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Research Article

Determining the Point of B-Cell Differentiation in the Bursa of Broiler Chicken Embryos with Phenotype Analysis

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Abstract

Background and Objective: A major B-cell differentiation event has been reported to occur in the bursa between Embryonic Days (ED) 15-19 and is detected by the upregulation in expression of a Lewis×(Lex) carbohydrate on developing B-cells using monoclonal antibodies. The goal of this project was to determine the onset of Lex expression in bursal B-cells in the Ross 708 broiler line. **Materials and Methods:** Frozen sections of pooled bursas were prepared from ED15 and ED19 and were evaluated by immunohistochemistry (IHC) using the anti-Lex and anti-chB6 antibodies. **Results:** In the embryonic bursa, very low levels of Lex expression was detected by ED15. However, the intensity of Lex expression increased markedly at ED19 and co-localized with a B-cell-specific marker in the developing bursal follicles. **Conclusion:** We conclude that repertoire development initiates between ED15 and ED19 in the embryonic bursa of the Ross 708 line, as has been shown in White Leghorn embryos in studies by others. This information is important for planning future B-cell development studies in this broiler line.

Key words: Bursa of Fabricius, embryonic development, Lewis X, monoclonal antibody, Ross 708 broiler embryos

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INTRODUCTION

The bursa of Fabricius is a gut-associated primary lymphoid tissue responsible for B-cell lymphopoiesis in the chicken[1]. The embryonic bursal mesenchyme expresses the chemokine CXCL12 to recruit B-cell progenitors between ED8-14[2]. The B-cell progenitors, termed prebursal stem cells have completed immunoglobulin (Ig) gene rearrangements and are identified by surface expression of the B-cell receptor (IgM) and a glycoprotein decorated with a sialyl Lewis x (sLex) carbohydrate[3]. Between ED 9-10, the precursors to the Bursal Secretory Dendritic Cells (BSDC) enter the bursal mesenchyme and migrate into the stratified luminal epithelium[4]. Upon entering the epithelium, the BSDC exhibit a dendritic morphology and induce the proliferation and differentiation of some of the cuboidal epithelial cells into a network of Reticular Epithelial Cells (REC)[5]. The development of the network of REC causes a separation of the epithelial layers forming a follicular structure. Between ED12 and ED14, the expression of CXCL12 is reduced in the mesenchyme but appears in the BSDC and may recruit the prebursal stem cells into the follicles[5]. The prebursal stem cells within the follicles initiate the B-cell development program by expression of the cytokine B-cell Activating Factor (BAFF) and its receptor[6]. The autocrine response to BAFF induces proliferation and leads to a population of $sLex^+/IgM^+$ cells, termed bursal B-cells, which will undergo Ig-gene diversification[7]. During the ED15-18 period, bursal B-cells undergo a change in surface phenotype from sLex expression to the expression of Lewis X (Lex)[8]. The change in surface carbohydrate expression identifies a major differentiation event at which repertoire development by immunoglobulin (Ig)-gene conversion initiates[7,9]. By hatching (ED21), B-cells are exported to secondary lymphoid tissues in the periphery[10].

The long-term goal of our lab is to identify the genes controlling the differentiation of $sLex^+$ bursal B-cells to the Lex^+ B-cell stage, with the onset of Ig-gene conversion. These basic B-cell differentiation studies will be important for future work to identify the bursal microenvironmental signals responsible for this development[11]. For these B-cell developmental studies we will use the Ross 708 broiler is widely used in the United States and is important to national food security. However, phenotypic analysis of bursal B-cell development has not been conducted with the Ross 708 broiler line. Therefore, the goal of this project was to confirm the timeline of Lex in this broiler line. This information will be critical to collect Ross 708 broiler bursas at the correct day of embryonic development for isolation of Lex^+ bursal B-cells for gene expression analysis.

MATERIALS AND METHODS

Experimental animals: Fertile eggs from a Ross yield plus×Ross 708 parent stock were incubated in a NOM 2000 Natureform incubator at the USDA-ARS Poultry Research Unit, Mississippi State location. Incubation conditions were 99.5°F and 55% relative humidity. This project was reviewed by the Mississippi State University Animal Care and Use Committee.

Immunohisto chemistry: Chicken embryos (15 embryos per timepoint) at ED15 and ED19 were euthanized by decapitation and bursa tissues were embedded in optimum cutting temperature medium contained in cryomolds (25×20×5 mm) and stored at -80°C. The bursas were grouped for the analysis. Based on bursal size, for ED15 each cryomold contained 3 bursas each and 2 bursas each for ED19. Ten-micron thick frozen sections (10 µM) were placed on Superfrost™ precleaned microscope slides (Fisher Scientific, Norcross GA, USA, No. 12-550-15), air dried and stored at -80°C. For immunohistochemistry, sections were equilibrated to room temperature, fixed in acetone/methanol (1:1) for 10 min, washed in PBS and then incubated in blocking buffer (5% nonfat dry milk in PBS with 0.05% Tween-20) for 1 hr at room temperature. The antibodies were diluted in blocking buffer, added to the sections and incubated overnight at 4°C in a humidified chamber. After incubation, sections were washed in PBS with 0.05% Tween-20, mounted with VECTASHIELD HardSet mounting (Vector Laboratories, Newark CA, USA, No. H-1500) and a Leica DMi8 confocal microscope (Leica Microsystems, Deerfield IL, USA) was used for qualitative evaluation of fluorescent staining. Sections were stained in duplicate with the primary antibodies and the appropriate isotype control antibodies.

Antibodies: The mouse anti-chB6 monoclonal antibody conjugated with Alexa Fluor 488 (IgG_1 isotype) [Southern Biotech, Birmingham AL, USA, No. 8395-30] was used at a concentration of 0.25 mg/mL. A mouse myeloma IgG_1 protein conjugated with Alexa Fluor 488 (0.25 µg/mL) [Southern Biotech, Birmingham AL, USA, No. 0102-30] was used as the isotype control antibody. The mouse anti-Lex monoclonal antibody conjugated with Alexa Fluor 647 (IgM isotype) [BD Biosciences, San Jose CA, USA, No. 560120] was used at a concentration of 2 µg/mL. A mouse IgM unrelated monoclonal antibody conjugated with Alexa Fluor 647 (2 µg/mL) [BD Biosciences, San Jose CA, USA, No. 560806] was used as the isotype control antibody.

RESULTS AND DISCUSSION

The goal of this pilot study was to identify the developmental window at which repertoire development initiates in the embryonic bursa of the Ross 708 broiler, as previous studies by others to determine the timepoint of this major differentiation event were conducted with White Leghorn embryos[3,8]. Therefore, based on those previous reports the embryonic bursa was sampled at ED15 and ED19 for phenotype analysis.

Figure 1 and 2 are representative photomicrographs of the phenotype analysis with immunohistochemistry. The chB6 antigen represents a pan-B-cell marker in chickens[12] and its detection by a monoclonal antibody was used to identify bursal B-cells in the tissue sections. The Lex antigen is expressed by bursal B-cells undergoing Ig-gene conversion[3]. The chB6 reactivity localized to the nascent follicle indicating that bursal B-cells have expanded by ED15 (Fig. 1a). Bursal B-cells at ED15 expressed low levels of the Lex protein (Fig. 1b and c). By ED19, the increased cellularity resulted in

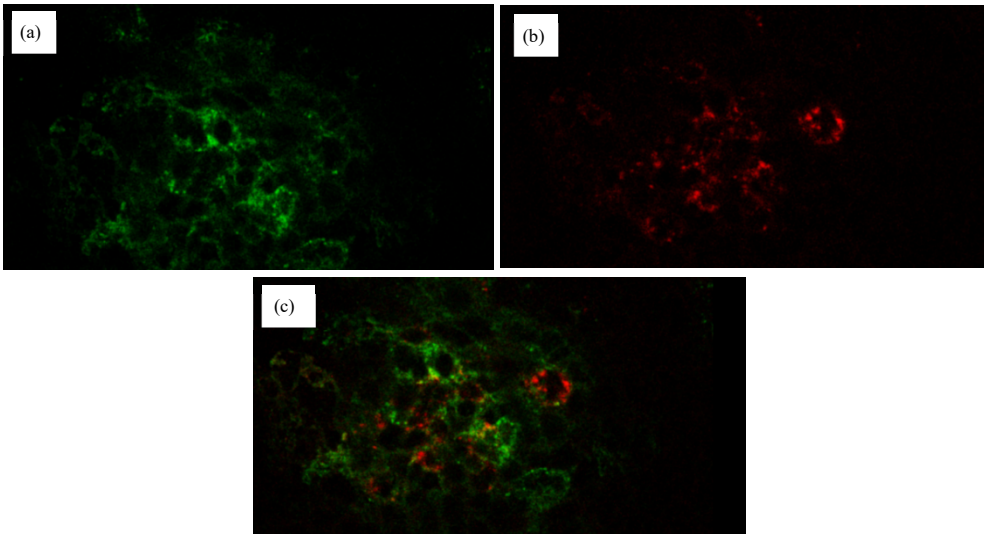

Fig. 1(a-c): Immunohistochemistry analysis of the ED15 embryonic bursa (a) Bursal B-cells detected with the anti-chB6 monoclonal antibody (100×magnification), (b) Weak expression of Lex in the nascent bursal follicle (100×magnification) and (c) Few bursal B-cells co-express Lex and chB6 (100×magnification)

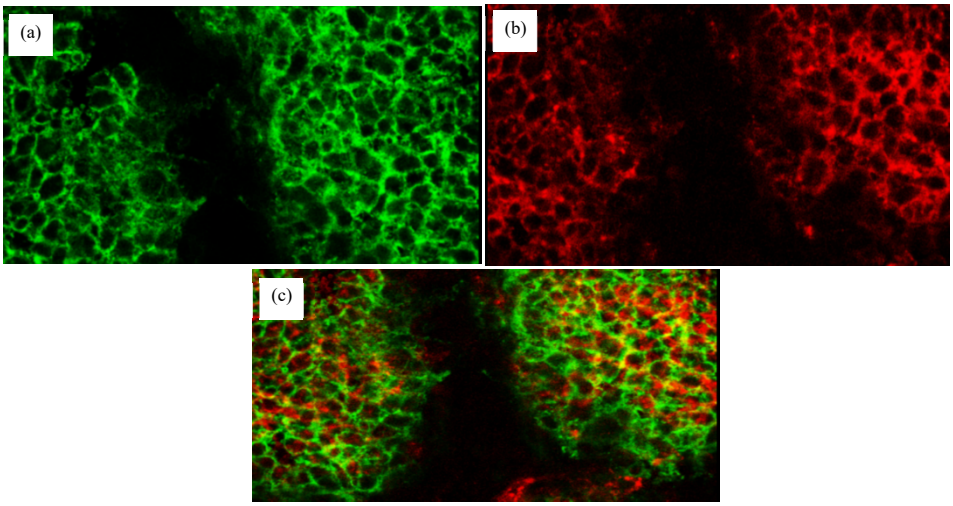

Fig. 2(a-c): Immunohistochemistry analysis of the ED19 embryonic bursa, (a) Bursal B-cells identified with the anti-chB6 monoclonal antibody (100×magnification), (b) Expression of the Lex protein in the bursal follicles. (100×magnification) and (c). Co-expression of chB6 and Lex on bursal B-cells (100×magnification)

strong fluorescence intensity of the staining for the chB6 and Lex proteins (Fig. 2a and 2b). At this developmental timepoint, bursal B-cells express high levels of both proteins (Fig. 2c).

Phenotype analysis with monoclonal antibody reagents has been used to characterize developing B-cell stages in both mammals and chickens[13,14]. In chicken B-cell development, the surface expression of the Lex protein identifies bursal B-cells that have differentiated and are undergoing Ig-gene conversion[10]. Indeed, antibody reagents recognizing the Lex epitope have been used in a number of studies examining bursal B-cell developmental mechanisms[15] or monitoring the recovery of the bursa after vaccination with the infectious bursal disease vaccine[16,17]. This pilot study confirms that ED19 is the optimal day of embryonic development for collecting Lex^+ bursal B-cells and also suggests that the Ross 708 and White Leghorn lines share B-cell development mechanisms, even though the lines were developed under different forms of genetic selection. In our future studies we will collect Ross 708 bursas at ED19 to ensure the isolation of Lex^+ bursal B-cells for our gene expression studies.

CONCLUSION

To verify that the major B-cell developmental timepoints in the Ross 708 line is equivalent to the White Leghorn lines is critical for planning future B-cell development studies with this broiler line.

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