELISA as soon as 4 DPI (195). Serum IgG levels typically peak by 21 DPI and high titers can be detected in the serum for several weeks (195). However, a correlation between circulating antibody titers and resistance to infection has not been definitively established.

Local anti-IBV antibodies in the upper respiratory tract are important for IBV immunity. Anti-IBV IgA antibodies can be detected in tracheal washes up to 44 days

following infection with IBV strain M41 (111). Interestingly, Cook *et al*. (54) found more IBV-specific IgA in the lachrymal fluids of chicken lines resistant to IBV, while antibody titers in tracheal washes were similar. The harderian gland contains a large age-dependent population of plasma cells and is the major source of immunoglobulins in the lachrymal fluid (15). Toro and Fernandez (273) demonstrated that in previously vaccinated chicks, specific lachrymal IgA levels correlated with resistance to re-infection following challenge with a moderately attenuated IBV strain. In a subsequent study, it was found that white leghorn chickens had a significantly higher and more homogenous serum IgG response between 5 and 9 DPI; and lachrymal IgA response between 5 and 14 DPI than broiler or brown leghorn chickens. These results suggest that local immune responses are perhaps a better indicator of immunoprotective status and immune response may vary with breed.

Several studies have been conducted to investigate the role of cell-mediated immunity in protection against IBV. Cytotoxic T lymphocytes (CTL) have been demonstrated in the spleen and peripheral blood following IBV infection using adherent cells as target cells and neutral red as an indicator of lysis (46). In fact, vigorous CTL responses associated with IBV infection correlate with an initial decrease in infection and clinical illness, and adoptive transfer of $CD4^-/CD8^+$ T cells bearing the αβ T cell receptor protected chicks from acute IBV infection following challenge with the nephrotropic Gray strain of IBV (248, 249).

CTL responses are MHC restricted and several studies have linked several MHC haplotypes to increased IBV susceptibility and resistance. Genetic differences in susceptibility to a mixture of IBV and *E. coli* have been demonstrated in inbred and

partially inbred chicken lines (34). In this study, line 7_2 white leghorns, which express standard MHC *B* complex haplotype *B*2, were more susceptible to respiratory disease caused by IBV and *E. coli* than $15I_5$ white leghorn lines expressing standard haplotype *B*15. Mortality rates of 83% and 47% in 7_2 and $15I_5$ lines, respectively, differed significantly. Moreover, mortality rates were significantly different than those observed in other breeds including Brown Leghorn, Light Sussex and Rhode Island Red chickens, which had mortality rates of 3.3%, 0% and 9%, respectively. In a more recent study using MHC congenic $15I_5$ white leghorn lines, day-old chicks were inadvertently vaccinated with a low attenuated IB virus (17). Birds expressing standard MHC *B* complex haplotypes *B*21 and *B*13 had a significantly higher mortality than birds expressing *B*15. Despite the significance of IBV in commercial broilers and layers, MHC-mediated IBV resistance or susceptibility has not been examined in commercial chickens.

Statement of research objectives

This dissertation consists of a three-part investigation of the pathogenesis and potential MHC associations of common Alabama poultry pathogens in commercial chickens possessing different MHC *B* complex haplotypes. Specific aims are as follows: 1) Identify potential MHC-associated differential susceptibility to naturally occurring bacterial skeletal disease in a select line of broiler breeder chickens located at a commercial facility in the southeastern United States, 2) Evaluate clinical and pathologic changes in 4-week-old, commercial broiler breeders experimentally inoculated with CAV and analyze select MHC *B* haplotypes for differences in disease susceptibility and subclinical immunosuppression, and 3) Characterize the pathogenesis of a virulent IBV

isolate from broilers in Alabama and analyze possible MHC *B* complex influence on disease susceptibility in a line of vaccinated and subsequently challenged commercial layer chickens expressing standard MHC *B* genotypes.

III. THE AVIAN MAJOR HISTOCOMPATIBILITY COMPLEX INFLUENCES BACTERIAL SKELETAL DISEASE IN BROILER BREEDER CHICKENS

Abstract. This study evaluated bacterial skeletal disease in conjunction with the major histocompatibility complex (MHC) in a genetically pure line of broiler breeder chickens. Chickens from six broiler breeder flocks were examined for skeletal lesions, bacterial pathogens and MHC genotype. During a ten-week period, eighty-eight, 9- to 21-week-old lame and 34 normal, age-matched controls were selected. Tenosynovitis, arthritis, and femoral or tibiotarsal (or both) osteomyelitis occurred in 86 of 88 (97.7%) lame chickens. Ninety-five bacterial isolates were obtained from 83 of 88 (94.3%) lame birds and 4 of 34 (11.8%) controls. *Staphylococcus* spp. was isolated from 72.6% of the skeletal lesions, predominantly *Staphylococcus aureus* (38.9%). MHC *B* complex genotypes were determined by hemagglutination for 88 lame birds, 34 controls and 200 randomly selected birds from each of the six flocks (1200 total). Combined Chi-square analysis revealed that the homozygous MHC genotypes *B* A4/A4 ($\chi^2 = 14.54$, $P = 0.0063$) and *B* A12/A12 ($\chi^2 = 42.77$, $P = 0.0001$) were over represented in the sample of symptomatic birds compared to random samples from the same flocks. The homozygous A4 and A12 MHC genotypes influenced flock Chi-square values more than the corresponding heterozygotes. An MHC *B* complex influence on bacterial skeletal disease was apparent in this line of broiler breeders.

Keywords: Chicken, genotype, major histocompatibility complex, osteomyelitis, *Staphylococcus aureus*, tenosynovitis

Abbreviations: CAV = chicken anemia virus; ELISA = enzyme linked immunosorbent assay; HE = hematoxylin and eosin; IBDV = infectious bursal disease virus; MHC; major histocompatibility complex; NBF = neutral buffered formalin

Generalized leg weakness and lameness are predominant economic and welfare concerns among poultry producers (183). Significant lameness has been historically attributed to various noninfectious dysplastic and metaplastic abnormalities, with less emphasis placed on the infectious counterparts (183). Recent commercial and experimental flock analyses however, revealed an increasing incidence of staphylococcal chondronecrosis with osteomyelitis in broilers (183). These analyses revealed that 20.4% of lame individuals in two commercial flocks had typical bacterial skeletal lesions that were primarily associated with *Staphylococcus aureus*. Originally described in pheasants and turkeys during the 1930s, staphylococcosis is now considered a major contributing factor in the development of crippling mortality and reduced production among commercial broiler chickens (12).

Despite the prevalence of the natural disease, staphylococcosis is difficult to reproduce experimentally. A recent successful model used *ad libitum* feeding protocols and immunosuppression induced by chicken infectious anemia virus (CAV) and infectious bursal disease virus (IBDV) (12, 181). This further supports the mechanism that multiple stresses and immunologic compromise are important pathogenic components for disease development (181). These immunologic responses are dependent

upon both humoral and cell mediated immunity and the major histocompatibility complex (MHC) is central to this regulation.

Genetic control of immunoresponsiveness has been confirmed with *Staphylococcus aureus* polyclonal activators of T-lymphocytes (59). Antigen recognition may be dependent on a single class-I or class-II MHC genotype, and specific genotypes are linked to the immunoregulation of various pathogens (143). Increased resistance or susceptibility to several common poultry diseases, including Marek's disease (18, 29), Rous sarcoma (247) and cecal coccidiosis (135) has been unequivocally linked to certain MHC genotypes. In addition, MHC associations with moderately and highly pathogenic strains of *Staphylococcus aureus* have been described in White Leghorn chickens (57). Albeit, staphylococcosis and MHC associations have not been established in broiler breeder chickens.

The objectives of this study were to evaluate lameness and skeletal lesions in a genetically pure line of broiler breeder chickens, identify bacterial pathogens from the lesions, and evaluate the association of MHC influence on the development of bacterial skeletal disease.

Materials and Methods

Broiler breeder chickens

Six, 9- to 21-week-old, broiler breeder flocks (*A* through *F*), in a genetically pure line designated Line A were studied. Flocks *A* through *D*, and *E* and *F* were located on farms 1 and 2, respectively, in a commercial breeder facility in the United States. All chickens were identified individually by wing band applied at hatch. The chickens were reared in solid-wall biosecure houses with cross-ventilation and evaporative cooling.

Male and female broiler breeders were kept in adjacent floor pens separated by mesh fencing with a population approximately 2000 females and 500 males per flock. They were fed standard, non-medicated, commercial starter and grower rations in conservative amounts to control body weight gain, and water was supplied *ad libitum.* The chickens were vaccinated for infectious bursal disease and Marek's disease at 1 day of age and for chicken infectious anemia at age 13 weeks. In addition, CAV-specific antibodies were not detected in flocks *F* and *E* at nine- and 11-weeks of age, respectively using a commercial enzyme-linked immunosorbent assay (ELISA) (Chicken anemia virus antibody test, IDEXX Laboratories, Westbrook, ME). Flocks *A* through *D* were immunized with live CAV vaccine prior to the initiation of the study.

Clinical Examination

During a ten-week period (mid-June through August), chickens were observed daily for evidence of gait abnormalities. Lameness was evaluated and semiquantitatively categorized on a five-point scale, similar to a previously described gait analysis protocol, as summarized in Table 2 (146). Chickens with lameness, reluctance to move or recumbancy, and age-matched, clinically normal control birds were selected from the general population, averaging four lame birds per day. Thirty-four control birds were selected from the general population. Numbers of lame and control birds were evenly distributed over all flocks.

Table 2. Categorization and lameness assessment among 88 lame broiler breeders

Lameness Categories and Criteria*	Number of lame chickens	
	Males (n = 56)	Females (n = 32)
1: Normal ambulation with no detectable abnormalities	0 (0%)	0 (0%)
2: Observable gait deficit, ambulatory and weight bearing	4 (7.0%)	2 (6.3%)
3: Gait deficit with reluctance to walk	14 (24.6%)	16 (50.0%)
4: Recumbent, difficult ambulation, able to support body weight when coerced	7 (12.3%)	5 (15.6%)
5: Recumbent, non-weight bearing and non-ambulatory	31 (54.3%)	9 (28.1%)

* Methodology for lameness categorization and semiquantitative assessment is modified from Kestin *et al*. (146)

Necropsy

The chickens were euthanatized by cervical dislocation and a necropsy was performed on each carcass. The bursa of Fabricius, spleen and thymus were examined for gross lesions and fixed by immersion in 10% neutral buffered formalin (NBF).

Before opening each carcass, the legs were removed to eliminate traumatic damage and reduce potential contamination of the femoral head. The coxofemoral joints were disarticulated by incising the skin over the greater trochanter, joint capsule and round ligament of the head of the femur. Surrounding musculature and tendinous attachments were bluntly dissected and the pelvic limbs were transported to a central laboratory for further dissection and subsequent bacterial culture in a clean environment. The pelvic limbs were inspected for gross lesions consisting of arthritis, tenosynovitis and osteomyelitis. If no gross lesions were found, one half of both the left and right proximal femur, tibiotarsus, and gastrocnemius and surrounding tendon sheathes were

fixed by immersion in 10% NBF. The remaining skeletal tissues were collected for bacterial culture.

Histopathology

Formalin-fixed tissues from six lame birds that had either negative bacterial cultures or no significant gross lesions and two *Staphylococcus* spp.-positive birds were histologically examined. Each femur and tibiotarsus was decalcified in 10% buffered formic acid for 36 hours. The decalcified bones, gastrocnemius and surrounding tendon sheathes, bursa of Fabricius, spleen and thymus were routinely processed, embedded in paraffin and sectioned at 4 - 6 μm. All sections were stained with hematoxylin and eosin (HE) (Gill's #2) using eosin phloxine counterstain. Definitive histomorphologic diagnosis of bacterial skeletal disease required coccoid bacterial colonies consistent with *Staphylococcus* spp. in the presence of inflammation in HE-stained sections.

Bacteriology

The cut surfaces of the proximal femur, tibiotarsus, gastrocnemius and surrounding tendons were dipped in 95% ethanol, flame sterilized and sampled with sterile Culturette™ swabs (Becton Dickinson Microbiology Systems, BD Diagnostic Systems, Sparks, MD). The swabs were streaked onto blood (BBL™ Prepared Media, BD Diagnostic Systems, Sparks, MD), mannitol salt (Difco Catapult, BD Diagnostic Systems, Sparks, MD) and MacConkey agar plates (BBL™ Prepared Media, BD Diagnostic Systems, Cockeysville, MD) and incubated aerobically in a CO_2 incubator for 24 hours at 37 ° C. Subsequently, a BBL™ Staphyloslide Latex Test (BD Diagnostic Systems, Sparks, MD) for *S. aureus* was performed on all colonies resembling

Staphylococcus spp. Individual bacterial isolates were then placed on tryptic soy agar slants (Difco Catapult, BD Diagnostic Systems, Sparks, MD) and incubated for additional growth to produce adequate amounts of pure isolates. The purified isolates were maintained in tryptic soy broth containing 20% glycerol (Difco Catapult, BD Diagnostic Systems, Sparks, MD) and stored at – 70 ° C. Gas chromatographic analysis was performed on all bacterial isolates utilizing standard extraction and characterization techniques outlined in the manufacturers' instructions (Hewlett-Packard Midi-system, Hewlett-Packard Company, Palo Alto, CA).

MHC *B* complex immunotyping by hemagglutination

Initially, a 2.0 ml sample of whole blood was obtained from each bird (n=122) via brachial venipuncture, diluted 1:10 (vol/vol) in 10% sodium citrate and stored at 4 ° C for subsequent MHC *B* complex hemagglutination immunotyping. Broiler *B* complex MHC genotypes were characterized by hemagglutination using antisera and methods established in the laboratory of Drs. Warren Johnson and Sandra J. Ewald (164). In addition to the selected population of 122 diseased and control birds, whole blood was collected from 200 randomly-selected, 20-week-old birds from each of the six flocks (n = 1200) for flock MHC *B* complex genotype distribution analysis.

Statistical analysis

The genotype distribution among lame individuals was statistically compared to the normal flock genotype distribution using Chi-squared analysis.

Results

Clinical signs and gross pathology

The mean age of all chickens (n = 122; 88 clinically lame and 34 age-matched control birds) was 12.8 weeks (range 9 - 21 weeks) and the majority (57 of 88; 64.7%) of lame individuals were male. The average body weight was 4.48 kg and 3.5 kg for males and females, respectively. Significant positive correlations between lameness and body weight or sex were not observed in this line of broiler breeders. The onset and progression of overt gait abnormalities, ranging from slight ambulation deficits to recumbancy, were insidious but rapid, often developing within 12 to 24 hours.

Table 3. Summary and distribution of gross lesions among 88 lame and 34 clinically normal, age-matched control broiler breeders

	Distribution[‡]		
Lesion*[†]	Males (n = 56)	Females (n = 32)	Controls (n = 34)
Osteomyelitis	40 (70.2%)	17 (50.0%)	0
Femur	39 (68.4%)	15 (44.1%)	0
Tibia	1 (1.8%)	2 (5.9%)	0
Arthritis	53 (93.0%)	26 (76.5%)	0
Coxofemoral	7 (12.2%)	4 (11.8%)	0
Stifle	23 (40.4%)	8 (23.5%)	0
Tibiotarsus	23 (40.4%)	14 (41.2%)	0
Tenosynovitis	30 (52.6%)	18 (52.9%)	0
Wing fracture	3 (5.3%)	5 (14.7%)	0
Valgal angular limb deformity	2 (3.5%)	2 (5.9%)	0
Costochondral widening	0 (0.0%)	1 (2.9%)	0
No gross lesions	1 (1.8%)	3 (8.8%)	34 (100%)

* Multiple lesions were observed in 40 of 56 (71.4%) males and 28 of 32 (87.5%) females.

[†] Bilateral lesions were observed in 48 of 88 (54.5%) of the lame birds.

[‡] Lesion distribution and severity did not significantly differ among MHC genotypes.

All age-matched, control birds were female (30 of 34; 88.2%) or missexed males (4 of 34; 11.8%). No lameness was detected in the 34 controls. Clinical assessment revealed increased expression of moderate (lameness score = 3) to severe (lameness score = 5) lameness among the selected population (Table 2).

In the lame chickens, gross lesions included osteomyelitis, arthritis and tenosynovitis; or combinations thereof (Table 3). Osteomyelitis, characterized by focal to locally extensive accumulations of fibrinonecrotic exudate with variable bone lysis, occurred most commonly in the proximal femoral physis (Fig. 2).

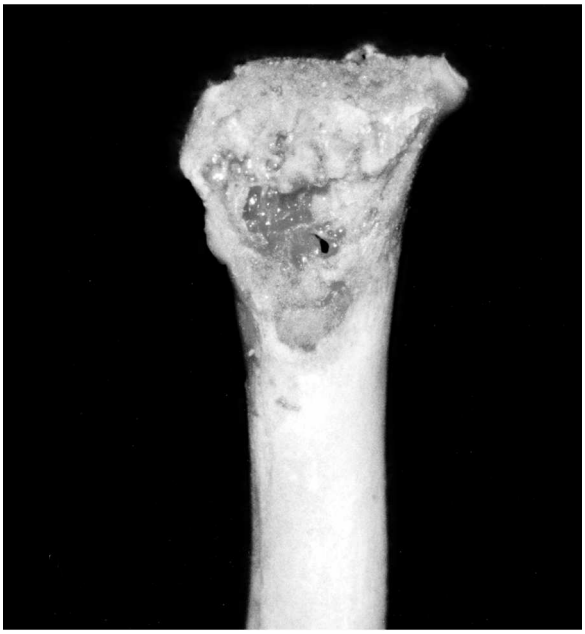

Fig. 2. Femur; broiler breeder chicken with femoral head and neck necrosis. Note the loss of normal femoral head and neck architecture, accumulation of fibrinonecrotic exudate along the proximal femoral epiphysis, absence of the articular cartilage and exposure of necrotic subchondral bone.

Similar osteolytic lesions were present in the tibiotarsus. Tibiotarsal osteomyelitis was often accompanied by separation of the articular cartilage, exposing the subchondral bone. Arthritic lesions in the coxofemoral, femorotibial and tibiotarsal joints consisted of

distention of the respective joint capsule with purulent to caseous exudate, and multifocal lysis of the articular cartilage. Similar caseous lesions occurred in the gastrocnemius and surrounding tendon sheathes. Nodular collections of purulent exudate filled the affected tendon sheath and the respective tendon was often hyperemic to hemorrhagic. The adjacent fascia was edematous and the gastrocnemius muscles had variable atrophy (Fig. 3). Other gross findings included a fractured wing, valgal angular limb deformities and costochondral widening. Four of the lame individuals and all controls had no gross lesions.

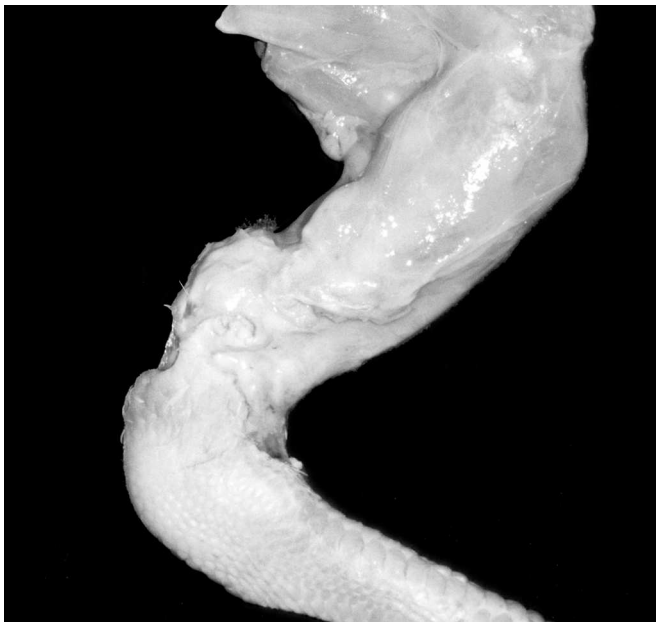

Fig. 3. Pelvic limb; broiler breeder chicken. Multinodular distention of the gastrocnemius and surrounding tendon sheathes with turbid exudate. There is marked atrophy of the gastrocnemius muscle.

Bacteriology

Culture of the proximal femoral physis, tibiotarsus, and gastrocnemius and surrounding tendon sheathes from each chicken (n = 122) yielded 95 bacterial isolates including *Staphylococcus* spp., *Escherichia coli* and several other species in 83 of 88

(94.3%) lame chickens and 4 of 34 (11.8%) controls (Table 4). A preliminary identification for *Staphylococcus aureus* was confirmed in 43 of 95 (45.3%) of the bacterial isolates with BBL™ Staphyloslide Latex agglutination. Gas chromatographic speciation confirmed the predominance of *Staphylococcus* spp. with *S. aureus* subgroup B being the most prevalent pathogen.

Table 4. Gas chromatographic speciation of 95 bacterial isolates obtained from lame broiler breeders and normal, age-matched controls

Bacterial Isolates	Distribution		
	Lame (n = 91)	Controls (n = 4)	Percentage (%)
Staphylococcus aureus subgroup A	7	0	7.3
Staphylococcus aureus subgroup B	34	3	38.9
Staphylococcus epidermidis	6	1	7.3
Staphylococcus hyicus	14	0	14.7
Staphylococcus spp.	8	0	8.4
Escherichia coli	8	0	8.4
Other enterics (*Enterobacter* spp. and *Salmonella* spp.)	12	0	12.6
Other species (*Bordatella avium*)	2	0	2.1

Histopathology

Histologic examination of skeletal tissues was performed for lameness determination among lame individuals with no gross lesions. Eighty-three of 88 chickens were positive for bacterial skeletal disease based on criteria established at gross necropsy and bacteriology. Formalin-fixed tissues from six culture-negative birds were histologically examined. Histomorphologic criteria confirmed staphylococcal osteomyelitis and tenosynovitis in 2 of 6 (33%) of the lame chickens that had either negative bacterial cultures and/or no significant gross lesions. Multiple physeal vessels

were occluded by coccoid bacterial colonies and were surrounded by heterophils, giant cell macrophages and cellular debris, which replaced the normal physeal architecture. In one bird, the gastrocnemius tendon and tendon sheath were expanded by a diffuse heterophilic infiltrate and cellular debris. Fibrosis occurred with secondary synovial cell hyperplasia and synechia formation. The spleen, thymus and bursa of Fabricius were histologically normal.

MHC association

At least 16 genotypes were represented in the lame and control individuals (Table 5).

Table 5. MHC *B* complex genotypes among lame and age-matched control chickens and overall flock distribution (*B* $A^{-/-}$)

	Distribution		
Genotype (*B* A-/-)	Lame (*n* = 88)	Controls (*n* = 34)	Flock analysis (*n* = 1200)
4/4	17	11	130
4/5	4		48
4/12	9	4	103
4/11	5	1	36
4/9	10	3	150
4/1	1	2	83
5/5	4		7
5/12	2		41
5/9	3		21
12/12	5		35
12/11	4	3	38
12/9	5	2	125
11/11	1	2	12
11/9	2	3	29
9/9	7	2	49
9/1	2	1	56
Unknown	7		112

A total of 28 different genotypes, resulting from hemagglutination utilizing seven different haplotype-specific antisera, were represented and evenly distributed among the six flocks (data not shown). Two MHC *B* complex haplotypes, A4 and A12, were associated with a significantly higher prevalence of lameness than were 14 other haplotypes (Table 6).

Table 6. Combined flock Chi-square analysis and the most influential MHC*B* complex genotypes in each flock

Flock	Classes*	Chi-Square Value	*P*	Most influential genotypes
A	14	33.08	0.002	4/5, 4/11
B	13	42.88	<0.001	12/12
C	15	29.23	0.010	12/12, 4/4
D	15	13.34	0.500	4/12, 5/12
E	14	21.02	0.072	4/11, 4/4
F	1	5.29		

* Number of genotypes represented

Frequency distribution and Chi-square analysis revealed that the homozygous genotypes *B* A4/A4 ($\chi^2 = 14.54$, $P = 0.0063$) and *B* A12/A12 ($\chi^2 = 42.77$, $P = 0.0001$) were overrepresented in the sample of lame birds compared to the random sample from the same flocks. The combined Chi-square values from each flock were significant, with A4 and A12 being prevalent in the most influential genotypes (Table 6). The homozygous individuals contributed greater to the respective Chi-square values than the corresponding heterozygotes (Table 7).

Table 7. Homozygous versus heterozygous influence on Chi-Square values

Flock	Homozygotes	Heterozygotes
A	0.32	1.49
B	11.58	0.15
C	9.10	1.32
D	0.12	0.31
E	0.42	0.07
F		
Sum	21.53	3.35
P	<0.001	0.512

Discussion

The avian MHC gene family is unequivocally associated with increased resistance or susceptibility to several common poultry pathogens, most notably Marek's disease virus (29, 113). There is a general paucity of information however, regarding both MHC associated differential immunity to bacterial infections and genetic control of immunoresponsiveness during natural disease outbreaks (157). This is the first report describing an MHC *B* complex influence on the development of bacterial skeletal disease in broiler breeder chickens, where *Staphylococcus* spp. was the predominant pathogen. Clinical signs and lesions were similar and consistent with natural and experimental staphylococcal osteomyelitis and tenosynovitis (12, 116, 260). Moreover, the present study parallels MHC mediated resistance after *S. aureus* challenge in inbred congenic lines of White Leghorn chickens (57).

Several models for staphylococcosis utilize either direct intracardiac or intravenous inoculation for experimental disease development (12, 116, 260). The respiratory tract or mucous membrane colonization likely serves as a common portal of entry during natural disease (12, 181). Experimental models for aerosol mediated

staphylococcal osteomyelitis require repeated high dose challenge, but often with inconsistent results (12, 181). Aerosol exposure in conjunction with virally induced immunosuppression and *ad libitum* feeding protocols enhanced the development of bacterial chondronecrosis and osteomyelitis (181). These results imply that the pathogenesis is multifactorial, involving interactions between the host immune system, environmental influences and bacterial virulence. Consequently, the aim of this study was to investigate the epidemiology of a natural skeletal disease with emphasis placed on the immunogenetic factors associated with disease development.

Two MHC *B* complex haplotypes, A4 and A12, were associated with increased susceptibility to bacterial skeletal disease within the examined flocks. This over-representation of select haplotypes among affected birds is suggestive of a potential MHC influence on the outcome of disease. The avian MHC, originally identified serologically as an extensively polymorphic erythrocyte antigen or blood group system, has three closely linked major loci, *B-F*, *B-L* and *B-G* (32, 145, 157, 159). The *B-F* determinants and *B-L* immune response genes are equivalent to the mammalian class-I and class-II genes, respectively. However, the *B-G* erythrocyte specific class IV alloantigens have not been identified in human or murine counterparts (160). Additional genes in the chicken MHC possess structural and functional homology with mammalian genes involved in lymphocyte activation and transporter associated with antigen-processing proteins (160). As such, the MHC genes are a crucial component in the initial antigen recognition and perpetuation of immune regulation.

Molecular surveys have mapped disease resistance to the *B-F*/*B-L* region (29, 113). The *B-F* and *B-L* MHC regions encode for cell surface molecules that act as

restriction elements for host cellular interactions, thus affecting immunoresponsiveness (282, 285). These associations have been studied with inbred congenic chicken lines that principally differ in MHC haplotype (2). Moreover, MHC mediated resistance to *Salmonella* spp. occurred in inbred congenic lines homozygous for the B4 haplotype (58). Age-dependent roles for chicken heterophil function are correlated with MHC enhancement of antibody production, complement activation and the production of acute phase proteins (43, 260). Thus, MHC-induced differential immunity to bacterial pathogens may be age related and involve genetic interactions between MHC and non-MHC loci.

The anatomic distribution of gross lesions among these lame broiler breeders was similar to previous reports (12, 116, 181, 260). The diagnosis of bacterial skeletal disease was established by consistent gross lesions, bacterial isolation or histologic lesions (or all). Osteomyelitis occurred most frequently in the proximal femoral physis and metaphysis, consistent with bacterial deposition in the terminal metaphyseal capillary network where laminar blood velocity is decreased (183). Localized extension into the surrounding joints, tendon sheathes and soft tissues commonly occur with severe osteomyelitis. For example, multiple lesions observed in the lame birds often consisted of osteomyelitis with arthritis of the corresponding joint or localized tenosynovitis (or both). Although gross lesions occurred in most lame chickens, histologic examination identified bacterial skeletal lesions in two of six lame birds with neither gross lesions nor bacterial isolates.

In addition to alterations in the host immune response, staphylococcal virulence factors are also related to septicemia and skeletal disease (12). Poultry isolates have

avian-specific phage groups and ecovars containing the *ermC* gene determinant (12, 183, 203). Most strains possess multiple virulence factors, including protein A, β-hemolysin, fibrinolysin and dermonecrotic toxin, which may act synergistically to elude the immune response.

In the present study, spontaneously lame broiler breeder chickens with bacterial osteomyelitis, arthritis and tenosynovitis were identified as a natural disease. The clinical and pathologic presentations were consistent with previously reported staphylococcosis in immature broiler breeders in commercial broiler production. The corresponding high incidence of bacterial infection in two significantly over represented genotypes indicate an MHC *B* complex influence on the expression of bacterial skeletal disease, with *Staphylococcus* spp. predominant. The current demonstration of differential immunity linked to the MHC *B* complex may provide insight into preventative measures for bacterial skeletal disease. Characterization of the mechanism of the MHC and host immunoresponsive influences on bacterial skeletal disease will require experimental disease reproduction and analysis within a larger, controlled population.

IV. ORAL INFECTION WITH CHICKEN ANEMIA VIRUS IN FOUR WEEK BROILER BREEDERS: LACK OF AN EFFECT OF MAJOR HISTOCOMPATIBILITY B COMPLEX GENOTYPE

Abstract. The pathologic consequences of chicken anemia virus (CAV) oral inoculation in 4-wk-old broiler breeders of different major histocompatibility *B* complex (MHC) genotypes were evaluated. MHC *B* complex was determined by hemagglutination and sequence-based typing. Clinical signs, serology, gross lesions, histopathologic analysis, and CAV genome quantification were used to evaluate disease progression. Clinical disease was not apparent in the inoculated broilers throughout the experimental period. At 14 days postinoculation, antibodies against CAV were detected in 26.4% (29/110) of the inoculated birds. The distribution of percent positive was 34.6% (9/26) and 32.3% (10/31) of the chickens with *B* A9/A9 and *B* A9/A4 MHC genotypes, respectively, and seroconversion in six other genotypes was 19% (10/53). These differences among MHC genotypes for specific seroconversion rate were not statistically significant. CAV genomes were detected in the thymus of 87.7% (93/110) of the inoculated birds with no statistically significant differences between MHC genotypes. Mild thymic lymphocytolysis, lymphedema, and medullary hemorrhage were observed in the inoculated chickens. Histomorphometric analysis showed that cortical lymphocyte-to-parenchyma ratios did not differ between inoculated and uninoculated groups or among

MHC genotypes. Similar findings have been reported previously in white-leghorn chickens of similar age, suggesting that broilers show a similar resistance to the effects of CAV infection at this age. The absence of significant clinical and pathological changes in the orally inoculated broilers at this age contrasts with CAV-associated thymus damage seen frequently in condemned commercial broilers at harvest.

Keywords: chicken anemia, broiler, pathology

Abbreviations: ANOVA = analysis of variance; CAV = chicken anemia virus; CL/P = cortical lymphocyte area to total parenchyma area; CTL = cytotoxic T lymphocyte; DPI = days postinfection; ELISA = enzyme linked immunosorbent assay; FRET = fluorescence resonance energy transfer; IBDV = infectious bursal disease virus; MD = Marek's disease; MHC = major histocompatibility complex; NK = natural killer, PCR = polymerase chain reaction; qPCR = quantitative polymerase chain reaction; SPF = specific pathogen free

Increased condemnations at slaughter and birds with extensive damage in primary immune tissues accompanied by chicken anemia virus (CAV) isolation or CAV detection by polymerase chain reaction (PCR), as well as significant associations between CAV and other diseases in Alabama broilers (104, 118) reveal a necessity for further work on CAV pathogenesis in broiler chickens. CAV causes transient aplastic anemia, thrombocytopenia, and generalized lymphoid atrophy in young chickens (184, 254, 263). Increased susceptibility to various viral, bacterial, and fungal pathogens has been associated with impairment of the host immune response following CAV infection (48,

230, 275, 294, 295, 312). More recently, Markowski-Grimsrud and Schat provided functional evidence that the immunosuppressive effect of CAV is associated with the impairment or absence of pathogen-specific cytotoxic T lymphocytes (175). Serologic data and detection of CAV genomes in commercial broilers suggest that CAV infection in broiler breeders occurs between weeks 4 and 6 of age, coinciding with decay of maternal immunity (255).

The pathogenesis of CAV infection has been elucidated by sequential virologic, pathologic, immunocytochemical, and immunologic studies of experimentally infected young white-leghorn chickens (90, 93, 94, 120, 133, 254, 287), and CAV has also been shown to induce thymic damage in white-leghorn and broiler breeders up to 10 wk of age (278). Infection in 3-wk-old white-leghorn chickens has been shown to induce immunosuppression without clinical disease (178, 179).

Resistance to infectious diseases is usually a complex genetic trait. Differences in susceptibility and immune responses against several agents have been reported to exist between different chicken inbred lines (34, 122, 279, 286). Immunoresponsiveness and outcome of disease have also been associated with the avian major histocompatibility *B* complex (MHC). The chicken MHC exerts a dramatic influence on resistance to a variety of pathogens, most notably Marek's disease (MD) virus (16, 29, 157). MHC-based genetic resistance/susceptibility against MD may depend, to a large degree, on MHC class I cell-surface expression, cytotoxic T-lymphocyte (CTL) response, and natural killer (NK) cell activity (85, 244). The presence of NK receptor gene(s) suggests that the chicken MHC may influence responsiveness of certain NK cells, which could be the basis for the yet-unexplained MHC association with the response to MD virus (144).

Susceptibility to and humoral immune responses associated with CAV infection may be linked to distinct MHC *B* complex haplotypes (39). Investigators found that chickens in a line with MHC *B* 13/13 had decreased seroconversion rates as compared with lines fixed for MHC *B*21 and MHC *B*19 haplotypes. These results suggest that genetic line differences, possibly including the MHC *B* complex, may influence susceptibility to CAV infection.

This study was aimed at evaluating clinical and pathologic changes in 4-wk-old broiler breeder chickens after oral inoculation with CAV and analyzing the possible MHC *B* complex influence on disease susceptibility.

Materials and Methods

Virus

The previously described low-passage CAV isolate 03-4876 was used (287). CAV 03-4876 titration was conducted in MDCC-MSB1 cells, as previously reported.

Chickens

Broiler breeders from commercial breeder line A (coded nomenclature to protect proprietary rights) were used. MHC haplotypes identified in this line include *B*A4 (*B*F/*B*L identical to *B*21), *B*A9, *B*A12, and *B*A1. The latter three haplotypes have been described in broiler lines but are not among haplotypes of leghorns (164). We determined the MHC haplotype frequencies in this broiler breeder line in the year 2000 to be A4 = 31.6%, A9 = 22.3%, A12 = 17.5%, A1 = 6.5%, A5 = 5.8%, A11 = 5.9%, and untypable = 10.4% (136). Therefore, in order to obtain a statistically adequate number of chickens representing MHC genotypes, 300 one-day-old broiler breeders were obtained from commercial breeding stock parents. Chickens were individually identified by wing

banding and maintained in isolated floor pens. All broiler chickens had maternally derived CAV antibodies as a result of CAV vaccination of the parent stock. All experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines.

Major histocompatibility *B* complex typing

At 3 wk of age, broiler *B* complex MHC genotypes were initially characterized by hemagglutination, as previously described, to select homozygotes of most frequent haplotypes (164). Based on results of this procedure, 113 birds were retained for the subsequent CAV inoculation. MHC *B* complex genotype assignments were later confirmed by DNA sequence-based typing of BF2 alleles, as recently described by Livant and Ewald (167). DNA was extracted from thymic cells using the high-pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. MHC *BF*2 exon 2 was amplified by PCR as described and nucleotide sequences were obtained by automated sequence analysis (Genomic Sequencing Laboratory, Auburn University, Auburn, AL) (167). Nucleotide sequence alignments were constructed using VectorNTI 9 ContigExpress sequence analysis and data management software (Invitrogen Corporation, Carlsbad, CA).

Experimental design

Ten chickens were euthanatized at 7 days of age and lobes of thymus obtained for DNA isolation and real-time quantitative polymerase chain reaction (qPCR). The chickens selected for CAV inoculation were serologically monitored by competitive enzyme linked immunosorbent assay (ELISA) as described below until confirmation of CAV maternal antibody decay. Chickens were orally inoculated at 4 wk of age with 2 ×

10^6 $TCID_{50}$ of CAV 03-4876. A total of 20 MHC *B* A9 and *B* A4 haplotype homozygote and heterozygote birds served as uninoculated controls. Body weights and hematocrits were recorded from each bird at 5, 11, and 14 days postinoculation (DPI). Statistical differences in body weights and hematocrits were evaluated by linear regression analysis and analysis of variance (ANOVA). All chickens were euthanatized and examined at necropsy at 14 DPI. Lobes of thymus were obtained from each bird and stored at −85 C for subsequent DNA isolation and qPCR. Thymic DNA was also used to confirm the major histocompatibility *B* complex genotype by sequence-based typing. Additional lobes of thymus were collected and fixed by immersion in 10% neutral buffered formalin for histopathologic evaluation.

Serology

Sera were collected from 20 birds at semiweekly intervals beginning at 3 days of age. Sera were tested for the presence of CAV-specific antibodies by competitive ELISA, using 10-fold dilutions, according to the manufacturer's recommendations (Idexx Laboratories, Inc., Westbrook, ME). Chickens were orally inoculated with CAV 03-4876 when maternal antibodies were no longer detectable (4 wk of age). Serum samples from all individual chickens were obtained at 14 DPI and tested for CAV antibodies as described above. Sera with s/n ratios <0.6 were considered positive. Significant differences in s/n ratios between inoculated and uninoculated control birds and between MHC genotypes in inoculated chickens were evaluated by ANOVA. Significant differences in percentage positive were evaluated by chi-square analysis.

Histopathology

Formalin-fixed sections of thymus were routinely processed, embedded in paraffin, sectioned at 4–6 μm, and stained with hematoxylin and eosin for histopathologic examination. A digital photomicrograph of a representative thymic lobe from each bird was taken at 40× magnification. To quantify lymphocytic depletion, histomorphometric analysis was conducted using ImageJ software version 1.29x (public domain, http://rsb.info.nih.gov/ij/), as previously described (287). Cortical lymphocyte area to total parenchyma area (CL/P) ratios were determined and statistically analyzed.

Virus detection and quantification by fluorescence resonance energy transfer-qPCR

DNA was extracted from thymic cells using the high-pure PCR template preparation kit (Roche Molecular Biochemicals) and DNA concentration was determined with the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) according to the manufacturers' recommendations. The number of CAV genomes in each sample was determined by fluorescence resonance energy transfer (FRET)-based real-time quantitative PCR (qPCR), as previously described, with a standard curve and negative control (no CAV DNA) included in each assay (288). The amount of DNA assayed (0.1–2 ng) was adjusted so the number of CAV genomes would fall within the standard curve for the qPCR based assay. Samples that yielded negative results with 0.1–2 ng DNA were retested using larger amounts (100–200 ng) of DNA. The limit of detection of the assay is approximately 1 CAV genome per 6000 cells. The number of CAV genomes per cell was calculated assuming that there were 2.5 pg of DNA per chicken cell (268). The $\log_{10}$ values were calculated for each sample and the geometric mean derived for each MHC *B*

haplotype. Differences of the geometric means between MHC *B* complex genotypes were statistically evaluated.

Results

MHC genotyping

Initial testing by hemagglutination suggested the presence of a previously unidentified MHC haplotype segregating at relatively high frequency in line A. The sera that are routinely used to identify known MHC haplotypes in this line indicated an improbable number of MHC homozygotes. Two reagents reacted weakly with a large number of samples that were otherwise positive for a single MHC type. On the basis of the serological typing, homozygotes were selected tentatively by choosing birds with the clearest reactions. Sequence-based typing of exon 2 of the *BF*2 locus demonstrated that some birds tentatively identified as MHC homozygous were actually heterozygous for a *BF*2 allele not previously detected in line A. Exon 2 of the new *BF*2 allele matches precisely a recently reported *BF*2 sequence named CC14a in Brazilian Caipira chickens (166). This novel *BF*2 allele was identified in combination with several other alleles, and the heterozygotes have been designated as CC14/X in figures and the table.

Serology

All tested chicks were seropositive for CAV-specific antibodies at 3 days of age, as expected, due to CAV vaccination of parent stock. CAV-specific antibody levels had significantly declined ($P < 0.05$) by 19 days of age. All chickens had achieved a CAV seronegative status (s/n ratio > 0.6) as determined by ELISA at day 26 of age, prior to inoculation with CAV 03-4876. At 14 days after CAV inoculation, CAV-specific antibodies were detected in 26.4% (29/110) of the exposed birds. Mean hematocrit values

and thymic cortical lymphocyte/total parenchyma ratios did not differ between inoculated chickens that were seropositive at 14 DPI and those that were seronegative. CAV-specific antibodies were found in 34.6% (9/26) and 32.3% (10/31) of the chickens of *B* A9/A9 and *B* A9/A4 MHC genotypes, respectively, while seroconversion in six other genotypes was 20.7% (11/53). Significant differences in mean s/n ratios were not observed between individual MHC genotypes (Table 8); however, significant differences ($P < 0.05$) were detected between inoculated and uninoculated control chickens. The uninoculated control birds maintained a negative CAV antibody status.

Table 8. Mean ± SD of body weights, hematocrits, and competitive ELISA CAV-specific antibody levels detected in 40-day-old broiler breeder chickens of different MHC *B* complex genotypes 14 days after oral inoculation with Alabama CAV isolate 03-4876

MHC genotype (*B* A-/-)	Mean ± SD		
	Body weight (g)	Hematocrit (%)	CAV antibody (s/n)
4/4	1835 ± 134.2	31 ± 1.5	0.8160 ± 0.2383*
9/9	1772 ± 142.6	31 ± 0.5	0.6593 ± 0.3122
9/4	1834 ± 171.8	32 ± 1.6	0.6790 ± 0.3392
12/12	1803 ± 134.8	32 ± 2.1	0.8283 ± 0.3339
12/4	1762 ± 111.3	33 ± 1.2	0.8354 ± 0.2136
12/9	1803 ± 193.9	32 ± 1.4	0.7328 ± 0.2562
12/1	1893 ± 211.4	31 ± 1.7	0.7195 ± 0.2941
CC14/X	1735 ± 215.5	32 ± 1.0	0.7172 ± 0.2464
Uninfected control	1701 ± 98.01	31 ± 1.4	0.8472 ± 0.1032

*Competitive ELISA was used. Lower values indicate the presence of antibody

Clinical signs

All chickens orally inoculated with CAV 03-4876 remained alert and no clinical signs were apparent throughout the experimental period. The average body weight at 14 DPI did not differ significantly ($P > 0.05$) between inoculated and uninoculated control chickens or among MHC genotypes (Table 8). Regression analysis indicated that the rate of body weight gain during the 14-day postinoculation period was a linear function of age

(r^2 = 0.9) and did not vary with genotype. Likewise, orally inoculated birds did not have reduced hematocrits at 14 DPI as compared with the uninoculated control birds ($P >$ 0.05). Hematocrits ranged from 24% to 35% at 3 DPI, 21% to 38% at 11 DPI, 21.5% to 46% at 14 DPI, and did not differ significantly ($P > 0.05$) among MHC genotypes (Table 8).

Gross and histopathologic findings

At 14 DPI, gross thymic lesions, characterized by distinct petechia and mild lobular atrophy, were evident in 13.6% (15/110) of the orally inoculated birds. These gross findings were consistent with the observed mild histologic lesions, including lymphocytolysis with apoptotic body formation, lymphedema, and medullary hemorrhage in scattered thymic lobules (Fig. 4).

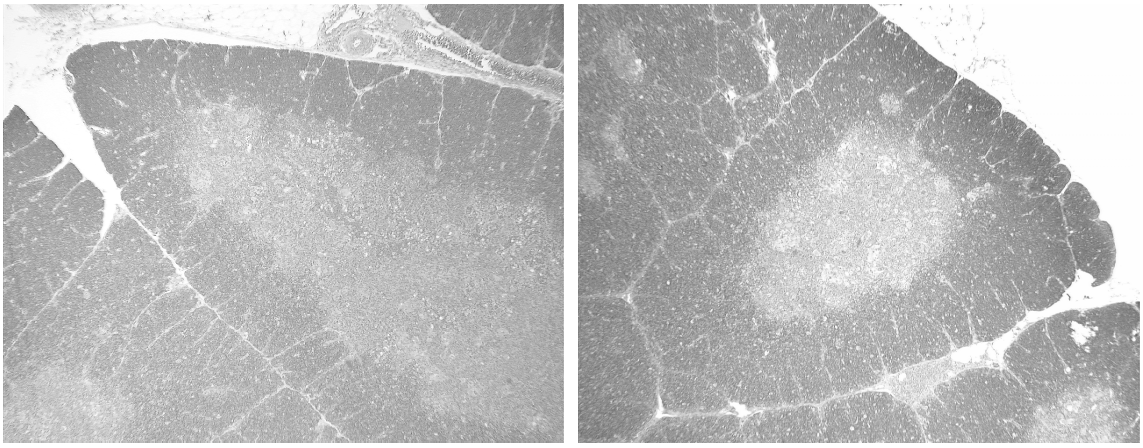

Fig. 4. Thymus of broiler breeder chicken 14 days after oral inoculation with Alabama CAV isolate 03-4876 (A) compared with an age-matched, uninfected control chicken (B). Mild cortical lymphocyte depletion can be observed in the CAV infected thymus. H&E. Bar = 300 μm

Thymic lymphocytic depletion was examined via histomorphometric analysis and expressed as the ratio of area of cortical lymphocytes to total parenchyma (CL/P). CL/P ratios ranged from 0.230 to 0.716, averaging 0.55. No significant differences were detected in CL/P ratios between inoculated and uninoculated control chickens (Fig. 5).

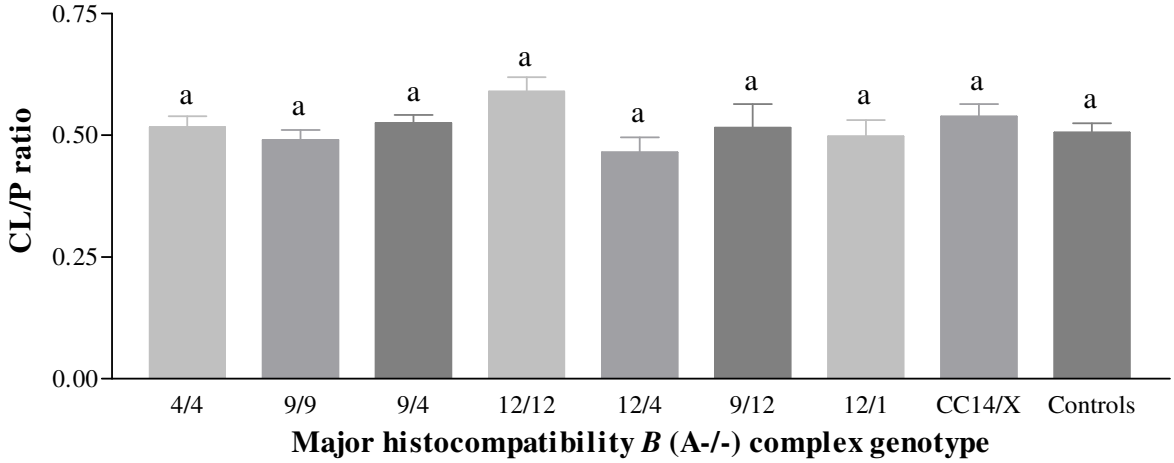

Fig. 5. Lymphocyte depletion expressed as the ratio of cortical lymphocytes/total lobular parenchyma area (CL/P) of the thymus of 6-wk-old broiler breeder chickens of different MHC *B* complex genotypes orally inoculated at 4 wk of age with CAV isolate 03-4876. Error bars indicate ± 1 SD (287, 288). No significant differences ($P < 0.05$) were found among MHC genotypes or between infected and uninfected groups.

However, variation in CL/P ratio was generally higher among the inoculated birds (0.230–0.716) as compared with uninoculated controls (0.395–0.622). Mean CL/P ratios in infected chickens did not significantly differ among distinct MHC genotypes. Gross and histologic lesions were not observed in the uninoculated controls.

Virus detection and quantification by FRET-qPCR

CAV genomes were not detected in thymus samples from chickens at 7 days of age, prior to CAV inoculation. CAV genomes were detected by FRET-qPCR in 87.7% (93/110) of the thymus samples obtained from all inoculated chickens at 14 DPI and

100% of samples obtained from inoculated chickens that were seropositive at 14 DPI. Fisher exact test indicated a significant difference ($P = 0.03$) between the number of samples with detectable CAV genomes between seropositive and seronegative inoculated chickens. A high dispersion in CAV genomes per cell ($\log_{10}$) levels, ranging from −3.9 to 3.2, was observed (Fig. 6).

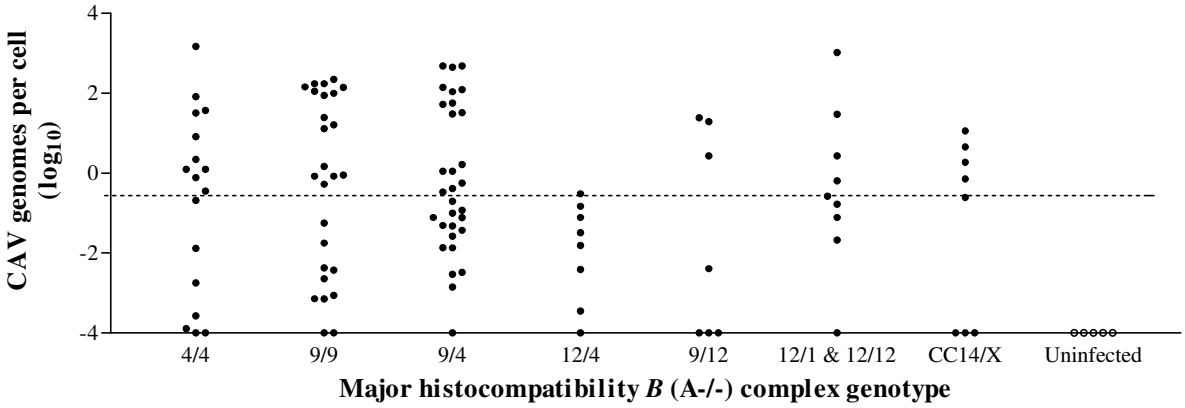

Fig. 6. CAV genomes per cell ($\log_{10}$) detected by FRET-qPCR in thymus of broiler breeder chicken groups of different MHC *B* complex genotypes orally inoculated at 4 wk of age with CAV isolate 03-4876. Because log values could not be calculated for 0, log values of −4 (below all positive samples) were assigned to samples with no detectable CAV genomes. The mean of $\log_{10}$CAV genomes per cell of all samples is −0.6275 (dashed line).

Linear regression analysis revealed a correlation between $\log_{10}$CAV genomes per cell and CAV antibody levels, as reflected by competitive ELISA s/n ratios in inoculated chickens ($R^2 = 0.44$, $P = 1.5 \times 10^{-14}$). Furthermore, the mean $\log_{10}$CAV genomes per cell was higher in inoculated chickens that were seropositive 14 DPI than in seronegative inoculated chickens (1.5 and −1.3, respectively; $P = 5 \times 10^{-14}$). While statistically significant differences were not observed between MHC *B* complex genotypes, the means of $\log_{10}$CAV genomes per cell were higher in *B* A9/A9 and *B* A9/A4 genotypes, 0.525

and 0.635, respectively, than in other genotypes. Within each of the *B* A9/A9 and *B* A9/A4 genotypes, the means of $\log_{10}$CAV genomes per cell were significantly higher ($P < 0.005$) in inoculated chickens that were seropositive 14 DPI than in inoculated chickens that were seronegative at this time. CAV genomes were not detected in the uninoculated control chickens.

Discussion

Alabama CAV isolate 03-4876, used herein to inoculate 4-wk-old broiler breeders, was originally obtained from commercial broilers exhibiting clinical disease. This isolate had been previously shown to be mildly pathogenic for 1-day-old specific pathogen-free white-leghorn chickens when inoculated orally with the same dose used in the present study (287). Others have reported that white-leghorn chickens orally or intramuscularly inoculated at 2–4 wk of age did not show significant reductions in body weight gain, hematocrit, or exhibit clinical signs at 14 DPI, which are frequently associated with CAV infection (92, 178, 238, 310, 313). In agreement with the results observed in white-leghorn chickens, broiler chickens of the present study showed absence of clinical signs.

Mild gross lesions as well as histologic lesions characterized by lymphocytolysis and lymphedema in thymic sections were detected in the CAV-inoculated broilers. These pathologic changes resulted in slight variation in CL/P among the inoculated birds. Similar findings have been reported previously in white-leghorn chickens infected with CAV at 3 wk of age, suggesting that broilers show a similar resistance to disease due to CAV at this age (178). These pathologic changes did not appear to be associated with MHC *B* complex genotype.

Following oral inoculation, CAV-specific antibodies were detected by ELISA at day 14 postinoculation in 26.4% of the birds. This low rate of seroconversion is in agreement with previous studies performed in white-leghorn chicks that have shown limited seroconversion at 14 days after CAV oral inoculation (287). In accordance with those previous results, presumably more birds would have shown seroconversion at a later stage after inoculation (287). The fact that no significant differences could be detected between MHC *B* complex genotypes for specific seroconversion rate suggests that the MHC haplotypes evaluated in the present study do not differ in this trait. However, we were unable to evaluate the *B*13 haplotype that was present in the white-leghorn line that others reported had a very low seroconversion rate (39).

CAV genomes were detected in the majority of inoculated birds; however, the number of CAV genomes was considerably less than we previously reported in orally inoculated, day-old chicks (287). The difference in the geometric mean of viral genomes/cell indicates approximately 5000-fold fewer CAV genomes/cell in the present study compared with chickens inoculated orally at day 1 of age. Furthermore, there is extensive variation in the number of CAV genomes ($\log_{10}$) detected in each genotype. There may be different explanations for this finding. We speculate that these lower viral concentrations may be associated with well-developed innate and local immune barriers (chickens 4 wk of age), which need to be overcome before achieving systemic and thymic infections. Alternatively, the low levels of CAV may have been a result of effects of maternally derived antibody below the level of detection of the competitive ELISA. Although antibody levels below the level of detection of this competitive ELISA are generally considered nonprotective, Markowski-Grimsrud and Schat (175) recently

demonstrated that anti-CAV maternal antibody status at 14 days of age had a profound effect on replication of CAV in leghorn chickens inoculated 2 wk later, although all chickens were seronegative by the competitive ELISA at the time of inoculation. Mild pathologic lesions are consistent with reduced CAV genome concentrations. Higher geometric mean of CAV genomes per cell were detected in *B* A9/A9 and *B* A9/A4 MHC genotypes but, because the differences were not statistically significant, this finding requires further investigation to allow valid conclusions.

Increased condemnation at broiler slaughtering has been reported to be accompanied by extensive thymic atrophy, bone marrow atrophy, and aplasia, and CAV detection by PCR (118). Serologic data and detection of CAV genomes in commercial broilers suggest that CAV infection in broiler breeders occurs between week 4 and 6 of age, coinciding with decay of maternal immunity (255). Our current findings in broilers, as well as findings by other authors in white-leghorn chickens (178), do not assist in explaining those observed under commercial conditions, which clearly suggest that CAV is actively causing disease and economic losses. Several possible explanations could account for such a discrepancy, i.e., concurrent diseases, synergistic immunosuppressive agents, and other stressors encountered in commercial operations. Evidence for such explanations is provided by reports in which severe lymphocytic depletion of the bursa, presumably due to infectious bursal disease (IBD), precedes severe lymphocytic depletion of the thymus associated with CAV infection in commercial chickens (117, 255). IBD has been shown experimentally to overcome age resistance and increase the severity of disease caused by CAV in white leghorns (238, 295, 312). Another possible explanation that arises from analyzing current and previous data is that the oral route may

not be a relevant route for CAV to induce disease in the field. In the present study, we used the oral route of inoculation based on the thesis that, under commercial conditions, this route would be a common natural route of CAV transmission in chickens of this age. However, our previous work, as well as work by others have shown that oral infection of very young leghorn chicks (even 1-day-old) induces mild CAV disease (178, 238, 253, 287). On the other hand, there is accumulating evidence that the virus persists in the tissues, even long after seroconversion (39, 188, 287, 311). Theoretically, amplification of CAV persisting in tissues of commercial chickens (initially acquired by the vertical or oral routes) would more closely mimic intramuscular inoculation, a route of CAV exposure that, most likely by circumventing innate immune responses, has proven to cause far more severe pathologic consequences in the infected chickens (275).

V. PATHOGENESIS OF INFECTIOUS BRONCHITIS VIRUS IN CHICKENS OF DIFFERENT MHC B COMPLEX GENOTYPES

Abstract. Infectious bronchitis (IB) disease progression in vaccinated chickens after challenge was evaluated in a commercial line of white leghorn chickens of different major histocompatibility *B* complex (MHC) genotypes. Chickens were vaccinated with an ArkDPI IB virus (IBV) vaccine and challenged with a homologous IBV field isolate (AL/4614/98) at 14 days later. MHC *B* genotypes were determined by DNA sequence-based typing of BF2 alleles. Clinical signs, histopathologic analysis, IBV-specific IgA in tears, and detection of IBV genomes in tears were used to evaluate disease progression. The overall incidence of clinical disease was significantly higher ($P = 0.044$) in chickens of the *B* 2/21 MHC *B* genotype than in *B* 2/15 MHC genotype birds. However, the severity and incidence of histologic lesions did not differ significantly with MHC genotype ($P = 0.14$). IBV-specific IgA was detected in tears of vaccinated and challenged chickens, and the frequency of antibody positive birds was significantly higher in the *B* 2/21 MHC *B* genotype at 3 ($P = 0.05$) and 10 ($P = 0.04$) days after challenge consistent with incidence of detectable disease in these birds. IBV genomes were present in the tears of vaccinated and challenged birds, and the incidence of detectable IBV genomes did not vary significantly with MHC *B* genotype. Collectively, these results indicated a discrete difference in susceptibility to IBV that might be attributed to the MHC genotype.

Keywords: Infectious bronchitis virus, lachrymal IgA, major histocompatibility complex, pathology

Abbreviations: ANOVA = analysis of variance; IB = infectious bronchitis; IBV = infectious bronchitis virus; CAV = chicken anemia virus; CTL = cytotoxic T lymphocyte; DPV = days post vaccination; ELISA = enzyme linked immunosorbent assay; MHC = major histocompatibility complex; MD = Marek's disease; NK = natural killer

Resistance to infectious diseases is usually a complex genetic trait. Differences in susceptibility to and immune responses against several agents have been reported in several inbred chicken lines (34, 122, 279, 286). Immunoresponsiveness and outcome of disease have also been associated with the avian major histocompatibility *B* complex (MHC). The chicken MHC differs from the typical mammalian MHC in that it exerts a dramatic influence on resistance to a variety of pathogens, most notably Marek's disease (MD) virus (16, 29, 157). MHC-based genetic resistance/susceptibility against MD may depend on MHC class I cell surface expression, cytotoxic T-lymphocyte (CTL) response, and natural killer (NK) cell activity (85, 144, 244). In previous work we demonstrated that the avian MHC influences bacterial skeletal disease in broiler breeder chickens (136). Differences in susceptibility to chicken anemia virus (CAV) infection have been described in non-congenic chicken lines that differ in MHC *B* haplotype (39). Investigators found that a line of chickens expressing MHC *B* 13/13 had decreased seroconversion rates compared to lines fixed for MHC *B*21 and MHC B19 haplotypes.

However, our most recent CAV studies performed in commercial broilers with 5 different MHC haplotypes did not demonstrate a MHC effect (135).

Both humoral and cell mediated immune responses seem to be relevant for protection against IBV. Presence of high titers of systemic antibodies correlate well with the absence of virus recovery from kidneys and genital tract (96, 173, 305) and protection against a drop in egg production (24). In addition, local IBV-specific IgA and IgG have been demonstrated in tracheal washes of infected chickens (70) and antibody-secreting cells have been detected in tracheal sections (201). The Harderian gland is the major source for lachrymal IgA and plays an important role in the development of vaccinal immunity. IBV-specific IgA has been demonstrated in the lachrymal fluid (54, 65, 276) and these IgA levels appear to be correlated with resistance to IBV re-infection (273). Cook *et al*. (54) found more IBV-specific IgA in the lachrymal fluids of chicken lines resistant to IBV. Variation in IBV-specific IgG levels in serum and IBV-specific IgA levels in lachrymal fluids has also been demonstrated in different chicken lines after ocular vaccination with IBV (279). However, Janse *et al*. (131) contended that local immunity to IBV in the trachea is mediated by T-cells. Both CD4+ and CD8+ T cells have been demonstrated in sections of trachea and lung of chickens infected with IBV (70, 131). Specific cytotoxic T lymphocytes (CTL) have been shown to be important for clearance of IBV infections in the non-immune host (248). Immunization with a plasmid encoding the IBV nucleocapsid protein, which induces IBV-specific CTL, but no neutralizing antibodies, protects chickens against IBV challenge (250). Furthermore, adoptive transfer of CD8+ T cells also protects against IBV challenge (249).

The aim of this study was to compare the susceptibility/resistance to IBV of two different MHC genotypes in a commercial layer line. A previous study in *B* congenic chicks vaccinated with a moderately attenuated IB vaccine at a day of age showed that chickens possessing the *B*15 haplotype were more resistant to IBV than chickens possessing *B*13 or *B*21 haplotypes (17). However, in outbred commercial populations, the vast majority of birds are MHC heterozygotes. All birds in the layer line selected for the present study were either *B* 2/15 or *B* 2/21 heterozygous MHC genotypes. Chickens are routinely vaccinated against IBV, yet outbreaks are common. Differences in susceptibility between chickens associated with MHC genotype might manifest in a response to vaccination or challenge, and might be amplified during responses to both vaccination and challenge. Thus, we vaccinated chickens before challenging them with a homologous IBV strain to increase the chances of detecting a difference in resistance between MHC genotypes. Resistance was evaluated by clinical signs and lesions, local antibody responses, and viral shedding.

Materials and Methods

Viruses

A commercially available ArkDPI vaccine strain was used following the recommendations of the manufacturer. In addition, a previously described wild IBV Ark-type isolate AL/4614/98 (280) was used for challenge. The S-1 gene of this isolate is similar to the ArkDPI strain (96% similarity both at the nucleotide and the deduced amino acid sequence) and more distant to other IBV strains and serotypes. Isolate AL/4614/98 and the ArkDPI vaccine strain have 92.4% antigenic relatedness (280).

Chickens

One hundred and twenty (120) 1-day-old chickens from a commercial white leghorn line were used. Standard MHC *B* haplotypes identified in this line include B2, *B*15 and *B*21. Approximate MHC genotype frequencies in this commercial layer line during 2005 were *B* 2/15 = 50% and *B* 2/21 = 50% with no homozygotes. Chicks were individually identified by wing banding and maintained in isolated cages with *ad libitum* access to feed and water. All day-old chicks had maternally derived IBV antibodies as a result of IBV vaccination of the parent stock. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an AAALAC accredited institution.

Experimental design

Sera were collected from all 120 chickens at 15 days of age and tested for the presence of IBV specific IgG by ELISA using 500-fold dilutions according to the manufacturer's recommendations (IDEXX Laboratories, Inc., Westbrook, ME). In accordance with the manufacturer's recommendations, sera with S/P ratios ≤ 0.2 were considered negative for anti-IBV specific antibodies. All birds seropositive at 15 days of age (S/P ratio > 0.2) were removed from the study. The 93 chickens with no detectable IBV antibodies (S/P ratio ≤ 0.2) were randomly assigned to the experimental groups indicated in Table 9. At 18 days of age, chickens in the vaccinated/challenged group were vaccinated by the ocular route with a live attenuated ArkDPI type IBV vaccine. Nonvaccinated/challenged and nonvaccinated/uninfected control chickens were housed in a separate isolated facility. Body weights of all chickens were recorded weekly and

evaluated by linear regression analysis and analysis of variance (ANOVA). At 14 days post vaccination (DPV) birds were challenged by intranasal and intraocular instillation with 100 µl containing $10^{5.83}$ embryo infectious doses 50% (EID_{50}) of IBV Ark-type isolate AL/4614/98. At this time birds of the nonvaccinated/challenged group (seven chickens) were commingled with previously vaccinated birds and challenged as well. Respiratory signs were assessed daily as described below. Lachrymal fluid samples were collected as described (277) at 5, 10, and 14 DPV, and 3, 6, and 10 days after challenge for both IBV-specific IgA detection by ELISA and IBV genome amplification and quantification (described below). Chickens were euthanized and examined for gross lesions at 10 days after IBV challenge and tracheal samples were obtained for histopathologic evaluation.

Clinical scores

Respiratory signs classified as crackles, wheezes, and rales (tracheal and/or nasal) were assessed daily in each individual bird by bringing the head of the bird near to the examiner's ear and listening. A five-point severity scale was assigned to each respiratory sign. A clinical score (maximum of 15 points) was obtained by adding the severity points for each respiratory sign. To include the duration of clinical signs in the score, an overall clinical score was computed by adding the clinical scores for all days. Differences in overall clinical scores between MHC genotypes were analyzed by Fisher's exact test.

Major histocompatibility B typing

MHC *B* complex genotypes were determined by DNA sequence based typing of *B-F2* alleles as previously described (167). Briefly, genomic DNA was extracted from blood, MHC BF2 exon 2 was amplified by PCR (167) and nucleotide sequences were

obtained by automated sequence analysis (Genomic Sequencing Laboratory, Auburn University, Auburn, AL). Nucleotide sequence alignments were constructed using VectorNTI 9 ContigExpress sequence analysis and data management software (Invitrogen Corporation, Carlsbad, CA).

IBV antibody detection in lachrymal fluid

All lachrymal fluid samples were tested for the presence of IBV specific IgA by ELISA as described (280). Absorbance values greater than two standard deviations from absorbances of tears from the nonvaccinated/uninfected control chickens were considered positive. Significant differences in incidence of antibody positive birds between vaccinated/challenged and nonvaccinated/challenged groups, and between MHC genotypes in the vaccinated/challenged group were determined by Fisher's exact test.

Histopathology

Formalin-fixed sections of trachea were routinely processed, embedded in paraffin, sectioned at 4 – 6 μm, and stained with hematoxylin and eosin for histologic examination. Sections were examined for the presence of mucosal epithelial necrosis, epithelial deciliation, lymphocytic infiltration, epithelial cell hyperplasia, and goblet cell hyperplasia. Each histologic parameter was scored by the study pathologist according to the following scale: 1 = normal; 2 = mild; 3 = moderate; 4 = marked; and 5 = severe. The summation of scores for each parameter comprised the total tracheal histologic index for each bird. Indices in vaccinated/challenged, nonvaccinated/challenged, and nonvaccinated/nonchallenged groups and of the two MHC genotypes within each group were statistically analyzed by Fisher's exact test.

Virus detection by RT-PCR

RNA was extracted from 10 individual tear samples from chickens of each MHC genotype at each collection day using the QIAamp RNA mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. IBV genomes were amplified by RT-PCR as described (280), but with the following modifications. A 229 bp cDNA fragment was amplified using primers corresponding to nucleotides 256-280 and 484-460 of the IBV Ark99 nucleocapsid (N) gene (GenBank accession number M85244). Samples of RNA pooled according to MHC genotype were initially assessed for presence of IBV RNA at each collection day. Pooled RNA samples were subjected to 29, 32, 35, 38 and 41 PCR cycles to determine the optimal number of cycles for semi-quantitative analysis of individual samples. RNA prepared from individual tear samples was evaluated for the presence and relative amount of IBV genomes using 25 PCR cycles following reverse transcription. PCR products were visualized by electrophoresis using a 0.75% Synergel/0.5% agarose gel, stained with SYBR® Green dye (Applied Biosystems, Bedford, MA), destained with distilled water and then photographed with the EDAS 290 gel imaging system (Eastman Kodak Company, Rochester, NY). Relative intensity of bands in negative gray-scale images was determined using ImageQuant 5.1 software (purchased from Molecular Dynamics, now GE Health Care Life Sciences). Background signal of the area of the gel containing a reaction without template was subtracted from each area of the gel containing PCR products.

Results

Clinical signs

Sequence-based typing of exon 2 of the BF2 locus identified 52 *B* 2/21 and 41 *B* 2/15 MHC *B* complex heterozygous chickens among the 93 chickens included in the study. As shown in Table 9, the mean body weight at 10 days after IBV challenge did not differ significantly ($P > 0.05$) between challenged and uninfected control chickens. Regression analysis indicated that the rate of body weight gain during the experimental period was a linear function of age ($r^2 = 0.9$) and did not vary with genotype. Clinical signs consisting of respiratory crackles, wheezes and tracheal/nasal rales were first detected in IBV challenged groups at 5 days post-challenge, peaked at 6 days, and progressively declined throughout the remainder of the experiment.

Following challenge, 93.3% (42/45) of the *B* 2/21, 75.0% (24/32) of the *B* 2/15 and 100% (7/7) of the nonvaccinated chickens developed clinical signs. The incidence of clinical disease in vaccinated/challenged chickens was significantly different between MHC genotypes ($P = 0.0437$) (Table 9).

Table 9. Distribution of MHC genotypes, body weights and clinical disease in Ark DPI vaccinated commercial layers subjected to wild Ark-type IBV challenge

Experimental group	MHC genotype	No. of chicks	Body weight[‡] (g)	Clinical disease*,[†] (%)
Vaccinated / challenged	*B* 2/21	45	498.3 ± 44.13	93.3[A]
	B 2/15	32	499.3 ± 41.63	75.0[B]
Nonvaccinated / challenged	*B* 2/21	3	492.6 ± 15.14	100
	B 2/15	4	477.8 ± 18.77	100
Nonvaccinated / uninfected	*B* 2/21	4	494.7 ± 24.58	0
	B 2/15	5	477.6 ± 22.59	0

* Incidence of clinical disease throughout a 10 day experimental period
[†] Different letters indicate significant differences MHC groups ($P = 0.0437$)
[‡] Mean body weight ± standard deviation at 10 days after challenge

Moreover, compared with *B* 2/15 chickens, a significantly higher percentage of *B* 2/21 birds ($P = 0.05$) had clinical scores ranging from 6 to 10 (Fig. 7). However, incidence of clinical scores ranging from 1 to 5 ($P = 0.93$) and those greater than 10 ($P = 0.68$) were not significantly different between MHC genotypes. Clinical scores were greater than 10 in all nonvaccinated/challenged birds. A significantly greater number of nonvaccinated/challenged *B* 2/15 ($P= 0.0276$) and *B* 2/21 ($P= 0.006$) birds had clinical scores greater than 10 as compared to vaccinated/challenged birds expressing the same MHC genotype. Throughout the experimental period, clinical disease was not observed in non-challenged nonvaccinated control birds.

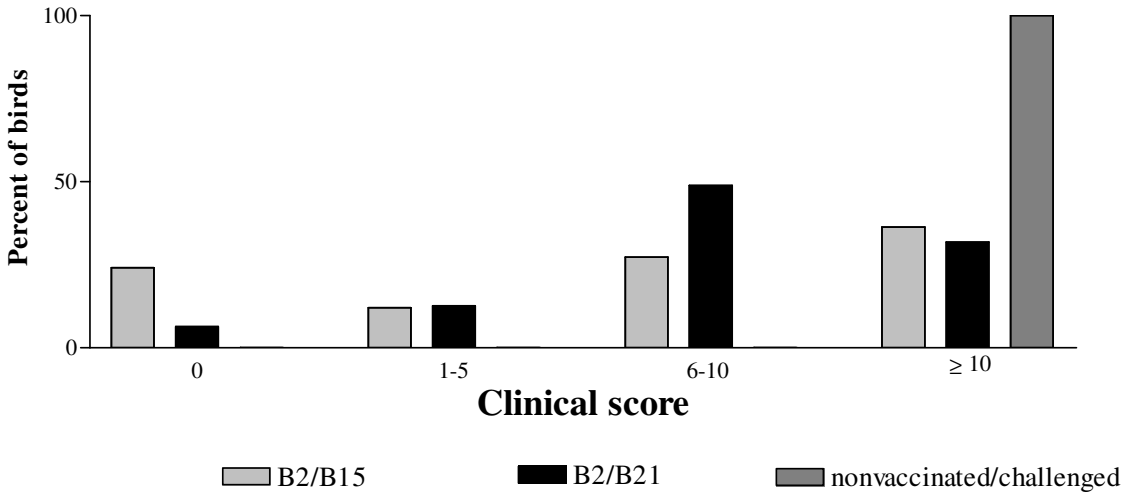

Fig. 7. Clinical scores in layer chickens of different MHC genotypes which were vaccinated with an ArkDPI strain and subsequently challenged with a wild Ark-type IBV isolate. Clinical scores were compared between MHC genotypes using Fisher's exact test, and significant differences ($P \leq 0.05$) are indicated by an asterisk.

Histopathologic findings

Epithelial necrosis, deciliation, lymphocytic infiltrates and hyperplasia were observed at day 10 after challenge in the tracheal mucosa. Differences in severity and

incidence of histologic lesions between MHC genotype were not detected ($P > 0.05$). The most consistent lesion in vaccinated and subsequently challenged chickens was mucosal thickening due to mononuclear cell infiltration. Some chickens had acute phase lesions characterized by loss of mucosal epithelium with deciliation, fibrinous exudation and heterophilic mucosal infiltrates. In addition, varying degrees of epithelial and goblet cell hyperplasia were evident. The total tracheal histologic indices, corresponding to the summation of individual lesion scores in each bird, did not differ significantly between the nonvaccinated/challenged and vaccinated/challenged groups ($P = 1.000$) or between chickens with different MHC genotypes in the vaccinated/challenged group ($P = 0.1438$).

IBV-specific IgA in tears

IBV-specific IgA was detected in tears after vaccination and subsequent challenge with IBV isolate AL/4614/98. Mean ELISA absorbance values did not vary significantly ($P > 0.05$) between nonvaccinated/challenged and vaccinated/challenged groups or with MHC genotype throughout the experimental period. The incidence of IgA positive birds was similar in both genotypes following vaccination; however, significant differences were observed following challenge (Fig. 8).

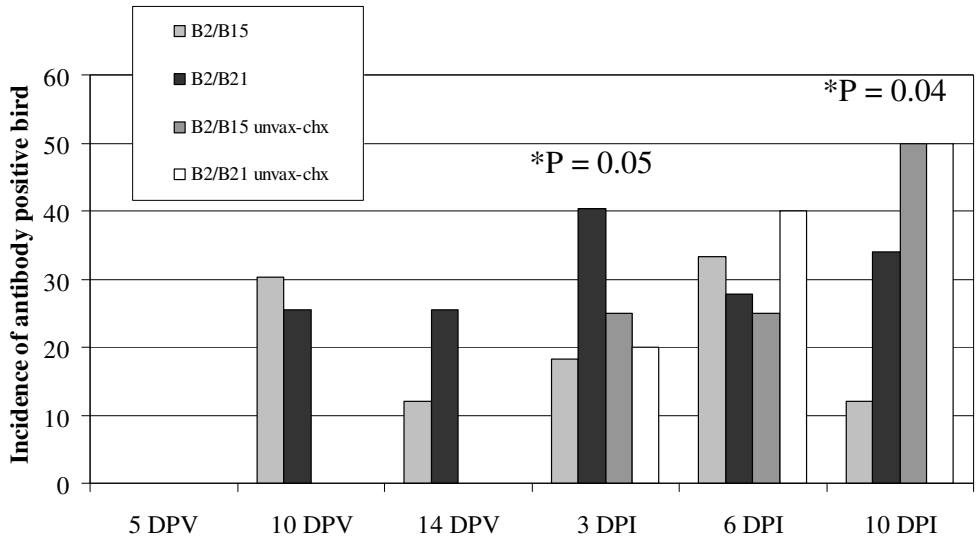

Fig. 8. IBV-specific IgA in lachrymal fluid of commercial layer chickens expressing different MHC genotypes following vaccination with a live attenuated ArkDPI IBV vaccine strain and after challenge with IBV isolate AL/4614/98. Significant differences in the incidence of antibody positive birds are indicated.

Compared with *B* 2/15 genotype birds, a significantly higher number of vaccinated and subsequently challenged *B* 2/21 birds had detectable lachrymal IBV-specific IgA at 3 ($P = 0.05$) and 10 days after challenge ($P = 0.04$). IBV-specific IgA was not detected in nonvaccinated birds prior to challenge. Following challenge of nonvaccinated chickens, similar increases in the incidence of antibody positive birds were observed in both genotypes. IBV-specific IgA was not detected in the uninoculated control group throughout the experimental period.

IBV in tears

IBV genomes were readily detected by RT-PCR in pooled tear samples from vaccinated birds expressing either MHC genotype at 5, 10, and 14 DPV and 3 and 6 days after challenge, but not at 10 days post challenge, even with over 40 PCR cycles (not shown). Individual tear samples from 10 randomly selected birds representing each MHC

genotype were evaluated for the presence and relative amount of IBV immediately prior to challenge (14 DPV), and at 3 and 6 days after challenge (Fig. 9).

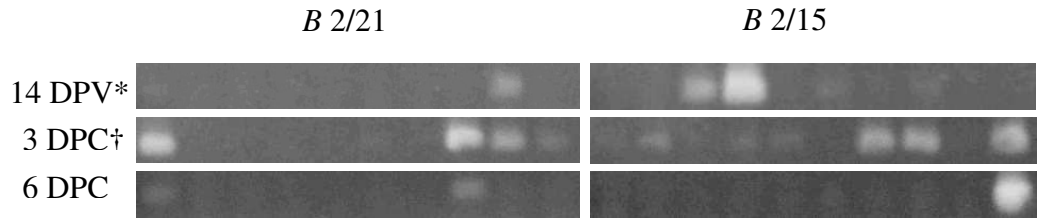

Fig. 9. RT-PCR detection of IBV genomes in tear samples from commercial layers of different MHC *B* complex genotypes following vaccination with a live attenuated Ark DPI IBV and challenge with IBV isolate AL/4614/98. *DPV = days post vaccination † DPC = days post challenge

Viral genomes were detected (produced an RT-PCR product visible in gels) in tears collected from 4 of 10 *B* 2/21 (40%) genotype birds and from 1 of 10 *B* 2/15 (10%) birds immediately prior to challenge. The relative amount of RT-PCR product varied greatly among individual chickens. At 3 days after challenge, virus was detected in 6 of 10 *B* 2/21 (60%) and 4 of 10 *B* 2/15 (40%) genotype birds. Virus was detected in 1 of 10 birds (10%) of each genotype at 6 days. The incidence of IBV detection did not differ significantly between MHC genotypes immediately prior to challenge (P= 0.30), or at 3 (P=0.66) or 6 (P=1.00) days after challenge. Although only a portion of tear samples collected 3 days after challenge generated visible RT-PCR products, image analysis indicated that all samples generated a signal above background. Comparison of relative intensities of RT-PCR product signals showed no significant difference (P=0.23) between MHC genotypes, even if samples with no visible RT-PCR product were excluded (P=0.62).

Discussion

The challenge IBV isolate AL/4614/98, obtained from a 1998 outbreak of respiratory disease in 40-day-old broilers, induced characteristic IB respiratory signs (42), but overall mild disease in a commercial line of white leghorn chickens. In the current study, neither nonvaccinated nor vaccinated chickens subjected to challenge with this isolate showed depression, mortality, severe nasal or ocular discharge, or a significant reduction in the rate of weight gain. This result was in agreement with the general observation that respiratory diseases under field conditions tend to be more severe than those reproduced experimentally (95). Respiratory signs of IB typically appear in susceptible birds within 18 to 36 hours after infection (42). However, in the present study, respiratory disease was first evident 5 days following challenge. These findings are similar to those previously reported in a vaccine efficacy study (141), where clinical signs were apparent in 20% of vaccinated birds by 5 days following challenge with a homologous ArkDPI isolate. Here, all of the nonvaccinated/challenged birds developed severe respiratory disease. Differences in the severity of respiratory disease between vaccinated and nonvaccinated birds following challenge with IBV isolate AL/4614/98 indicates that vaccination with the live attenuated ArkDPI vaccine induced some extent of protection. These findings suggest that immune responses had indeed been activated by homologous vaccination prior to challenge in the chickens.

The incidence of respiratory disease was significantly less in vaccinated *B* 2/15 (75%) than in *B* 2/21 (93.3%) chickens ($P = 0.044$), suggesting that birds of the MHC *B*15 haplotype are more resistant to IBV infection than those expressing the *B*21 haplotype. Consistent with the overall incidence of disease, clinical score evaluation also

indicated that respiratory disease was significantly more severe in *B* 2/21 birds. These findings are in agreement with other studies showing increased resistance to IBV in *B*15 haplotype birds compared to other MHC *B* haplotypes including *B*2 and *B*21 (17, 34, 55). Studies conducted in IBV susceptible lines have demonstrated that line 7_1 chickens, possessing the MHC *B*2 haplotype, are more susceptible to IB than line 15I5 birds (*B*15) (34, 207). In a more recent retrospective comparison among inbred and congenic lines, mortality was significantly greater in the 7_1 (*B* 2/2), and congenic 15.P-13 (*B* 13/13) and 15.N-21 (*B* 21/21) lines than in 15I5 (*B* 15/15) birds following inappropriate administration of an attenuated IBV vaccine strain (17).

Because we found differences both in incidence and severity of IBV clinical signs between MHC genotypes, we expected to also find histologic differences between MHC genotypes. Nakamura *et al*. (201) found that deciliation, degenerative and hyperplastic changes, and lymphocytic infiltration could be detected histologically by 2 days after IBV infection. These lesions were readily observed in the trachea at 9 days and showed a tendency to decline afterwards. According to Gouffaux *et al*. (95) diffuse lymphocytic infiltration peaks at 7 days after IBV infection and declines to baseline by 21 days. In previous studies in our laboratory, significant histologic differences between IBV AL/4614/98-infected and uninfected SPF chickens were detected at day 9 after challenge (280). Based on these observations, we chose to evaluate histologic changes 10 days after challenge. However, in the present study, histologic differences were not detected between MHC genotypes when tracheas were examined 10 days post challenge. We speculate that histological differences among MHC *B* haplotypes might have been detected at other times post-challenge. In the present study, we chose a time for

histological examination when we expected to observe lesions, but might detect differences if duration of disease was different between MHC haplotypes.

IBV-specific IgA was detected in lachrymal fluids by ELISA following vaccination and challenge in chickens of either MHC type, but the incidence of antibody positive birds was significantly higher ($P < 0.05$) in *B* 2/21chickens than *B* 2/15 chickens at 3 and 10 days after challenge. *B* 2/21 genotype birds had both a higher incidence of lachrymal IgA and a higher overall incidence of clinical signs. However, the presence of antibody did not correlate with differences in clinical disease between genotypes at those individual time points. In this study we did not measure the levels of IgA; only presence or absence. IgA responses depend substantially on B cell and MHC restricted CD4+ lymphocytic activity. Thus, measuring the levels of IgA may have better assisted our interpretations.

Virus was not detected in all vaccinated birds after challenge, and the number of birds with detectable virus declined throughout the remainder of the study. These findings are in accordance with other studies that have shown that challenge of vaccinated birds with homologous virus results in much lower titers of recovered challenge virus, and for a shorter period than in nonvaccinated birds (280). IBV genomes were detected in lachrymal fluids by RT-PCR following vaccination and up to 6 days after challenge. Even though not statistically significant, more *B* 2/21 chickens had detectable IBV genomes after both vaccination and challenge. This finding is consistent with the higher incidence of clinical disease in the *B*21 chickens.

In the immune response against oncogenic diseases MHC-restricted CTL responses are essential. Marek's disease provides the most dramatic example. In the

present study we detected only slight differences in the outcome of IBV infection in chickens differing in MHC genotypes. The discrete difference may be due to complex interactions including both humoral and cell mediated immune responses and is only partially restricted by the MHC.

VI. CONCLUSION

Employing genetic based mechanisms for disease resistance is a plausible approach for sustainable control of infectious disease in the commercial poultry industry. Continued emergence of virulent pathogens and the limitations on the use of chemotherapeutics emphasize the importance of breeding for disease resistance. Genetic heterogeneity among various immune response mechanisms constitutes the major immunologic basis for resistance variations. The MHC is a central mediator for immune responses against a diverse repertoire of diseases, and is a readily selectable genetic trait that can strongly influence disease development by conferring either resistance or susceptibility to disease.

The research presented in this dissertation constitutes a three-part investigation that was conducted to potentially define MHC mediated resistance to several common pathogens in the poultry industry. In the first study, MHC-associated resistance to bacterial skeletal disease was demonstrated in a commercial line of broiler breeder chickens. Specifically, two MHC *B* complex haplotypes, A4 and A12 were associated with increased susceptibility to bacterial skeletal disease. *Staphylococcus aureus* was the predominant pathogen, and clinical and pathologic lesions observed in the selected line of commercial broiler breeders were similar to previous reports. Differential immunity linked to the MHC *B* complex may provide insight for controlling natural outbreaks of bacterial skeletal disease; however, further characterization of the exact immune

mechanisms responsible for the observed differences is necessary prior to implementation of these results by the broiler industry.

In the second experiment, commercial broiler breeders expressing select MHC *B* complex genotypes were experimentally inoculated with CAV at 4-weeks of age. This study was important from two vantages: potentially defining MHC mediated resistance to CAV and further elucidating pathogenic mechanisms associated with subclinical immunosuppressive effects of CAV infection in older birds. While MHC influence on disease susceptibility could not be substantiated, higher mean levels of virus were demonstrated in the thymuses of A9/A9 and A9/A4 MHC *B* haplotype chickens. Further investigations in a larger population may provide statistical inference necessary for valid conclusions. CAV is most likely spread horizontally by the oral route; however, in this study, experimental oral inoculation was not associated with clinical disease or immunosuppression. These results imply that concurrent diseases including synergistic immunosuppressive agents and environmental stressors play an important role in the pathogenesis of CAV in older chickens.

The final study focused on the pathogenesis of IBV in a line of commercial layers with two distinct heterozygous MHC *B* complex genotypes, B2/B15 and B2/B21. Vaccination with a homologous IBV strain provided some protection against subsequent challenge. The incidence of clinical disease was significantly less in B2/B15 than in B2/B21 genotype birds; however, correlations between MHC genotypes and histologic lesions, detectable IBV-specific IgA and virus in tears could not be established. MHC-associated differences in resistance to IBV have been demonstrated in congenic and inbred lines, suggesting that genetic variability within these commercial layers may be

associated with the lack of correlations in this study. Demonstration of MHC mediated disease resistance and characterization of CTL responses in this line of commercial chickens will require evaluation of homozygous individuals within a larger controlled population.

Genetic selection for disease-resistant MHC genotypes can be a beneficial addition to a comprehensive disease control program. Continually emerging and evolving pathogens, and the limitations on the use of antimicrobials in the food animal industry, emphasize the advantage of breeding for genetic resistance. Recommendations for ongoing or similar studies include:

1) Evaluate additional haplotypes for bacterial skeletal disease, CAV and IBV resistance. The studies presented in this dissertation involved a limited number of haplotypes, and strong MHC associations were not identified. However, substantial associations may exist with haplotypes that were not examined.
2) Identify MHC associations with other pathogens, particularly emerging infectious diseases such as avian influenza.
3) Characterize immunologic factors contributing to resistance and susceptibility differences. The MHC exerts broad effects on various immune responses including antibody and cell mediated responses. Significance in disease resistance may be elaborated by using marker-assisted selection by introgression of resistant quantitative trait loci. The immune system interacts with individual pathogens in a distinct manner. Therefore, simultaneously evaluating different markers is essential to identifying genetic traits that may account for resistance to a variety of pathogens.

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