IN SITU B-LYMPHOCYTE APOPTOSIS AND PROLIFERATION DURING ONTOGENY OF THE NEONATAL CHICKEN BURSA OF FABRICIUS

A Senior Honors Thesis

By

STACY ERIN GRANBERG

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2000

Group: Cell Biology II

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ABSTRACT

In Situ B-Lymphocyte Apoptosis and Proliferation During Ontogeny of the Neonatal Chicken Bursa of Fabricius. (April 2000)

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Apoptosis, or programmed cell death, is believed to be the mechanism for depletion of lymphocytes recognizing self antigens following clonal expansion in the bursa of Fabricius. Further, some evidence suggesting that recognition of foreign antigens, presented by the bursal follicular epithelial cells, may promote retention and expansion of selected clones by protection from apoptosis. While bursal apoptosis has previously been shown to increase following *in vivo* exposure to glucocorticoids, the microanatomical site of induced or normal apoptosis has not been unequivocally established. Presently, we adapted the existing TUNEL (Terminal Deoxynucleotidal Transferase Mediated dUTP Nick End Labeling) assay for use with neonatal bursae. Similar to previous reports, TUNEL revealed that normal apoptosis is preferentially, but not completely, ongoing in bursal follicular cortical cells. Administration of a single dose of either a synthetic glucocorticoid (dexamethasone) or androgen (19-nortestosterone) failed to cause follicular lymphocyte depletion and increased

apoptosis per unit of area at the time points evaluated post-administration (6 or 24 hrs). However, administration of either steroid increased the interfollicular epithelial thickness, a change usually associated with edema, within 6 hr following treatment. Additionally, administration of the androgen 19-Nortestosterone significantly decreased the number of proliferating cells as detected using mouse anti-PCNA as a primary immunohistochemical antibody. These findings extend existing data suggesting that normal bursal apoptosis occurs to a greater degree in the bursal follicular cortex although steroid-mediated lymphocyte apoptosis was not observed in these experiments. Unexpectedly, a very low incidence (0.26%) of follicles predominantly containing apoptotic lymphocytes was observed in normal bursal tissues. While the reason for the presence of bursal follicles consisting of predominantly apoptotic cells is not known, possible reasons are intriguing. Future elucidation of the underlying physiological or pathophysiological processes responsible for this phenomenon may lead to important clues for more complete understanding of humoral immune system ontogeny.

DEDICATION

I would like to dedicate this thesis to Mom and Dad.

I cannot thank you enough for believing in me and supporting me in everything that I do. I love you both.

ACKNOWLEDGEMENTS

To begin, I would like to thank my advisor, Billy Hargis. Thank you for giving me the opportunity and the environment to learn and grow. I cannot express how much I appreciate the time, the many lessons, and the extra effort you have given to help me complete this project.

I also would like to thank Dr. Luc Berghman for teaching me immunocytochemistry, and for letting me be welcome in his lab. Additionally, I truly appreciate Dr. David Caldwell for taking the time to answer my many questions and supporting this project. I also would like to thank Dr. Ian Tizard for sharing his expertise in immunology with me.

Denise Caldwell, thank you for helping me all along the way, and for allowing me to stain slides when I should have been washing dishes.

Randy Moore, have been a wonderful friend, and an invaluable resource. I appreciate you tremendously.

Heather Seelye, you have a gift as an encourager. Thank you for blessing my life with cheerfulness.

Debbie Stanush, thanks for being around to talk about anything, and for always having great advice. You will be a great veterinarian.

To "the guys": JackMac, Ruben, Koyle, Justin, and Blair - you all make work fun. Thank you for your friendship, and I hope none of you ever start taking life too seriously.

Finally, I especially want to thank my fiancé, Jack. Thank you for helping me in a million ways, from helping me with slides when I work late, to making sure I ate during the day. I could not have finished this without your love and support.

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CHAPTER I

INTRODUCTION

THE CHICKEN BURSA OF FABRICIUS

The existence of the bursa of Fabricius (BF) in chickens provides a unique advantage for study of the humoral immune system. Although it was once believed to be the site of semen deposition, as described by Hieronymus Fabricius in the late 16th century (Adelman, 1967), in the 1950's the true function of the BF was discovered accidentally by two graduate students. When Timothy Chang attempted to produce antiserum in bursectomized chickens for use in a microbiology laboratory class, he found that he could not. Bruce Glick, who was studying the growth of the bursectomized chickens, with Chang, hypothesized that the BF was necessary for antibody production. Further experimentation confirmed the hypothesis, and the two submitted their results in a manuscript to *Science*. However, *Science* rejected the manuscript titled "The Role of the Bursa of Fabricius in Antibody Production" claiming that more study of the mechanism was necessary (Glick 1991,1994). The paper was accepted for publication in *Poultry Science* (Glick *et al.,* 1956) and provides the first description of the role of the

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This thesis follows the style and format of *Poultry Science.*

BF in humoral immune system development. These initial studies (Glick *et al.,* 1956, Cooper *et al.,* 1965, 1966) provided the first understanding that two distinct but interrelated arms of the immune system, cell mediated immunity and humoral immunity exist in vertebrate species.

EARLY EMBRYOLOGICAL DEVELOPMENT

The development of the BF begins in 4 day embryos on the lower large intestine as an outgrowth of the urodeal membrane (Ratcliffe, 1989). Though some stem cells colonize the bursa at this time, the majority of pre-bursal stem cells (PBSC) colonize the bursa from day 7e to day 14e (Houssaint *et al.,* 1976). B-Lymphocyte stem cells are committed to the B-cell lineage before they enter the bursa (Houssaint *et al.,* 1989, 1991). Evidence that PBSC are of the B-lymphocyte lineage includes the presence of Bu-1 cell surface antigen and rearranged light (V-J) and heavy (V-D-J) chain immunoglobulin gene segments. Recently, a new criteria was uncovered. The presence of sialyl Lewis x, a cell surface carbohydrate molecule was discovered during embryonic stages on PBSC prior to "homing" to the bursa. This molecule is a sialylated fucosylated lactosamine, and is capable of being bound by members of the selectin family of adhesion molecules (Masteller *et al.,* 1995a). Between day 15e and day 18e, the sialyl Lewis x molecule changes to a Lewis x molecule. This event is correlated with cells which have undergone gene conversion events, and which are no longer able to seed the BF. In other words, once a cell expresses Lewis x, it has changed from a PBSC to a

post-bursal stem cell (Masteller *et al.*,1995b). In the BF 8,000 to 12,000 follicles exist (Olah and Glick, 1978), but only 2-4 PBSC are responsible for colonizing each follicle during embryonic life (LeDourin *et al.*, 1984). Stem cells that express functional IgG then proliferate logarithmically day 14e until hatch (Masteller and Thompson, 1994). This results in a doubling of the number of cells every 10 hours. This rate of proliferation slows to a doubling time of once per week after hatch (Ratcliffe,1989).

STRUCTURE OF THE BURSA OF FABRICIUS

The BF is a dorsal diverticulum of the proctodeum, and is a characteristic only of the phylogenetic Class *Aves* (Glick, 1995). It is a blind pouch lined with 11 to 14 primary plicae (folds) and 6 to 7 secondary plicae. Each follicle within the BF actually provides a distinct microenvironment. Follicles are connected to the bursal lumen by a tuft of cells comprising the follicle associated epithelium (FAE). These cells are capable of pinocytosis and draw material from the lumen of the bursa into the follicle (Olah and Glick, 1992; Davenport and Allen, 1995). Each follicle also contains a cortex and a medulla, which are formed about day 20e (Ratcliffe, 1996). Both follicular compartments contain lymphocytes, lymphoblasts, macrophages, and plasma cells. However, the central medulla also contains reticular and secretory dendritic cells (Glick, 1988). The interfollicular epithelium (IFE) separates the cortex and medulla in each follicle, and is an extension of the basal lamina which divides the FAE from the medulla (Bockman and Cooper, 1973). The cortex and medulla also contain distinct cell

populations, though some cells may migrate between the compartments. Cortical lymphocytes make up a population of proliferating cells, while the medullary lymphocytes are proliferating less, but are more mature (Grossi *et al.*, 1974). The BF regresses at puberty, after seeding the peripheral lymphoid organs with B-lymphocytes (Ratcliffe, 1989). Between four (Weill and Reynaud, 1987) and six weeks of age (Toivanen and Toivanen, 1973), birds generally have developed a competent humoral immune system. After development of a competent humoral immune system, an age associated involution of the bursa occurs (Lydyard *et al.*, 1976). The age at which this involution begins is variable genetically, and ranges from 3 weeks (Bellamy and Mohamed, 1982) to 12 weeks (Naukkarinen and Sorvari, 1984).

ANTIGEN DIVERSITY

Gene conversion events in the bursa are responsible for providing antigen diversity, which allow B-lymphocytes to recognize specific epitopes on specific antigens. Gene conversion begins between 15e and 18e, and can continue for up to 12 to 16 weeks of age (McCormack *et al.*, 1989). Although gene rearrangement must occur in PBSC before colonizing the bursa, it does not provide enough antigen diversity because chickens only possess one V gene segment, one J segment, and 10 to 16 different D gene segments (Weill and Reynaud, 1987; Masteller and Thompson, 1994). Therefore, a process of interchromosomal gene conversion in both the light and heavy chains is necessary. The necessary diversity is provided by the random multiple insertions of V

pseudogenes. Approximately 25 V pseudogenes exist for both the light and heavy chains of the IgG molecule (Weill and Reynaud, 1987). They are downstream from where the V-(D)-J gene segments initially rearrange, but during gene conversion events, segments of the pseudogenes are inserted upstream from the rearranged V-(D)-J segments (Masteller and Thompson, 1994). Gene conversion is responsible for the determination of antigenic specificity of individual lymphocytes, and each lymphocyte can undergo 7 to 10 gene conversion events while in the BF (Weill and Reynaud, 1987). Bursal secretory dendritic cells (BSDC) are dark mesenchymal cells that are hypothesized to play a part in the B-cell maturation process (Glick and Olah, 1993). BSDC are first found in the bursa day 9-10e (Olah *et al.*, 1986), have a characteristic secretory morphology (Olah *et al.*, 1979; Olah and Glick, 1978, 1987), and expresses cell surface IgG (Olah *et al.*, 1991). Currently, the product secreted by BSDC and the source of the cell surface IgG have not been identified. A hypothetical mechanism proposed by Glick and Olah (1993) described the possible roles BSDC play in clonal expansion and gene conversion. In this proposed mechanism, pre B-cells expressing IgM would interact with IgG present on the surface of BSDC, stimulating them to undergo clonal expansion, or proliferation. The interaction would also release a cytokine that induces gene conversion events. Although this is a strictly hypothetical model, it could explain how B-cells diversify and differentiate.

EFFECT OF ENVIRONMENT ON NEONATAL BURSA OF FABRICIUS FUNCTION

The bursa functions as a peripheral lymphoid organ by drawing antigens from the environment into the lumen (Sorvari and Sorvari, 1977; Ekino *et al.*,1985; Naukkarinen and Hippelainen, 1989; Ekino *et al.*, 1995). Cloacal drinking is the reflex mechanism by which environmental antigens are physically drawn into the lumen (Sorvari *et al.*, 1975). Antigens in the bursal lumen are phagocytized by the follicle associated epithelium and presented to medullary B-cells (Jeurissen *et al.*, 1994). Vaccination by cloacal drinking has been demonstrated, and this mechanism is the proposed means of obtaining immunologic diversity against environmental antigens (Sorvari and Sorvari, 1977). Repeated experiments have shown that bursectomy, either by surgical or chemical means, severely injures the humoral immune system (Warner *et al.*,1969; Glick and Olah, 1984; Ratcliffe, 1989; Cooper *et al.*, 1969). Additionally, ligation of the bursal duct, reducing antigen stimulation, significantly slows the maturation of immune reactivity in bursal lymphocytes (Ekino *et al.*, 1985).

Although B-cells can mature and differentiate without the presence of environmental antigens in the bursa (Lydyard, *et al.*, 1976), environmental antigens do influence the lymphocytes developing in the BF (Boyd *et al.*, 1987). Two distinct populations of B-cells exist in the periphery. One group is very short lived (Paramithiotis and Ratcliffe, 1993) and may represent a naive population of B-cells (Ratcliffe and Jacobsen, 1994). After differentiation and maturation in the BF cells can

migrate from the cortex directly into lymph vessels located adjacent to the follicles. These cells never enter the follicular medulla, and are thought to comprise the short-lived group of cells in the periphery. The second population of B-cells are characterized by a longer half-life in the periphery, and are thought to be the result of exposure to cloacally derived and bursal captured antigens. These cells are thought to emigrate from the medulla (Paramithiotis and Ratcliffe, 1993). Perhaps these long lived cells are selected to protect against pathogens found in the environment of young chicks (Ratcliffe and Jacobsen, 1994).

As BF regresses at puberty, it must seed the peripheral lymphoid organs prior to involution. It is thought that antigen exposure to developing B-lymphocytes in the BF can influence the emigration of B-lymphocytes. Interestingly, only 1-5% of all bursal lymphocytes produced ever actually emigrate to the periphery (Ratcliffe, 1989). The difference between the rapid rate of cell division in the BF, and the low levels of emigration implies that most bursal cells die *in situ* (Jacobsen *et al.*, 1996). *In situ* cell death, also known as programmed cell death, is apoptosis (Cohan *et al.*, 1992). Apoptosis is unlike necrosis, which is "accidental" cell death. Necrosis involves injury to a cell, which usually results in cell lysine, and a subsequent inflammatory response (Schwartzman and Cidlowski, 1993). Eventually, affected cells are broken into numerous small vesicles which are phagocytized without lysis (Cohen et al., 1992). Clearly, apoptosis is generally considered to be cell death that in some way is beneficial to the organism. Some investigators consider that bursal apoptosis may reflect B-lymphocytes that are deselected because of gene conversion events that allow

self-recognition by expressed immunoglobulins through a yet unknown mechanism (Motyka and Reynolds, 1991; Nieman *et al.*, 1994; Paramithiotis *et al.*, 1995).

The first event in apoptosis is a rapid intracellular increase in Ca^{2+} ions, which activate certain endogenous nucleases (Cohen *et al.*, 1992; Schwartzman and Cidlowski, 1993). Endonucleases are enzymes responsible for cleaving DNA, one of the hallmarks of apoptosis. DNA in apoptotic cells is broken by endonucleases in specific regions between histones or nucleosomes. The DNA wound in a nucleosome is approximately 180-200 base pairs in length, and breaks in the DNA occur in regions that are multiples of 180 base pairs (Cohen *et al.*, 1992; Schwartzman and Cidlowski, 1993). In fact, this characteristic is a marker for apoptotic cells using gel electrophoresis (Schwartzman and Cidlowski, 1993). Cells undergoing apoptosis lose up to 30% volume, and the nucleus is degraded, although other organelles remain intact. The cell then undergoes zeiosis, which results in the microscopic morphological appearance of boiling, or blebbing of the cell membrane. Apoptotic bodies, or small membrane bound vesicles, then form from the cell, and are quickly engulfed by macrophages, or sometimes neighboring cells (Cohen *et al.*, 1992). There are some hypotheses suggesting mechanisms by which the macrophage recognizes the epithelial cells. Possibilities include: a receptor on the macrophage surface, an integrin on the macrophage surface, and/or recognition of an exposed phosphotidyl serine molecule on the surface of the apoptotic bodies (Schwartzman and Cidlowski, 1993).

Apoptosis potentially may serve several purposes, including aiding in organ formation in embryos, a response to events that may damage DNA, such as radiation, or

eliminating neutrophils in inflamed areas. Apoptosis in lymphocyte populations is believed to result in deletion of self recognizing cells to prevent auto immune responses. Apoptotic cells in the BF have differing characteristics depending on whether they reside in the cortex or medulla. Apoptotic cells in the cortex are derived from precursor cells that are dividing quickly, but medullary apoptotic cells die 12-24 hours after the last cell division (Jacobsen *et al.*,1996). Evidence has shown that possibly loss of IgG expression may be a criterion for initiation of apoptosis of B-lymphocytes within the BF cortex. An alternative hypothesis is that apoptotic cells in the medulla were not selected on the basis of antigen selection, and as a result were "deselected" by apoptosis (Paramithiotis *et al.*,1995). The present manuscript will examine several new aspects related to selection and recruitment of neonatal chick B lymphocytes.

CHAPTER II

IN SITU DETECTION AND QUANTIFICATION OF BURSA OF FABRICIUS CELLULAR PROLIFERATION OR APOPTOSIS IN NORMAL OR STEROID-TREATED NEONATAL CHICKS

INTRODUCTION

The bursa of Fabricius in chickens is the sole site of B-lymphocyte maturation and differentiation (Glick, 1988). In a unique process by which antigens are presented to the developing immune system, follicle associated epithelial cells lining the bursal lumen phagocytize antigens from both the large intestine and the environment through a mechanism known as cloacal drinking (Ekino *et al.*, 1985; Glick, 1977). When material from the environment comes in contact with the chicken vent, this material is immediately internalized into the cloaca and moved into the lumen of the bursa of Fabricius by retrograde transport. Once in the lumen of the bursa of Fabricius, particulate material is engulfed by specialized phagocytic cells lining the bursal lumen, known as the follicle associated epithelium (Glick 1977). In this manner, antigens are presented to developing B-lymphocytes in the follicular medulla, while these cells are undergoing the process of gene conversion, a process allowing for antigenic diversity which allows each mature lymphocyte to recognize only one specific epitope (Ratcliffe, 1989). It is known that mature B-lymphocytes from both the medulla and cortex can

emigrate to the blood and peripheral lymphoid organs, where they await activation by contact with the specific antigen which they are individually capable of recognizing (Paramithiotis and Ratcliffe, 1994). Thus, this emigration to, and colonization of, peripheral lymphoid organs and tissues provides a means for continued ability to respond to new antigens and maintainance of humoral immunity once the bursa of Fabricius regresses at puberty (Paramithiotis and Ratcliffe, 1993). Despite the fact the future B-lymphocyte repertoire of the bird is dependent upon these emigrated cells which escape the bursal microenvironment, only 5% of all B-lymphocytes ever emigrate to the periphery, the rest of the cells die *in situ* (Glick, 1988; Paramithiotis and Ratcliffe, 1994). The process by which B-lymphocytes die *in situ* without inflammation is called apoptosis (Jacobsen *et al.*, 1996; Paramithiotis *et al.,* 1995; Motyka and Reynolds, 1991). Apoptosis, or programmed cell death, is much different than necrosis, the latter causing cell lysis. In apoptosis, the process begins when linker DNA between nucleosomes is cleaved by a lysosomal deoxyribonuclease. Cellular chromatin then condenses, and the nucleus fragments. As the endoplasmic reticulum expands and joins the cytoplasmic membrane, small membrane bound vessicles known as apoptotic bodies begin to form. Apoptotic bodies are subsequently phagocytized by macrophages, or surrounding specialized epithelial cells (Schwartzman and Cidlowski, 1993; Williams, 1991; Cohen and Duke, 1992).

Previous research on apoptosis of B-lymphocytes in the bursa of Fabricius utilized an agarose gel electrophoresis assay or "ladder assay" for detection of apoptosis. As DNA fragmentation is one of the earliest signs of apoptosis, and as fragmentation

occurs at regular intervals in the genome, DNA from apoptotic cells appears as multiple bands at regular 180-200 bp fragment sizes on gel electrophoresis. This "ladder pattern" of fragmentation develops because the cleavage of linker DNA occurs between nucleosomes, which are each 180-200 base pairs long. The assay is sensitive as long as both strands of DNA are severed, and the population of cells is primarily apoptotic (Cohen and Duke, 1992).

A more recently developed assay detects *in situ* apoptosis within individual cells . This Terminal Deoxynucleotidal Transferase Mediated dUTP Nick End Labeling (TUNEL) method of detecting apoptosis uses immunohistochemical methods to detect the occurrence of apoptosis in specific microanatomical areas of tissue. The TUNEL assay is more sensitive than other detection methods because it uses labeled nucleotides which bind to free 3' hydroxyl groups which are only present in nicked or broken strands of DNA. Using antibodies and substrates specific for the nucleotide label allows the apoptotic cells to be identified using light or fluorescent microscopy (Sanders and Wride, 1996). Additionally, in order to detect proliferating cells, we used an immunohistochemical assay which detects Proliferating Cell Nuclear Antigen (PCNA). The primary antibody used in this assay labels the antigen, which is present in the nuclei of mitotic cells. (Krude, 1999: Liu *et al.*, 1998).

Studies by Compton (et al., 1990) indicated that systemic glucocorticoid treatment of 4 weeks chickens induced a marked regression in bursal tissue weight, and also increased DNA degradation (9% within 6 hours of treatment). He also determined that cell viability was unchanged at 6 hours, but began to decline by 24 hours post

treatment. Apoptosis in these studies was observed and quantified using the ladder assay (Compton *et al.*, 1990).

Presently, we have adapted the TUNEL assay for use with neonatal bursa of Fabricius tissue in order to evaluate the effect of glucocorticoid or androgen treatment *in situ*.

MATERIALS AND METHODS

Experimental Design

Experiment 1: One-day-old Leghorn chicks were obtained from a commercial hatchery and were maintained in standard plastic chick boxes on paper liners under brooder lamps for 24 hours. Chicks were not provided feed or water during this holding period. 90 chicks were randomly assigned to three groups. Each treatment was administered subcutaneously in a 0.25 ml injection volume. Group 1 was administered Lactated Ringer's Injection[1], group 2 received 3.5 µg Dexamethasone[2], and group 3 received 35µg Dexamethasone[2]. At 6 and 24 hours post treatment, 10 birds were euthanized from each group by cervical dislocation.

Experiment 2: One-day-old Leghorn chicks were obtained from a commercial hatchery and were maintained in plastic chick trays under brooder lamps for 24 hours as described above. Ninety chicks were randomly assigned to three equal groups.

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[1] USP Abbott Laboratories, Chicago, IL 60064
[2] Phoenix Scientific, Inc., St. Joseph, MO, USA

Ninety chicks were randomly assigned to three equal groups. Each treatment was administered subcutaneously in a 0.25 ml injection volume. Group 1 was administered sterile saline[1], group 2 received 0.035 µg Dexamethasone[2], and group 3 received 4.16 mg 19-Nortestosterone[3] (Catalog no. N 7252). At 6 and 24 hours post treatment, 10 birds were euthanized by cervical dislocation from each group.

Histology

Immediately following euthanasia, bursae were dissected from each chick, fixed in 4% buffered paraformaldehyde, and routinely processed in paraffin. Thin sections (5µm) of each tissue were sliced from each block, mounted on glass, and assayed using either the Terminal Deoxynucleotidal transferase (TdT) mediated dUTP nick end labeling (TUNEL) assay for detection of apoptosis, or the Proliferating Cell Nuclear Antigen (PCNA) assay for detection of proliferating cells, as described below.

TUNEL Assay

The process by which slides were sectioned and prepared for immuno-staining has been previously described (Harlow and Lane, 1988). Briefly, tissue sections were rehydrated in a series of xylene and ethanol solutions, then incubated at 37°C for one hour with Proteinase K[3] (Catalog no. P 2308) (10-20mg Proteinase K in 10 mM Tris HCl, pH 7.4-8.2) in a humidified chamber. Slides were then rinsed in 1M Phosphate Buffered Saline (PBS). TUNEL Enzyme (Catalog no. 1767 305) and Label Solution

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[1] Lactated Ringer's Injection, USP Abbott Laboratories, Chicago, IL 60064
[2] Phoenix Scientific, Inc., St. Joseph, MO, USA
[3] Sigma Chemical Company, St. Louis, MO 63178
[4] Boehringer Mannheim Corporation, Indianapolis, IN 46250-0414

(Catalog no. 1767 291)[4] were mixed and applied to the tissues, which were incubated again in the humidified chamber for one hour at 37°C. Slides were rinsed thoroughly in PBS, then in Tris buffered saline (TBS). Monoclonal mouse Anti-Flourescein Isothiocyanate antibody[3] (Catalog no. F 8771) (FITC), diluted 1:400 in TBS, was applied and incubated on the bursal tissue sections at room temperature (RT) for 2-3 hours, then rinsed with TBS. Tissues were then incubated with streptavidan conjugated peroxidase[3] (Catalog no. S 5512) diluted to 1:600 in TBS for 30 minutes at RT, and rinsed in TBS. A hydrogen peroxide and diaminobenzidine[3] (Catalog no. P8001) (DAB) substrate solution (80 µl H_2O_2 and 25 mg DAB in 200 ml of 50 mM Tris buffer, pH 7.4) was applied for 20 minutes to stain the nuclei of apoptotic cells. After developing with the DAB substrate, tissues were dehydrated in a series of 3 ethanol baths and 2 xylene baths for 5 minutes each (Harlow and Lane, 1988).

PCNA Assay

The process by which slides were sectioned and prepared for immuno-staining has been previously described (Harlow and Lane, 1988). Briefly, tissue sections were rehydrated in a series of xylene and ethanol baths, and then incubated for 30 minutes at room temperature with 10% H_2O_2 diluted in methanol to quench endogenous peroxidase activity. After rinsing thoroughly with TBS, then tissues were incubated for 30 minutes with normal goat serum in order to block non-specific antigen binding sites. The tissues were then incubated overnight with the primary antibody Mouse Anti-Proliferating

[3] Sigma Chemical Company, St. Louis, MO 63178

Cell Nuclear Antigen (Catalog no. P8825) (PCNA)[3] at a 1:1000 dilution in TBS. Slides were rinsed in TBS, and the secondary antibody biotinylated goat Anti-mouse IgG[5] (Catalog no. 115-065-146) at a 1:600 dilution was incubated on the tissue for 2 to 3 hours. Tissues were rinsed in TBS then incubated with streptavidan conjugated peroxidase[3] (Catalog no. S 5512) diluted to 1:600 in TBS for 30 minutes at RT, and rinsed in TBS. A hydrogen peroxide and diaminobenzidine[3] (Catalog no. P8001) (DAB) substrate solution (80 µl H_2O_2 and 25 mg DAB in 200 ml of 50 mM Tris buffer, pH 7.4) was applied for 20 minutes to stain the nuclei of apoptotic cells. After developing with the DAB substrate, tissues were dehydrated in a series of 3 ethanol baths and 2 xylene baths for 5 minute each (Harlow and Lane, 1988).

Data Collection

Photomicrographs of two randomly selected follicles per sample were obtained and subjected to morphometric analysis[6] to determine follicular area and interfollicular epithelial thickness. Occurrence of apoptosis was quantified by counting the number of apoptotic cells and normal cells in each follicle. Numbers of apoptotic or normal cells per unit area were determined by dividing the counted number of cells by the determined follicular area. Means of each tissue were used as independent samples for statistical analysis.

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[3] Sigma Chemical Company, St. Louis, MO 63178
[5] Jackson Immuno Research, West Grove, PA 19390
[6] Jandel Scientific (1996)

Statistical Analysis

All data were analyzed within time points using the General Linear Model procedure for ANOVA. Statistically different means were further separated using procedure for ANOVA. Statistically different means were further separated using Duncan's multiple range test (Luginbuke and Schlotzhaver, 1987).

RESULTS AND DISCUSSION

The present report is the first to enumerate numbers of normal and apoptotic follicles within the neonatal chick bursa of Fabricius. In experiment 1, we observed that the control bursal follicles contained an average of 17.6 apoptotic cells per 100 um^2, while either dose of dexamethasone caused a numerical, but non-significant ($p<0.05$), increase in the number of apoptotic cells observed per unit of bursal follicular area (Table 1). In the second experiment, we again evaluated the effects of dexamethasone or the androgen 19-nortestosterone. In experiment 2 (Table 2) an average of 29 apoptotic cells per 100 um^2 are found in normal bursal follicles (control, 6 hrs post-treatment). This apoptotic cell frequency was found to comprise 12% of the total follicular cell population. Similarly, we detected an average of 21 apoptotic cells per 100 um^2 of follicular area in control bursae at 24 hrs post-treatment (Table 2). Interestingly, we also detected a significant decrease in the number of proliferating lymphocytes in bursa tissues treated with 19-Nortestosterone compared to control tissue (Table 3). In fact, all three treatment groups at least doubled the number of proliferating cells from 6 to 24

Table 1. Enumeration of apoptotic cells per unit area in control or Dexamethasone treated day old chicks 6 hours post-administration.

Treatments[1]	Apoptotic Cells per 100 μm^2
Control[2,5]	17.58±0.90[7]
0.1 mg/kg Dexamethasone[3,6]	19.99±2.06
1.0 mg/kg Dexamethasone[4,6]	24.76 ±2.71

[1] All treatments were administered subcutaneously in a 0.25 mL injection volume.
[2] Sterile saline.
[3] 0.035 mg per chick diluted in sterile saline.
[4] 0.0035mg per chick diluted in sterile saline.
[5] n=8
[6] n=6
[7] Means in this column did not test to be significant.

Table 2. Enumeration of apoptotic and normal cells per unit area and interfollicular thickness in control, Dexamethasone, or 19-Nortestosterone treated day old chicks.

Treatments[1]	Hours Post Treatment	Apoptotic Cells per 100 μm²	Normal Cells per 100 μm²	Interfollicular Epithelial Thickness (μm)
Control[2,5]	6	28±3.33	235±27.85	8.06±0.69 [b]
Dexamethasone[3,5]	6	25±3.15	184±16.57	8.68±0.79 [b]
19-Nortestosterone[4,6]	6	32±7.64	275±38.99	12.68±1.92 [a]
Control[2,5]	24	21 ±1.43	188±12.32	7.54±0.76 [x]
Dexamethasone[3,5]	24	21 ±3.42	166±18.31	10.77±1.51 [xy]
19-Nortestosterone[4,5]	24	27 ±6.07	164±12.03	12.64±1.26 [y]

[A-b] Means in columns within time periods with no common superscript differ significantly.
[X-y] Means in columns within time periods with no common superscript differ significantly.
[1] All treatments were administered subcutaneously in a 0.25 mL injection volume.
[2] Sterile saline.
[3] 35 μg per chick diluted in sterile saline.
[4] 4.16 mg 19-Nortestosterone suspended in 20% absolute ethanol and 80% polyethylene glycol.
[5] n=6
[6] n=5

Table 3. Enumeration of proliferating cells per unit area in control, Dexamethasone, or 19-Nortestosterone treated day old chicks.

Treatments[1]	Hours Post Treatment	Proliferating Cells per 100 μm²
Control[2,5]	6	75.75±3.83[a]
Dexamethasone[3,5]	6	28.28 ±1.42[ab]
19-Nortestosterone[4,6]	6	24.48 ±4.28[b]
Control[2,5]	24	157.07 ±12.64[a]
Dexamethasone[3,5]	24	126.80 ±7.20[ab]
19-Nortestosterone[4,5]	24	76.04 ±6.35[b]

[A-b] Means in columns within time periods with no common superscript differ significantly.
[X-y] Means in columns within time periods with no common superscript differ significantly.
[1] All treatments were administered subcutaneously in a 0.25 mL injection volume.
[2] Sterile saline.
[3] 35 μg per chick diluted in sterile saline.
[4] 4.16 mg 19-Nortestosterone suspended in 20% absolute ethanol and 80% polyethylene glycol.
[5] n=6
[6] n=5

hours, and the androgen treatment group remained significantly lower at all both time intervals.

It may be somewhat surprising to note that even at this early age more than 10% of developing lymphocytes are apparently destined for apoptosis. Although the mechanisms are not completely known, Paramithiotis and Ratcliffe (1993) have suggested that much of the early apoptosis within the bursa of Fabricius is due to recognition of self antigen. In this hypothesis, those cells producing antibody against self antigens are directed, through an incompletely understood mechanism, to undergo apoptosis, presumably to prevent the eventual induction of auto immune disease. In a previous investigation, Compton et al. (1990) found that systemic administration of 0.02 mg, 0.06 mg, 0.1 mg, 0.5 mg, and 1 mg dexamethasone to 4 week old male broiler chicks caused a relative increase in apoptosis in bursal tissue using the gel electrophoresis-based ladder assay at 4, 6, 24, 48, and 72 hours following administration of the synthetic corticosteroid. In contrast, the present investigation using a dose of approximately 1 mg/kg dexamethasone in day-of-hatch chicks did not induce a detectable increase in either the total number of apoptotic cells nor in the relative number of apoptotic cells per unit of area at 6 or 24 hours post-treatment. Similarly, the androgen 19-Nortestosterone, at the dosage and times chosen for the present study, failed to induce measurable apoptosis as compared to control chicks, but significantly suppressed intrafollicular cell proliferation. As both dexamethasone and 19-Nortestosterone increased bursal interfollicular thickness, a lesion commonly

associated with mild edema, presumably the dosages chosen for each steroid had some physiologic or pharmacologic effect during this study.

Reasons for the apparent discrepancy between the study of Compton (1990) and the present manuscript are not known. It is possible that differences in age sensitivity to the effects of glucocorticoids could be important with regard to apoptotic cell frequency within the bursa of Fabricius. Alternatively, it is possible that rapid phagocytosis by the reticuloendothelial system within the bursa of fabricius is capable of rapid removal of individual apoptotic cells, quickly removing affected cells without removing the associated DNA fragments. Investigations of these possibilities are ongoing in our laboratory.

CHAPTER III

OBSERVATION AND INTERPRETATION OF OCCASIONAL FOLLICLES WITH UNIFORM HIGH INCIDENCE OF APOPTOTIC CELLS IN THE NEONATAL CHICK BURSA OF FABRICIUS

INTRODUCTION

The bursa of Fabricius in chickens is the sole site of B-lymphocyte maturation and differentiation (Glick, 1988). In a unique process by which antigens are presented to the developing immune system, follicle associated epithelial cells lining the bursal lumen phagocytize antigens from both the large intestine and the environment through a mechanism known as cloacal drinking (Ekino *et al.*, 1985; Glick, 1977). When material from the environment comes in contact with the chicken vent, this material is immediately internalized into the cloaca and moved into the lumen of the bursa of Fabricius by retrograde transport. Once in the lumen of the bursa of Fabricius, particulate material is engulfed by specialized phagocytic cells lining the bursal lumen, known as the follicle associated epithelium (Glick 1977). In this manner, antigens are presented to developing B-lymphocytes in the follicular medulla, while these cells are undergoing the process of gene conversion, a process allowing for antigenic diversity

which allows each mature lymphocyte to recognize only one specific epitope (Ratcliffe, 1989). It is known that mature B-lymphocytes from both the medulla and cortex can emigrate to the blood and peripheral lymphoid organs, where they await activation by contact with the specific antigen which they individually capable of recognizing (Paramithiotis and Ratcliffe, 1994). Thus, this emigration to, and colonization of, peripheral lymphoid organs and tissues provides a means for continued ability to respond to new antigens and maintainance of humoral immunity once the bursa of Fabricius regresses at puberty (Paramithiotis and Ratcliffe, 1993). Despite the fact the future B-lymphocyte repertoire of the bird is dependent upon these emigrated cells which escape the bursal microenvironment, only 5% of all B-lymphocytes ever emigrate to the periphery, the rest of the cells die *in situ* (Glick, 1988; Paramithiotis and Ratcliffe, 1994). The process by which B-lymphocytes die *in situ* without inflammation is called apoptosis (Jacobsen *et al.*, 1996; Paramithiotis *et al.*, 1995; Motyka and Reynolds, 1991). Apoptosis, or programmed cell death, is much different than necrosis, the latter causing cell lysis. In apoptosis, the process begins when linker DNA between nucleosomes is cleaved by a lysosomal deoxyribonuclease. Cellular chromatin then condenses, and the nucleus fragments. As the endoplasmic reticulum expands and joins the cytoplasmic membrane, small membrane bound vesicles known as apoptotic bodies begin to form. Apoptotic bodies are subsequently phagocytized by macrophages, or surrounding specialized epithelial cells (Schwartzman and Cidlowski, 1993; Williams, 1991; Cohen and Duke, 1992).

Previous research on apoptosis of B-lymphocytes in the bursa of Fabricius utilized an agarose gel electrophoresis assay or "ladder assay" for detection of apoptosis. As DNA fragmentation is one of the earliest signs of apoptosis, and as fragmentation occurs at regular intervals in the genome, DNA from apoptotic cells appears as multiple bands at regular 180-200 bp fragment sizes on gel electrophoresis. This "ladder pattern" of fragmentation develops because the cleavage of linker DNA occurs between nucleosomes, which are each 180-200 base pairs long. The assay is sensitive as long as both strands of DNA are severed, and the population of cells is primarily apoptotic (Cohen and Duke, 1992).

A more recently developed assay detects *in situ* apoptosis within individual cells . This Terminal Deoxynucleotidal Transferase Mediated dUTP Nick End Labeling (TUNEL) method of detecting apoptosis uses immunohistochemical methods to detect the occurrence of apoptosis in specific microanatomical areas of tissue. The TUNEL assay is more sensitive than other detection methods because it uses labeled nucleotides which bind to free 3' hydroxyl groups which are only present in nicked or broken strands of DNA. Using antibodies and substrates specific for the nucleotide label allows the apoptotic cells to be identified using light or fluorescent microscopy (Sanders and Wride, 1996). Presently, we have adapted the TUNEL assay for use with neonatal bursa of Fabricius tissue in order to evaluate the distribution of apoptosis within the chicken bursa of Fabricius.

MATERIALS AND METHODS

Experimental Design

One-day-old Leghorn chicks were obtained from a commercial hatchery and were maintained in standard plastic chick boxes on paper liners under brooder lamps for 24 hours. Chicks were not provided feed or water during this holding period. Birds were euthanized by cervical dislocation.

Histology

Immediately following euthanasia, bursae were dissected from each chick, fixed in 4% buffered paraformaldehyde, and routinely processed in paraffin. Thin sections (5μm) of each tissue were sliced from each block, mounted on glass, and assayed using either the Terminal Deoxynucleotidal transferase (TdT) mediated dUTP nick end labeling (TUNEL) assay for detection of apoptosis.

TUNEL Assay

The process by which slides were sectioned and prepared for immuno-staining has been previously described (Harlow and Lane, 1988). Briefly, tissue sections were rehydrated in a series of xylene and ethanol solutions, then incubated at 37°C for one hour with Proteinase K[1] (Catalog no. P 2308) (10-20mg Proteinase K in 10 mM Tris HCl, pH 7.4-8.2) in a humidified chamber. Slides were then rinsed in 1M Phosphate Buffered Saline (PBS). TUNEL Enzyme[2] (Catalog no. 1767 305) and Label

[1] Sigma Chemical Company, St. Louis, MO 63178
[2] Boehringer Mannheim Corporation, Indianapolis, IN 46250-0414

Solution[2] (Catalog no. 1767 291) were mixed and applied to the tissues, which were incubated again in the humidified chamber for one hour at 37°C. Slides were rinsed thoroughly in PBS, then in Tris buffered saline (TBS). Monoclonal mouse Anti-Flourescein Isothiocyanate antibody[1] (Catalog no. F 8771) (FITC), diluted 1:400 in TBS, was applied and incubated on the bursal tissue sections at room temperature (RT) for 2-3 hours, then rinsed with TBS. Tissues were then incubated with streptavidan conjugated peroxidase[1](Catalog no. S 5512) diluted to 1:600 in TBS for 30 minutes at RT, and rinsed in TBS. A hydrogen peroxide and diaminobenzidine[1](Catalog no. P8001) (DAB) substrate solution (80 ml H_2O_2 and 25 mg DAB in 200 ml of 50 mM Tris buffer, pH 7.4) was applied for 20 minutes to stain the nuclei of apoptotic cells. After developing with the DAB substrate, tissues were dehydrated in a series of 3 ethanol baths and 2 xylene baths for 5 minutes each (Harlow and Lane, 1988).

Data Collection

Using a light microscope at 20x magnification, 15 tissue sections, each obtained from tissue derived from different animals, were examined. All follicles were counted, and follicles appearing to consist primarily of apoptotic cells were also enumerated for each sample. From this data we determined the percentage of follicles consisting primarily of apoptotic cells for each tissue. Additionally, in tissues where follicles consisting primarily of apoptotic cells were observed, photomicrographs of each affected, and 4 randomly-selected normal follicles were produced for each tissue. These

[1] Sigma Chemical Company, St. Louis, MO 63178
[2] Boehringer Mannheim Corporation, Indianapolis, IN 46250-0414

photomicrographs were analyzed using commercial morphometric analysis[3]software for determination of individual follicular areas apparent in the sections. The average measurement of each follicle type within each independent tissue sample was considered as a single measurement for statistical analysis.

RESULTS AND DISCUSSION

Measurements made in the present study were prompted by the casual observation of occasional follicles consisting primarily of apparently apoptotic cells in preliminary studies. The overwhelming majority of bursal follicles in tissues derived from this chick population consist primarily of non-apoptotic cells, with occasional intrafollicular apoptosis noted. We enumerated the entire number of follicles in each sample, as well as the primarily apoptotic follicles. These data indicated that out of 3132 total follicles, only 0.26%, or 8 follicles, were predominantly, if not entirely, composed of apoptotic cells. We also observed that the apoptotic follicles seemed to be consistently smaller than the normal follicles. When all affected follicles and randomly selected normal follicles were measured using morphometric analysis software, normal follicles were approximately 10-fold larger than the predominantly apoptotic follicles. The average apoptotic follicle was 1,364.81 square microns, and the average normal follicle measured 14,445.84 square microns.

[3] Jandel Scientific(1996)

While the reason for the presence of bursal follicles consisting of predominantly apoptotic cells is not known, possible reasons are intriguing. Future elucidation of the underlying physiological or pathophysiological processes responsible for this phenomenon may lead to important clues for more complete understanding of humoral immune system ontogeny. Within an affected follicle, it may be reasonable to consider that all cells are dying for the same reason, and are at the same stage of the same ontogenic pathway. Perhaps all of the cells are from the same precursor B- cell. If that single precursor cell had a functional defect, recognized self antigen, or had another integral mistake, a uniform signal for deletion would seem appropriate. A possible mechanism for the existence of a large population of clones recognizing self antigen within a single follicle could be inappropriate proliferation prior to deselection event(s) triggering apoptosis (e.g. late recognition of self recognition). Alternatively, it is possible that affected cells within a single follicle could be infected with a virus, or that these cells were inappropriately signaled by an aberrant paracrine signal. Regardless, the existence of follicles consisting predominantly of apoptotic cells suggests that some error of B-cell ontogeny occasionally occurs and must be corrected by essential destruction of the entire follicle. Future elucidation of mechanisms regarding this phenomenon are necessary for more complete understanding of B-cell ontogeny in the chicken bursa of Fabricius.

CONCLUSIONS

The research reported in this manuscript is the first *in situ* enumeration of normal and apoptotic lymphocytes and follicles within the neonatal chicken bursa of Fabricius, the site of B-lymphocyte ontogeny in birds. This research also examined the effects of exogenous administration a selected androgen or corticosteroid on *in situ* apoptosis within the neonatal bursa of Fabricius. While neither steroid significantly altered apoptosis at the post-administration times measured in these experiments, significant increases in interfollicular epithelial thickness was observed due to either steroid, suggesting that effective physiological or pharmacological doses of these treatments were administered. Interestingly, administration of the androgen 19-nortestosterone significantly decreased the numbers of proliferating cells, while not affecting numbers or ratios of apoptotic bursal lymphocytes.

Unexpectedly, a low frequency (0.26%) of follicles containing mostly apoptotic lymphocytes was observed in normal neonatal bursal tissues in these studies. Follicular areas of affected follicles were approximately 10% of normal appearing follicles as determined by morphometric analysis, suggesting that these entire follicles are undergoing atresia. While the reason for the presence of bursal follicles consisting of predominantly apoptotic cells is not known, possible reasons are intriguing. Within an affected follicle, it may be reasonable to consider that all cells are dying for the same reason, and are at the same stage of the same ontogenic pathway. Perhaps all of the cells

are from the same precursor B- cell. If that single precursor cell had a functional defect, recognized self antigen, or had another integral mistake, a uniform signal for deletion would seem appropriate. A possible mechanism for the existence of a large population of clones recognizing self antigen within a single follicle could be inappropriate proliferation prior to deselection event(s) triggering apoptosis (e.g. late recognition of self recognition). Alternatively, it is possible that affected cells within a single follicle could be infected with a virus, or that these cells were inappropriately signaled by an aberrant paracrine signal. Regardless, the existence of follicles consisting predominantly of apoptotic cells suggests that some error of B-cell ontogeny occasionally occurs and must be corrected by essential destruction of the entire follicle. Future elucidation of mechanisms regarding this phenomenon are necessary for more complete understanding of B-cell ontogeny in the chicken bursa of Fabricius.

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Figure 1A. Light photomicrograph (20X) of bursa of Fabricius tissue from non-treated day-of-hatch chicks processed using the TUNEL assay for demonstration of apoptotic cells (brown-staining nuclei). Bursal tissues were fixed in 4% buffered paraformaldehyde solution, paraffin-embedded, sectioned (5 μm), rehydrated and subjected to TUNEL assay. Occasional apoptotic cells are distributed within the follicle with increased frequency of occurrence in the follicular cortex.

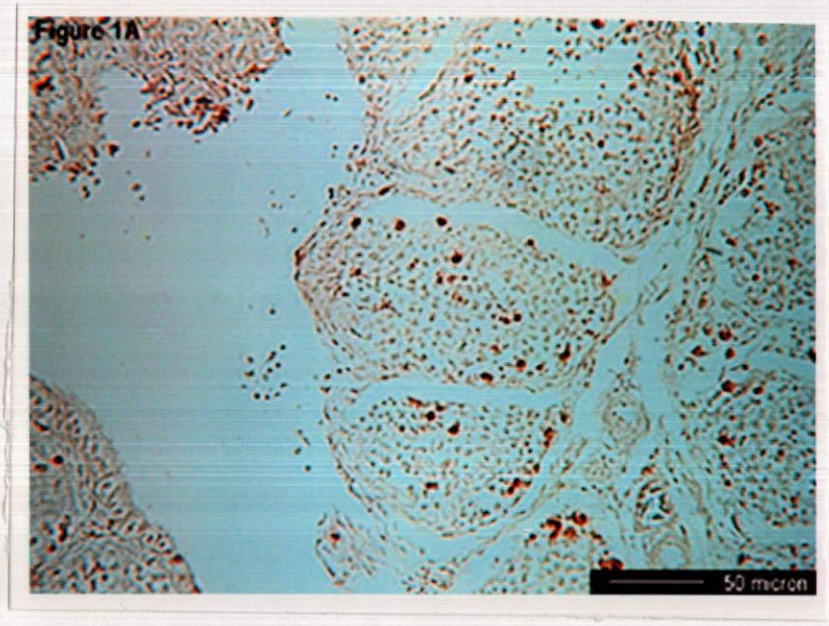


Figure 1B. Light photomicrograph (40x) of the section described above (1B).

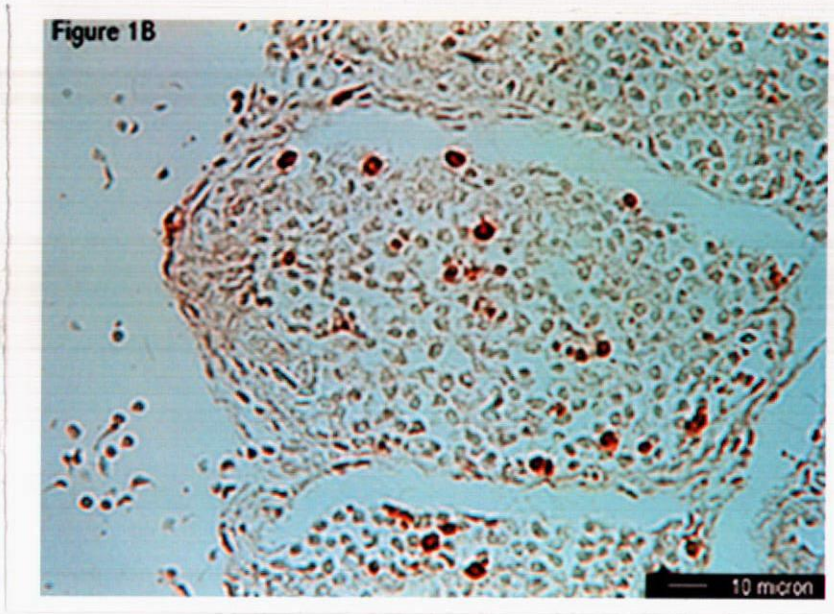


Figure 2A. Light photomicrograph (20X) of bursa of Fabricius tissue from a non-treated day-of-hatch chick, processed using the PCNA assay for demonstration of proliferating cells (brown-staining cells). Bursal tissues were fixed in 4% buffered paraformaldehyde solution, paraffin-embedded, sectioned (5 μm), rehydrated and subjected to PCNA immunostaining assay. Proliferating cells are distributed throughout the follicle with increased frequency of occurrence in the follicular cortex.

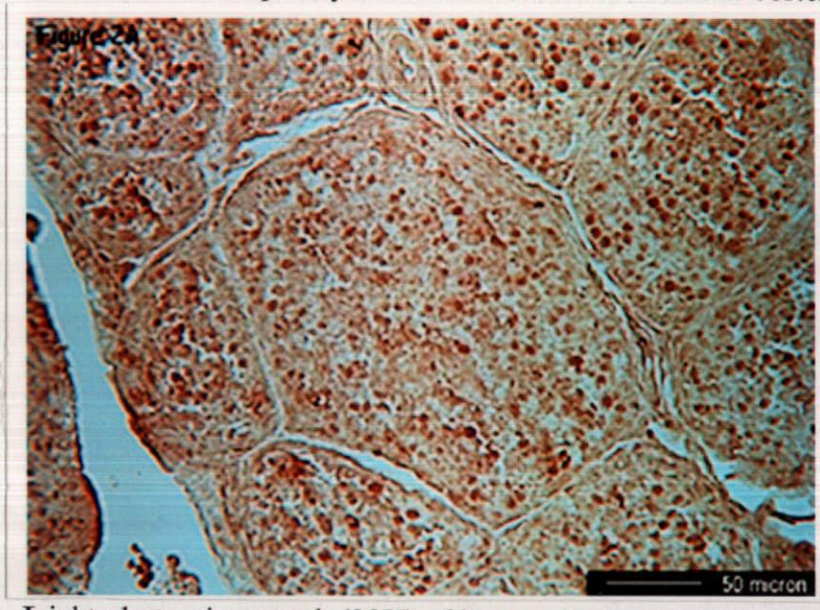


Figure 2B. Light photomicrograph (20X) of bursa of Fabricius tissue from a day-of-hatch chick treated with 4.2 mg the androgen 19-nortestosterone, processed using the PCNA assay as described above. Proliferating cells are distributed throughout the follicle with increased frequency of occurrence in the follicular cortex. Notice the treatment-related decrease in the frequency of identifiable proliferating cells as compared to similar tissue derived from an untreated chick (Figure 2A).

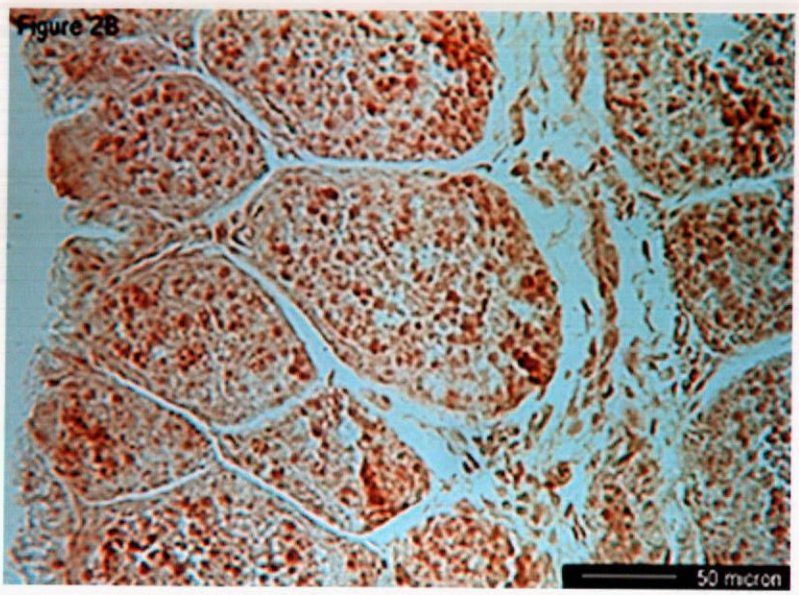


Figure 3A. Light photomicrograph (20X) of bursa of Fabricius tissue from non-treated day-of-hatch chicks processed using the TUNEL assay for demonstration of apoptotic cells (brown-staining nuclei). Center dark-staining follicle is an example of the occasional predominantly apoptotic follicle (only 0.26% of follicles exhibited this level of apoptosis). Follicles consisting predominantly of apoptotic cells were approximately 10% of the area of normal-appearing follicles based on morphometric analysis. Bursal tissues were fixed in 4% buffered paraformaldehyde solution, paraffin-embedded, sectioned (5 μm), rehydrated and subjected to TUNEL assay.

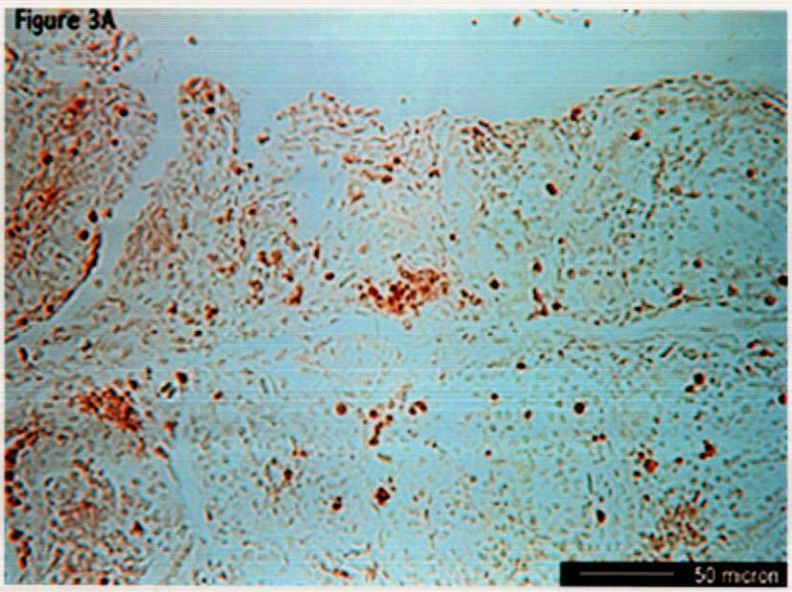
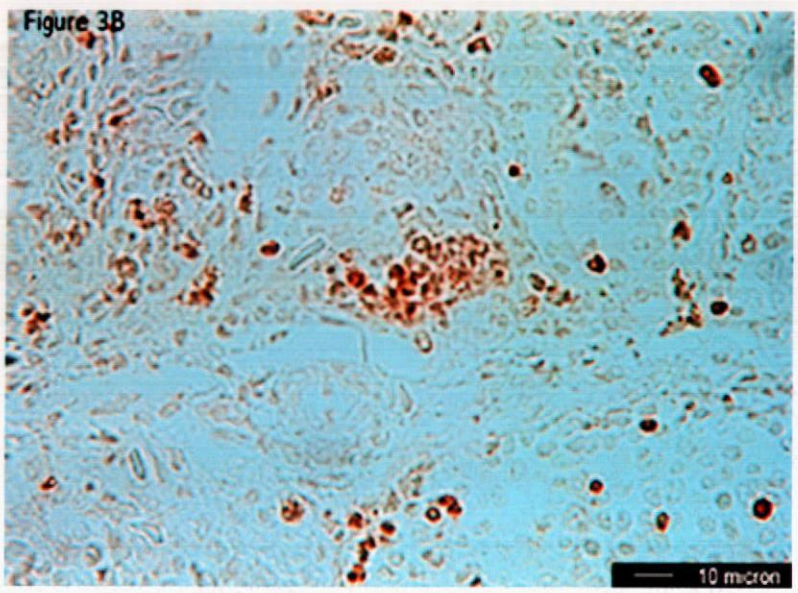


Figure 3B. Light photomicrograph (40x) of the section described above (3B).



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