IMMUNOPATHOGENESIS OF AVIAN LEUKOSIS VIRUS SUBGROUP J IN WHITE LEGHORN CHICKENS

By

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AN ABSTRACT OF A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pathobiology and Diagnostic Investigation

2001

Professor Willie M. Reed

ABSTRACT

IMMUNOPATHOGENESIS OF AVIAN LEUKOSIS VIRUS SUBGROUP J IN WHITE LEGHORN CHICKENS

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Avian leukosis virus subgroup J (ALV-J) was first described in the early 1990s in England as the cause of myeloid leukosis. Since its discovery, the virus is now spread worldwide and broiler breeder companies are trying to rid their breeder flocks of the infection. Accidental infection has also occurred in commercial laying chickens. The overall goal of this research was to determine the pathogenesis of ALV-J infection in white leghorn chickens. The objectives were: 1) Compare the pathogenicity of ALV-J in various lines of white leghorn chickens; 2) Determine the effects of endogenous virus 21 on the pathogenesis of ALV-J in white leghorns; and 3) Determine the tissue tropism of ALV-J and the susceptibility of the B cell to ALV-J induced transformation in white leghorns.

For objective 1, chickens from four genetic lines of white leghorn were inoculated with the U. S. prototype of ALV-J, ADOL Hc1, at either the day of hatch or as 7-day-old embryos. At 4, 10, 20 and 30 weeks of age, chickens were tested for ALV-J- induced viremia and antibody, packed cell volumes, and differential white blood cell counts. At 4 and 10 weeks of age, 5 chickens from each treatment group and of each line were humanely euthanized and bursal tissues were examined for follicle transformation. All chickens that died and those that survived the experiment were examined for tumor formation. Microscopic examination of the grossly affected tissues was also performed

to confirm the diagnosis of tumors. Of all the lines of chickens inoculated with ALV-J at day of hatch, only Line 0 developed antibodies and cleared the virus. Chickens of all other lines had various degrees of success in developing antibody and clearing the virus. The primary tumors observed were lymphoid leukosis (LL) and hemangiomas, regardless of treatment or genetic line of chicken.

For objective 2, the effect of endogenous leukosis virus 21 (EV21) on the pathogenesis of ALV-J induced infection and disease was examined. F_1 progeny from a cross between ADOL line 0.44-EV21+ males and ADOL $15B_1$ females were hatched and characterized as late feathering or early feathering by the length of the primary feathers. Chicks were inoculated with stain ADOL Hc1 of ALV-J at day of hatch. At 4, 10, 18 and 31 weeks of age, chickens were tested for ALV-J viremia and antibody. All birds that died and those that survived the experiment were necropsied. Chickens harboring EV21 mounted a weak antibody response as compared with those lacking EV21. Although the incidence of tumors was surprisingly low for birds harboring EV21, overall LL was diagnosed in five birds and hemangioma in one bird.

Tissue tropism and B cell transformation assays were studied in chickens of line $15I_5$ X 7_1, a highly susceptible line to ALV-induced infection and tumors. Chicks were inoculated as 7-day-old embryos with strain ADOL Hc1 of ALV-J. At 2 and 6 weeks of age, various tissues were tested by immunohistochemistry utilizing a monoclonal antibody specific for gp85. Viral antigen was found in all tissues examined except skeletal muscle. At 4 and 10 weeks of age, bursal tissues were examined for the presence of transformed follicles using the methyl green pyronine stain. Results indicated that ALV-J could transform bursal follicles. Infection with ALV-J may be manifested as LL.

I want to dedicate this body of work to my family.

ACKNOWLEDGMENTS

I would like to thank everyone involved in this project. To my husband, Orlando, for all your love and support (all those extra miles you drove so I could do this). To my son, Jonathan, for making me realize that there is life besides grad school. To the members of the Tuskegee Michigan State family, thanks for all the fun times and support for my time spent here and especially Dorothy Reed, Claudette Buckingham, and Ruby Perry for taking care of Jonathan when I needed your help. To Pat Lowrie for the motherly advise when I first came to MSU as a Vetward Bound Student and when I returned as a grad student. To the scientists and technicians at the USDA ADOL lab, thank you for all your help, input and insight in tumor virus biology and life in general. To Melanie Flesberg for all your help in bleeding what seemed like thousands of birds, your patience with all my "dumb" questions and all the "chick" parties in necropsy, thanks a million and I'll remember "the only good chicken is a dead chicken". To the former graduate students of ADOL, Carol Cardona, Ping Wu and Hsiao-Ching Lui, thanks for all your help, after hours grad student pity parties, inspiration and friendship. To Sharon Thon, for your photographic and slide making skills, thanks for all your help. To my church family at Friendship Baptist Church, thanks for all your prayers and support, especially Marnise Roberts, Dr. Charles Lowrie, and the extraordinary women's group. And finally to my committee, Dr. Willie Reed, Dr. Aly Fadly, Dr. Mick Fulton, Dr. Margo Holland and Dr. Scott Fitzgerald for all the support, mentoring, and guidance you offered me during this process and Dr. Jon Patterson for joining my committee at the end. Once again, thank you very much for this opportunity to grow professionally and personally.

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30g	30 gauge
Ab	antibody
ADOL	Avian Disease and Oncology Laboratory
AEV	avian erythroblastosis virus
ALSV	avian leukosis sarcoma virus
ALV	avian leukosis virus
ALV-A	avian leukosis virus subgroup A
ALV-J	avian leukosis virus subgroup J
AMV	avian myeloblastosis virus
ART-CH	avian retrotransposons-chicken
att	attachment site
C	centigrade
CA	capsid
CEF	chicken embryo fibroblasts
COFAL	complement fixation for avian leukosis
c-myc	cellular myc gene
DNA	deoxyribonucleic acid
EAV	ancient endogenous virus
env	envelope gene
ev	endogenous virus locus
EV21	endogenous virus 21
FeLV	feline leukemia virus
g	gram
gag	group specific antigens gene
gp37	glycoprotein 37
gp85	glycoprotein 85
gs	group specific antigen
HEM	hemangiomas
HPRS	Houghton Poultry Research Station
IN	integrase
IU	infectious units
Kb	kilobases
kD	kilodalton
LDLR	low density lipoprotein receptor
LL	lymphoid leukosis
LPD	lymphoproliferative disease
LTR	long terminal repeats
MA	matrix
MGP	methyl green pyronine
ml	milliliter
ML	myelocytomatosis or myeloid leukosis
NC	nucleocapsid
nm	nanometer
p27	protein 27

PB	primer binding
PCR	polymerase chain reaction
PCV	packed cell volume
pol	polymerase
PP	polypurine
T_3	triidothyronine
T_4	$_L$-thyroxine
R	repeat
REV	reticuloendotheliosis virus
RNA	ribonucleic acid
RNase H	ribonuclease H
RPRL	regional poultry research laboratory
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SAIDS	simian immunodeficiency syndrome
SEAR	subgroup E receptor
src	sarcoma gene
SU	surface protein
TM	transmembrane protein
TNFR	tumor necrosis factor receptor
tRNA	transfer ribonucleic acid
tv-a	tumor virus-a
tv-b	tumor virus-b
tv-c	tumor virus-c
U3	unique sequences at the 3' end of genome
U5	unique sequences at the 5' end of genome
V+, A-	virus positive, antibody negative
V+, A+	virus positive, antibody positive
VI	virus isolation
VN	virus neutralization
WBC	white blood cell

CHAPTER 1

Introduction and Literature Review

I. *Retroviridae*

A. *Introduction*

The *Retroviridae* is a large family of viruses that primarily infect mammals. Retroviruses are characterized as an enveloped virion with a dimer ribonucleic acid (RNA) genome, composed of two identical single stranded RNA molecules. The RNA molecule has a plus sense orientation meaning that it can serve as a template for translation of proteins. However, retroviruses use a deoxyribonucleic acid (DNA) intermediate stage before transcribing RNA for protein translation. The genome ranges from 7 to 13 kilobases (Kb) in length. The genomic structure that is conserved in all retroviruses includes the 5' R and U5 regions, group specific antigens (*gag*), polymerase (*pol*), envelope (*env*) genes and the 3' U3 and R regions. Some viruses have additional genes while others have the basic three. These additional genes account for the larger genomes (Coffin 1992).

B. *Morphology*

Retroviruses have a distinct morphology when viewed ultrastructurally. Four types of particles can be found. The A Type particles are only found inside cells. They have a clear center that is surrounded by a shell. They can be intracytoplasmic or intracisternal. The B Type particles have doughnut shaped cores when they bud and eccentrically located cores within a budded particle. The C Type particle has a crescent

shaped core at budding and centrally located cores in virions. The D Type particles have more elongated electron dense cores in the virions (Weiss *et al.* 1982).

C. ***Major Viral Genes and Encoded Proteins***

There are three major genes identified in all retroviruses, encoding a number of essential proteins for the life cycle of the virus. Italics are usually used to name a gene and two to three capital letters for proteins. The *gag* gene encodes proteins that are the major structural elements of the capsid. There are three GAG proteins common to all retroviruses. Some make a fourth GAG protein. First are the matrix (MA) proteins that line the inner face of the virion envelope. MA proteins are involved in budding of the virus. The capsid (CA) protein forms the core shell of the virion (Dickson *et al.* 1982; Dickson *et al.* 1985). In most retroviruses, the CA protein is the detectable antigen and is the basis for identification assays. The nucleocapsid (NC) is the protein closely associated with the genomic RNA. It has been reported to promote RNA-RNA duplex formation (Prats *et al.* 1988; Prats *et al.* 1990) which is presumed necessary for genome-primer association and genome dimerization. At the end of the *gag* gene, the protease enzyme is encoded. It is responsible for all the proteolytic cleavages that generate the mature GAG and POL proteins during viral maturation.

The *pol* gene is 3' downstream of the *gag* gene and encodes for two major proteins, reverse transcriptase (RT) and integrase (IN). The main function of the POL proteins is viral synthesis and integration into the host DNA. The RT product includes enzymes needed for DNA synthesis—RNA directed DNA polymerase, DNA directed DNA polymerase, and ribonuclease H (RNase H). Each of these enzymes is necessary to create double stranded DNA from the single stranded RNA viral genome. However, the

error rate in RNA directed DNA polymerase is high when compared to DNA-DNA polymerase (Battula and Loeb 1976). This error rate is the cause of the variability found in retrovirus genomes. The RT is highly conserved among retroviruses. The IN protein has two enzymatic functions, DNA cleavage and strand transfer (Grandgenett and Mumm 1990). It accomplishes these functions by cleaving the host DNA target and the viral DNA 3' ends then ligating the viral and cellular DNA together (Katzman *et al.* 1989; Rice *et al.* 1996; Katz *et al.* 1998).

The envelope or *env* gene encodes the surface proteins that are needed for recognition of specific cell receptors. The polypeptide precursor is created by RNA splicing and then further processed into two proteins, the surface protein (SU) and the transmembrane protein (TM). The SU protein is a larger protein and is glycosylated. The SU protein also includes the site for host-cell receptor interaction with the virion (Coffin 1992) and host ranges that help classify closely related viruses into subgroups in certain species (Weiss *et al.* 1982). The TM protein is smaller and is the C-terminal cleavage product. It serves as an anchor for the SU protein complex to the viral envelope and mediates fusion of the envelope to the host cell membrane (Coffin 1992).

D. ***Long Terminal Repeats***

The process of reverse transcription creates repeated sequences at the ends of the proviral genome, referred to as long terminal repeats or LTRs. These are terminal noncoding regions that are essential in *cis* for viral replication. They are composed of three regions, the U5, a unique sequence near the 5' end of the genome, R, a short sequence directly repeated at each end of the genome that is used during reverse transcription, and U3, a unique sequence that contains signals used by the provirus to

regulate transcription and processing of transcripts at the 3' end of the genome. After reverse transcription has occurred the regions are fused together in the following order U3-R-U5 and are found at each end of the resulting proviral genome (Coffin 1992).

E. ***Groups of Retroviridae***

1. **Avian Leukosis-Sarcoma Virus (ALSV) Group**

This group includes of both exogenous and endogenous viruses of domestic fowl (Payne 1992). These viruses fall into the C-Type virion and their genomes encode the basic three genes, *gag, pol,* and *env.* Sometimes the exogenous viruses carry oncogenes like *src* (sarcoma) found in Rous sarcoma virus (Parker *et al.* 1981). The viruses are further divided into ten subgroups (A-J) according to host range, viral envelope glycoprotein antigens, and viral interference patterns (Duff and Vogt 1969; Okazaki *et al.* 1975; Dorner *et al.* 1985; Bova *et al.* 1988; Payne *et al.* 1992). However, viruses of ALSV that affect chickens belong to six subgroups (A, B, C, D, E and J) (Payne *et al.* 1991; Payne 1992).

2. **Mammalian C-Type Virus Group**

Viruses of this group also include exogenous and endogenous viruses from many different mammals, including rodents (Kozak and Ruscetti 1992), carnivores (Hardy Jr. 1993), and other avian viruses not included in ALSV group. (Barbacid *et al.* 1979; Gazit *et al.* 1979). These viruses are similar to the ALSV group with simple genomes and there are many oncogene-containing strains that have been described (Coffin 1996). Examples of viruses in this family are murine leukemia viruses, feline leukemia virus (FeLV), reticuloendotheliosis virus (REV) and lymphoproliferative disease of turkeys (LPD). Mouse viruses are classified by species distribution of their receptors: *xenotropic* viruses

use receptors found in most species except mice, *ecotropic* viruses replicate only in mice cells, and *polytropic* and *amphotropic* viruses use different receptors found on mouse and non-murine species (Kozak and Ruscetti 1992; Coffin 1996). FeLVs are classified into subgroups A, B, and C. Subgroup B contains the endogenous viruses (Hardy Jr. 1993; Coffin 1996).

3. **B-Type Virus Group**

The only infectious agents in this group are the endogenous and exogenous mouse mammary tumor viruses (Kozak and Ruscetti 1992; Coffin 1996). These viruses also have simple genomes with the addition of the *sag* gene for superantigen activity (Coffin 1996) and no oncogene has been described with the viruses. Mammary carcinomas are the sequela to infection with these viruses.

4. **D-Type Virus Group**

This group includes both endogenous and exogenous viruses from primates and sheep (Coffin 1996). These also are simple genomic viruses with no oncogene-containing members. Examples include Mason-Pfizer monkey virus, simian immunodeficiency syndrome (SAIDS) virus, and Jaagsiekte disease virus in sheep (York *et al.* 1992). B and D-Type viruses are closely related and both form intracytoplasmic A particles (Coffin 1996).

5. **HTLV-BLV Group**

This group only includes exogenous viruses of humans and cattle. This group of viruses contains at least two genes that are for gene expression, *tax* and *rex*, besides the basic three genes and no oncogenes. Examples of this group include bovine leukemia

virus (a B cell lymphoma) and human T-cell leukemia virus –1 and –2 (a T-cell lymphoma) (Coffin 1996).

6. **Lentivirus Group**

Viruses in this group are complex exogenous viruses that infect humans, feline, bovine, primates, sheep and goats. The prototypes of these viruses were called "slow viruses" because of the slow progression of the disease and very long incubation periods. Various other genes are found in the genome besides the basic three. *tat* and *rev* are most common with *nef* and *vif* also occurring in some of the viruses as well as *vpr* and *vpu*. Examples from this group include human immunodeficiency virus-1 and –2, simian immunodeficiency virus, feline immunodeficiency virus, bovine immunodeficiency virus, Visna/maedi virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus. These viruses mainly induce neurological and immunological diseases versus neoplastic disease that can manifest with the previously mentioned groups.

7. **Spumavirus Group**

This group is referred to as the "foamy" viruses because of the vacuolation in cell culture that occurs following inoculation (Coffin 1996). Infection with these viruses is not associated with any known disease (Loh 1993; Coffin 1996). They are complex viruses with additional proteins encoded at genes *bel*1, *bel*2, and *bel*3. Examples of this group are simian foamy virus, human foamy virus, and feline syncytium-forming virus. They are among the largest of the retroviruses with approximately 11,000 nucleotides (Maurer and Flugel 1988; Loh 1993).

8. **Unclassified fish retroviruses**

Retroviruses of fish have recently been described (Martineau *et al.* 1992; Petry *et al.* 1992; Eaton *et al.* 1993; Bernard and Bremont 1995; Hart *et al.* 1996; Zhang *et al.* 1996; LaPierre *et al.* 1999). Examples are walleyed dermal sarcoma virus, walleyed epidermal hyperplasia virus and snakehead fish virus. Walleyed dermal sarcoma virus is now the largest retrovirus with approximately 13,000 nucleotides (Martineau *et al.* 1992). The lengths of the genomes sequenced so far indicate that other proteins are present but have not been fully characterized (LaPierre *et al.* 1999).

II. *Avian Leukosis-Sarcoma Viruses and Diseases*

A. *Introduction*

The term, lymphoid leukosis (LL), was first coined by Biggs (1961) and Campbell (1961). This name superseded *visceral lymphomatosis* that Jungherr (1941) had termed the disease in 1941. The earliest case of a leukotic disease was first report by Roloff (1868) and Caparini (1896) who described fowl leukemia. Confusion with Marek's Disease, a neoplastic disease involving T cells, was cleared away when the etiological agent for Marek's Disease was finally discovered in 1967 (Churchill and Biggs 1967; Nazerian *et al.* 1968). Burmester (1947) provided proof of a viral etiology for LL.

In 1908 the first reported experimental transmission of erythroblastosis was documented (Ellerman and Bang 1908). Later, both Burmester and coworkers (1959) and Beard (1963) describe the viral etiology and pathogenesis of various strains of leukosis viruses causing erythroblastosis. Strains are called avian erythroblastosis viruses

or AEVs. Experimental transmission of myeloblastosis was first demonstrated by Schmeisser (1915). The most investigated leukosis virus strain is the BAI-A AMV (Payne and Fadly 1997). Pentimalli reported the first description of myelocytomatosis in 1915. Most strains that cause myelocytomatosis such as MC29 and HPRS-103 also cause other types of neoplasms (Mladenov *et al.* 1967; Beard 1980; Payne *et al.* 1992).

Nephromas and nephroblastomas as well as other types of tumors are caused by ALV. Nephromas are usually categorized as either adenomatous or carcinomatous while nephroblastomas are of the complex Wilm's tumor type (Payne and Fadly 1997). The only viruses that can experimentally induce nephroblastomas are BAI-A AMV (Burmester *et al.* 1959; Beard 1980), MAV-2 (N) (a myeloblastosis associated virus)(Watts and Smith 1980) and virus strain 1911(Payne *et al.* 1993). Nephromas can be induced by a variety of well characterized laboratory viruses as well as field strains.

Osteopetrosis was first described in the 1920's (Pugh 1927) as sporadic diffuse osteoperiostitis. Other names for the disease include Marble bone and thick leg disease (Payne and Fadly 1997). In 1938, Jungherr and Landauer suggested the term *osteopetrosis gallinarium*. They were also the first to reproduce the disease and noted that it was associated with LL with some frequency (Jungherr and Landauer 1938). Several other researchers were able to reproduce the disease without LL tumors (Campbell 1963; Franklin and Martin 1980; Hirota *et al.* 1980).

B. ***Disease Incidence and Distribution***

Leukosis-sarcoma viruses are found worldwide but actual clinical disease incidence is quite low. Subgroup A, B and, most recently, J of ALV are found in commercial flocks in the United States in the field with subgroup A ALV more common

than subgroup B (Payne and Fadly 1997). All subgroups of ALV can be detected either by virus isolation or antibody assays (Spatar *et al.* 1975; Payne and Fadly 1997). Egg- and meat-type chickens as well as other species such as pheasants, partridges and quail, are susceptible to infection with ALV. Natural infection of Japanese quail, pigeons, geese, and Pekin and Muscovy ducks has not been reported (Chen and Vogt 1977; Vogt 1977). However, the susceptibility of experimental infection with ALV has been documented in these species as alternative models for infection (Nehyba *et al.* 1990; Geryk *et al.* 1996; Salter *et al.* 1999; Trejbalova *et al.* 1999). Endogenous retroviral genes can be found in cells from almost all chicken lines and some other species of galliform birds. These genes can be complete or defective viruses of subgroup E (Robinson 1978; Crittenden 1981; Smith 1987; Crittenden 1991; Dimcheff *et al.* 2000). They are inherited in a Mendelian fashion at discrete loci on the chromosome and termed endogenous viral loci or *ev* loci.

The incidence of actual clinical LL in commercial chickens has decreased over the years due to eradication programs. There are occasional losses that can be up to 30% (Jordan 1990). Most losses are noted in egg laying chickens and breeder stocks. Subgroup J viruses cause myelocytomatosis and has resulted in significant economic losses for broiler breeder flocks in the United States and abroad (Payne *et al.* 1992; Fadly and Smith 1997; Fadly 1998; Fadly and Smith 1999). Field cases of erythroblastosis and myeloblastosis are very uncommon (Payne and Fadly 1997). Other tumors that occur frequently with ALV infection include hemangiomas (25%) and renal tumors (19%) (Campbell and Appleby 1966) in broilers. Osteopetrosis occurs sporadically and males tend to be more affected than females (Payne and Fadly 1997).

C. ***Etiology***

1. **Classification**

The avian leukosis-sarcoma viruses as well as all other retroviruses are characterized by the presence of the enzyme reverse transcriptase. However, not all of those viruses have complete genomes and various terms have been used to describe the viruses. The terms "defective" and "complete" describe retroviruses that either lack some aspect of the viral genome and cannot replicate without a helper virus or a complete virus that can replicate without help. The term "acutely transforming" describes viruses that cause neoplasms in a short period of time, usually in a few days to weeks. These viruses carry a viral oncogene that allows for transformation and tumor formation quickly. (Weiss *et al.* 1982). An example of an acutely transforming complete virus is Rous sarcoma virus. It contains all the genes necessary for replication and *v-src*, a viral oncogene (Duesberg *et al.* 1977). The opposite term, "slowly transforming", describes viruses that take many weeks to months for tumor development. These viruses do not carry a viral oncogene and have to insert their genomes near a host proto-oncogene. (Weiss *et al.* 1982)

2. **Subgroups**

Six of the ten different subgroups of ALV are found in chickens: A, B, C, D, E and J. Classification of ALV into various subgroups is based on assays that determine host range, interference patterns, and viral envelope antigens. Host range is determined by growth of virus in embryo fibroblasts from various avian species. Interference patterns look at interactions between members of different and same groups. Viral envelope antigens are detected by viral and serum neutralization tests (Weiss *et al.* 1982).

Subgroups F and G viruses are endogenous and are found in three species of pheasants, ring-necked (Hanafusa and Hanafusa 1973; Fujita *et al.* 1974), golden (Fujita *et al.* 1974), and Lady Amherst (Hanafusa *et al.* 1976). Subgroup G viruses, isolated by Hanafusa *et al* (1976), had different gs (group specific) antigen from the rest of ALSVs and the viral proteins were also different from ALV and REV. On this basis they were placed in a new class termed pheasant virus (Hanafusa *et al.* 1976) but retained the Subgroup G designation. Subgroup H is an endogenous virus group isolated from Hungarian partridge (Hanafusa *et al.* 1976). Subgroup I is also an endogenous virus group isolated from Gambel's quail (Troesch and Vogt 1985). Various defective strains of ALSVs lack an envelope gene and thereby have no subgroup. They can replicate with help of helper viruses and they take on the designation of the helper virus' envelope (Payne and Fadly 1997).

3. **Morphology**

The diameter of the ALV particle is 80-120 nm. It is spheroidal in shape with knobbed spikes projecting from the envelope. The spikes are about 8nm in diameter. The freshly budded virion is not mature until the cleavage of the GAG precursor protein which results in an electron dense central core (Coffin 1996). Figure 1.1 illustrates a schematic drawing of retroviruses. The genome consists of two single-stranded molecules of RNA with capping at the 5′ end and polyadenylation at the 3′ end. The buoyant density in sucrose is characteristic for C-type retroviruses at values of 1.15-1.17g/ml (Robinson and Duesberg 1968; Bauer 1974).

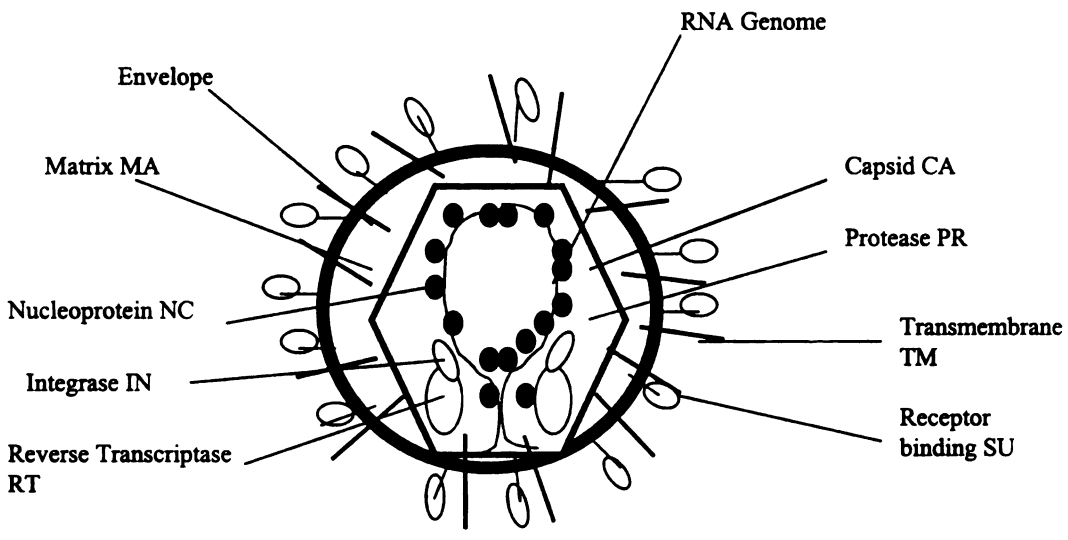

Figure 1.1. A schematic drawing of a retrovirus virion illustrating various locations of structure and proteins. Adapted from *Fields Virology* 3rd ed.

D. ***Virus Replication***

Retrovirus replication involves the enzyme reverse transcriptase that makes the group unique. The first interaction between the virus and the host cell is attachment of the virion to the cell via a specific cell surface receptor. Interactions between the receptor and the envelope glycoproteins allow or prevent viral entry. The virion glycoproteins are arranged such that the TM protein or glycoprotein 37 (gp37) serves as an anchor for the SU protein or glycoprotein 85 (gp85) via disulfide linkages (Sommerfelt 1999).

The receptor for ALV-A has been defined and recently mapped on the chicken genome. ALV-A receptor is genetically related to the human low-density lipoprotein receptor (LDLR) (Bates *et al.* 1993; Young *et al.* 1993). The receptor mapped to the *tv-a* (tumor virus A subgroup) locus (Bates *et al.* 1998) which also confers susceptibility to infection for subgroup A. Subgroups B, D and E susceptibilities are linked to the *tv-b*

locus. The receptor for subgroups B and D is CAR1 and appears to be a member of the tumor necrosis factor receptor (TNFR) family (Brojatch *et al.* 1996). The gene for CAR1 was recently mapped to the *tv-b* locus (Smith *et al.* 1998). Subgroup E ALV's receptor (SEAR) was discovered and cloned from turkey cells (Adkins *et al.* 1997) and is almost identical to CAR1 (Adkins *et al.* 2000). Both receptors are located in the *tv-b* locus and only differ in animo acids at residue 62 (Adkins *et al.* 2000). Subgroup C ALV receptor is located in the *tv-c* locus (Crittenden 1991) but no gene has been identified yet. The receptors for the remaining subgroups have not been identified.

The envelope protein exists as a homotrimer (three SU and TM subunits covalently bonded). After the SU protein binds with the appropriate receptor, the current model of entry is a conformational change of the SU protein and fusion to the cell membrane. The following proposed model for fusion involves the trimeric envelope subunits binding to the host cell receptor. More receptors migrate to the area and are bound to the SU subunits, resulting in a cooperative conformational change in both SU and TM subunits. The activated fusion peptide is inserted into the host membrane and the receptor is released by the SU subunits. TM subunits can laterally diffuse to form a fusion pore (Damico *et al.* 1998). This all takes place independently of pH. This model is similar for the influenza virus hemagglutinin glycoprotein except that a low pH is needed (White 1990). After the fusion pore is complete, the virion core is released into the cell cytoplasm (Hunter and Swanstrom 1990). What happens to the various capsid proteins after fusion is not well defined (Coffin 1996).

The next step is to begin creating DNA from the RNA viral genome. In 1964, Temin (1964,1976) suggested the idea that a provirus was necessary for RNA tumor

viruses such as ASLV to reside in infected cells as DNA. In 1970 Temin isolated the polymerase that created DNA from RNA templates using Rous sarcoma virus (Temin and Mizutani 1970) thereby proving his hypothesis of a provirus existence. To begin synthesis of the DNA, the RT (p68) enzyme needs a primer to attach the nucleotides to when transcribing the RNA. In all retrovirus genomes, a specific tRNA molecule is base paired, an 18 nucleotide sequence, to the primer binding (PB) region on the viral genome (Fu *et al.* 1997). For all ASLVs, it is tRNA tryptophan ($tRNA^{Trp}$) (Faras *et al.* 1974; Faras *et al.* 1974; Waters *et al.* 1975; Waters and Mullin 1977).

Once synthesis begins, the RT enzyme goes towards the 5' end of the viral genome, transcribing the U5 and R regions into minus strand DNA using the RNA dependent DNA polymerase activity. The newly created RNA:DNA hybrid is acted upon by the RT's RNase H activity which degrades RNA in RNA:DNA hybrids (Moelling *et al.* 1971). This allows for base pairing to occur with the 3' R region and the newly synthesized R in the minus strand DNA. This strand transfer or "jump" allows continued synthesis of the minus strand DNA. The capsid structure and the presence of nucleocapsid, NC, protein help facilitate the alignment after the "jump" (Allain *et al.* 1994). The remainder of the RNA template is transcribed into minus-strand DNA and degraded by the RNase H activity except for a polypurine (PP) tract located just 5' to the U3 region. This tract resists the RNase H activity and leaves the RNA bound to the DNA to serve as a primer for synthesis of the plus-strand DNA (Resnick *et al.* 1984; Smith *et al.* 1984; Coffin 1996). The resultant minus-strand of DNA looks like this: 3'—PB-*gag*-*pol*-*env*-PP-U3RU5-$tRNA^{Trp}$--5'.

The plus-strand synthesis begins using the RNA PP region not digested by the RNase H activity as a primer. The RT, now using the DNA dependent DNA polymerase activity to transcribe the plus-strand DNA molecule, begins to elongate from the primer to the 5' end of the template including the tRNA that encodes for the original PB site. Elongation stops at the correct site due to a modified base in the tRNA, a m^1A residue, that cannot be copied (Coffin 1996). This allows for perfect alignment after the second "jump" to the PB region on the 3' end of the minus-strand DNA. Once the double stranded DNA provirus is complete; the remaining RNA primers are removed by the RNase H activity of the RT (Coffin 1996). Figure 1.2 summarizes the viral RNA to proviral DNA conversion. The error rate in incorporating incorrect nucleotides with reverse transcriptase is quite high when compared to DNA polymerase because it lacks a 3' to 5' exonuclease proofreading mechanism. RT has an error rate of about one nucleotide per 9,000-17,000 nucleotides for ASLV (Battula and Loeb 1976) as compared to DNA polymerase from *Escherichia coli*, which has an error rate of one in 100,000 nucleotides (Battula and Loeb 1976). This error may have contributed to the highly variable strains of ALV-J subgroup that have recently been described (Venugopal *et al.* 1998; Venugopal 1999; Silva *et al.* 2000).

The next step is integration of the proviral genome into the host cell. How the DNA gets into the nucleus has not been fully described for ASLVs. Murine leukemia virus (MLV) can be found in the cytoplasm for many hours before they appear in the nucleus. It seems that mitosis is important for MLV to get into the nucleus and integration occurs in post replication DNA (Haihosseini *et al.* 1993). The same is not true for lentiviruses. Controversial evidence suggests that nuclear localization signals in

MA (Bukrinshy *et al.* 1993) or Vpr (Heinzinger *et al.* 1994) direct the preintegration DNA through the intact nuclear membrane (Bukrinsky *et al.* 1992). Two other forms of provirus exist in the nucleus. They are covalently closed circles with one or both LTR (Coffin 1996). Once believed to be intermediates in integration (Panganiban and Temin 1984), now it is known that they are functionally dead ends of aberrant transcripts that cannot be integrated (Randolph and Champoux 1993).

The terminal ends of the LTRs are highly conserved in all retroviruses (Varmus and Brown 1989). The LTR's outside edges have inverted repeats that serve as the retroviral attachment (att) site which function in integration (Varmus and Brown 1989). The repeats are about 20 nucleotides long and the terminal four nucleotides are 5'-AATG...CATT-3' (Varmus and Brown 1989). The site of integration in the host DNA has been determined to be random for retroviruses as a whole group (Coffin 1996). However, when examining B cell lymphomas in chickens, the integration site seems to be in the first intron of c-*myc* (Robinson and Gagnon 1986). The integrase enzyme, IN (p32), cleaves the two terminal nucleotides off leaving a –OH on the 3' ends of the proviral DNA. IN also cleaves the host DNA in a straggered manner to allow strand transfer of the proviral DNA into the host DNA. Once inserted, the host cell machinery fills the the gaps and ligates the ends together. Overall, this process results in duplication of host DNA at the ends of the proviral DNA (Luciw and Leung 1992).
Expression of the provirus is now controlled by the host cell systems. The LTRs have the signals that are recognized by the cellular transcription machinary. The 5' LTR acts as the promoter for viral RNA synthesis by RNA polymerase II, the same enzyme that creates mRNA. All retroviruses have a TATA box in the U3 region, 20-30 base pairs

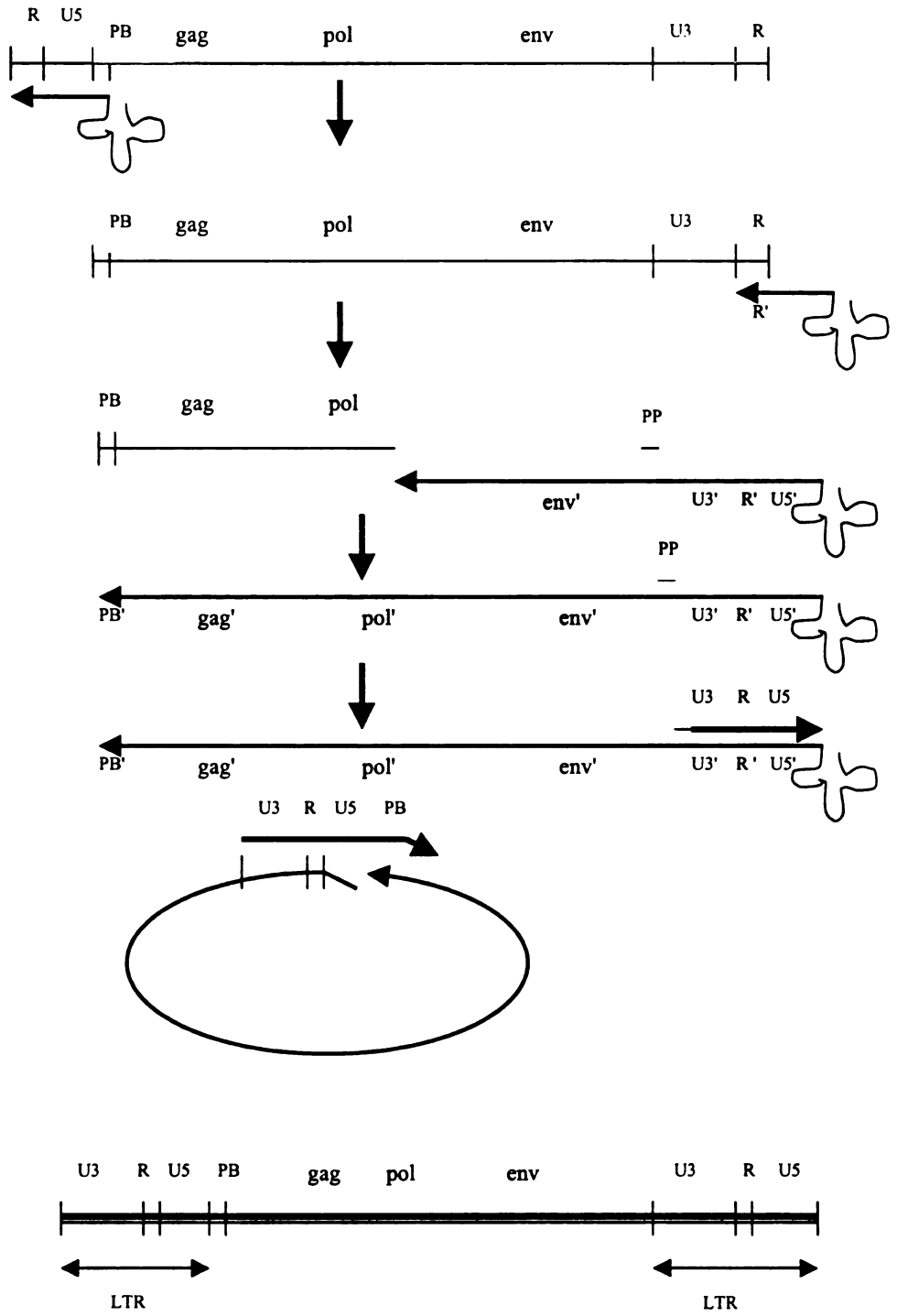

Figure 1.2. Summary of reverse transcriptase activity. Adapted from *Fields Virology* 3rd ed.

upstream from the initiation site (Lewin 1990). The TATA-binding protein (TBP) recognizes the TATA box and binds. Upstream from the TATA box is a region called the CCAAT box, which is important in determining the efficiency of the protomer (Lewin 1994). There are also enhancer elements still further upstream in the U3 region. The role for enhancers is thought to be to increase the numbers of transcription factors in the area of the promotor (Lewin 1994).

Once all the transcription factors have been recruited to the area of the promoter and put into place, the polymerase can create RNA transcripts (viral genome). The newly synthesized RNAs are capped on the 5' ends with a 7-methylguanosine designated as 7mG and methylated by host enzymes (Stoltzfus 1988). A polyadenylation (poly(A)) site in 3' U3 adds a poly(A) tail to the end of the transcripts. The 5' U3 poly(A) site is not found in the transcripts of ALSVs (Coffin 1996).

The newly synthesized RNA transcript can now travel down three different pathways. First is to be genomic RNA that gets packaged into the new virions. Second is to be messager RNA (mRNA) for translation of gag encoded polyproteins. Third is to be precursors for subgenomic viral transcripts (Varmus and Swanstrom 1982; Varmus and Swanstrom 1985; Stoltzfus 1988). The subgenomic messages are created by splicing the leader sequence from the 5' end to an acceptor site within the genome. This results with all spliced transcripts with the same 5' and 3' ends as the viral genome (Luciw and Leung 1992). The *env* gene is expressed from a spliced transcript for all retroviruses (Luciw and Leung 1992). The 5' donor site is usually located in the leader sequence upstream from *gag* in most retroviruses; for ALSV it is found six codons into *gag* (Coffin 1996). What determines which pathway the RNA goes is unknown.

The *gag* and *pol* genes are translated into proteins via the typical fashion of the ribosomal subunits binding to the capping group and then scan the RNA for the GAG AUG start codon. At the end of *gag* is a translational terminator. *pol* is in a different reading frame (Coffin 1996) from *gag* and requires a frame shift in the –1direction in order for the ribosome subunits to continue downstream and translate POL proteins (Coffin 1996). This slippage occurs about 5% of the time (Jacks *et al.* 1988) when the tRNA recognizes a specific sequence in the ribosomal acceptor site. For ALSV these sequences are A AAU UUA plus a psuedoknot structure after the seven nucleotides (Chamorro *et al.* 1992).

The ENV protein is synthesized on polyribosomes associated with the rough endoplasmic reticulum. All retroviral ENV precursor proteins stay anchored to the membrane by the hydrophobic membrane spanning domain near the carboxy end (Perez and Hunter 1987). Modifications take place in the Golgi body. Then the ENV precursor protein is cleaved into SU(gp85) and TM (gp37) (Hunter and Swanstrom 1990). The cleavage always takes place at a characteristic amino acid sequence. For example, in RSV, the sequence is Arg/Lys-x-Lys-Arg (Dong *et al.* 1992).

Virion assembly is becoming more understood for retroviruses because of the research on HIV. There are two mechanisms of assemby depending on the retroviral group. With B-, D-type, and spumavirus groups, capsid assembly occurs in the cytoplasm while the others assemble at the cell membrane (Coffin 1996; Sakalian and Hunter 1998). How the capsid precursor protein gets to the assembly site is still unknown, but is mediated by the MA domain of the GAG (Kräusslich and Welker 1996). Once the GAG and GAG-POL precursors are at the plasma membrane, self assembly into

capsids occurs (Sakalian and Hunter 1998). The genomic RNA also needs to get to the plasma membrane area in order to be included with in the virion. How this occurs is also unknown but is believed to migrate with the viral proteins(Kräusslich and Welker 1996). Two copies of the viral RNA genome are packaged (Berkowitz *et al.* 1996). The packaging signal lies within the 5' untranslated region of the genome and is termed Ψ (Mann *et al.* 1983). And a specific host cell tRNA is incorporated in the virion before budding occurs (Faras *et al.* 1974; Waters and Mullin 1977). For all ASLVs, it is $tRNA^{Trp}$ (Faras *et al.* 1974; Faras *et al.* 1974; Waters *et al.* 1975; Waters and Mullin 1977).

Three regions in the GAG protein have essential functions for budding of the virus to occur (Craven and Parent 1996; Sakalian and Hunter 1998). They are designated M, I, and L. M, the membrane binding domain, is located at the amino terminus of the GAG protein (Nelle and Wills 1996; Verderame *et al.* 1996). Any mutations in this area prevent assembly from occuring (Wills *et al.* 1991). I domain or the interaction domain functions in holding GAG proteins in place near one another for assembly to occur (Craven and Parent 1996). I appears to be located in the NC domain of GAG and confers density to the virion by interaction of NC and RNA, directing the alignment of proteins (Craven and Parent 1996). The L or late domain is essential for budding or releasing of the particle from the cell membrane (Wills *et al.* 1994). It is located near the MA cleavage site, in the p2b region of GAG (Craven and Parent 1996). How the L domain functions is unknown in the budding mechanism (Craven and Parent 1996).

After the particle buds from the cell membrane, it undergoes maturation to become infectious. The viral protease (PR) enzyme is responsible for cleavage of the

GAG polyprotein into its three major proteins, CA (p27), MA (p19) and NC (p12). Once the cleavage has taken place, the virion is mature and infectious (Vogt 1996).

E. ***Endogenous Viruses***

A unique feature of retroviruses is their ability to be inherited in the germline in a relatively stable manner (Coffin 1982). There are three well-established retrovirus-like families in the normal chicken genome plus a newly described family. These families include the endogenous viral or *ev* loci, the EAV or ancient endogenous viral elements (Dunwiddie *et al.* 1986; Boyce-Jacino *et al.* 1992), and avian retrotransposons of chickens or ART-CH (Gudkov *et al.* 1992; Nikiforov and Gudkov 1994). There are about fifty copies of EAV and ART-CH in a normal chicken genome. The newly described family is the ev/J family (Benson *et al.* 1998; Ruis *et al.* 1999). Just recently ev/J family was determined to be a member of EAV by sequence analysis (Sacco *et al.* 2000). There is also a highly repetitive element known as CR1 (Stumph *et al.* 1984).

There have been at least 29 different *ev* loci identified (Payne and Fadly 1997) and there is, on average, 5 *ev* loci per chicken (Rovigatti and Astrin 1983). Endogenous viruses are assigned the subgroup E designation. They can be complete infectious virions or defective viruses that partially express proteins from the *gag* gene and/or *env* gene. Sometimes they are transcriptionally silent like *ev*-1 (Coffin 1982). Protein expression from these *ev* loci can interfere with interpreting diagnostic tests like the enzyme-linked immunosorbent assay (ELISA) and complement-fixation test for ALV (COFAL) as well as the chick helper factor (chf) test, because they will give positive reactions. The prototype virus for subgroup E is RAV-0. It was first described as originating from spontaneous production in line 7 chicken embryo cells (Vogt and Friis 1971).

The phenotypic character of endogenous viruses has been determined by what viral products are expressed in uninfected cells. The first example is gs, which refers to group specific antigens encoded by the *gag* gene. Dougherty and Di Stefano (1966) described the first endogenous virus in a COFAL assay on uninfected cells. To be classified as gs+, the cells must have the *ev*3 locus (Astrin and Robinson 1979; Coffin 1982). Another example is the chf. To be chf+, cells must produce endogenous subgroup E ENV proteins that complement *env* gene defective RSV(-) to allow production of infectious particles without a helper virus (Vogt 1967). Several loci encode for the chf+ phenotype, *ev*3, *ev*6, and *ev*9 (Payne and Fadly 1997).

One *ev* locus, *ev21*, has become very important for the poultry industry. It encodes for a complete virus known as EV21. EV21 has been mapped to the Z chromosome and is linked to the dominant sex linked gene K which regulates slow feathering (Warren 1925). Late feathering can be differentiated from early feathering and is commonly used for sex determination of newly hatched chicks by chicken breeders and growers (Warren 1930). EV21 can be transmitted congenitally to the progeny and therefore increases the susceptibility of chicks to exogenous ALV (Harris *et al.* 1984; Bacon *et al.* 1988; Smith and Fadly 1988; Smith *et al.* 1991).

Endogenous viruses rarely cause tumors because of the weak promoter activity of the LTR (Motta *et al.* 1975; Payne and Fadly 1997). The endogenous viral loci can be beneficial or detrimental to the host depending on whether they are expressed or not by creating resistance or tolerance (Payne and Fadly 1997). Also, endogenous viral genes have been shown to be nonessential for chicken survival (Astrin *et al.* 1979). Chickens that lack *ev* genes are designated line 0 (Crittenden and Fadly 1985). Line 0 chickens are

useful research tools for chickens experiments as well as providing cells lacking *ev* loci for biological assays. Line 0 chicken embryo fibroblasts (CEF) are resistant to infection with subgroup E viruses and are designated as C/E.

F. ***Pathogenesis and Pathogenicity***

1. **Natural and Experimental Hosts**

Chickens are the natural hosts for all subgroups of ALV (Payne 1987). Other species like pheasants, partridges and quail are also natural hosts but only for certain subgroups. Turkey cells are susceptible to ALV A through E and J (Payne *et al.* 1992) but rarely do turkeys develop tumors after infection. Experiments with subgroup A ALV and turkeys inoculated as embryos or at hatch show that turkeys develop non-neoplastic lesions like inflammation and lymphoid proliferation in various visceral organs (El-Mubarak *et al.* 1983). Osteopetrosis had been reported in turkeys experimentally (Holmes 1964). Just recently, turkeys were experimentally infected and developed tumors with strain 966, an acutely transforming ALV-J (Venugopal *et al.* 2000). Rous sarcoma virus has the largest host range and can result in tumor formation in chickens, turkeys, pigeons, ducks, guinea fowl, pheasants, rock partridges, and Japanese quail (Payne and Fadly 1997).

2. **Transmission**

Exogenous ALVs can be transmitted in two ways: horizontally by bird to bird contact, either direct or indirect, and vertically from hen to progeny via the egg (Cottral *et al.* 1954; Rubin *et al.* 1961; Rubin *et al.* 1962). Vertical transmission is very important in terms of epidemiology for virus spread. Vertical transmission results in chicks that are viremic tolerant that can spread the infection to their hatch mates. Viremic chicks

maintain the infection and transmit virus to the next generation. Horizontal infection is thought to be important also in maintaining a vertical transmission rate because sometimes a small percentage of highly susceptible chickens become viremic tolerant when exposed to virus soon after hatch. Together, these two methods of transmission allow the virus to maintain itself in the chicken population (Payne and Bumstead 1982).

Endogenous ALVs are transmitted genetically in the germ cells of both male and females and by contact among susceptible chickens. For subgroup E viruses that are defective and cannot give rise to infectious particles, they can still express some proteins that influence how the chicken responds to infection with an exogenous ALV (Crittenden *et al.* 1982). If the endogenous virus can produce a complete infectious virus, for example EV21, then it can be transmitted like an exogenous ALV. Many chickens are resistant to such infections because of the endogenous viral genes within their genome (Crittenden 1981; Crittenden and Astrin 1981).

There are four serological categories in mature chickens with regards to ALV infection: 1-virus negative, antibody negative (V-, A-); 2-virus negative, antibody positive (V-, A+); 3-virus positive, antibody positive (V+, A+); 4-virus positive, antibody negative (V+, A-) (Rubin *et al.* 1961; Rubin *et al.* 1962). Category 1 contains birds that are in an infection free flock and genetically resistant birds in a susceptible flock. Chickens that are genetically susceptible and in an infected flock fall into the other three categories. Most will be V-, A+ and a small percentage will be V+, A-. The remaining category consists of birds in the midst of seroconversion when tested. The V+, A- chickens transmit virus to the majority of their progeny (Rubin *et al.* 1962; Payne *et al.* 1982). These congenitally infected embryos fall into the V+, A- category once they

hatch. A small percentage of the V-, A+ hens can transmit the virus to their progeny on an intermittent basis, which may be due to a low antibody titer in those hens (Tsukamoto *et al.* 1992).

Viral shedding in the oviduct comes from the albumen secreting glands (Payne and Fadly 1997). This results with virus in the albumen but does not necessitate that the chick will be virus positive when hatched. Several studies have shown that $1/8^{th}$ to ½ of the embryos were infected from eggs with virus in the albumen (Spencer *et al.* 1977; Payne *et al.* 1982; Tsukamoto *et al.* 1992). This may be due to maternal antibodies in the yolk that were sufficient enough to neutralize the virus. Another explanation for this phenomenon, is inactivation of the virus during storage before incubation. Storing the fertile eggs at various temperatures resulted in decreased viral titers in the albumen, thus lowering the prospect of the developing embryo to be infected (Fadly and Okazaki 1979).

3. Host and Environmental factors

The age of the host at exposure determines the rate of development of tumors. Usually as the host ages, resistance to tumor formation increases (Burmester *et al.* 1960). However, route of inoculation also is important. Administering the virus via the oral or nasal cavities in young birds can lead to tumors but usually not after three weeks of age. Using an intravenous route, birds are susceptible for longer periods of time (Burmester *et al.* 1960). Females are more susceptible to LL than males. Testosterone seems to increase resistance (Burmester and Nelson 1945) to tumor formation. The genotype of a bird is also important and will be discussed later.

4. **Viral/Cellular Oncogenes**

Viruses that carry oncogenic or *onc* sequences have been isolated from many animal species. The sequences were traced back to cellular genes that fall into one of several categories: growth factors, growth factor receptors, signal transduction, intracellular tyrosine kinases, serine/threonine kinases, and transcription factors (Gordon 1985; Lewin 1994). Viral and cellular sequences are termed as *v-* and *c-* oncogene, respectively. The differences between a *v-onc* and *c-onc* usually is that *c-onc* contains introns and is expressed at low levels while a *v-onc* does not have introns and is expressed in high levels (Lewin 1994).

Examples of *v-oncs* in avian retroviruses are *src, myc, myb, erb,* and *rel.* The first four are found in ASLVs and *rel* is found in strain T of reticuloendotheliosis virus. Rous sarcoma virus was the first retrovirus isolated and characterized along with its oncogene, *src* (Bishop and Varmus 1982). The protein was named $pp60^{v\text{-}src}$. It is characterized as an intracellular tyrosine kinase (Bishop and Varmus 1982; Lewin 1994). There are at least eight subfamilies of tyrosine kinase. The *c-src* family of proteins is activated by many different signals. Some include mitosis, growth factors, cytokines, and G proteins (Erpel and Courtneidge 1995). The proteins that SRC interacts with demonstrate its multifunctionality. They include proteins involved in cytoskeleton organization, cell to cell adhesion, cell to cell communication, cell to substrate adhesion, and RNA processing (Brown and Cooper 1996).

What makes *v-src* different than *c-src* is the truncation and replacement of sequences encoding the COOH terminus so that Y527 (Tyrosine 527) is deleted (Parson and Weber 1989). This conserved sequence and constant position serves as the major site

of phosphorylation because without it, the *v-src* protein has increased kinase activity with no inhibitory regulation that phosphorylation at Y527 provided (Cooper *et al.* 1986). By examining what pathways *c-src* protein interacts with in the cell, the over production of the protein *v-src* could easily cause uncontrolled cell growth and transformation.

The oncogene *v-myc* was determined to be responsible for transformation by the virus MC29 that causes myelocytomatosis (Sheiness *et al.* 1978; Reddy *et al.* 1983; Enrietto and Hayman 1987). A fusion protein of GAG-MYC which has 452 N terminal residues encodes the transforming protein and 416 of these residues come from exons 2 and 3 of *c-myc* (Reddy *et al.* 1983). MC29 that has *v-myc*, is a transforming defective virus because part of *gag*, all of *pol* and most of *env* are missing (Reddy *et al.* 1983).

The cellular component *c-myc* was first described in 1980 (Sheiness *et al.* 1980). *c-myc* belongs to the *myc* family of genes which include B-*myc*, L-*myc*, N-*myc*, and s-*myc*. It encodes the transcription factor c-MYC, which interacts with another protein MAX to control gene expression (Dang *et al.* 1999). There are over forty target genes that c-MYC can act upon as an up regulator or down regulator for cell growth (Dang *et al.* 1999). Because of the vast number of target genes that regulate cell growth affected by *c-myc*, the potential for cell transformation is high if the highly regulated *c-myc* becomes unregulated for some reason. While most of the research on *c-myc* as a proto-oncogene has been in human carcinogenesis, viral induction of *c-myc* was described in the early 1980's (Crittenden and Kung 1984). In chickens infected with avian leukosis virus, the integration of the proviral DNA upstream of *c-myc* can cause transformation of B cells due to the strong promoter properties in the LTR (Crittenden and Kung 1984).

Avian myeloblastosis virus (AMV) contains the oncogene *v-myb* along with avian E26 leukemia virus. Both are defective viruses. In AMV, most of the *env* gene has been replaced by *v-myb* (Duesberg *et al.* 1980). There are ten substitutions in *v-myb* compared to *c-myb* in the shared region (Gerondakis and Bishop 1986). These substitutions affect the phenotype of the transformed myeloid cells (Introna *et al.* 1990). In the DNA binding region, there are four amino acid substitutions in *v-myb* that affect the ability of the MYB protein to transform different cell types, to control specific genes, and to be controlled by other host proteins (Introna *et al.* 1990). Truncation of *c-myb* can cause transformation. Truncation of the N-terminus only is highly transforming while truncation of C-terminus only is weakly transforming (Lipsick and Wang 1999). As the immature cells of the hematopoietic and lymphoid systems go through their differentiation process, *c-myb* is expressed on the cells. As they become mature, this expression decreases rapidly (Chen 1980; Westin *et al.* 1982). It is also expressed in other tissues that can become cancerous. Therefore, the narrow spectrum of tumors seen with AMV may be due to the mutations specific to *v-myb* versus *c-myb* having a limited potential as an proto-oncogene (Lipsick and Wang 1999).

The final *v-oncs* are found in avian erythroblastosis virus (AEV). It is *v-erb* that is two separate oncogenes, *v-erbA* and *v-erbB*. Both genes are necessary for full transformation of erythroblasts because *v-erbA* does not cause transformation itself, but increases the transformation activity of *v-erbB* (Lewin 1991). Recent research indicated that *v-erbA* expression has two different effects on target cells: it prevents the erythroblasts that have been transformed by *v-erbB* from spontaneous maturation into

erythrocytes and expands the range of conditions that allow the erythroblasts to propagate (Lewin 1991).

The cellular homolog is *c-erbA* which encodes the nuclear receptor for thyroid hormone (Sap *et al.* 1986; Weinberger *et al.* 1986; Sawyers *et al.* 1991). The oncogenic gene *v-erbA* is a 75 kDa fusion product of *gag* and *v-erbA* (Thormeyer and Baniahmad 1999). Functionally, the two proteins are different: c-erbA protein binds to triiodothyronine (T_3) but v-erbA protein has little affinity for T_3 ligand. It can no longer be stimulated to activate transcription of the avian erythrocyte anion transporter gene (Zenke *et al.* 1990). The truncation at the 3' end of *v-erbA* is responsible for this loss because that region had been mapped to the very end of the 3' end of *c-erbA (Zenke et al. 1990).*

The gene *c-erbB* encodes the epidermal growth factor receptor (Sawyers *et al.* 1991). The *v-erbB* product has lost the regulation of expression by lacking the ligand binding domain and therefore tyrosine protein kinase function of *v-erbB* may be constitutively activated (Kris *et al.* 1985).

5. Non-neoplastic Conditions

Sometimes when chickens are exposed at a young age to certain strains of ALV, other disease manifestations besides neoplastic conditions can occur. Examples include hepatitis, anemia, immunodepression and wasting with infection of RAV-1, RAV-60, MAV-2 and subgroups B and D (Crittenden *et al.* 1982; Smith 1986). The immunodepression was described as decreased lymphoid cells in the bursa and spleen, decreased antibody response, and hypergammaglobulinemia (Smith 1986).

In addition to tumor disease conditions, ALV infection has been shown to decrease egg production in layer breeder hens (Gavora *et al.* 1980; Payne and Fadly 1997). Various researchers have determined that ALV infection can also affect growth and performance of the chickens (Gavora *et al.* 1980; Crittenden *et al.* 1983). In males, ALV in the semen does not affect sperm production, but quality may be affected slightly (Segura *et al.* 1988).

G. ***Viral Induced Neoplasms of Chickens-Gross and Microscopic Lesions***

1. **Marek's Disease**

Marek's disease (MD) is caused by an alpha herpesvirus. It induces lymphoid tumors in visceral organs. It also causes paralysis and in the case of infection with very virulent viruses, bursal and thymic atrophy are the only lesions seen after death. There are vaccines available to control MD tumors.

Gross lesions can consist of a single lesion or a combination of the following lesions. There is unilateral enlargement (2-3X normal size) of peripheral nerves, which are edematous and have grey to yellow discoloration. Lymphoid tumors can occur in one or more organs resulting in enlargement due to diffuse cellular infiltrate or due to nodular formation. The tumors are grey to white in color. (Riddell 1987; Calnek and Witter 1997).

Microscopically, MD tumors are composed of pleomorphic lymphoid cells. The neoplastic cells have been determined to be mostly T cells, with a few B cells admixed with a few plasma cells. There is also lymphocytic infiltration in nerves, lymphoid cell cuffing in the cerebellum, infiltration in the feather follicles by lymphoid cells, and sometimes, lymphoid infiltration in the iris resulting in a lesion known as "Marek eye".

MD can also affect the bursa, causing interfollicular tumors with a pleomorphic lymphoid cell population (Riddell 1987; Calnek and Witter 1997).

2. **Reticuloendotheliosis**

Reticuloendotheliosis is a group of diseases caused by a retrovirus in the Mammalian Type C group called reticuloendotheliosis virus (REV). REV causes three syndromes: An acute reticulum cell neoplasm, a chronic lymphoid neoplasm, and a runting syndrome. The chronic lymphoid neoplasm and runting syndrome is caused by nondefective REVs in nature.

With REV infection in chickens there can be two forms of the lymphoid neoplastic disease, a bursal form and a nonbursal form. Grossly, the lymphoid tumors can be diffuse or nodular and grayish white in appearance. The bursal form mainly affects the bursa of Fabricius and liver. The neoplastic cells are B cells, which makes this form of REV indistinguishable from LL. The nonbursal form resembles Marek's disease with thymus, heart, liver and spleen affected with lymphomas. The neoplastic cells have been determined to be T cells (Riddell 1987; Witter 1997).

3. **Lymphoproliferative Disease**

Lymphoproliferative disease of turkeys (LPD) is a disease cause by a retrovirus in the Mammalian Type C group of viruses. Grossly, the predominant lesion is splenomegaly. The spleen can be pale pink and mottled or contain miliary white to gray foci. Sometimes these foci occur in pancreas, kidneys, thymus, lungs, heart, gonads, and intestinal wall. There can be peripheral nerve enlargement (Riddell 1987; Biggs 1997).

Microscopically, the characteristic lesion is lymphoproliferation of pleomorphic cells like lymphocytes and lymphoblasts, plasma cells and macrophages scattered throughout the lesion. Nerve lesions are more focal in distribution than diffuse and consist of the same pleomorphic cells (Riddell 1987; Biggs 1997).

4. **Lymphoid Leukosis**

Grossly visible tumors can be seen in various visceral organs but most commonly affected organs are liver, spleen and bursa of Fabricius. Tumors are smooth, soft and glistening. Cut surface appears to be slightly gray to off white. Neoplasms can be nodular, miliary or diffuse in distribution or a combination of these. The nodules can range from 0.5 mm to 5 cm in diameter and occur by themselves or in clusters. The miliary form can be seen best in the liver as numerous, small nodules no larger than 2mm in diameter evenly distributed throughout the organ parenchyma. The diffuse form causes the organ to be uniformly enlarged with a slight gray color and a very friable texture (Whiteman and Bickford 1989; Jordan 1990; Payne and Fadly 1997).

Microscopically, all lymphoid leukosis tumors are focal and multicentric in origin. Organs are affected diffusely with coalescing foci. The cells displace and compress the normal parenchyma rather than infiltrate between normal cells. The cell type consists of large, lymphoblastic cells that are all in the same stage of development, i.e., a monomorphic population. The cytoplasm is very basophilic and the nucleus has prominent nucleoli and margination and clumping of chromatin (Riddell 1987; Payne and Fadly 1997). There have been two reports of viral inclusion bodies seen in the myocardium (Gilka and Spencer 1985; Nakamura *et al.* 1988).

5. **Erythroblastosis**

Diffuse enlargement of the liver and spleen, and sometimes the kidneys, are the most characteristic gross lesions of erythroblastosis. These organs are usually cherry red to deep mahogany in color and are soft and friable. The liver may be finely mottled due to degeneration surrounding the central veins of the lobules. The bone marrow is hyperplastic, very soft or watery, cherry red to deep mahogany and often has hemorrhages. The affected birds appear anemic and have petechial hemorrhages in various organs (Jordan 1990; Payne and Fadly 1997).

Microscopically, the bone marrow can have rapidly proliferating erythroblasts that fail to mature in the blood sinusoids. In advanced cases, there is little to no adipose tissue and small islands of myelopoiesis with sheets of homogeneous erythroblasts filling the marrow. When organs are involved, changes are usually due to hemostasis with accumulation of erythroblasts in the sinusoids and capillaries. As the sinusoid distends, it results in pressure atrophy of the parenchyma. The cells always remain intravascular unlike LL or myeloblastosis. The erythroblast has a large round nucleus with fine chromatin and one to two nucleoli and abundant amounts of basophilic cytoplasm. The cell itself is irregular shaped and can have pseudopodia (Riddell 1987; Payne and Fadly 1997).

6. **Myeloblastosis**

Gross lesions of myeloblastosis include enlarged and friable parenchymal organs. There can be gray diffuse tumor nodules in the liver and occasionally in other visceral organs. The bone marrow is firm and reddish gray to gray. With advanced cases, grayish infiltrations in the liver, spleen and kidneys are diffuse and give the organ a mottled

appearance or sometimes a granular appearance. There is usually an anemia that may cause paleness to be noted in the comb as well as hemorrhage from feather follicles due to clotting deficiencies (Jordan 1990; Payne and Fadly 1997).

Microscopic lesions include massive intravascular and extravascular accumulations of myeloblasts and some promyelocytes in the parenchyma of various organs. There is infiltration and proliferation outside the sinusoids and around portal tracts in the liver. The bone marrow has extensive myeloblastic activity in the extrasinusoidal areas. The myeloblast is a large cell with slightly basophilic cytoplasm and a large nucleus with 1-4 acidophilic nucleoli which may not stain well (Riddell 1987; Payne and Fadly 1997).

7. **Myelocytomatosis**

Gross lesions of myelocytomatosis consist of tumors that are characteristic and usually easily recognizable. The tumors occur on the surface of bones and near cartilage, although any organ can be affected. They usually develop on the costochondral junctions of the ribs and the inner sternum. Sometimes the cartilaginous bones of the mandible and nares can be affected. Flat bones of the skull also can be affected. Myelocytomas are dull, yellow-white, soft and friable or caseous, and diffuse or nodular. They sometimes have a thin layer of bone covering them that can be broken easily (Jordan 1990; Payne and Fadly 1997).

Microscopically, the tumors consist of uniform myelocytes with little stroma. Their nuclei are large, vesicular and eccentrically located. A distinct nucleolus is common also. The cytoplasm is filled with eosinophilic spherical granules. The cells are

similar to myelocytes found in normal bone marrow (Riddell 1987; Payne and Fadly 1997).

8. **Nephromas and Nephroblastomas**

There are two types of ALV-induced tumors of the kidney: 1) nephroblastomas of the complex Wilm's, and 2) adenomas or carcinomas. Grossly, the tumors can range from small grayish nodules embedded in the parenchyma to large, yellowish gray lobulated masses that replace most of the normal kidney tissue. The tumors can be pedunculated and connected by a fibrous vascular stalk. The larger tumors are often cystic and can occur in both kidneys (Payne and Fadly 1997).

Nephromas microscopically can vary greatly. When the tubules are affected, there can be primitive abnormal glomeruli among the abnormal tubules. Frequently cystic adenomas are seen. Hemangiomas and endotheliomas sometimes occur within the nephroma (Riddell 1987; Payne and Fadly 1997).

Nephroblastomas have great variation histologically. Both epithelial and mesenchymal elements are affected to varying degrees. The epithelial structures can vary from enlarged tubules with invaginated epithelium with malformed glomeruli to irregular masses of distorted tubules to large, cuboidal undifferentiated cells with little to no tubular formation (Riddell 1987; Payne and Fadly 1997). There may be islands of keratinizing stratified squamous epithelial structures (pearls), bone, or cartilage (Ishiguro *et al.* 1962). Metastases of these tumors are rare.

9. **Osteopetrosis**

Gross lesions include changes in the diaphysis of the tibia and/or tarsometatarsus. Other bones that can be affected are other long bones, pelvic bones, shoulder girdle and

ribs. The lesions are typically bilaterally symmetrical and begin as pale yellow foci against the normal bone. The periosteum is thickened and the abnormal bone is spongy. The lesion is circumferential and advances to the metaphysis, resulting in a fusiform looking bone. The lesion can vary in severity from a slight exostosis to massive asymmetric enlargement with almost complete occlusion of the bone marrow cavity (Jordan 1990; Payne and Fadly 1997).

Microscopically, the periosteum is thickened over the lesion due to increased number of basophilic osteoblasts. There is a size increase and irregularity of the haversian canals. There is also an increase in size and number of lacunae and their positioning is altered. Osteocytes are numerous, large and eosinophilic and the new bone is basophilic and fibrous (Riddell 1987; Payne and Fadly 1997).

10. **Hemangioma**

Grossly, hemangiomas can appear as dark red, circumscribed and raised nodules on the skin or surface of visceral organs. When the tumor ruptures in an organ, blood clots are found in the peritoneal cavity. Also the chicken will appear pale and anemic. Exsanguination can occur.

Microscopically, the neoplasms are composed of blood distending channels lined by endothelial cells. The endothelium may proliferate as dense solid masses, or may form a latticework with small capillary-sized spaces or large vessels supported by collagenous cords. (Riddell 1987; Payne and Fadly 1997).

H. ***Immunity***

Both humoral and cell-mediated immunity are involved with ALV infection. After becoming infected from hatch-mates or their surroundings, most chicks have a

transient viremia then develop virus-neutralizing antibodies that rise to a high titer and persist throughout the life of the bird (Rubin *et al.* 1962; Solomon *et al.* 1966). Antibodies to ALV are passed from the dams to progeny, and passive immunity lasts for 3-4 weeks after hatch (Payne and Fadly 1997). The cytotoxic T lymphocyte activity has been shown to influence immunity to ALV (Bauer *et al.* 1976).

Reports on Rous sarcomas illustrate that a tumor-bearing host will also respond to tumor-associated cell surface antigens or TASAs. This response serves to retard growth of the tumor or cause regression. How this process works is not understood well. Immunodepression has been associated with ALV infection in some instances. However, it has also been documented that B and T cell functions are normal with a subgroup A infection (Fadly *et al.* 1982) and, most recently, that heterophil, macrophage and lymphocyte functions in chickens with a subgroup J infection are the same as uninfected controls (Stedman *et al.* 2000).

I. ***Genetic Resistance***

1. **Cellular Resistance to Infection**

Genetic cellular resistance to ALV infection means that the virus cannot infect the cell. Various reports suggested that a single autosomal dominant gene was responsible for controlling the susceptibility to RSV (Prince 1958; Waters and Burmester 1961). This was later proven with subgroup A RSV infection using various assays (Crittenden *et al.* 1964; Crittenden *et al.* 1971). It is now known that independent autosomal loci control the responses to infection by subgroups A, B, and C viruses. They are called *tva*, *tvb*, and *tvc*, respectively. These are the same loci that have genes that encode the various receptor proteins for those subgroups mentioned in a previous section. There is some

evidence of a linkage between *tva* and *tvc* loci (Payne and Pani 1971). Subgroup D viruses are controlled by the *tvb* locus (Pani 1975). Each locus has alleles for susceptibility and resistance with resistance being recessive (Crittenden 1968). These loci are inherited in a simple Mendelian fashion.

Inheritance of subgroup E ALV resistance is much more complicated. There are two autosomal loci involved, *tve* and i^e (Payne *et al.* 1971). The I^e gene is dominant for resistance and can block susceptibility when the *tve* susceptible allele is present. However, it has been reported that *tvb* alleles are required for susceptibility to subgroup E infection and there is a controversy over the existence of the *tve* locus (Crittenden *et al.* 1973; Pani 1974; Pani 1976). One study suggests that the i^e locus is actually an *ev* locus that expresses ENV glycoproteins which block the receptor for subgroup E infection (Robinson *et al.* 1981).

Genetic resistance to subgroup J ALV has not been documented in chickens. However, host range assays demonstrated that several avian species were resistant to infection (Payne *et al.* 1991; Payne *et al.* 1992). These avian species include Common pheasant, Japanese Green pheasant, Golden pheasant, Japanese quail, guinea fowl, Pekin and Muscovy ducks and geese (Payne *et al.* 1992).

2. **Resistance to Tumor Formation**

Genetic resistance to tumor formation has mainly been examined using Rous sarcoma virus. The major histocompatibility complex (MHC) or B complex in chickens was determined to influence the outcome of RSV inoculation in the wing web beginning in the 1970's. The B locus was involved with regression of tumors induced by RSV

(Collins *et al.* 1977). A MHC-linked gene allowed regression of RSV-induced tumors, R-Rs-1. Its allele, r-Rs-1, allowed progression of the tumor (Schierman *et al.* 1977).

As more knowledge was discovered about the B locus, three regions were determined to make up the locus, the B-F, B-G and B-L regions (Pink *et al.* 1977). Three different laboratories reported results that the gene that controlled regression lies within the B-F region (Collins and Briles 1980; Plachy and Benda 1981; Birkmeyer and Nordskog 1982). It also seems that the MHC gene associated with regression also restricted metastasis of the tumors (Collins *et al.* 1977). Recent research confirms that a gene or genes in the B-L/B-F regions or closely linked regions are responsible for the regression of Rous sarcomas (Auclair *et al.* 1995).

Genetic resistance to LL tumor development is less understood that genetic resistance to infection (Crittenden 1975; Schierman and Collins 1987). However, resistance documented in RPL line 6 chickens has been attributed to the bursal cells and their intrinsic ability to become infected or not (Purchase *et al.* 1977).

J. ***Diagnosis***

1. **Virus Isolation**

Virus isolation used to be carried out by chick inoculation, either subcutaneously, intramuscular, intraperitoneal, or intracerebral (Payne and Fadly 1997). These are slow and expensive ways to isolate virus. Currently, virus isolation is carried out in cell culture, which is faster and more economical. Suitable sample materials for virus isolation include plasma, serum, tumors, whole blood, peripheral blood mononuclear cells (Fadly and Witter 1998), infected organs, feces (Burmester 1956), albumen or 10-day-old embryos (Spencer *et al.* 1977), and feather pulp (Spencer *et al.* 1983). To

perform virus isolation in cell culture the correct chick embryo fibroblasts (CEF) are necessary. To detect all endogenous and exogenous ALVs, CEFs of the genotype C/O (susceptible to all subgroups) are necessary. To detect only exogenous ALVs, CEFs of the genotype C/E (cells resistant to subgroup E only) are necessary. There are various CEFs that have resistance to various subgroups. An example is ALV6 CEFs which are C/AE (resistance to subgroups A and E) developed by Crittenden and Salter (Salter and Crittenden 1989; Crittenden and Salter 1990; Salter and Crittenden 1991) via transgenics. Recently, a genetically engineered cell line that is resistant to subgroup J ALV (C/J) has been developed (Hunt *et al.* 1999).

Because most ALVs do not produce cytopathic effects in cell culture, a method for indirect detection of the virus is essential. Most diagnostic tests for ALV use detection of gs antigen p27, the viral capsid protein, as an indicator for the presence of the virus. The most common tests used for detection of p27 are COFAL (Complement Fixation for Avian Leukosis) (Sarma *et al.* 1964) and ELISA.

The most commonly used test for detecting p27 is the enzyme-linked immunosorbent assay or ELISA (Smith *et al.* 1979; Tsukamoto *et al.* 1991). ELISA kits for detection of p27 are now commercially available. Commercially available rabbit anti-p27 IgG and rabbit anti-p27 conjugated to horse-radish peroxidase can be used for in-house preparation of ELISA plates. Using ELISA, ALV or gs antigen can be detected directly on samples or indirectly on cell culture inoculated with samples. Interpretation of the results of the direct ELISA for p27 must be done with care due to the possibility of detecting endogenous p27 from *ev* loci. Serum has been shown to be unsuitable for use in direct assay for p27 (Payne *et al.* 1993).

2. **Serology**

Samples of plasma, serum or egg yolk can be used for antibody determination (Payne and Fadly 1997). The virus neutralization test is the most sensitive test for detecting antibodies to ALV. This test can be conducted in microtiter plates. The correct indicator virus for the desired subgroup and known positive antibody should be used. Equal volumes of sample and virus are mixed together and incubated for 45minutes. The entire mixture is then placed on CEFs. After culture for 7-9 days, the p27 ELISA is used on the lysates to determine the presence of p27. If the ELISA test is negative, there are neutralizing antibodies in that sample. If the ELISA test is positive, there are no neutralizing antibodies in the sample (Fadly and Witter 1998). Direct method for detecting ALV antibody has been developed using the ELISA (Smith *et al.* 1986). Kits for detection of antibodies of subgroups A, B and J ALV are commercially available. False positives can occur with microtiter virus neutralization for various technical reasons and cross-reactions can occur if endogenous virus antibody is present (Fadly and Witter 1998).

3. **Phenotypic Mixing Assays**

Samples of plasma or serum are suitable test materials for phenotypic mixing (PM) assays. The test requires C/O and C/E CEFs and virus stocks of RSV(Rous-associated virus –0) [RSV (RAV-0)]. RAV-0 is the prototype subgroup E ALV. C/O CEFs are co-cultured with RSV (RAV-0) for 1 day. The test sample is added and cultured for 5-7 more days. When discrete foci form from RSV (RAV-0) transformation, supernatant fluid is collected and centrifuged. The supernatant is then placed on C/E CEFs to detect RSV (with sample ALV envelope), not the original RSV (RAV-0), and

cultured for 5-7 days. Production of foci is considered positive (Okazaki *et al.* 1975; Fadly and Witter 1998). This assay forms the basis for the non-producer (NP) cell activation test (Rispens and Long 1970; Rispens *et al.* 1970). The NP activation test requires NP cells that have been transformed by defective RSV strain (RSV Bryan high-titer strain) and do not produce virus that is detected in tests using C/E CEFs. A Japanese quail cell line transformed by an envelope defective BH RSV, (R(-)Q), is also needed. Test samples are inoculated onto susceptible CEFs and co-cultivated with NP cells. The test is positive for ALV by testing for the presence of RSV. The R(-)Q cell test uses non producing R(-)Q cells and C/E cells infected with the test sample. Cocultivation of the two cell lines activates the R(-)Q cells to produce infectious RSV having the envelope of the sample exogenous ALV (Crittenden *et al.* 1979).

4. **Detection of Viral Nucleic Acids**

Two main methods for detecting viral nucleic acids include Southern blotting and hybridization and polymerase chain reaction (PCR). There are reported PCR methods for detection of proviral DNA for subgroups A and J (Van Woensel *et al.* 1992; Smith *et al.* 1998; Smith *et al.* 1998; Silva *et al.* 2000). However, these tests require that no mutations have occurred where the primers anneal in order to amplify the desired sequences. If there is a mutation that interferes with the primer, the test will permit false negatives. Southern hybridization uses specific probes labeled radioactively to detect proviral DNA in tissues or tumors (Weiss *et al.* 1982).

5. **Immunohistochemical Tests**

Direct and indirect fluorescent antibody tests can be used to detect antigen in infected cell cultures (Kelloff and Vogt 1966; Payne *et al.* 1966). Other

immunohistochemical staining methods have been used to detect group-specific antigen in a variety of tissues (Di Stefano *et al.* 1973; Gilka and Spencer 1984; Schnegg *et al.* 1994; Arshad *et al.* 1997; Arshad *et al.* 1999). Various staining methods include avidin-biotin complex and soluble enzyme immune complex. Each can use various enzymes to convert the chromogens into a colored product like horseradish peroxidase, alkaline phosphatase, glucose oxidase or beta-galactosidase. Using antibodies against gs antigen (p27) may also detect endogenous viral p27.

6. **Differential Diagnosis**

Differential diagnosis of tumors of poultry must include three diseases, avian leukosis, Marek's disease, and reticuloendotheliosis. In the 1970s, lymphoproliferative disease of turkeys was also included in the differential diagnosis of tumors in Europe and Israel.

MD causes paralysis. In the case of infection with very virulent viruses, bursal and thymic atrophy are the only lesions seen after death. MD can cause mortality in birds as young as four weeks of age and as old as 20 weeks, sometimes older. To reach a diagnosis of either MD or LL, one must evaluate the history of the flock, age of the flock, clinical signs, gross and microscopic lesions. There are various sources of information that can help a clinician or pathologist make that decision (Page *et al.* 1969; Siccardi and Burmester 1970; Yamamoto *et al.* 1972; Fadly and Witter 1998).

Historically, there have been few reports of RE infection causing neoplastic disease in the United States (Crittenden *et al.* 1979; Witter and Crittenden 1979; Hayes *et al.* 1992; Drew *et al.* 1998; Miller *et al.* 1998). In other parts of the world, it occurs more frequently (Sasaki *et al.* 1993; Witter 1997; Payne 1998). There was a recent report of

vaccine contamination with REV that resulted in lymphomas in commercial chickens (Fadly *et al.* 1996).

To determine if the bursal tumors seen with REV are caused by REV or ALV, one must complete virologic assays or conduct PCR on the tumors to determine which etiologic agent is the cause (Aly *et al.* 1993; Fadly and Witter 1998).

K. ***Prevention and Control***

Because there is no effective or practical treatment or vaccination for prevention of ALV infection, eradication is the preferred method of control. The key to eradication is to break the vertical transmission from dam to progeny. To establish an ALV-free flock, chicks must come from hens that are not infected or do not shed the virus, then are reared and maintained in a clean environment (Payne and Fadly 1997). Many methods to produce ALV-free flocks have been recommended. The use of immune, non-virus shedder hens to repopulate a flock is one method. These antibody-positive hens are selected on the assumption that they would be less likely to shed virus than hens without antibody. Three chicks per hen are tested for the presence of virus and hens that are negative are used to repopulated the flock (Hughes *et al.* 1963). Another method to produce an ALV negative flock is to choose hens that are non-immune, non-virus shedders. Here the assumption is that hens without antibody have not been exposed or infected by the virus, therefore, they are less likely to be intermittent shedders than antibody positive hens (Levine and Nelsen 1964). A third method to produce an ALV negative flock is to select nonviremic hens regardless of antibody status. This method could take up to four generations before becoming leukosis free and infection was not ruled out (Zander *et al.* 1975).

The application of eradication programs to commercial flocks has been based on information on relationships between hens, eggs, embryos and chicks (Spencer *et al.* 1977). Three associations came out of this research: 1) egg albumen may include gs antigen and exogenous ALV, and both are usually present together; 2) there is a strong association between ALV or gs antigen in egg albumen and ALV in vaginal swabs; and 3) there is an association between ALV in vaginal swabs or albumen and ALV in newly hatched chicks and embryos (Spencer *et al.* 1977). Several tests can be used to detect virus in swabs but it is well known that not all birds will be identified with one test (Payne and Fadly 1997). Therefore, depending on the company's philosophy about eradication or reduction of shedding, their eradication programs would be different.

An example of a complete eradication program involves four steps: 1) negative dams (negative vaginal swabs or egg albumen tests) to select fertile eggs (Okazaki *et al.* 1979; Fadly *et al.* 1981; Payne *et al.* 1982; Crittenden *et al.* 1984); 2) small group rearing and avoid contact transmission between groups (Fadly *et al.* 1981); 3) test chicks for virus by biologic assay or by PCR and discard all positive chicks and their pen mates (Okazaki *et al.* 1979; Fadly *et al.* 1981; Fadly *et al.* 1981; Payne *et al.* 1982; Payne and Howes 1991); 4) rear ALV-free groups in isolation.

Because chicks are most susceptible to infection, various good management practices can help reduce the chance of infection. Thorough disinfection of all incubators, hatchers, brooding houses, and equipment with a detergent will disrupt the viral envelope and render the virus inactive (Payne and Fadly 1997).

Another method for prevention and control is to select breeders that are genetically resistant to ALV. Birds that lack the receptor for a certain subgroup cannot

become infected with that subgroup. See previous section on Genetic Resistance. At least one primary broiler breeder company has selected chickens resistance to subgroup A through genetic selection (McKay 2000).

The use of vaccines to control ALV has not been successful at all. Burmester tried to inactivate ALV by various methods and could not produce an antibody response to the inactivated product. Attempts to produce a modified live or attenuated ALVs also have failed in that all strains tested produced tumors (Okazaki *et al.* 1982). Recently, recombinant ALVs expression various envelope proteins have been created as vaccines (McBride and Shuman 1988; Chebloune *et al.* 1991; Wright and Bennett 1992).

L. ***Subgroup J Avian Leukosis Virus***

1. **Introduction**

In 1991 the first description of a novel subgroup (J) of ALV (ALV-J) affecting chickens was documented in the United Kingdom (Payne *et al.* 1991). The isolates came from commercial meat-type chickens. The acronym HPRS, which stands for Houghton Poultry Research Station, was assigned to each isolate plus a number. Isolate HPRS-100 was isolated from tissues of a line 20 chicken with ascites syndrome. Isolates HPRS-101 and 102 were isolated from vaginal swabs of normal hens from line 20. Isolate HPRS-103 was isolated from a vaginal swab from a normal hen of line 23. Isolated HPRS-104 was isolated from a myeloid leukosis tumor from a bird in line 20. Payne's group conducted various tests such as interference assays, virus neutralization assays and host range assays, on these novel subgroup viruses to determine what subgroup they belonged (Payne *et al.* 1991). The results concluded that these isolates, HPRS-100-104, were members of the first new subgroup described since 1970 (Vogt 1977). HPRS-103 was

identified as the prototype. Since then, various reports of subgroup J infection indicated that it has spread worldwide (Fadly and Smith 1997; Fadly 1998; Fadly and Smith 1999; Nakamura *et al.* 2000; Payne 2000).

2. **Subgroup Designation**

The host range of HPRS-103 strain of ALV-J has been defined (Payne *et al.* 1992). Cell cultures from various avian species (Vogt 1977; Troesch and Vogt 1985) including two jungle fowl species were used to define host range of the new isolate. Cell cultures from line 0 chickens, Japanese quail, ring-necked pheasant, domestic guinea fowl, turkey, Pekin duck, Muscovy duck, domestic goose, red jungle fowl and Sonnerat's jungle fowl were used in this host range study. The host range focus assay (Spencer 1987) was conducted by creating RSV pseudotypes with the prototype virus for subgroups A, B, C, D, E, and HPRS-103, the prototype for ALV-J. Of all the species tested only chicken, red jungle fowl, Sonnerat's jungle fowl, and turkeys were susceptible to RSV (HPRS-103) infection. Because the new isolate RSV (ALV-J) failed to grow in remaining cell cultures, the presence of subgroup F, G, and I were ruled out.

The new subgroup was confirmed when sequence analysis of the *env* gene revealed that is was different from all other ALV subgroups that affect chickens (Bai *et al.* 1995). Genetic analysis of the gp85 protein (SU) revealed many deletions, amino acid substitutions, and amino acid insertions when compared to subgroups A-E. The gp85 of HPRS-103 had only a 40% overall average identity with subgroups A-E gp85s while A-E have 80-85% identity to each other (Bai *et al.* 1995). These differences led the researchers to confirm the novel subgroup is different and should be identified as J.

3. **Disease manifestations**

a. **Neoplastic conditions**

To determine the oncogenic properties of subgroup J ALV, Payne and collaborators used the HPRS-103 prototype and various commercial lines of meat-type chickens and experimental strains of Leghorn chickens (Payne *et al.* 1991; Payne *et al.* 1992). They reported a variety of tumors with myeloid leukosis (ML) most common, followed by renal adenomas, hemangiomas, histocytic sarcomas, granulosa cell tumor, mesothelioma, pancreatic adenocarcinoma, and unclassified leukemias. The myeloid leukosis seen was similar to myelocytomatosis caused by MC29, an acutely transforming virus (Mladenov *et al.* 1967). Most of the tumors developed in meat-type chickens with few tumors in the Leghorn strains (Payne *et al.* 1991; Payne *et al.* 1992).

In the field, various ages of meat-type chickens have been affected. Fadly and Smith (1997, 1999) reported that flocks from great grandparents, grandparents, parents, and broilers haven been affected and as young as four weeks of age with myeloid leukosis. Other field observations include histocytic sarcomatosis in the spleen and sometimes liver with ML (Arshad *et al.* 1997). A case report from Japan, thoroughly described the lesions seen histologically in the bone and ossifying cartilage of the trachea and larynx (Nakamura *et al.* 2000). They described the marked proliferation of myelocytes in the marrow of various bones and periosteum. Also noted was proliferation of myelocytes in the dura mater of the spinal cord or periosteum of the vertebrae that caused pressure atrophy of the cord.

Just recently, a report on the experimental induction of erythroblastosis with subgroup J infection was published (Venugopal *et al.* 2000). Acutely transforming virus

strains were isolated from field cases of erythroblastosis and given to day-old chicks or 11-day-old embryos. The earliest cases of myelocytomatosis and erythroblastosis occurred at 22 and 23 days post inoculation in chickens infected as embryos. Similar findings regarding *in vitro* transformation with ALV strain 966 (Payne *et al.* 1993) were noted with these strains even though *in vivo* they transformed erythroblasts. Moscovici and others (Moscovici *et al.* 1981) reported that leukemic viruses often select one target cell lineage to transform *in vitro* while *in vivo* multiple lineages can be affected. The report by Venogopal *et al* did not determine whether *erbA* or *erbB* oncogenes were included in the genomes of these acutely transforming virus strains of ALV-J.

A report of experimental infection of turkeys with strains HPRS-103 and 966 ALV-J was recently published (Venugopal *et al.* 2000). The results indicate that day old turkey poults can become infected with subgroup J and seroconvert at about 10 weeks post-infection. No tumors were observed in this group most likely due to the short time period. However, turkey poults inoculated with the acutely transforming strain 966 did develop tumors within 4 weeks of age, the most common time period. Nine of the 12 poults that received the virus intravenously developed tumors while zero out of eight poults inoculated intraperitoneally with virus developed tumors. The tumors were described as multiple discrete nodules in the liver. Other gross lesions included enlargement of the kidneys and white foci on the surface of the spleen. Microscopically, the lesions in the liver consisted of myeloblasts in the parenchyma and blood vessels. These tumors were similar to tumors produced by the MC29 virus in turkeys (Schaff *et al.* 1978).

b. **Non-neoplastic Conditions**

Although there have been anecdotal remarks that infection with ALV-J is responsible for a variety of conditions such as immune depression, increased flock variability and poor feed conversion, no reports have been published to document these remarks. The only report of body weight suppression is from an experimental flock of broiler breeders and their congenitally infected progeny (Stedman and Brown 1999). Results from this report demonstrated that congenital ALV-J infection affected weight gain as compared to uninfected, age-matched controls as early as one week of age. They also noted that the infected chicks had delayed maturation and feather development. An explanation for this difference in weight gain offered by Brown *et al* (Brown *et al.* 2000) suggested that the differences in $_L$-thyroxine (T_4) levels between the two groups was related to the development of hypothyroidism in ALV-J infected birds. These birds had less T_4 measured in their sera when compared to uninfected birds which, then led to differences in weight gain between the groups.

4. **Theories of Evolutionary Beginnings**

Genetic analysis revealed that ALV-J has interesting sequences (Bai *et al.* 1995). Bai and others detected an E element that had only been detected in sarcoma viruses (Schwartz *et al.* 1983) and had an *env* gene similar to sequences found in the EAV family of endogenous viruses (Boyce-Jacino *et al.* 1989; Boyce-Jacino *et al.* 1992). EAV E51 is an *env* gene that is defective in the SU region due to stop codons and frame shifts (Boyce-Jacino *et al.* 1992) and are very closely related to the *env* gene of HPRS-103 as determined by high stringency Southern blotting and sequence analysis (Bai *et al.* 1995). This would explain the 40% identity with other ALV gp85s. The E element is found just

upstream to the 3' LTR. It has only been detected in replication competent RSVs before and never in ALVs. The function of the E element is unknown. They also discovered an insertion of a redundant TM segment that is truncated just downstream from the whole TM (gp37). Bai and coworkers (1995) believe that a recombination event occurred with either EAV E51 or similar sequences to create HPRS-103. Also, their analysis provided possible origins for other parts of the HPRS-103 genome from other exogenous ASLVs.

Work by two different groups have identified the sequences similar to E51 that are present in subgroup J ALV. Benson *et al* (1998) named these sequences ev/J. From their work, they identified between 6-10 copies of the ev/J proviruses per genome and that some of the elements segregate in the chicken population. Further work by the same group (Ruis *et al.* 1999) determined that ev/J isolates are proviruses that have very little variation between them, demonstrate a weak similarity to ASLVs, retain enough sequences that encode for assembly, budding and/or infectivity. RNA can be expressed by some members which encoded GAG and ENV related polypeptides in chick embryo cells (Ruis *et al.* 1999). They also noted that the LTRs of ev/J and ART-CH only had four different base pairs in the R and U5 regions when compared.

Work by Smith *et al* (1999) determined that the EAV-HP sequence is different from E51 and most likely was recombined with in producing HPRS-103. They also determined that EAV-HP was in the genome in relatively few copies. They mapped EAV-HP loci to 4 chromosomes on the chicken genome map, 1, 3, 4, and W. Further work with sequence analysis compared ev/J and EAV-HP and found them to be virtually identical (Sacco *et al.* 2000). They also determined that EAV-HP has 96% identity with ART-CH on the 5' end of the genome and 99% identity with ART-CH on the 3' LTR R

and U5. Another interesting point they found was the EAV-HP U3 is a weak promoter of transcription.

This subgroup is the first one for ASLV to arise by recombination events between endogenous and exogenous viruses (Smith *et al.* 1999). However, this is not the first documented case for retroviruses in general. Feline leukemia viruses have been reported to evolve in this manner (Roy-Burman 1995). This report demonstrated that endogenous feline retroviruses were able to recombine with exogenous viruses in the envelope region and result in new viruses, among other traits, that are antigenically different from existing viruses.

5. **Antigenic Variants**

Antigenic variants have been documented not only in avian retroviruses but also in all retroviruses as well with other animal and human viruses. Chubb and Biggs (1968) reported that viruses within the same subgroup could be antigenically different based on antisera. Fadly and Smith (1997, 1999) documented this phenomenon with subgroup J isolates HPRS-103 and ADOL Hc1, the U. S. prototype. Work by Benson *et al* (1998) determined that U. S. isolates ADOL Hc1, ADOL R5-4 and HPRS-103, the European prototype arose from a common ancestor and not as separate recombination events.

Two reports have been published about the variability of subgroup J *env* gene and how it can result in antigenic variants (Venugopal *et al.* 1998; Silva *et al.* 2000). Venugopal and others (1998) examined 12 ALV-J viruses in order to determine that they were variants from HPRS-103. Only two viruses reacted with the neutralization assays while nine were PCR positive (Smith *et al.* 1998). All reacted with a specific monoclonal antibody to gp85 made from HPRS-103. They suggest that the variants arose due to

selection pressure most likely caused by immune responses. The United States group looked at envelope genes from various ALV-J isolated from different farms (Silva *et al.* 2000). They noted that the U. S. strains were more related to themselves than to the European strains. Also, the U. S. strains seem to continue mutating at a fairly high rate. These changes cause difficulty in diagnosis using traditional methods such as virus neutralization assays (which virus to choose as indicator virus), or whether ELISA kits for antibody based on the gp85 region will it detect all variants of ALV-J.

Diagnosis of subgroup J ALV can be difficult because of the many variants of the virus. Virus isolation will detect all subgroups in samples containing infectious virus particles. Direct ELISA for antibody detection (Venugopal *et al.* 1997) has limited value due to the antigenic variation (Venugopal *et al.* 1998; Venugopal 1999; Silva *et al.* 2000). PCR tests that have been published detect proviral DNA in the samples (Smith *et al.* 1998; Smith *et al.* 1998; Silva *et al.* 2000). PCR tests run the risk of false negatives due to mutations in the proviral DNA where the primers are designed to anneal. RT-PCR (reverse transcriptase-polymerase chain reaction) has been documented to detect ALV-J (Smith *et al.* 1998) along with nested RT-PCR which increases the sensitivity of the test (Garcia *et al.* 2000). However, all these PCR based tests may not detect the virus if there are mutations in sites of the genome where primers must anneal.

A recently published report examined infection rates in congenitally infected commercial broiler breeders raised in experimental conditions (Witter *et al.* 2000). They wanted to determine when was the optimal time for testing and detecting dams that would transmit virus to their progeny and what types of conventional tests would best detect shedder dams. From their results, 95% of the hens that would transmit virus to progeny

were detected using routine assays for virus and GSA in the albumen. However, the remaining 5% were difficult to detect, if at all, using the assays in the report (Witter *et al.* 2000). This remaining percentage is important for the breeding companies due to the high rate of horizontal transmission of ALV-J after hatch.

M. ***Research Objectives***

Most of the research on ALV-J has been conducted with meat-type chickens because ALV-J has been associated with major problems in meat-type chickens. Fadly (1998) reported one case of ALV-J-like induced disease and tumors in commercial laying chickens in North America. The reason for this outbreak in egg-laying chickens was co-mingling of infected meat-type chickens with commercial layer chickens in the hatchery. Furthermore, in the Netherlands, experiments were conducted to determine susceptibility of two lines of commercial layers, a brown Leghorn line and a white Leghorn line, to ALV-J infection (Albers and Derkx 2000). Studies conducted by Payne *et al* (1991, 1992) demonstrated that laying chickens are susceptible to ALV-J infection but less likely to develop tumors from the infection.

The overall research goal for the current work was to determine the pathogenesis of ALV-J infection in white Leghorn chickens. The objectives were to: 1) compare pathogenicity of subgroup J ALV in various lines of white Leghorn chickens; 2) determine the effects of host factors on the pathogenesis of subgroup J ALV in white leghorns; and 3) determine the tissue tropism of ALV-J in white leghorns and the susceptibility of the B cell to transformation by ALV-J.

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CHAPTER 2

Response of Four Genetic Lines of White Leghorn Chickens to Infection with Subgroup J Avian Leukosis Virus

I. Abstract

The response of four lines of white leghorn chickens to infection with ALV-J was studied. The lines of chickens evaluated were lines 0, $15I_5$, 7_1, and 6_3. Chickens of each line were inoculated as either 7-day-old embryos or at the day of hatch. Uninoculated chickens from each line were also included as controls. The two treatment groups involving each line received the ADOL-Hc1 strain of ALV-J at a dose of 10^4 infectious units per chicken. At 4, 10, and 30 weeks of age, samples for virus isolation and virus neutralization were collected. At 4 and 10 weeks of age, bursal tissue was collected for methyl green pyronine (MGP) staining and examined for ALV induced pre-neoplastic bursal lesions, also known as transformed follicles. Tumor incidence and type were recorded for each line and treatment group. At 4, 10, 20, and 30 weeks of age, hematological assays of packed cell volume (PCV) and white blood cell (WBC) differentials were performed. Subgroup A ALV served as positive controls for the experiment using the highly susceptible $15I_5$ X 7_1 cross of chickens with the exact same protocol as ALV-J inoculated groups. The embryo inoculated groups remained viremic tolerant throughout the entire study. Groups inoculated at day of hatch had various serological responses. Line 0 developed neutralizing antibodies and cleared the virus by 10 weeks of age. The other three lines had varying degrees of success in developing neutralizing antibodies and viral clearance, with some birds at 30 weeks of age still harboring the virus in the presence of neutralizing antibodies. Results from the bursal transformation assays indicated that ADOL-Hc1 could induce transformed follicles in white leghorn chickens. PCVs did not differ between lines or treatment groups. Lymphocytes were the most common cell type detected for all lines, regardless of virus treatment used. Heterophils followed lymphocytes as the second most frequently detected cell type while eosinophils and monocytes comprised a very small portion of the cell counts. Differential WBC counts were difficult to interpret for all groups due to the variability of cell counts among the control birds. Tumor type and incidence varied. LL

and hemangiomas were the main manifestations with a total of 14 birds diagnosed with each. There was only one bird diagnosed with myelocytomatosis.

II. **Introduction**

Avian leukosis virus is the most common retrovirus that causes neoplasms in poultry. ALVs have been classified into subgroups (A-I) based on host range in embryo fibroblasts of different avian species, interactions between virus-specific cell receptors and viral envelope glycoproteins and serum neutralization assays (Fadly and Witter 1998). ALV causes a variety of neoplasms and other production problems. A novel subgroup J was first isolated in the United Kingdom in the early 1990s (Payne *et al.* 1991) and subsequently isolated in the United States (Fadly and Smith 1997). ALV-J causes myeloid leukosis as its main manifestation while other subgroups cause LL. ALV-J seems to manifest tumor formation more readily in broiler breeder chickens, whereas infection of white leghorn chickens results in a lower incidence of tumor formation (Payne *et al.* 1991; Fadly and Witter 1998). White leghorn chickens naturally infected with subgroup J-like ALV also develop tumors (Fadly 1998).

Much is known about subgroups A and B ALVs in leghorn chickens. Disease is usually typified by bursal transformation and metastasis of transformed B lymphocytes to the liver, spleen and other visceral organs after a long period of time (Payne and Fadly 1997). Early lesions include hemangiomas /hemangiosarcoma of the liver, lung and kidney (Payne and Fadly 1997). Problems such as testicular atrophy or gonad non-maturation can lead to production losses. Broiler breeders infected with ALV-J tend to have tumor formation on the keel or sternum, ribs, liver and kidneys (Payne *et al.* 1991; Payne and Fadly 1997). These tumors are characterized as myelocytomatosis and are histologically distinct from LL. Renal tumors of adenomas or carcinomas are also common. Ages of birds affected by tumor formation and increased mortality usually begins at 15 weeks. ALV-J induced tumors have been noted in broiler parent flocks as

young as four weeks of age (Fadly and Smith 1997). Payne *et al* (1992) experimentally infected various genetic lines of meat-type and leghorn chickens. They determined that line 0 chickens (leghorns in general) had a lower incidence of tumor formation than the meat type lines of chickens. They also noted the lack of LL in the leghorns experimentally inoculated with HPRS-103. Other lines involved in Payne's study were Brown leghorn, line 15I, line 6_1 and line N plus three lines of meat-type chickens. The objective of this study was to determine the response of four genetic lines of white leghorn chickens to infection with strain ADOL-Hc1 (U.S. prototype of ALV-J). The response of chickens to infection with ALV-J was compared to that of line $15I_5$ X 7_1 infected with ALV-A.

III. METHODS AND MATERIALS

A. **Chickens:** Five lines of white leghorn chickens from the USDA, Avian Disease and Oncology Laboratory, East Lansing, MI were used. They included line 0, $15I_5$, 7_1, 6_3 and F_1 of line $15I_5$ X 7_1. Line 0 is not an inbred line, it lacks endogenous viral (*ev*) loci, is resistant to ALV-E infection, and susceptible to infection and tumor development by ALV-A, -B, and –C. Line $15I_5$ is an inbred line that contains *ev* loci and is susceptible to ALV infection and tumor development. Line 7_1 is an inbred line that contains *ev* loci and undefined for ALV-A infection and tumor formation. Line 6_3 is an inbred line that contains *ev* loci and is susceptible to ALV-A infection but resistant to tumor development (Bacon *et al.* 2000). The breeder flocks for all lines are free of many avian pathogens, including ALV, as determined by routine serological surveys. Chickens were housed by line and treatment and kept in plastic isolators with positive airflow for 30 weeks and given food and water *ad libitum*.

B. **Viruses:** Strain ADOL-Hc1of ALV-J (Fadly and Smith 1999) was used to infect chickens of lines 0, $15I_5$, 7_1, and 6_3. The titer of the virus stock was 10^5 infectious units

(IU)/ml. Rous-associated virus-1 (RAV-1), a subgroup A ALV, with a titer of 10^6 IU/ml was used to infect the F_1 progeny of $15I_5$ X 7_1 chickens. Each treatment group was given 10^4 IU/chicken either via the yolk sac or intraperitoneally of the assigned virus.

C. **Experimental Design:** Five lines of chickens were divided into three treatment groups per line comprising a total of 15 groups. Group 1 in each line (75 embryos/line) was inoculated as 7-day-old embryos via the yolk sac using a 30g 1/2-inch needle. Group 2 in each line (50 chicks/line) was inoculated intraperitoneally at day of hatch using a 30g 1/2 inch needle and group 3 in each line (20 chicks/ line) served as uninoculated controls. RAV-1 infected chickens ($15I_5$ X 7_1) served as the positive control for Groups 1 and 2. At hatch (embryo inoculated only-10 chicks/line), 4, 10 and 30 weeks of age, whole blood samples were collected and plasma was used for virologic assays. The experiment was terminated at 30 weeks of age. All birds were necropsied either at time of death or at termination of the experiment.

Bursal transformation was determined by methyl green pyronine (MGP) staining. At 4 and 10 weeks of age, five chickens from each of the fifteen groups were randomly chosen and euthanized. Bursal tissue was collected and plica separated and placed flat in cassettes, usually 2 to 3 cassettes per bird. Bursal tissues were fixed in 4% paraformaldehyde in phosphate buffered saline solution. Tissues were embedded in paraffin, sectioned, and stained with MGP stain (Siccardi and Burmester 1970; Carson 1990) and examined histologically.

D. **Virological and serological assays**: Virus isolation (VI) and virus neutralization (VN) assays were conducted on all plasma samples at each sampling period using line 0 (C/E) CEF. Briefly, VI was performed using 100 microliters of sample plasma added to CEF grown in 4% calf serum Leib McCoy media containing penicillin, streptomycin and amphotericin B plus 0.04IU of heparin per plate. Media was changed the following day

to 1% calf serum Leib McCoy media containing above antibiotics and fungicide. Cultures were allowed to grow for 7- 9 days. Plates were frozen and thawed twice (Fadly and Witter 1998). Cell lysates from CEF cultures were tested by the gs antigen p27 ELISA (Smith *et al.* 1979). VN assays were performed according to procedures by Fadly *et al* (1998). Briefly, test samples were diluted 1:5 with Leib McCoy media without serum and heat inactivated for 30 minutes at 56° C. Dilute virus stocks of RAV-1 or ADOL-Hc1 were added such that each plate of a 96 well microtiter plate received 500-1000 viral particles/ml. Equal volumes of test samples were added to the microtiter plates and incubated for 45 minutes. Appropriate positive and negative control samples were included. Cells were added ($5X10^4$ cells/well) in 4% calf serum Leib McCoy containing antibiotics and fungicide, and incubated 7-9 days at 37° C. Cultures were frozen and thawed twice and the cell lysates were tested for the presence of p27 by ELISA. VN assays were performed separately with both RAV-1 and ADOL-Hc1 for indication of accidental cross contamination.

E. **Hematological assays**: At 4, 10, 20 and 30 weeks of age, differential WBC counts and PCVs were performed. Blood smears were stained with a Wright stain using an automated stainer (Wescor 7100 Aerospray TM Slide Stainer). Differential WBC counts were done manually and based on counts of 100 WBC using a light microscopic at 40X. PCVs were performed using microhematocrit tubes (Oxford Labware by Sigma) and spun for 3 minutes. Each tube was hand read using the Lancer Critocap Microhematocrit Capillary Tube Reader card.

F. **Pathology:** Necropsy was performed on all birds that died during the experiment and at termination. Tissue samples for histology were taken from birds with gross tumor formation. Normal tissue samples taken from control birds from each line included liver, spleen, gonads, intestines, heart, lung, and kidney. All samples were fixed in 10% neutral

buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

G. **Statistical analysis**: Statistical analysis was performed with the SAS 8.0 program. Viremia and antibody data, bursal transformation data, and tumor incidence data were all examined with a chi-square test. Differential WBC counts for each line of chicken were analyzed by the Wilcoxon Rank-Sum test due to the nonparametric distribution of the data. Additional analysis was performed using the 2-way analysis of variance (ANOVA) with the Dunnett's t Test when comparing control lines to treatment groups lines for differences in cell means and comparing line 0 to the other three lines within treatment groups for differences in cell means. Results were considered significant at a level of $P \leq 0.05$ for chi-square, Wilcoxon Rank-Sum and 2-way ANOVA tests. Results were considered significant when within the 95% confidence interval for the Dunnett's t Test.

IV. RESULTS

A. **Viremia and antibody assays**: The whole blood sampling of the embryo inoculated groups consisted of 10 chicks randomly selected from each line at hatch. At hatch, 48 out of 50 were VI positive. The two negative chicks, one line 0 and one line 7_1 were inoculated with ALV-J. VI and VN results for 4, 10, and 30 weeks sampling periods are presented in Table 2.1. The embryo inoculated group, regardless of line of chicken or virus, were viremic tolerant (viremia and lacking antibody) the entire experiment. All ten ALV-A infected positive control chicks sampled at hatch were positive. Two other ALV-A embryo inoculated positive control chickens seroconverted at 10 weeks of age indicating that inoculation of those two chickens as embryos was not successful. However, they became infected soon after hatch as all birds in that group were virus positive at four weeks of age. The negative control groups in each line were negative for

virus and antibodies throughout the experiment. Day of hatch ALV-J infected chickens had variation in viremia and antibody production along the different genetic lines. Line 0 responded sooner with antibody production at four weeks, 27% Ab +, followed by line 7_1 with 17% Ab +, line 6_3 with 6% Ab + and none positive in line $15I_5$. Line 0 was also able to neutralize the virus sooner than other lines. By ten weeks, there were no line 0 VI+ chickens. Interestingly, line $15I_5$ developed neutralizing antibodies but could not neutralize circulating virus as demonstrated by the fact that all remaining chickens at 30 weeks were VI+ (30/30) and 13/30 also had neutralizing Ab. Lines 6_3 and 7_1 also had varying degrees of success in producing neutralizing Ab but could not completely neutralize circulating virus as demonstrated by the presence of virus at 30 weeks in the presence of neutralizing Ab. ALV-A, day of hatch inoculated chickens began to test positive for antibodies at 4 weeks of age (3%). By 10 weeks, 96% tested positive for antibody with 5/27 still virus positive. At the end of the 30 week time period, all chickens had died.

B. **Bursal Transformation:** Bursal follicle transformation is detectable by using a MGP staining technique (Carson 1990). The transformed follicles appear pyroniphilic (more eosinophilic) compared to surrounding follicles. Also, the normal architecture is disrupted such that the cortex and medulla are indistinguishable from each other. Results are presented in Table 2.2. The bursal tissue sample was considered positive if one follicle had evidence of transformation after examining all sides for that chicken. Negative controls had no transformations in any line. Line $15I_5$ had a 40% transformation rate in the day of hatch inoculated group at 10 weeks versus none in its embryo inoculated counterpart. Line 0 had a 20% rate at 4 weeks in each group that transformed, while line 7_1 day of hatch inoculated group and line 6_3 embryo inoculated group had a 20% transformation rate at 10 weeks. ALV-A inoculated chickens had a 60% follicle transformation rate at 4 weeks of age regardless of time of virus inoculation. At 10

weeks, both embryo and day of hatch inoculated chickens had a 100% bursal follicle transformation rate.

C. **Hematological assays**: The PCV results did not demonstrate any differences between lines of chickens or treatment group. Results are presented in Table 2.3. There was an occasional chicken that had a low PCV of 13-17 but no overall anemia could be detected for any treatment group or genetic line of chicken.

Differential WBC counts for all five lines indicated that lymphocytes were the most common cell type counted regardless of genetic line, treatment protocol, or virus. The one exception was for the 30 week old line 0 control chickens where heterophils were more numerous on a percent average basis. Heterophils were the next most common cell type. Eosinophils and monocytes had minimal cell counts with usually no more than an average of 2 cells per 100 examined regardless of line or treatment group.

Results of differential WBC counts are in Figures 2.1-2.20. Line 0 control chickens mean percent for lymphocytes was increased compared to day of hatch inoculated birds and embryo inoculated chickens for the 4 week time period (61% versus 60% and 51% respectively) and the10 week time period (62% versus 59% and 53% respectively). At 20 and 30 weeks, the results were reversed with day of hatch inoculated chickens (66% and 60%) and embryo inoculated chickens (86% and 79%) having increased lymphocytic mean percents compared to the control chickens counts (65% and 44%) (Figure 2.2). With regards to heterophils (Figure 2.1), the mean percent for control line 0 chickens were decreased compared to day of hatch and embryo inoculated chickens at the 4 week time period (37% versus 38% and 47%) and 10 week time period (33% versus 39% and 46%). At 20 and 30 weeks, control chickens (34% and 56%) have increased heterophil mean percents

Table 2.1: Development of ALV-J or ALV-A induced viremia and antibody in five lines of white leghorn chickens inoculated as 7 day old embryos or at day of hatch.

			VI and Ab Testing--No.+/No. tested (%)	
Age	Group	Line	VI	Ab
	1	Line 0	40/40 (100)[1]	0/40 (0)[2]
	2		13/44 (30)[1]	12/44 (27)[2]
	3		0/20 (0)	0/20 (0)
	1	Line $15I_5$	40/40 (100)	0/40 (0)
	2		44/44 (100)	0/44 (0)
	3		0/19 (0)	0/19 (0)
4 weeks	1	Line 63	21/21 (100)	0/21 (0)
	2		16/16 (100)	1/16 (6)
	3		0/21 (0)	0/21 (0)
	1	Line 7_1	3/3[a] (100)	0/3 (0)
	2		18/18 (100)	3/18 (17)
	3		0/18 (0)	0/18 (0)
	1	Line $15I_5$ X 7_1	45/45 (100)	0/45 (0)
	2		34/34 (100)	1/34 (3)
	3		0/16 (0)	0/16 (0)
	1	Line 0	26/26 (100)[3]	0/26 (0)[4]
	2		0/37 (0)[3]	28/37 (76)[4]
	3		0/15 (0)	0/15 (0)
	1	Line $15I_5$	34/34 (100)	0/34 (0)[5]
	2		36/36 (97)	13/37 (35)[5]
	3		0/14 (0)	0/14 (0)
10 weeks	1	Line 63	15/15 (100)	0/15(0)
	2		11/12 (92)	1/12 (8)
	3		0/18 (0)	0/18 (0)
	1	Line 7_1	2/2 (100)	0/2 (0)[6]
	2		3/14 (21)	13/14 (93)[6]
	3		0/11 (0)	0/11 (0)
	1	Line $15I_5$ X 7_1	34/35 (97)[7]	2/35 (6)[8]
	2		5/27(19)[7]	26/27 (96)[8]
	3		0/11 (0)	0/11 (0)

Table 2.1 Continued

Age	Group	Line	VI	Ab
30 weeks	1	Line 0	7/7 (100) [9]	0/7 (0) [10]
	2		0/28 (0) [9]	26/28 (93) [10]
	3		0/6 (0)	0/6 (0)
	1	Line $15I_5$	17/17 (100)	0/17 (0) [11]
	2		30/30 (100)	13/30 (43) [11]
	3		0/9 (0)	0/9 (0)
	1	Line 6_3	8/8 (100)	0/8 (0)
	2		4/5 (80)	1/5 (20)
	3		0/12 (0)	0/12 (0)
	1	Line 7_1	1/1 (100)	0/1 (0) [12]
	2		5/6 (83)	6/6 (100) [12]
	3		0/5 (0)	0/5 (0)
	1	Line $15I_5$ X 7_1	1/1 (100)	0/1 (0)
	2		nt [a]	nt
	3		0/5 (0)	0/5 (0)

Group1, inoculated as embryos; group 2, inoculated at one day of age; group 3, uninoculated controls.

a: all but three chicks accidentally died in first week of life due to management problems.

b: all chickens in this group died before 30 weeks of age.

Entries with the same number in superscript are statistically different from each other with a p-value of <0.05 for that assay.

Table 2.2: Bursal transformation induced by ALV in five lines of white leghorn chickens inoculated as 7 day old embryos or at day of hatch.

Age at Testing--No. +/No. tested (%)

	Embryo		Day of hatch	
Line of chicken	**4 weeks**	**10 weeks**	**4 weeks**	**10 weeks**
Line 0	1/5 (20)	0/5(0)	1/5(20)	0/4(0)
$15I_5$	0/5(0)	0/5(0)	0/5(0)	2/5(40)
7_1	0/1(0)	0/1(0)	0/5(0)	1/5(20)
6_3	0/5(0)	1/5(20)	0/4(0)	0/5(0)
$15I_5$ X 7_1	3/5 (60)	5/5 (100)	3/5 (60)	5/5 (100)

compared to day of hatch (33% and 39%) and embryo inoculated chickens (13% and 21%).

Line $15I_5$ control chickens had increased mean percent heterophil count for all time periods when compared to day of hatch and embryo inoculated chickens (Figure 2.5). Embryo inoculated chickens heterophil counts were increased at 4 (23%) and 10 (18%) weeks compared to day of hatch inoculated chickens (5% and 8% respectively). At 20 and 30 weeks the day of hatch inoculated group (13% and 12%) is slightly increased compared to the embryo inoculated group (11% and 11%). Control chickens had decreased mean percent lymphocyte counts for all time periods when compared to day of hatch and embryo inoculated chickens (Figure 2.6). Day of hatch inoculated chickens mean percent lymphocyte counts were increased at 4 and 10 weeks (95% and 91%) compared to embryo inoculated birds (74% and 81%). The groups switch at 20 and 30 weeks with the embryo inoculated chickens (88% and 89%) slightly increased compared to day of hatch inoculated chickens (86% and 87%).

Line 7_1 control chickens had decreased mean percent heterophils for all time periods when compared to day of hatch inoculated chickens (Figure 2.9). With regards to the mean percent lymphocyte counts, the day of hatch inoculated chickens were decreased compared to control chickens(Figure 2.10). Due to the very low number of chickens in the embryo inoculated group, no comparisons were made at any time period.

Line 6_3 control chickens had increased mean percent heterophil counts at 4, 10 and 30 weeks of age compared to day of hatch and embryo inoculated chickens (Figure 2.13). At 20 weeks, controls were equal to day of hatch chickens for heterophils (4.8%) while embryo inoculated chickens were increased (5.7%). For mean percent lymphocyte counts (Figure 2.14), the control chickens were decreased at 4, 10 and 30 weeks of age when compared to day of hatch and embryo inoculated chickens. At 20 weeks, the controls (95%) were equal to the day of hatch inoculated (95%) and slightly increased compared to the embryo inoculated (94%).

The cross $15I_5$ X 7_1 (ALV-A infected) control chickens had decreased mean percent heterophil counts compared to day of hatch and embryo inoculated chickens at all time periods (Figure 2.17). However, the control chickens had increased mean percent counts of lymphocytes at all time periods when compared to day of hatch and embryo inoculated chickens (Figure 2.18). Comparisons at 30 weeks only consisted of controls and embryo inoculated chickens because all remaining day of hatch chickens had died. Analysis of the 30 week data was not performed due to the small number of embryo inoculated chickens left at this time period.

Each line was analyzed for differences between control group and inoculated treatment groups. There were significant statistical differences found between line 0 control chickens and embryo inoculated chickens for heterophil and lymphocyte counts at weeks 4,10, 20 and 30 (Figures 2.1 and 2.2) and for monocytes between control chickens and day of hatch chickens at week 10. Line $15I_5$ had significant statistical differences between control chickens and both day of hatch inoculated chickens and embryo inoculated chickens for heterophil counts at weeks 10, 20 and 30, lymphocyte counts at weeks 4, 10, 20, and 30, and eosinophil counts for weeks 4, 10, and 30 (Figures 2.5-2.7). Line 6_3 had significant statistical differences between the control chickens and both day of hatch inoculated chickens and embryo inoculated chickens for heterophil and lymphocyte counts at weeks 4 and 10, and eosinophil counts at week 30 (Figures 2.13-2.15). In line 7_1 there were significant statistical differences between control and day of hatch chickens for heterophil and lymphocyte counts at weeks 4, 10 and 30, eosinophil counts at week 4 and monocytes at weeks 4 and 20. Due to the low number of line 7_1 embryo inoculated chickens that survived to 4 weeks of age, comparisons were made only between control and day of hatch groups (Figures 2.8-2.12).

Statistical analysis of the differential WBC counts revealed that when all 4 lines of ALV-J infected chickens are combined together for that specific treatment group, there were significant differences seen for heterophils between the control lines and both day of

hatch and embryo inoculated lines at 4 and 30 weeks and there were differences between the control lines and embryo inoculated lines at 10 and 20 weeks (Figures 2.21). For lymphocytes, there were significant differences between the control lines and both day of hatch and embryo inoculated lines at 4 and 30 weeks and there were significant differences between the control lines and embryo inoculated lines at 10 and 20 weeks (Figures 2.22). For eosinophils, there were significant differences between the control lines and embryo inoculated lines at 4 weeks (Figure 2.23). For monocytes, there significant differences between the control lines and both day of hatch and embryo inoculated lines at 4 and 10 weeks and there were significant differences between control lines and embryo inoculated lines at 30 weeks (Figure 2.24).

Comparing the four lines, (lines 0, $15I_5$, 6_3, and 7_1) for differences between cell counts was conducted by comparing each line to line 0 results for each treatment group and time period. Line 0 was chosen for comparison because it is only non-inbred line in the study. In the control group, there were significant differences in heterophil and lymphocyte average percentages noted at 4, 10, and 30 weeks between line 0 and all three remaining lines and at 20 weeks between line 0 and $15I_5$ and 6_3 (Figures 2.25-2.26). For eosinophils, there were significant differences noted at 20 weeks between line 0 and 6_3 and at 30 weeks between line 0 and $15I_5$ and 6_3 (Figure 2.27). For monocytes, there were significant differences noted at 4 and 30 weeks between line 0 and 6_3 and at 10 weeks between line 0 and $15I_5$ (Figure 2.28). Comparing day of hatch inoculated line 0 to the three remaining day of hatch inoculated lines revealed significant differences in the heterophil and lymphocyte average percentages at all time periods (Figures 2.29-2.30). For eosinophils, there were significant differences noted at 4 weeks between line 0 and line $15I_5$, at 20 weeks between line 0 and lines $15I_5$ and 6_3, and at 30 weeks between line 0 and $15I_5$ (Figure 2.31). For monocytes, there were significant differences between line 0 and lines $15I_5$ and 6_3 at 10 weeks (Figure 2.32). Due to the low number of embryo inoculated line 7_1 chickens that survived to 4 weeks,

no analysis for line 7_1 compared to line 0 embryo inoculated chickens will be noted on Figures 2.33-2.36 although line 7_1 data was included. Comparing embryo inoculated line 0 to embryo inoculated lines $15I_5$and 6_3 revealed significant differences in the heterophil and lymphocyte average percentages at all time periods (Figure 2.33-2.34). For eosinophils, there were no significant differences noted at any time period. For monocytes, there were significant differences between line 0 and 6_3 (Figure 2.36).

D. **Histopathology**: All chickens that died during the experiment and at termination were subjected to necropsy. All gross tumors were noted and tissue samples taken for microscopic examination. Results are presented in Table 2.4. In treatment group 1 line 0, there was one chicken with erythroblastosis and one chicken with myeloid leukosis. The other lines had hemangiomas and renal tumors as the main non-myeloid or non-leukosis manifestation as well as LL. The single chicken with myeloid leukosis had involvement of the heart, lung, liver and kidney. In treatment group 2, besides tumors, line 0 and $15I_5$ had some male chickens with atrophic testes at 30 weeks. Line 6_3 had no tumor formation. One line 0 chicken developed a chondrosarcoma. Group 1 ALV-A infected chickens developed the greatest number of tumors overall, as expected. There were 28 chickens with LL and 4 with hemangiomas. Group 2 ALV-A infected chickens again developed the most tumors with 23 developing LL, one with myelocytomatosis, three with hemangiomas and one developed renal tumors. Negative control chickens did not develop tumors.

Table 2.3: Packed cell volume values in five lines of white leghorn chickens inoculated with ALV-J or ALV-A as 7 day old embryos, at day of hatch, or uninoculated controls

Age	Group	Line	# of samples	Range	Average
	1	Line 0	15*	24-33	30
		Line $15I_5$	38*	26-41	36
		Line 6_3	19	33-43	29
		Line 7_1	3	27-33	31
		Line $15I_5$ x 7_1	44 *	17-39	31
	2	Line 0	44	26-38	32
		Line $15I_5$	44	36-44	40
4 weeks		Line 6_3	15*	32-40	35
		Line 7_1	19	28-35	30
		Line $15I_5$ x 7_1	34	28-35	31
	3	Line 0	20	31-39	35
		Line $15I_5$	19	35-40	37
		Line 6_3	21	33-41	37
		Line 7_1	18	29-35	31
		Line $15I_5$ x 7_1	16	30-38	34
	1	Line 0	26	18-39	27
		Line $15I_5$	33*	32-38	35
		Line 6_3	15	27-35	31
		Line 7_1	2	19-30	25
		Line $15I_5$ x 7_1	35	17-37	29
	2	Line 0	35	22-34	28
		Line $15I_5$	37	26-38	34
10 weeks		Line 6_3	12	27-40	31
		Line 7_1	14	17-36	31
		Line $15I_5$ x 7_1	27	23-42	32
	3	Line 0	15	29-33	31
		Line $15I_5$	14	33-38	35
		Line 6_3	18	30-35	33
		Line 7_1	13	29-33	31
		Line $15I_5$ x 7_1	11	29-34	32
	1	Line 0	7	14-45	28
		Line $15I_5$	17	30-35	40
		Line 6_3	8	28-46	36
		Line 7_1	1	28	28
		Line $15I_5$ x 7_1	1	40	40
	2	Line 0	27	25-50	36
		Line $15I_5$	30	13-53	40
30 weeks		Line 6_3	5	32-46	42
		Line 7_1	6	26-43	33
		Line $15I_5$ x 7_1	0^	nt	nt
	3	Line 0	6	30-50	39
		Line $15I_5$	9	35-50	43
		Line 6_3	0*	nt	nt
		Line 7_1	8	30-52	39
		Line $15I_5$ x 7_1	5	30-40	36

* Indicates fewer samples evaluated due to human error.

^ No samples tested because all birds had died by 30 weeks of age.
nt- not tested.
1- Embryo inoculated
2- Day of hatch inoculated
3- Uninoculated controls

Table 2.4: Diagnosis of ALV induced tumors in five lines of white leghorn chickens inoculated with ALV-J or AVL-A as 7 day old embryo or at day of age.

Group	Line	# with Tumor/#at risk[a]	LL[b]	ML[c]	HEM[d]	RT[e]	Other[f]
1	Line 0	11/15	7	1	1	2	1
	$15I_5$	7/17	0	0	4	1	2
	6_3	1/5	1	0	0	0	0
	7_1	1/1	0	0	1	0	0
	$15I_5$ X 7_1	28/29	28	0	4	0	0
2	Line 0	7/29	2	0	4	0	1
	$15I_5$	6/31	4	0	3	0	1
	6_3	0/5	0	0	0	0	0
	7_1	2/6	0	0	1	1	0
	$15I_5$ X 7_1	26/26	23	1	3	1	0
3	Line 0	0/6	0	0	0	0	0
	$15I_5$	0/8	0	0	0	0	0
	6_3	0/12	0	0	0	0	0
	7_1	0/8	0	0	0	0	0
	$15I_5$ X 7_1	0/5	0	0	0	0	0

Group 1, inoculated as embryos. Group 2, inoculated at day of age, group 3, uninoculated controls.
a: # at risk=# died with tumors + # survived to end of experiment
b: LL=lymphoid leukosis
c: ML=myelocytomatosis
d: HEM=hemangioma
e: RT=renal tumors
f: Other= chondrosarcoma, erythroblastosis, fibromas

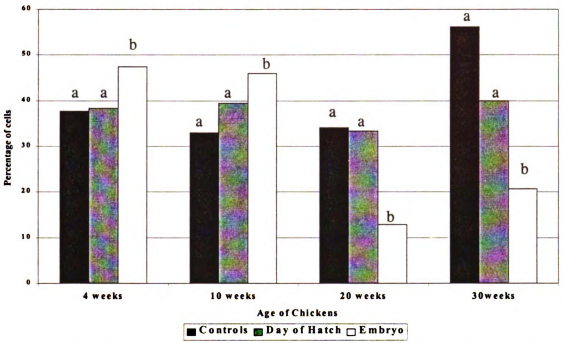


Figure 2.1: Line 0 heterophil comparison. Mean percent number of heterophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

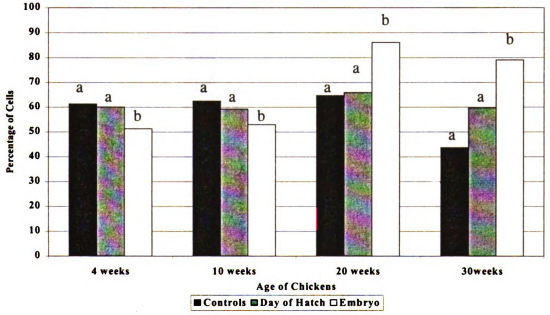


Figure 2.2: Line 0 lymphocyte comparison. Mean percent number of lymphocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

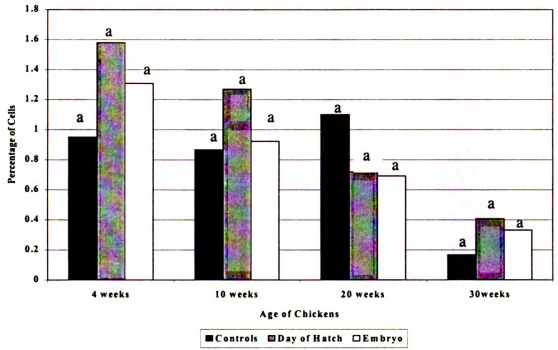


Figure 2.3: Line 0 eosinophil comparison. Mean percent number of eosinophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

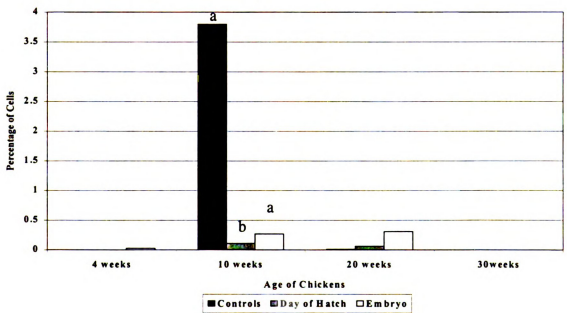


Figure 2.4: Line 0 monocyte comparison. Mean percent number of monocytes over time. Change in letters indicates a statistically significant difference between the control group and indicated treatment group at that time period.

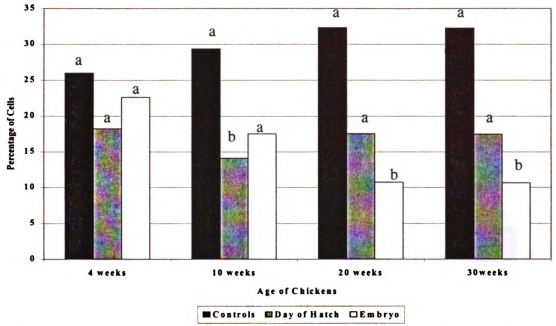


Figure 2.5: Line $15I_5$ heterophil comparison. Mean percent number of heterophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

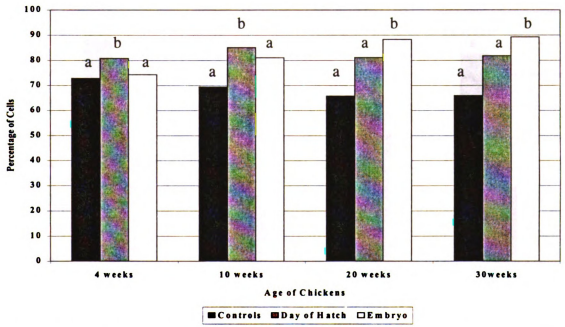


Figure 2.6: Line $15I_5$ lymphocyte comparison. Mean percent average for lymphocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

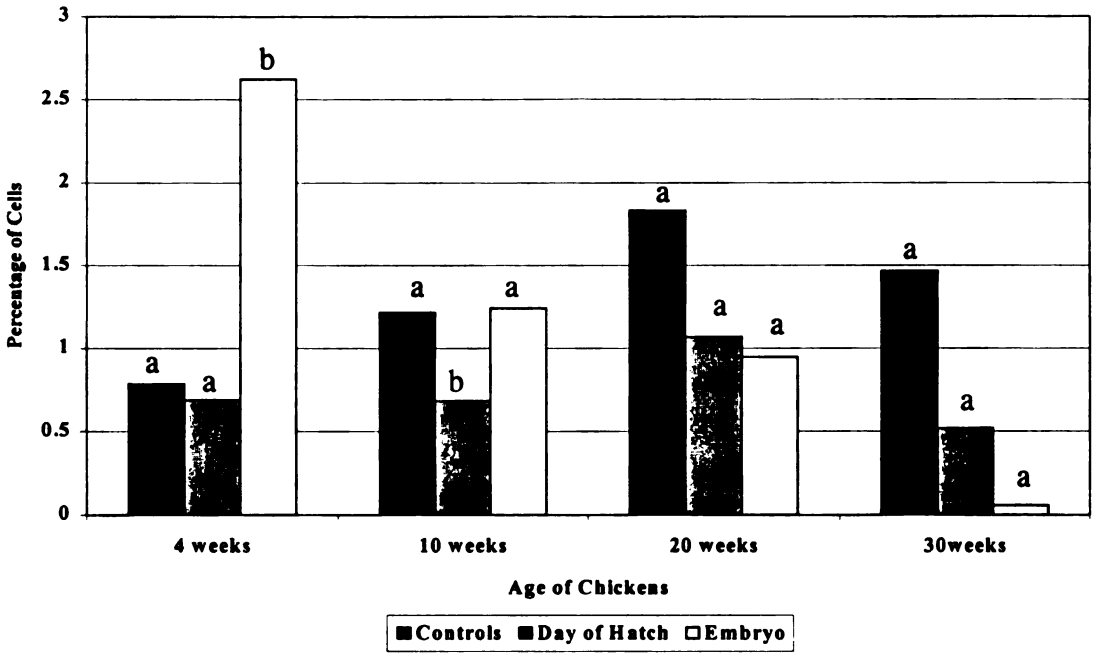


Figure 2.7: Line $15I_5$ eosinophil comparison. Mean percent number of eosinophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

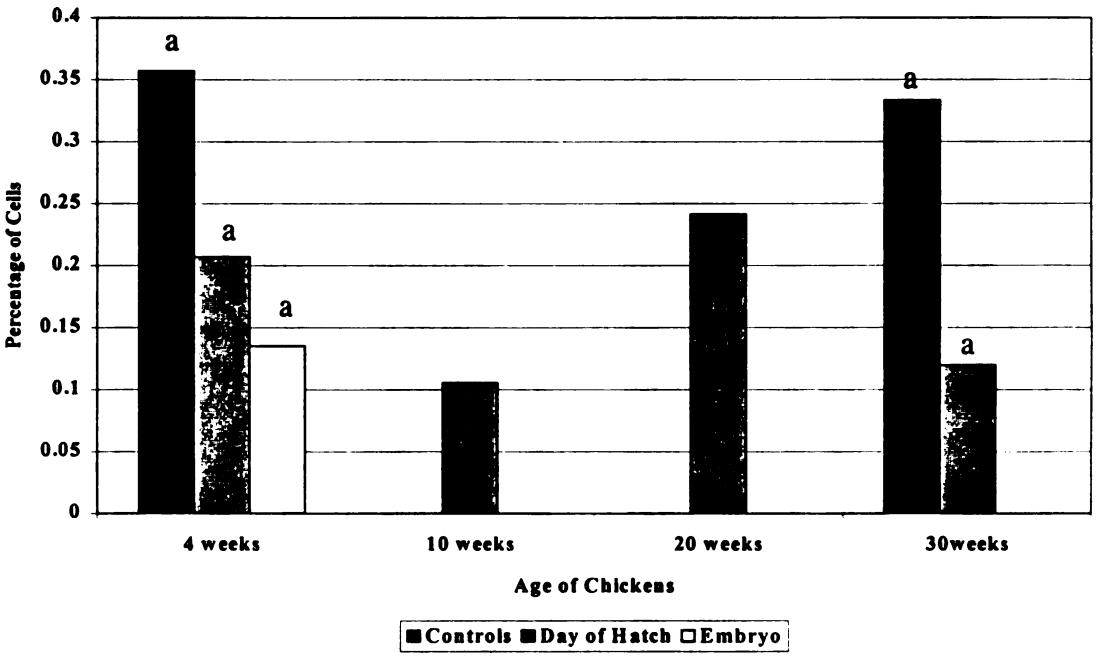


Figure 2.8: Line $15I_5$ monocyte comparison. Mean percent number of monocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

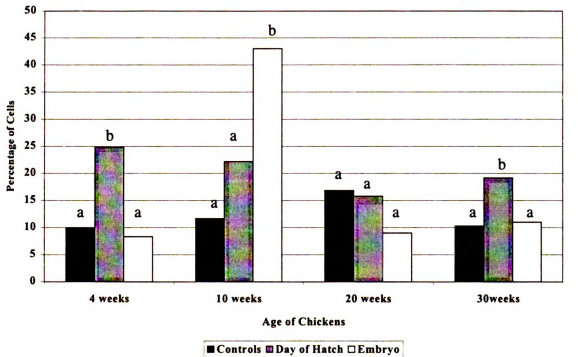


Figure 2.9: Line 7_1 heterophil comparison. Mean percent number of heterophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

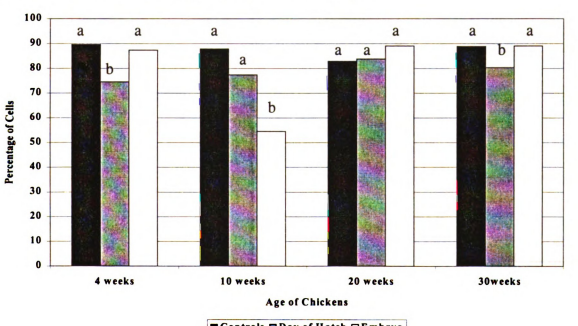


Figure 2.10: Line 7_1 lymphocyte comparison. Mean percent number of lymphocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

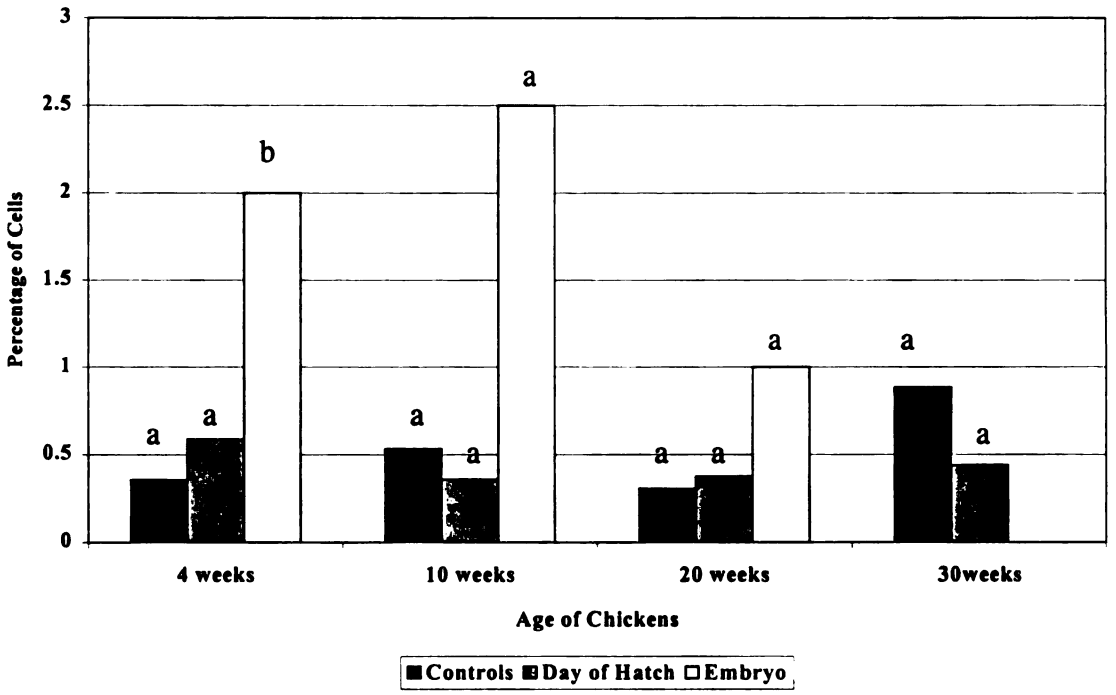


Figure 2.11: Line 7_1 eosinophil comparison. Mean percent number of eosinophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

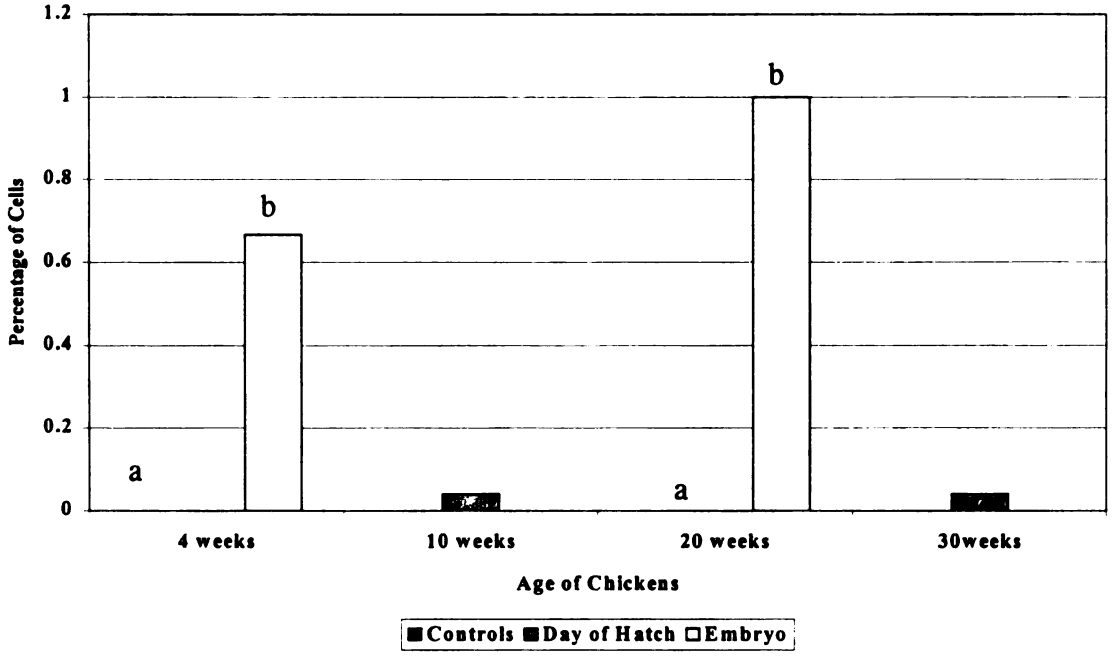


Figure 2.12: Line 7_1 monocyte comparison. Mean percent number of monocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

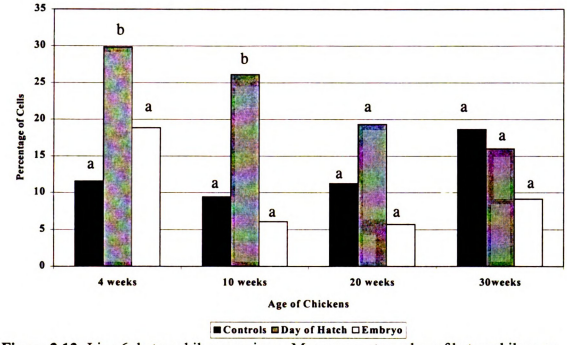


Figure 2.13: Line 6_3 heterophil comparison. Mean percent number of heterophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

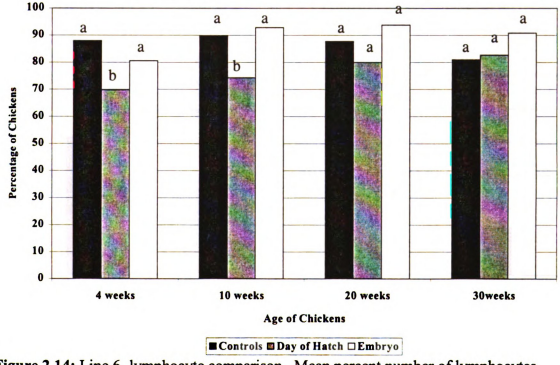


Figure 2.14: Line 6_3 lymphocyte comparison. Mean percent number of lymphocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

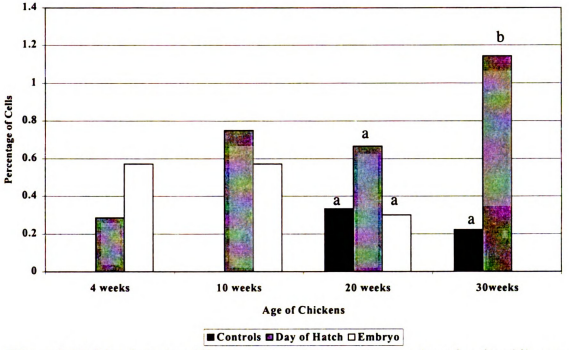


Figure 2.15: Line 6_3 eosinophil comparison. Mean percent number of eosinophils over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

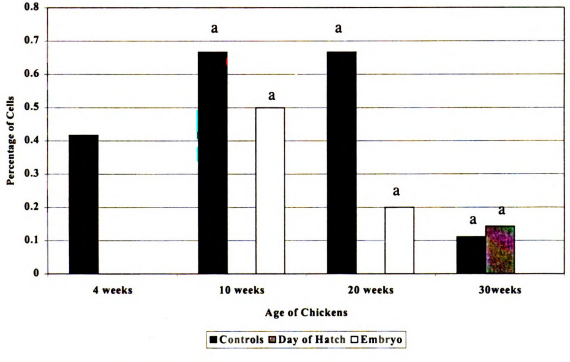


Figure 2.16: Line 6_3 monocyte comparison. Mean percent number of monocytes over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

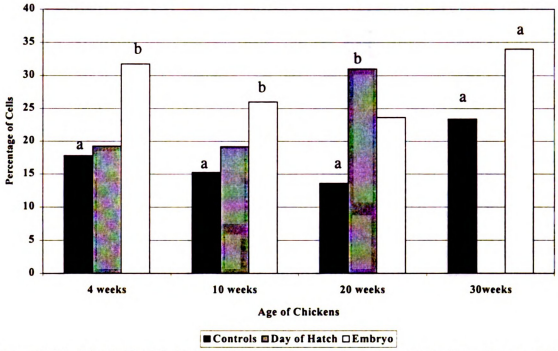


Figure 2.17: Line $15I_5 \times 7_1$ heterophil comparison. Mean percent number of heterophils over time. These birds were infected with ALV-A. There were no birds left at 30 weeks of age in Day of Hatch group. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

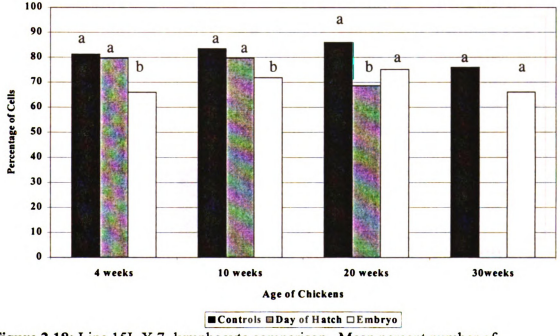


Figure 2.18: Line $15I_5 \times 7_1$ lymphocyte comparison. Mean percent number of lymphocytes over time. These birds were infected with ALV-A. There were no birds left at 30 weeks of age in Day of Hatch group. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

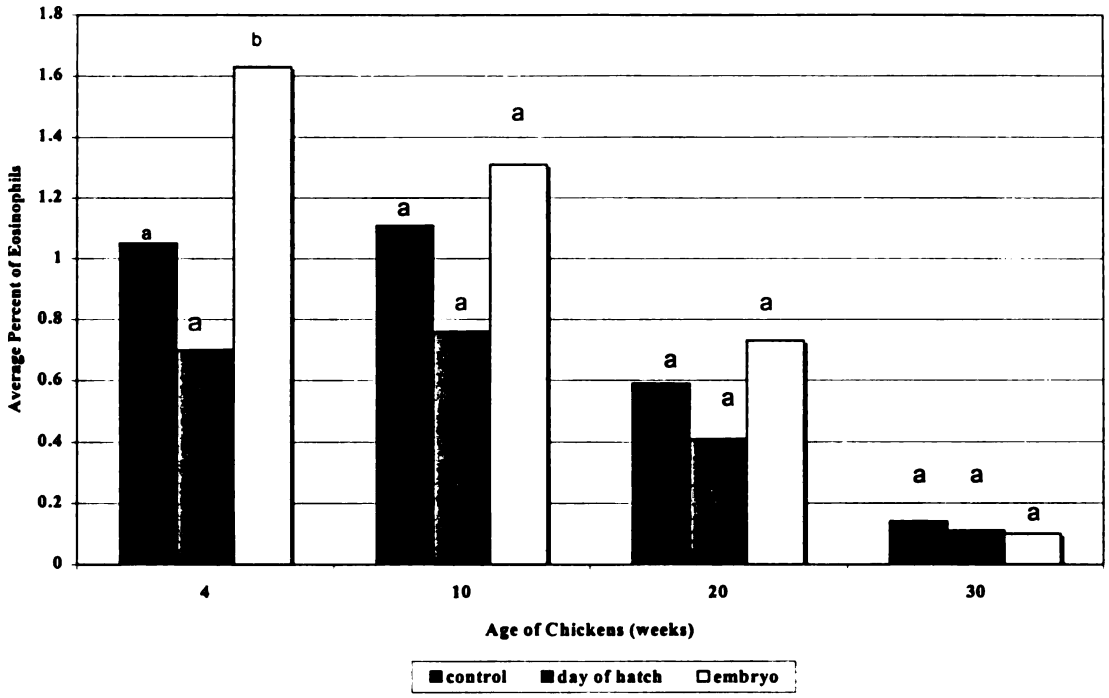


Figure 2.23: Eosinophil percent comparison of ALV-J infected lines combined over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

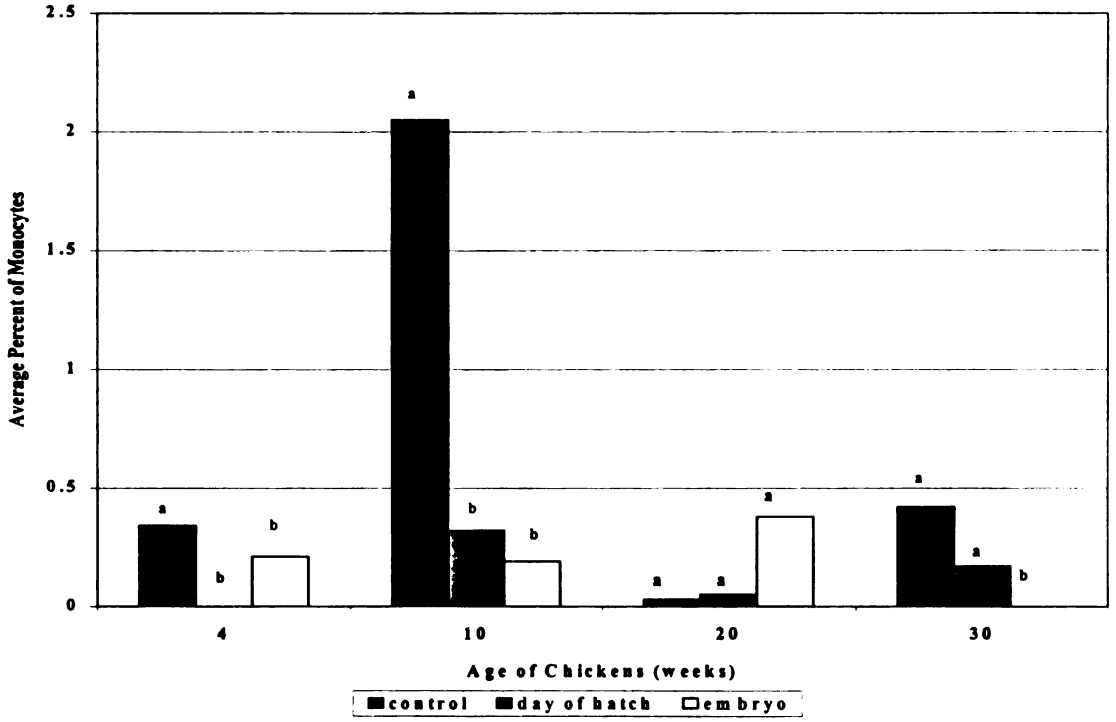


Figure 2.24: Monocyte percent comparison of ALV-J infected lines combined over time. Change in letters indicates a statistically significant difference between the control group and the indicated treatment group at that time period.

Figure 2.25: Control group average percent for heterophils. Change in letters indicated a statistically significant difference when compared to line 0.

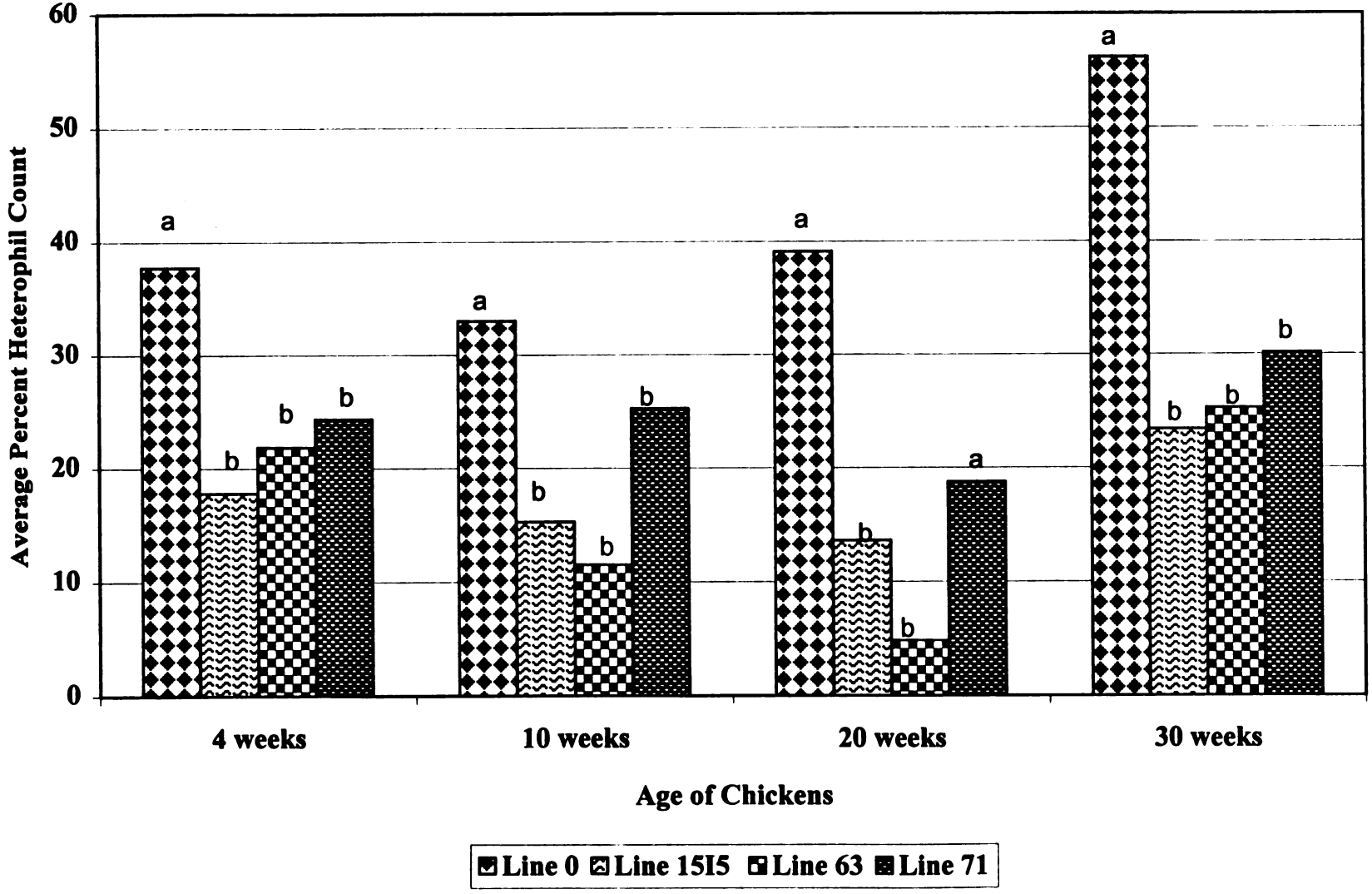

60
50
40
30
20
10
0
Average Percent Heterophil Count
a
b
b
b
a
b
b
b
a
b
b
a
a
b
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 63
Line 71

Figure 2.26: Control group average percent for lymphocytes. Change in letters indicated a statistically significant difference when compared to line 0.

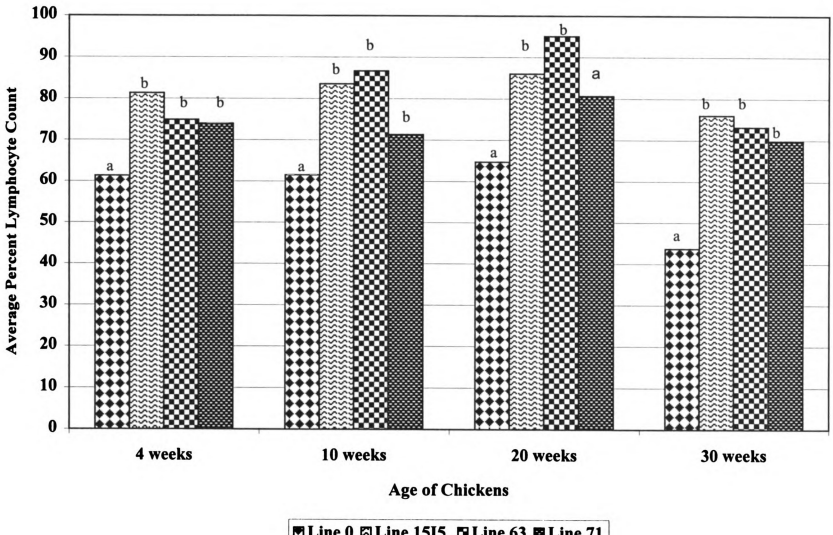

Average Percent Lymphocyte Count
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
a
b
b
b
a
b
b
a
a
b
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 63
Line 71

Figure 2.26: Control group average percent for lymphocytes. Change in letters indicated a statistically significant difference when compared to line 0.

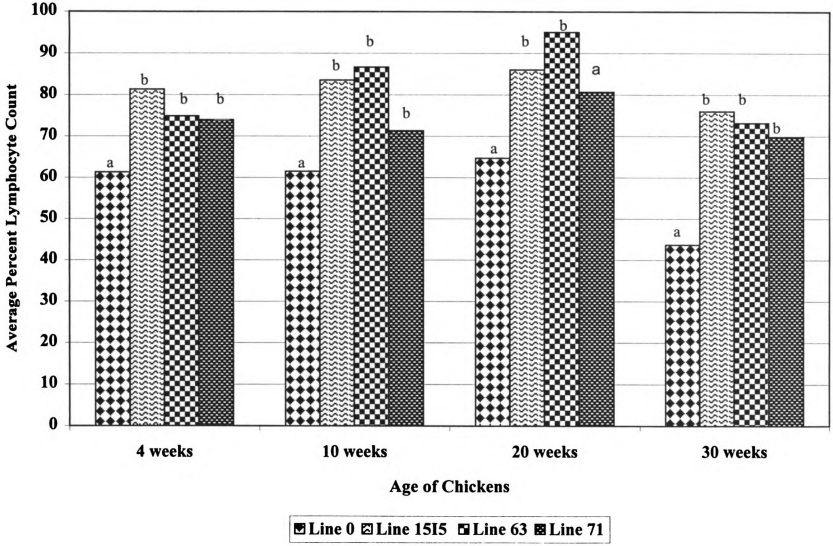

100
90
80
70
60
50
40
30
20
10
0
Average Percent Lymphocyte Count
a
b
b
b
a
b
b
b
a
b
b
a
a
b
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 63
Line 71

Figure 2.27: Control group average percent for eosinophils. Change in letters indicated a statistically significant difference when compared to line 0.

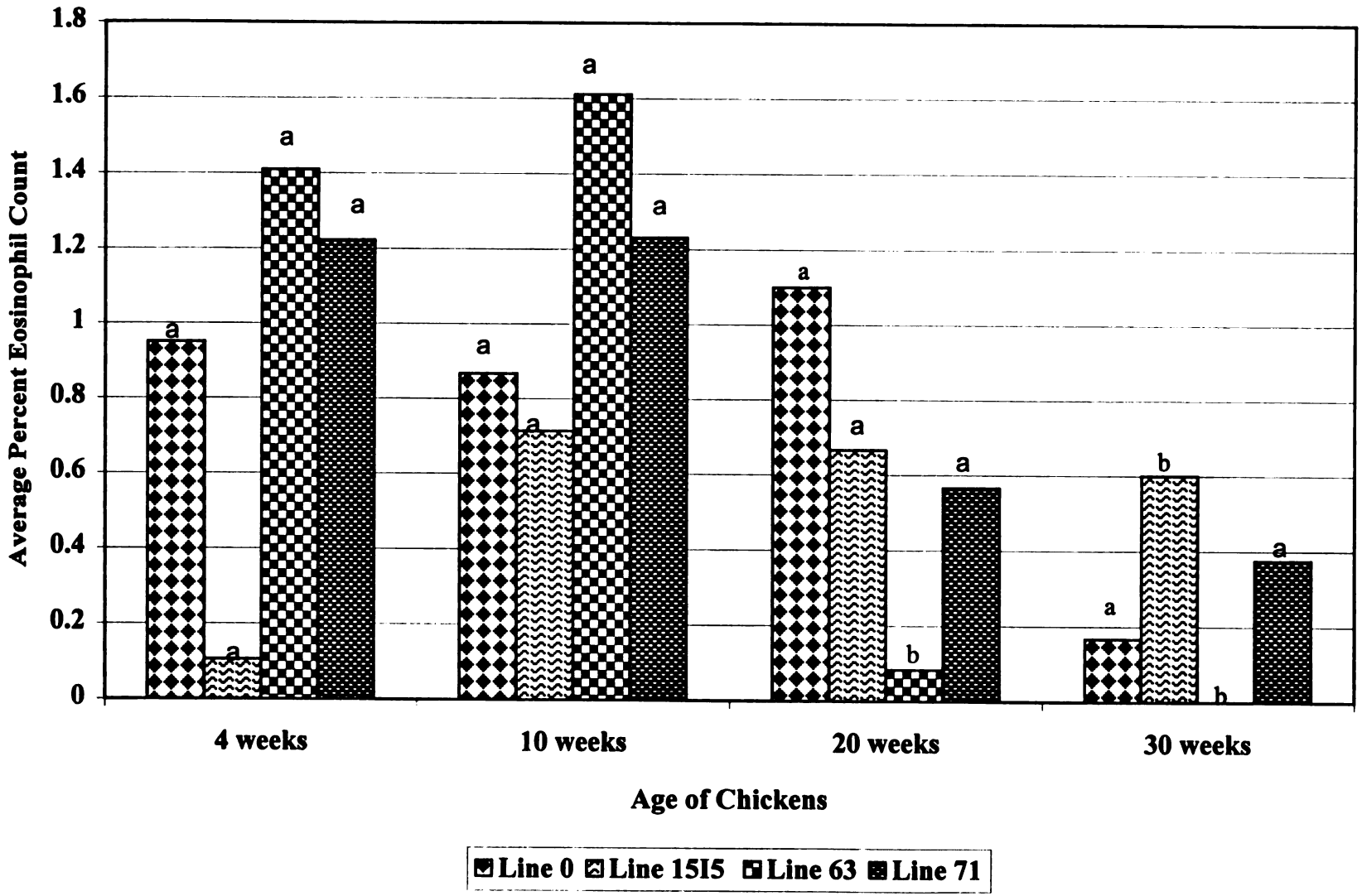

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Eosinophil Count
a
a
a
a
a
a
a
a
a
a
a
b
a
b
a
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 63
Line 71

Figure 2.27: Control group average percent for eosinophils. Change in letters indicated a statistically significant difference when compared to line 0.

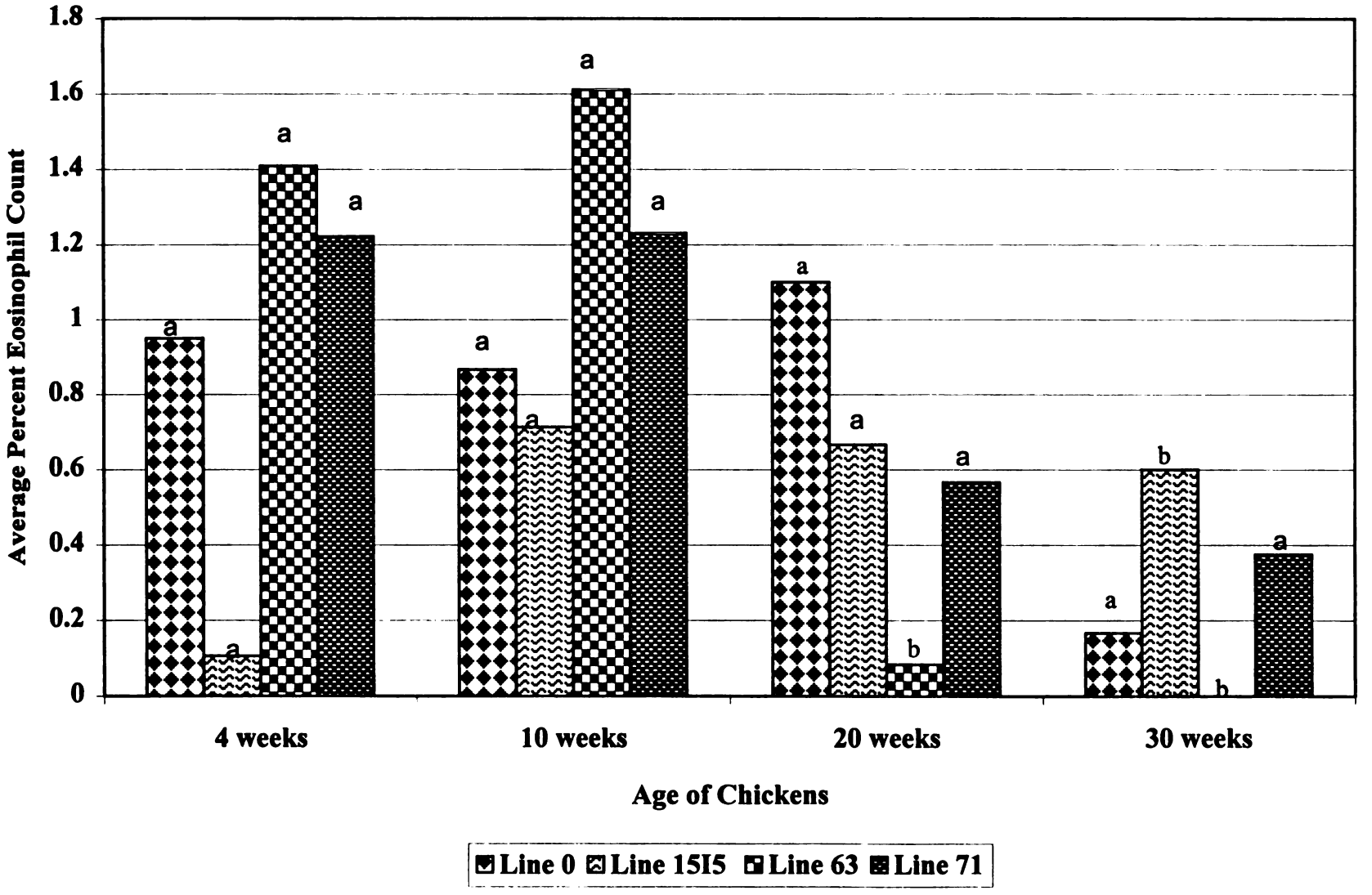

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Eosinophil Count
a
a
a
a
a
a
a
a
a
a
a
b
a
b
b
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 63
Line 71

Figure 2.28: Control group average percent for monocytes. Change in letters indicated a statistically significant difference when compared to line 0.

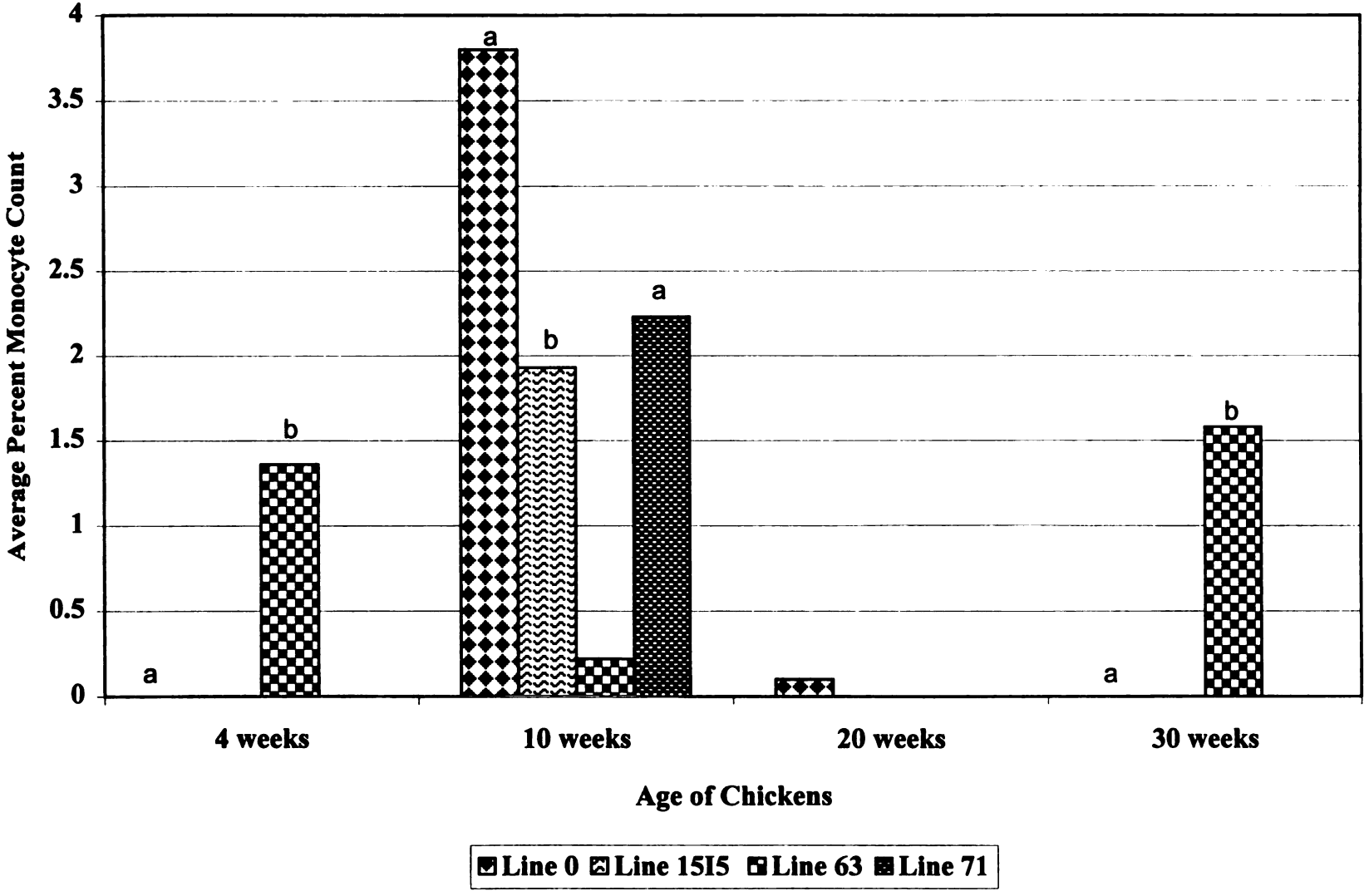

Average Percent Monocyte Count
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
a
b
a
a
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 63
Line 71

Figure 2.28: Control group average percent for monocytes. Change in letters indicated a statistically significant difference when compared to line 0.

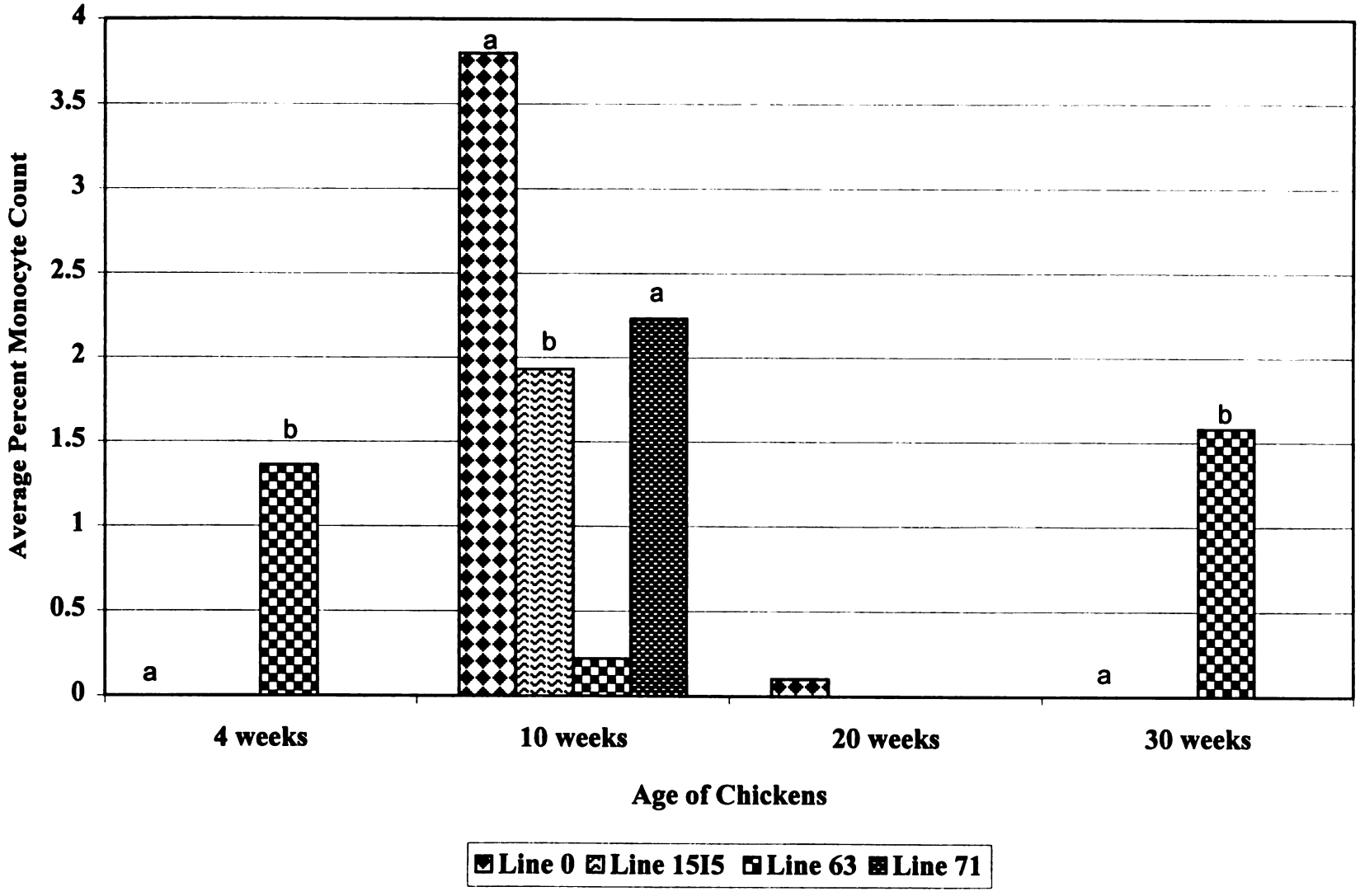

Average Percent Monocyte Count
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
a
b
a
a
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 63
Line 71

Figure 2.29: Day of hatch inoculated group average percent for heterophils. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0.

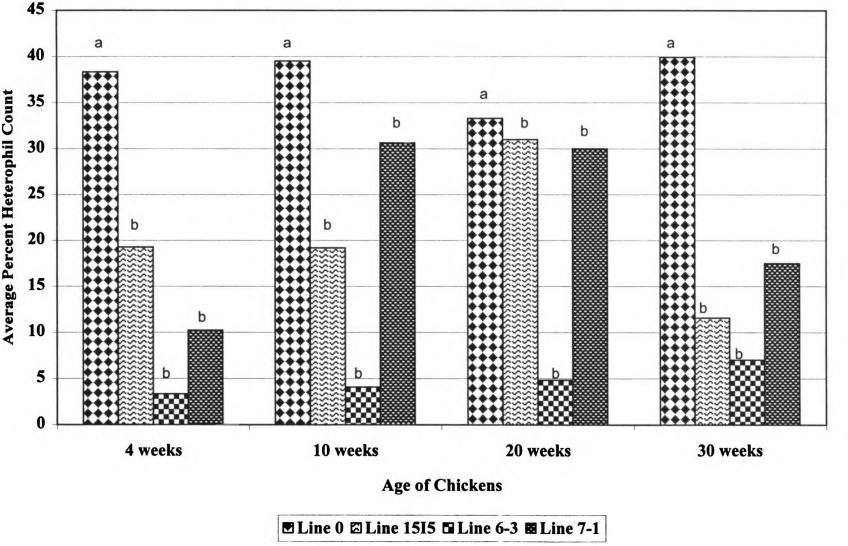

45
40
35
30
25
20
15
10
5
0
Average Percent Heterophil Count
a
b
b
b
a
b
b
b
a
b
b
b
a
b
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 6-3
Line 7-1

Figure 2.30: Day of hatch inoculated group average percent for lymphocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0.

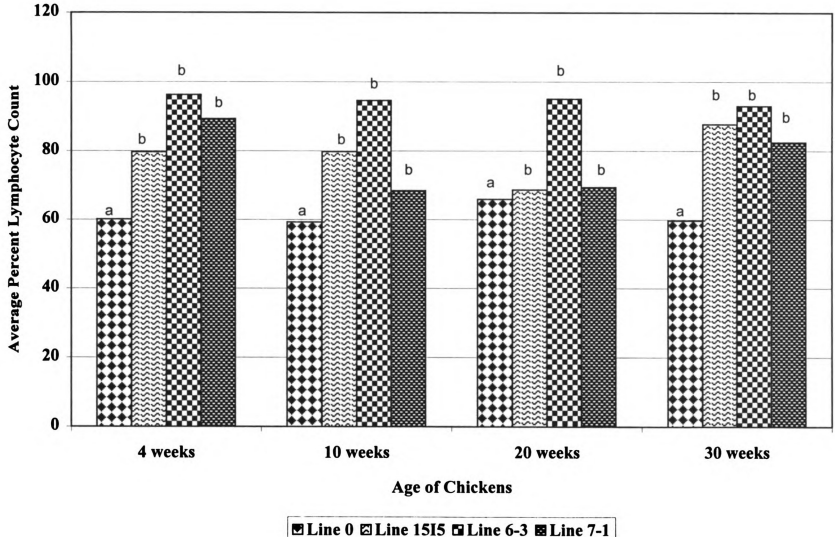

120
100
80
60
40
20
0
Average Percent Lymphocyte Count
a
b
b
b
a
b
b
b
a
b
b
b
a
b
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 6-3
Line 7-1

Figure 2.31: Day of hatch inoculated group average percent for eosinophils. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0.

Average Percent Eosinophil Count
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
a
a
a
a
b
a
b
a
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 1515
Line 6-3
Line 7-1

Figure 2.32: Day of hatch inoculated group average percent for monocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0.

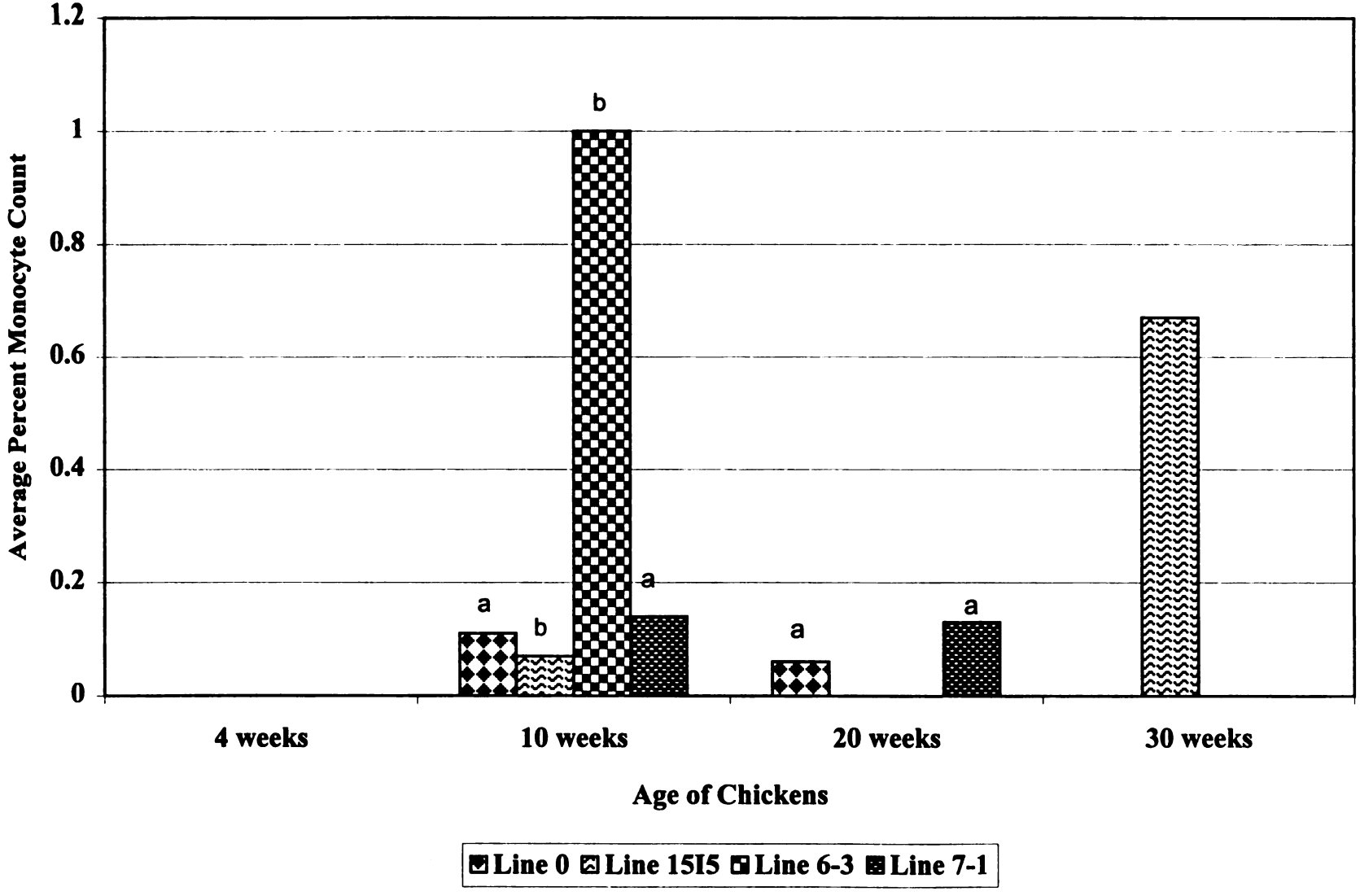

1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Monocyte Count
a
b
b
a
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.32: Day of hatch inoculated group average percent for monocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0.

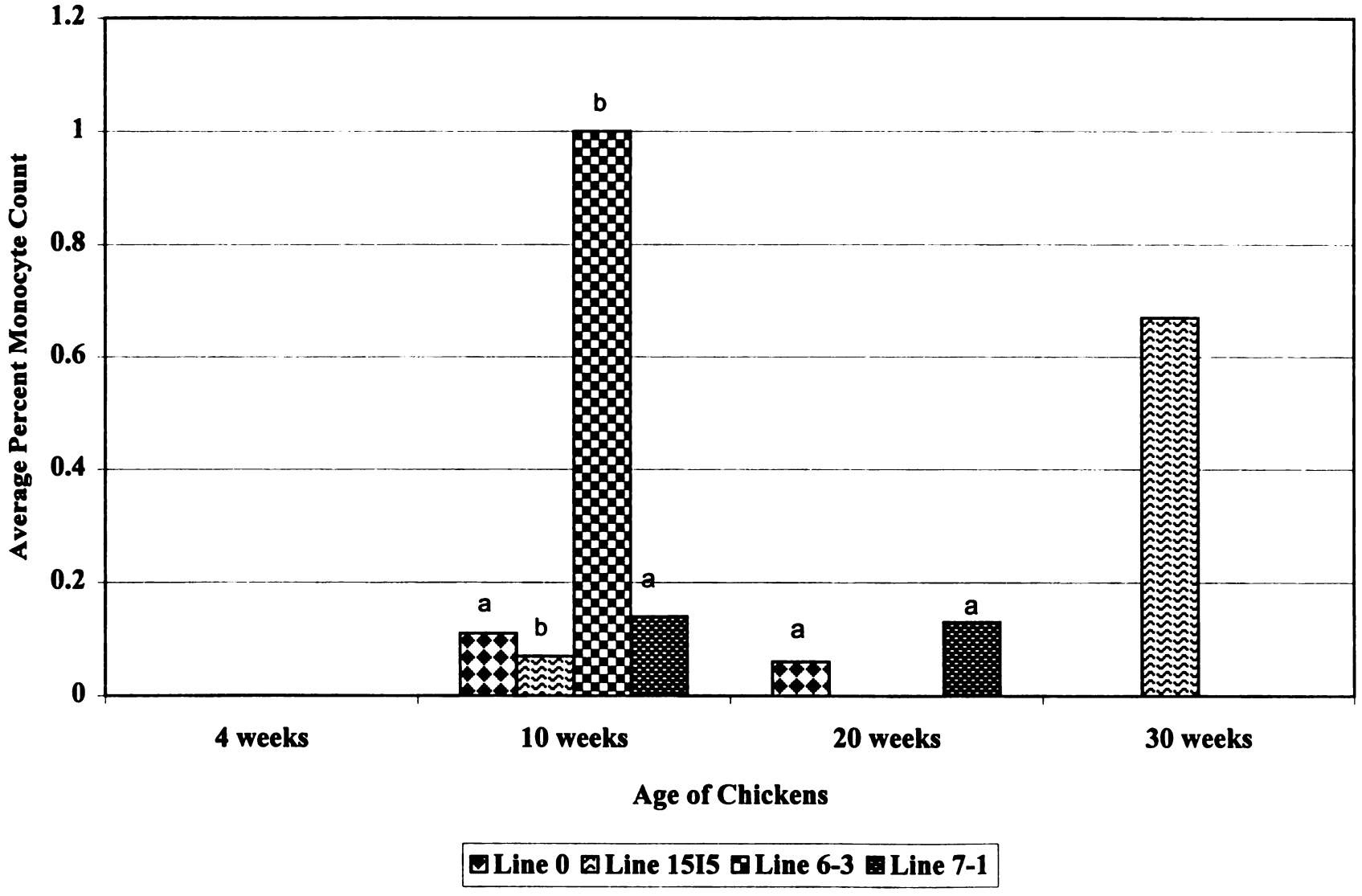

1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Monocyte Count
a
b
b
a
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.33: Embryo inoculated group average percent for heterophils. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicated a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.

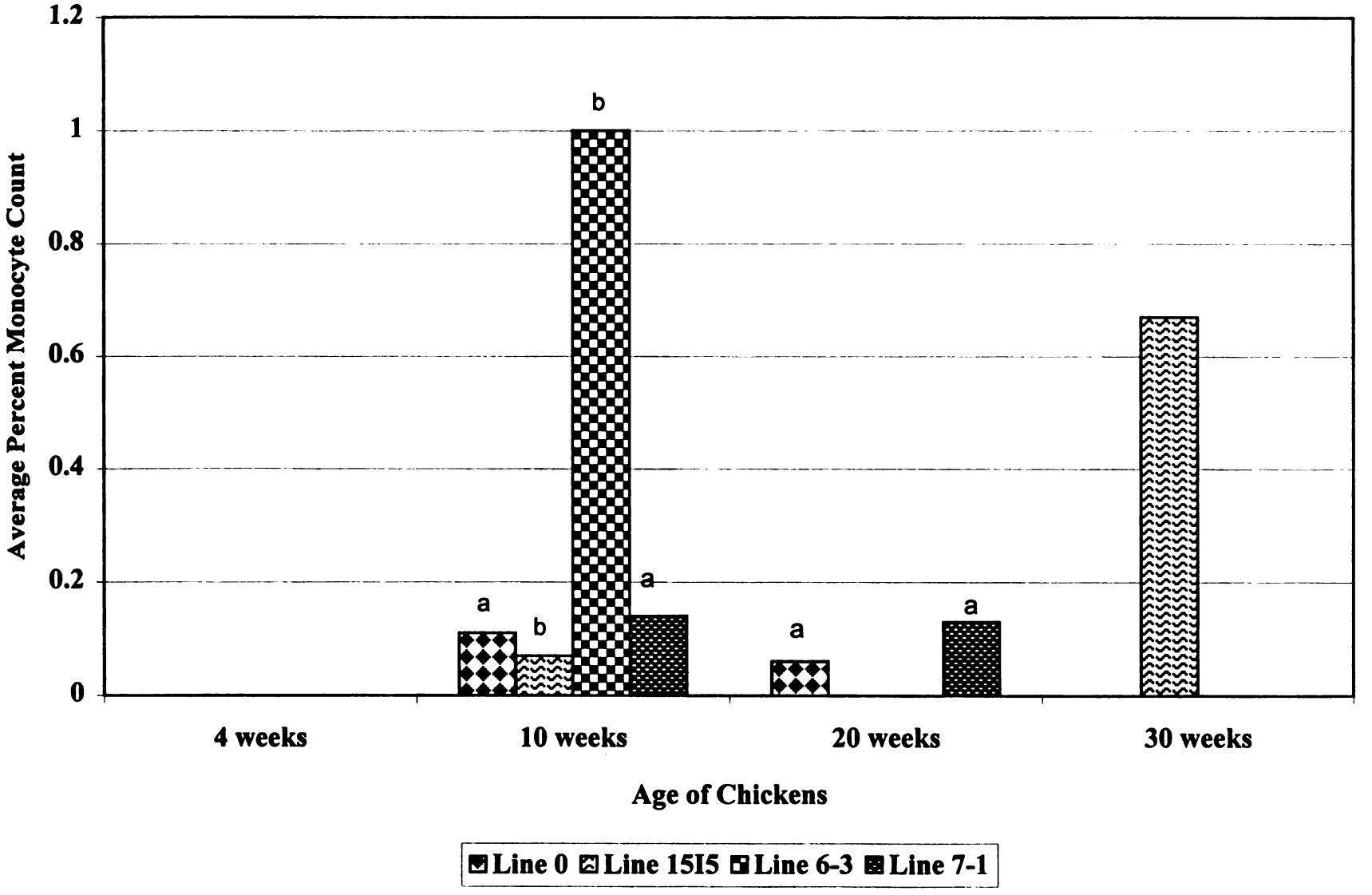

1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Monocyte Count
a
b
b
a
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.33: Embryo inoculated group average percent for heterophils. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicated a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.

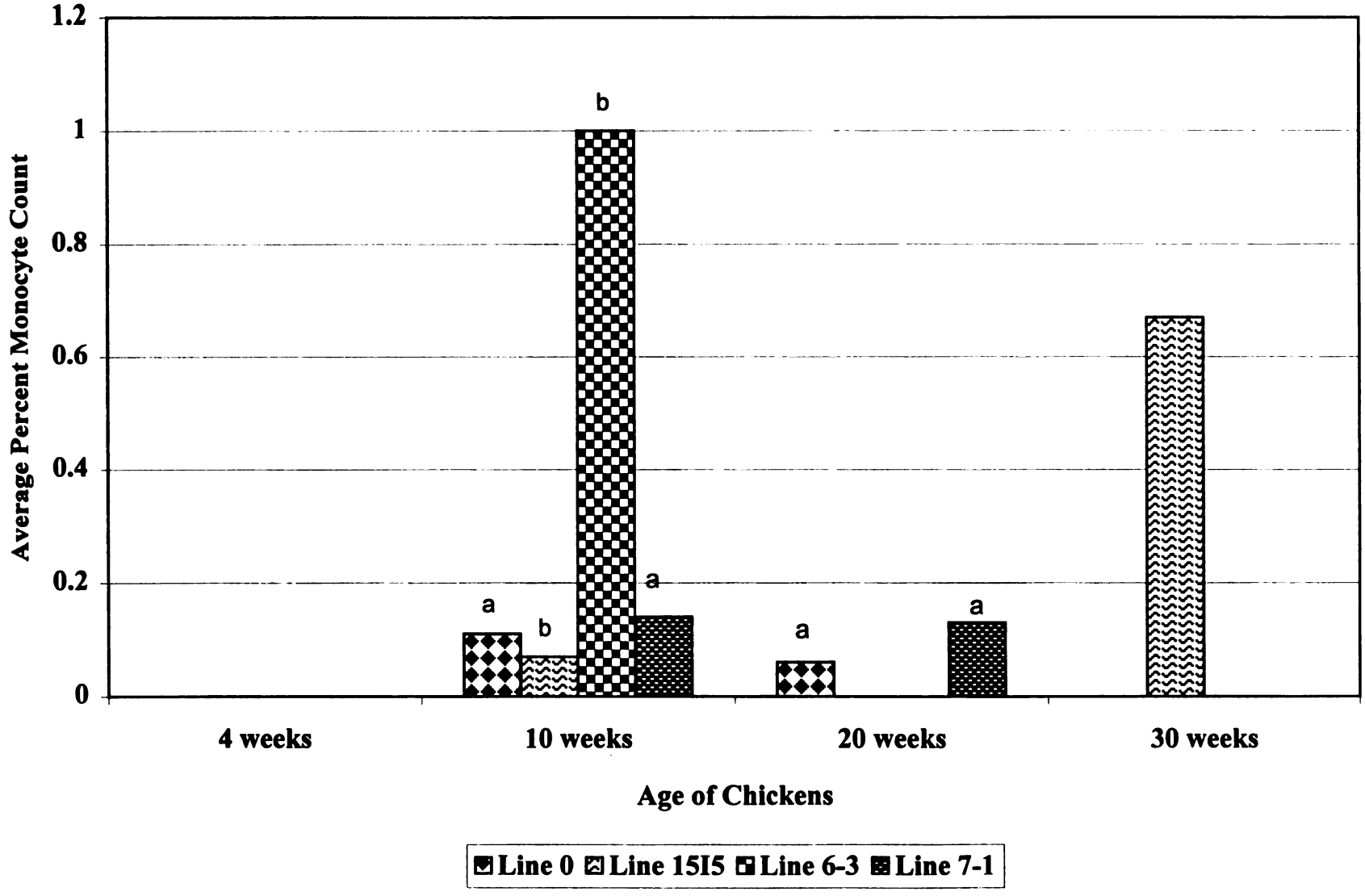

1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Monocyte Count
a
b
b
a
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.34: Embryo inoculated group average percent for lymphocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicated a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.

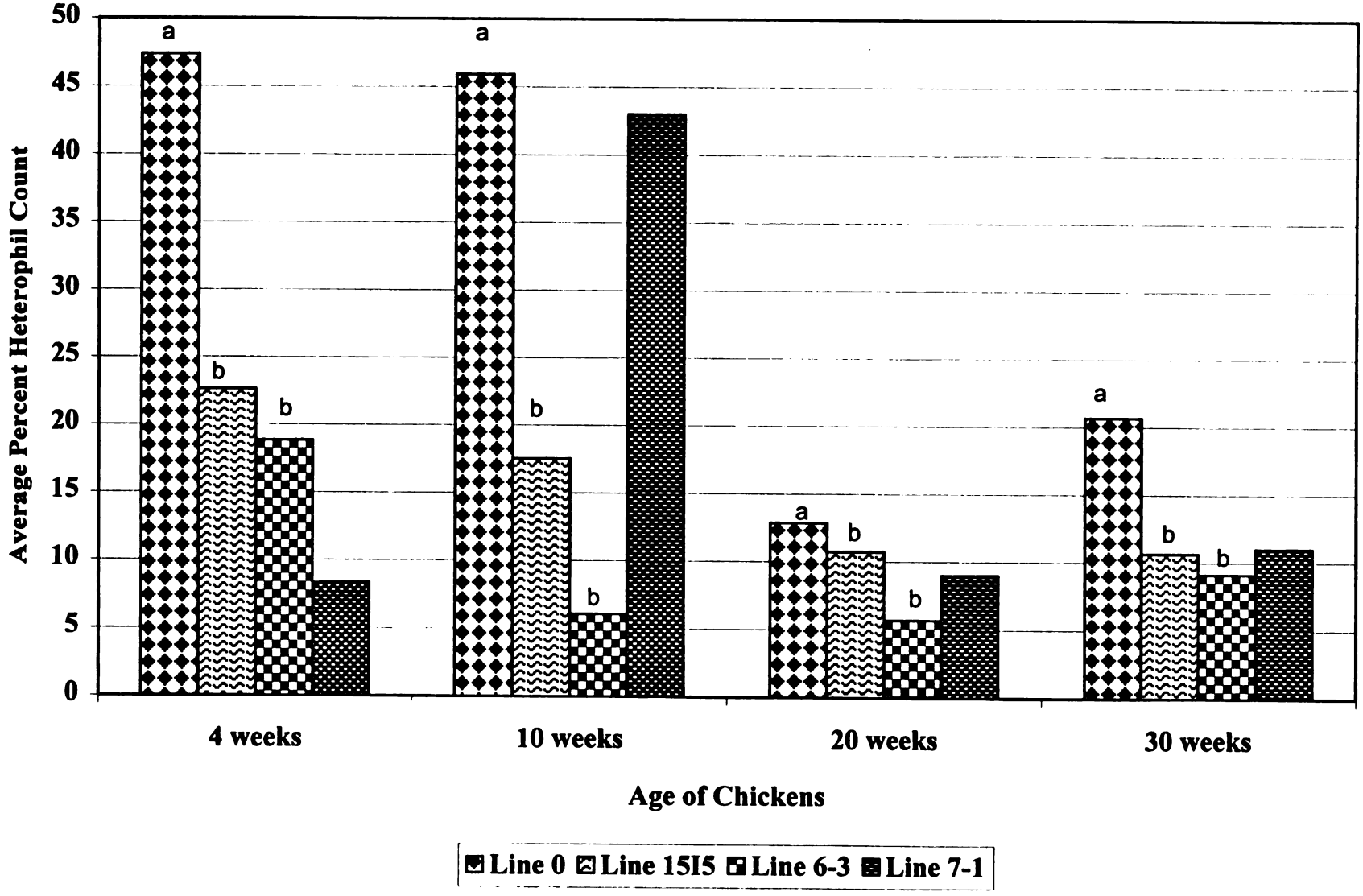

Average Percent Heterophil Count
50
45
40
35
30
25
20
15
10
5
0
a
b
b
a
b
b
a
b
b
a
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.34: Embryo inoculated group average percent for lymphocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicated a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.

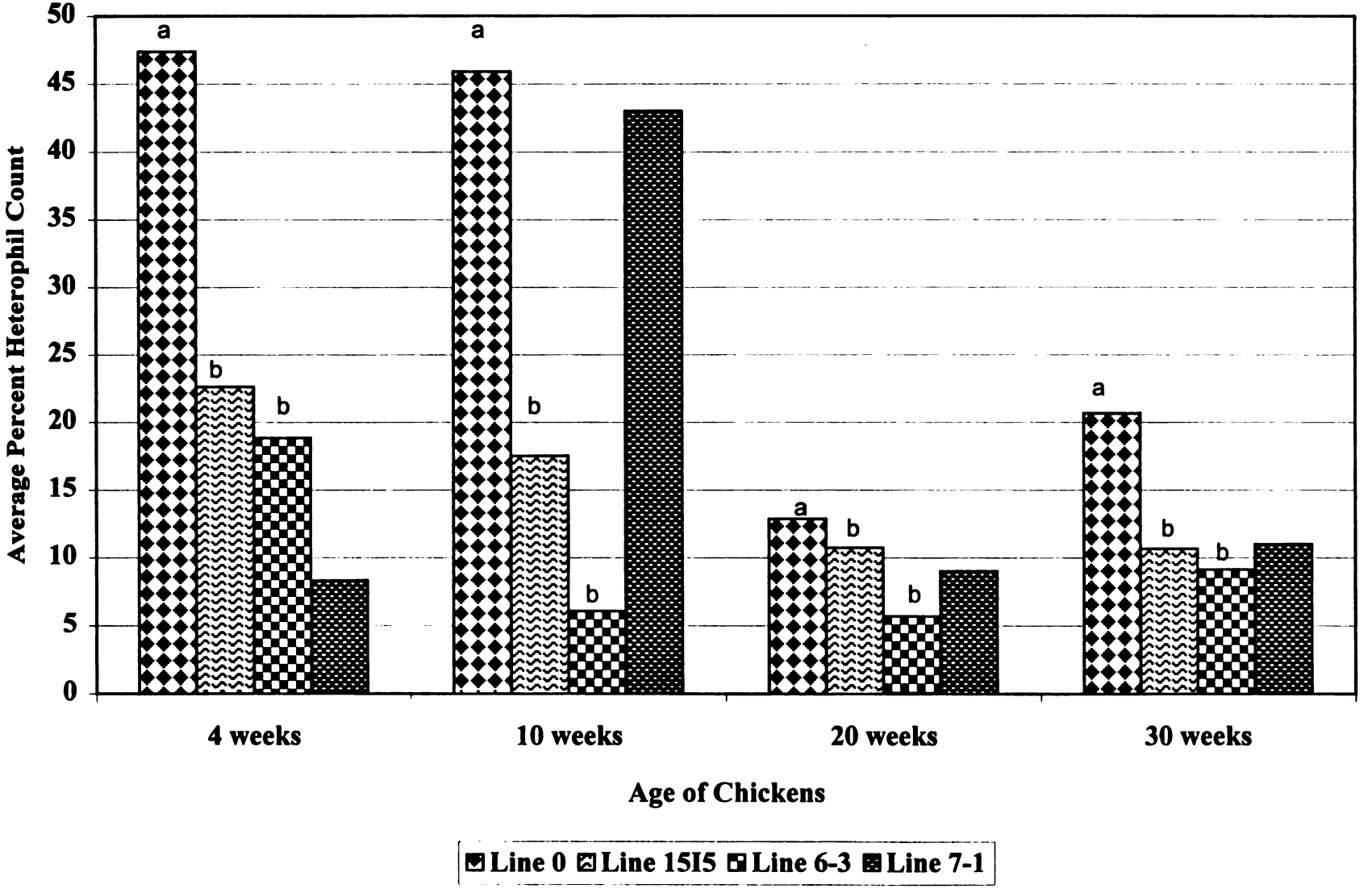

Average Percent Heterophil Count
50
45
40
35
30
25
20
15
10
5
0
a
b
b
a
b
b
a
b
b
a
b
b
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.35: Embryo inoculated group average percent for eosinophils. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicated a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.

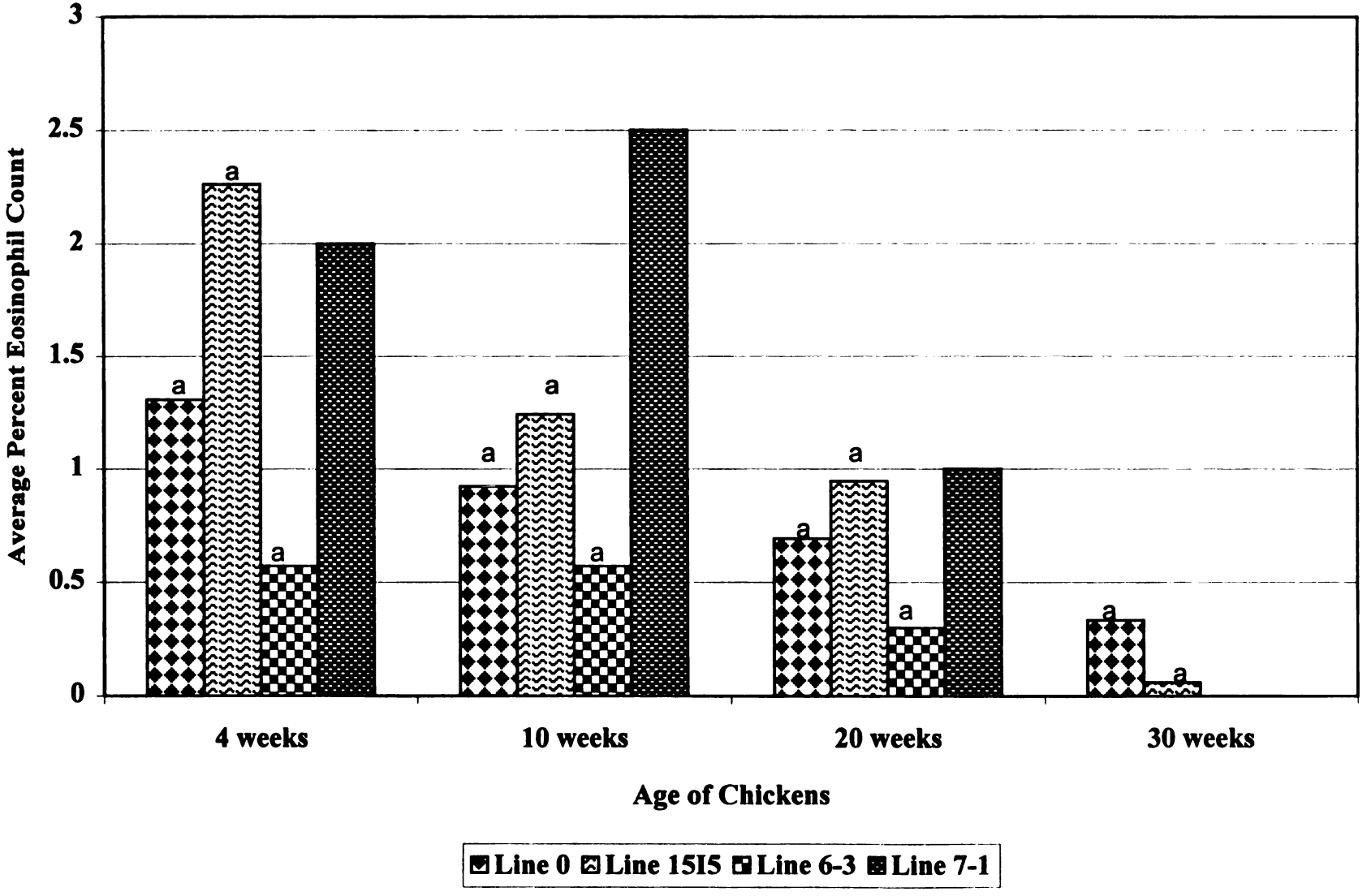
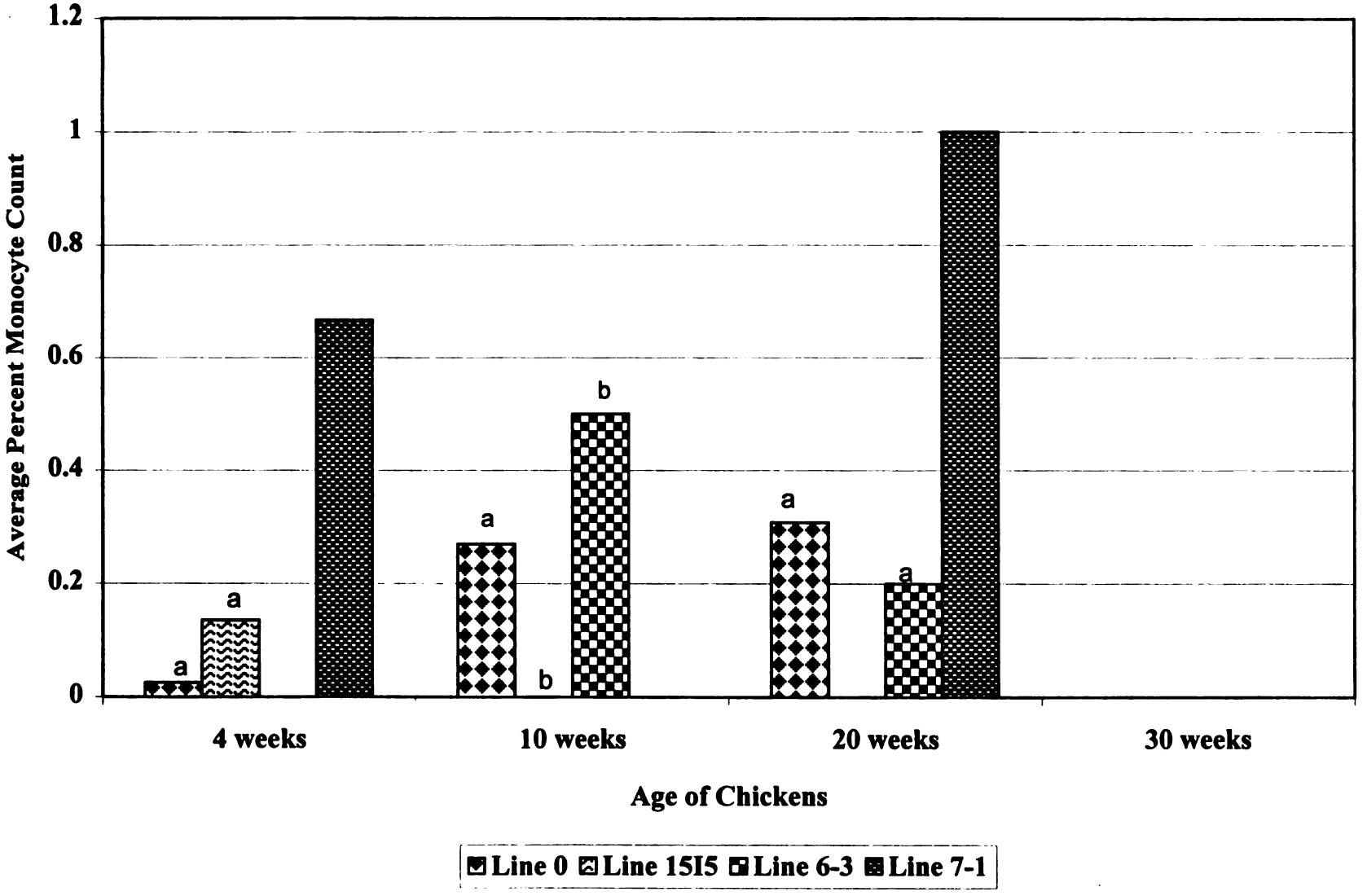

3
2.5
2
1.5
1
0.5
0
Average Percent Eosinophil Count
a
a
a
a
a
a
a
a
a
a
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

Figure 2.36: Embryo inoculated group average percent for monocytes. Lines $0,15I_5$, 6_3, and 7_1 were infected with ALV-J. Change in letters indicates a statistically significant difference when compared to line 0. Line 7_1 was not included in analysis due to insufficient numbers of birds.


1.2
1
0.8
0.6
0.4
0.2
0
Average Percent Monocyte Count
a
a
a
b
b
a
a
4 weeks
10 weeks
20 weeks
30 weeks
Age of Chickens
Line 0
Line 15I5
Line 6-3
Line 7-1

V. *DISCUSSION*

In this study, we repeated some of the work of Payne *et al* (1992) utilizing some of the same genetic lines of chickens infected with an antigenically different strain of ALV-J. Our studies were conducted with the antigenically distinct U. S. strain, ADOL-Hc1, whereas Payne's work was conducted with the HPRS-103 strain of ALV-J first isolated in the U. K. Antibodies made against HPRS-103 do not neutralize ADOL-Hc1 while antibodies against ADOL-Hc1 neutralize HPRS-103 (Fadly and Smith 1997; Fadly and Smith 1999). Venugopal *et al* (1998) also noted antigenic variability of 12 field isolates compared to HPRS-103. Only two of those field isolates were neutralized by HPRS-103. The 12 isolates were determined to be ALV-J by PCR (Smith *et al.* 1998). Silva *et al* (2000) also described the genetic variability among U. S. isolates in the envelope region. Since there is antigenic differences between the two prototypes, this study was conducted to determine if there are differences in response to infection with a different strain of virus in various genetic lines of chickens. The highly susceptible F_1 progeny of $15I_5$ X 7_1 infected with ALV-A served as the positive control.

Experimental inoculation of embryos before the immune system has developed is one way to reproduce natural congenital infection with ALV. Congenitally infected chicks with ALV usually remain viremic tolerant throughout their lives, serving as a source of infection for hatch mates. The results obtained in this experiment demonstrated the same outcome of viremic tolerance in four genetic lines of white leghorn using the U. S. prototype of ALV-J. However, the results were dramatically different in chickens inoculated at day of hatch compared to those inoculated as embryos. Day of hatch inoculated line 0 chickens were able to seroconvert and neutralize circulating virus by 10 weeks of age. Line $15I_5$ had all chickens (30/30) harboring virus at 30 weeks of age while 13/30 had detectable antibody at 30 weeks of age. Line 7_1 had virus detected in 5/6 chickens and antibody detected in 6/6 chickens at 30 weeks of age. Line 6_3 had limited antibody detected while the majority of the chickens harbored virus at 30 weeks of age.

Previous experiments done with ALV-A (RAV-1) and different genetic lines of chickens with various *ev* genes develop antibodies at a slower pace than line 0 which lacks *ev* genes (Crittenden *et al.* 1987; Crittenden 1991). The ev genes in chickens have been studied and have been shown to interfere with the immune system when challenged with exogenous ALVs (Crittenden *et al.* 1987). The chickens in this experiment had *ev* genes (except line 0) and our results supported previous work which demonstrated that the *ev* genes interfere with the immunological response to ALV infection.

The four virus negative chickens in Group 1, ALV-J (lines 0 and 7_1) and ALV-A embryo inoculated, illustrated that embryo infection is not always 100%. The inoculum in these cases may not have had enough viral particles to establish an infection. One line 0 chick and one line 7_1 chick ALV-J Group 1 were not virus positive and did not survive the first week of life. The two, ALV-A, Group 1, chickens were exposed to the virus at hatch from their infected hatch mates and were not detected until 10 weeks when seroconversion occurred. They were not a part of the original 10 chicks that were randomly selected to test at day of hatch for the presence of virus. Because detection of the antibody did not occur until 10 weeks of age and only one chicken had cleared the virus, they were kept in the experiment.

Tumor susceptibility in ALV infection does not correlate to serological status and the ability to produce antibodies (Payne and Fadly 1997). The loci tvA and *tv*B (tumor virus subgroup A and B) are important in determining the cellular resistance or susceptibly of the bird to infection (Payne and Fadly 1997). However, this is not always the case. For example, line 6_3 is susceptible to infection by subgroup A but rarely develop tumors, mainly due to lack of target cells in the bursa of Fabricius, not because of the tvA or *tv*B loci (Purchase *et al.* 1977). This perhaps will hold true with subgroup J infection. Only one chicken in the Line 6_3 groups developed a tumor and it was of lymphoid origin and only one chicken had bursal transformation at ten weeks of age. Both of these line 6_3 chickens were in the embryo inoculated treatment group. Line 7_1 is

undefined for ALV-A infection and tumor formation. In this study, line 7_1 was susceptible to infection and tumor formation with ALV-J. There was bursal transformation at 10 weeks (20%) with three chickens (one embryonically challenged and two day of hatch challenged) developing tumors. Inoculation as embryos or at day of hatch can also influence tumor incidence (Fadly *et al.* 1987). Viremic tolerant chickens are more likely to die from LL than chickens that have antibody (Rubin *et al.* 1961). This is due to the lack of antibody response, allowing the circulating virus to continuously replicate in embryo inoculated chickens and therefore increase the chance of integration by the proviral DNA next to a proto-oncogene. Line 0 and $15I_5$ illustrated this phenomenon when inoculated with ALV-J as embryos or at day of hatch (Table 3). The other two lines did not have enough chickens survive to the end of the experiment to make a valid statistical comparison. Line $15I_5$ X 7_1 is highly susceptible to ALV-induced tumor formation. This was evident by the fact that 96% (28/29) of the chickens in the embryo inoculated group developed tumors and 100% (26/26) of chickens inoculated at day of hatch developed tumors before 30 weeks of age. Both groups had a few chickens with more than one tumor type present.

Bursal transformation due to infection with subgroup A ALV demonstrates the ability of the virus to integrate next to a proto-oncogene (usually *c-myc*) in B cells and result in LL. The MGP staining technique was used to demonstrate the presence of RNA within a cell, which indicates that the cell is active in the cell cycle. When retroviruses integrate next to a proto-oncogene, the strong promoters within the LTRs direct the over expression of the downstream host genes. The overexpressed proto-oncogene protein disregards the normal cellular growth regulatory system within that cell (Lewin 1994). These cells are candidates for transformation and stain brightly eosinophilic with the MGP stain due to the large amounts of RNA being produced. The results from these studies indicates that ALV-J can transform bursal follicles and may account for the lymphoid leukosis manifestation that was observed as a predominate lesion in the white

leghorns used in this study (Table 3). The oncogene involved was not determined in this study. With regards to the ALV-A infected chickens, they responded as expected with 100% of the bursas examined having transformed follicles. The highly susceptible line $15I_5 \times 7_1$ allows the integration of the proviral DNA near *c-myc*, resulting in a high frequency of bursal follicle transformation.

Differences in WBC counts among chickens may be difficult to interpret due to the wide range of normal white blood cell counts (Lucas and Jamroz 1961; Sturkie and Griminger 1976; Campbell 1988). Most authors agree that in order to identify ill birds, large differences from the normal leukogram must be noted (Lucas and Jamroz 1961; Sturkie and Griminger 1976; Campbell 1988). Comparisons were made only on a mean percent basis because a complete blood count was not performed. Therefore, no analysis on actual decreases or increases in cell counts was performed. Within control Group 3, there were statistical differences between all genetic lines of chickens for heterophil and lymphocyte levels at all sampling periods. These differences could be due to the genetics of each line (Lucas and Jamroz 1961; Shen *et al.* 1984) or due to the overall health status of the chickens. The control chickens may have had incidental physical injuries that could have influenced their leukograms. Various authors explain the differences seen on many factors like age, sex, nutritional status, environment, and degree of stress (Lucas and Jamroz 1961; Sturkie and Griminger 1976; Campbell 1988). Since all chickens in this study were hatched on the same day and housed in similar isolators and fed the same feed, some of those factors may have little influence on the variation seen. Sex and degree of stress may have a strong influence on the outcome in addition to genetics. It should be noted that the time of sampling may be considered stressful for the subjects due to handling, restraint, and catching the chickens in the isolators. Although there were significant statistical differences noted, these cannot be explained by the viral infection alone. One interesting observation is that with all the chickens that died with LL induced

by ALV-J or ALV-A, there were no lymphoblastic cells in blood smears even though there was lymphoblastic cell infiltrates in various tissues.

Although ALV-J is mainly encountered in broiler breeder chickens, this study along with earlier work by Payne *et al* (1992) demonstrates that white leghorn chickens are susceptible to infection and tumor formation under experimental conditions. The main tumors encountered in this study were LL and hemangiomas, while Payne did not report the presence of LL in the leghorns or meat type chickens. The differences were probably not due to genetic differences among experimental birds. Three lines of white leghorn chickens that were common to both experiments have very similar genetic backgrounds. Differences may be due to the use of antigenically different viruses. Determining the receptor on the target cells may offer additional insight to explain the different tumor manifestations seen (myeloid versus lymphoid).

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CHAPTER 3

Influence of Endogenous Virus 21 (EV21) on the Response of White Leghorn Chickens to Infection with Subgroup J Avian Leukosis Virus

I. **Abstract:**

The influence of endogenous virus 21 (EV21) on the response of White Leghorn chickens to infection with ALV-J was studied. F_1 progeny from the cross EV21+ males with $15B_1$ females were hatched, characterized as late feathering or early feathering by the length of the primary feathers, and inoculated at day of hatch with the U. S. prototype of ALV-J, ADOL Hc-1. Confirmation of feathering typing was performed at 10 weeks of age by the R2 alloantisera assay. Whole blood samples for virus isolation and neutralization assays were collected at 4, 10, 18 and 31 weeks of age. Necropsy was performed on all birds that died during the experiment and at termination. Gross tumor samples were collected for microscopic examination. Birds harboring EV21 were more likely to be ALV-J virus positive at all sampling periods than birds lacking EV21. The incidence of ALV-J antibody in chickens lacking EV21 was higher than in chickens harboring EV21. Birds harboring EV21 were also more viremic tolerant at the end of the experiment than birds lacking EV21. Tumor development was minimal for both groups. LL was the most common tumor type.

II. **Introduction**

There are many avian retrovirus-like elements in the normal chicken genome. They include the endogenous virus (*ev*) loci, avian retrotransposon-chickens or ART-CH, and endogenous avian virus or EAVs (Dunwiddie *et al.* 1986; Boyce-Jacino *et al.* 1992; Nikiforov and Gudkov 1994). Recently, a novel element has been reported in the literature, ev/J by Benson *et al* (1998). However, later work demonstrated that ev/J and EAV-HP were virtually identical (Sacco *et al.* 2000). The *ev* loci are related to subgroup

E avian leukosis virus (ALV) and are either complete and infectious or defective viruses (Robinson 1978; Crittenden 1981; Crittenden and Astrin 1981; Smith 1987). Twenty-nine *ev* loci have been located and identified thus far, (Gudkov *et al.* 1986; Smith 1987). Rovigatti and Astrin (1983) determined that a normal chicken averages about five *ev* loci in its genome. Depending on whether the *ev* loci are complete or defective, and what defects are present, these birds may react positively to various diagnostic tests without being infected with exogenous retrovirus.

One such important *ev* locus is the *ev21* locus. It encodes for a complete infectious subgroup E ALV known as EV21. EV21 has been mapped to the Z chromosome and is linked to the dominant sex linked gene K which regulates slow or late feathering (Warren 1925). Late feathering can be differentiated from early feathering and is widely used for sex determination of chicks in the poultry industry (Warren 1930). However, there have been reports of reduced production and increased leukosis mortality in female progeny from dams with the K gene (Lowe and Garwood 1981; Harris *et al.* 1984). In studies by Bacon *et al* (1988) and Harris *et al* (1984), the *ev21* gene is expressed as a complete infectious endogenous virus, which can be transmitted to the progeny congenitally and result in increased susceptibility to infection by exogenous ALVs because of immunologic tolerance.

Subgroup J ALV, first described in the early 1990s by Payne *et al* (1991), mainly causes myelocytomatosis in broiler breeder chickens. Although meat type chickens are mainly affected by ALV-J, there have been reports of white leghorn chickens developing myelocytomatosis due to subgroup J-like infection (Fadly 1998). Experimental studies using leghorn chickens inoculated with subgroup J have demonstrated that they are

susceptible to infection and can develop tumors (Payne *et al.* 1992; chapter2). The objective of this study was to determine the role of EV21 on the response of white leghorn chickens to infection with ALV-J using the U. S. prototype virus ADOL-Hc1.

III. METHODS AND MATERIALS

A. **Chickens**: Two lines of chickens from the Avian Disease and Oncology Laboratory in East Lansing, MI were used. These were males of the Regional Poultry Research Laboratory (RPRL) line 0.44-EV21+ (a late feathering line) and females of the early feathering RPRL $15B_1$ line (Bacon *et al.* 2000). The F_1 progeny are either late feathering chicks (both male and female) that are EV21+ or early feathering chicks (also both male and female) that are EV21-. The breeder line 0.44-EV21+ (EV21+) male chickens are detected by feather phenotype and blood typing using the R2 alloantiserum assay (Bacon *et al.* 1996). The breeder flocks for the two lines are free from many avian pathogens, including exogenous ALV, as determined by routine serological surveys. Chicks were separated into early or late feathering groups by length of primary feathers on both wings. Birds were housed in plastic isolators according to feather phenotype for 31 weeks and given food and water *ad libitum*.

B. **Virus:** Strain ADOL-Hc1, the U. S. prototype, of ALV-J was used (Fadly and Smith 1999). The virus titer of the virus stock was determine to be 10^5 infectious units (IU)/ml. Each treatment group received 10^4 IU/bird intraperitoneally at day of hatch.

C. **Experimental Design:** 82 day-old chicks from the previously mentioned cross were separated into early or late feathering groups according to feather phenotype. All birds were inoculated at day of hatch intraperitoneally with 10^4 IU/bird with a 30g-½ inch needle. At 4, 10, 18 and 31 weeks of age, whole blood samples were collected and the plasma was used for VI and VN assays. At 10 weeks of age red blood cells and/or plasma was used to perform the R2 assay for endogenous virus to confirm feather phenotype (Bacon *et al.* 1996; Bacon 2000). Termination of the study was conducted at 31 weeks of age. All birds that died during the experiment and those that survived to the end of the experiment were necropsied.

D. **Virological and serological assays**: Virus isolation (VI) and virus neutralization (VN) assays were conducted on all plasma samples at each sampling period using line 0 (C/E) CEF. Briefly, VI was performed using 100 microliters of plasma added to 4% calf serum Leib McCoy media containing penicillin, streptomycin and amphotericin B plus 0.04IU of heparin per plate and CEFs in suspension. Media was changed the following day to 1% calf serum Leib McCoy media containing above antibiotics and fungicide. Cultures were allowed to grow for 7- 9 days. Plates were frozen and thawed twice (Fadly and Witter 1998). Cell lysates from CEF cultures was tested by the gs antigen p27 ELISA (Smith *et al.* 1979). VN assays were performed according to procedures by Fadly and Witter (1998). Briefly, test samples were diluted 1:5 with Leib McCoy media without serum and heat inactivated for 30 minutes at 56° C. Dilute virus stocks ofADOL-Hc1 was added such that each well of a 96 well microtiter plate received 500-1000 viral particles/ml. Equal volumes of test samples were added to the microtiter plates and

incubated for 45 minutes. Appropriate positive and negative control samples were included. Cells ($5X10^4$ cells/well) were added in 4% calf serum Leib McCoy containing antibiotics and fungicide, and incubated 7-9 days at 37° C. Cultures were frozen and thawed twice. The cell lysates were tested for the presence of p27 by ELISA. The R2 alloantiserum assay was performed as described by Bacon *et al* 1996, 2000.

E. **Pathology:** Necropsy was performed on all birds that died during the experiment and at termination. Tissue samples for histology were taken from birds with gross tumor formation. All samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

F. **Statistical Analysis:** Statistical analysis was performed using the SAS 7.0 program. Viremia and antibody data were analyzed with a chi-square test. Results were considered significant at a level of $P < 0.05$.

IV. RESULTS

A. **Virological and serological assays**: The results of the serologic assays performed at the various sampling periods are presented in Table 3.1. The first sampling period, 4 weeks post inoculation, demonstrated that 93% of the chickens harboring EV21 were viremic whereas only 43% of the chickens lacking EV21 were viremic. This difference continued at all sampling periods for birds harboring EV21 compared to birds lacking EV21 where viremia was present at a much lower level. A difference in antibody production occurred at 10 weeks with 16% of the chickens harboring EV21 having

detectable levels of antibody versus 83% of the chickens lacking EV21 having antibody to ALV-J. The 18 weeks sampling period had the highest percentage of chickens with antibody to ALV-J with 54% chickens harboring EV21with detectable antibody and 98% of chickens lacking EV21 with detectable antibody.

Table 3.1: Development of ALV-J induced viremia and antibody in white leghorn chickens harboring or lacking EV21.

Age at Testing –No. +/No. Tested (%)

	4 weeks		10 weeks		18 weeks		31 weeks	
Type of chicken	**VI[a]**	**AbJ[b]**	**VI**	**AbJ**	**VI**	**AbJ**	**VI**	**AbJ**
Harboring EV21	37/40 (93)[1]	0/40 (0)[3]	37/40 (93)[1]	6/38 (16)[3]	20/37 (54)[1]	19/37 (51)[3]	21/35 (60)[1]	15/35 (43)[3]
Lacking EV21	17/40 (43)[2]	0/40 (0)[3]	9/40 (23)[2]	33/40 (83)[4]	2/40 (5)[2]	39/40 (98)[4]	5/38 (13)[2]	28/38 (74)[4]

[a]virus isolation performed on line0 (c/e) CEF for ALV-J detection only
[b]antibody presence to ALV-J as determined by VN assays
[1,2] Numbers change when results are significant at a level of $P \leq 0.05$ for virus isolation at that time period.
[3,4] Numbers change when results are significant at a level of $P \leq 0.05$ for antibody detection at that time period.

Table 3.2 contains the virus positive birds from Table 3.1 and displays them in regards to antibody status. In chickens harboring EV21, the number of birds characterized as virus positive, antibody negative (V+, A-) decreased over the time course of the study. However, at 31 weeks there were still 19/35 (54%) V+, A- chickens. These chickens were considered viremic tolerant. With regards to chickens lacking EV21, there was a sharp decrease from 4 to 10 weeks (45% to 5%) in V+, A- chickens

which should be expected as birds seroconvert and clear the virus. Interestingly, two birds (5%) were V+, A- at 31 weeks in this group and can be considered viremic tolerant.

The R2 alloantisera assay performed at 10 weeks of age for the detection of endogenous ALV virus revealed that 100% of chickens characterized as early feathering by feather phenotype were negative for endogenous virus. Thirty-nine out of 40 chickens characterized as late feathering by feather phenotype were positive for endogenous ALV virus. The one chicken mis-categorized by feather phenotype was euthanized after the 10 week sampling due to severe leg paralysis.

Table 3.2: Characterization of ALV-J positive chickens with regards to antibody status

		Age at Testing—No. +/No. Tested (%)			
Type of chicken	**Serological status**	**4 weeks**	**10 weeks**	**18 weeks**	**31 weeks**
Harboring EV21	V+, A-[a]	37/40(93)	25/38(66)	18/37(49)	19/35(54)
	V+, A+[b]	0/40(0)	1/38(3)	2/37(5)	2/35(6)
Lacking EV21	V+, A-	17/40(43)	2/40(5)	1/40(3)	2/38(5)
	V+, A+	0/40(0)	7/40(18)	1/40(3)	3/38(8)

[a]Virus positive, antibody negative
[b]Virus positive, antibody positive

A. **Pathology**: Four birds were diagnosed with LL on gross examination. One additional bird was diagnosed with LL microscopically. Two other birds had suspect microscopic lesions of LL in the spleen. These two birds were not included in the analysis. One other bird was diagnosed with hemangioma by gross examination. In regards to EV21 status, 1/38 (2.6%) at risk chickens lacking EV21 developed tumors while 5/36 (13.8%) at risk chickens harboring EV21 developed tumors. At risk is defined

as the number of chickens that died with tumors plus the number of chickens that survived to termination.

V. DISCUSSION

Endogenous viruses have been known to interfere with antibody development to exogenous leukosis viruses (Halpern *et al.* 1975; Crittenden *et al.* 1982; Crittenden and Fadly 1985). There are several reports regarding EV21 virus and its influence on the response to exogenous ALV infection in chickens exposed after hatch (Smith and Crittenden 1988; Fadly and Smith 1991; Gavora *et al.* 1995; Fadly and Smith 1997). Most of these reports used a subgroup A ALV as the exogenous virus for inoculation followed by serological assessment for antibody production. The purpose of this study was to determine the influence of EV21on the response of white leghorn chickens to infection with ALV-J.

The data presented (Table 3.1) demonstrated that birds harboring EV21 were significantly more likely to be viremic at the 4 time periods tested than birds lacking EV21. The largest difference in the level of viremia occurred at 10 weeks of age. These findings are consistent with other reports (Smith and Crittenden 1988; Gavora *et al.* 1995; Fadly and Smith 1997) that examined the differences in viremia between birds harboring and lacking EV21 following infection with subgroup A ALV. The delayed ALV antibody response seen at 10, 18 and 31 weeks of age in chickens harboring EV21 supported earlier studies by Crittenden (1982), Crittenden and Fadly (1985), and Halpern (1975) that indicated that endogenous viruses interfere with antibody production.

A large percentage of ALV-J viremic tolerant chickens were observed in this study (Table 3.2) with most occurring in chickens harboring EV21. This characteristic occurred at the 10, 18 and 31 weeks of age sampling periods. The marked difference in response by the two groups of chickens can be attributed to the presence or lack of EV21. Smith and Crittenden (1988) also noted the tolerance inducing effect of EV21 on RPL-40 (ALV-A field isolate) viremia, cloacal shedding, and neutralizing antibodies in chickens congenitally infected with EV21. Crittenden *et al* (1982, 1984) demonstrated that Rous-associated virus 0 (RAV-0) which is encoded at the *ev*2 locus, can also induce immunologic tolerance along with the *ev*3 locus with subgroups A and B ALV. The data presented here can further substantiate the effects of EV21 on responses of white leghorn chickens infected with exogenous ALVs. The data suggests that the use of antibody tests for ALV surveillance may not detect the true ALV-J infection status in late feathering leghorns due to the lack of antibody production.

The lack of tumor formation in this experiment was unexpected. Explanations as to why this occurred include some resistance of the genetic line used in this study, the genetic make up of the ALV-J virus, or the combination of both factors. White leghorn chickens can develop tumors when infected with ALV-J but at a lower rate than meat type chickens (Payne *et al.* 1992). Previous experiments using the same genetic cross and subgroup A ALV yielded tumors in up to 32% of infected birds regardless of EV21 status (Fadly and Smith 1997). In that report, only the genetic transmission of EV21 increased the incidence of tumors following ALV infection at hatch, not contact exposure to EV21. Smith and Fadly (1988) evaluated the influence of congenital transmission of EV21 on the incidence of tumors and concluded that congenital transmission of EV21 did

have an effect on tumor formation. Another report by Smith and Crittenden (1988) examined genetic resistance and the susceptibility of late feathering females on transmission of EV21 to the progeny congenitally. They determined that cellular resistance to subgroup E ALV in the late feathering dams limited congenital transmission of EV21 to the progeny. Due to the fact that the EV21+ males used in the cross for the progeny used in this study have a line 0 genetic background, they may have contributed some cellular resistance to the progeny (Smith and Crittenden 1988). ALV-J has EAV viral sequences in the envelope gene (Bai *et al.* 1995; Benson *et al.* 1998) and together with the genetic resistance acquired from the male parent, it may have influenced the manner in which ALV-J induces tumor formation. Although tumor formation was low, there was correlation with previous reports (Smith and Crittenden 1988; Smith and Fadly 1988; Fadly and Smith 1997) in that the chickens lacking EV21 had fewer tumors than chickens harboring EV21, 2.6% versus 13.8%, respectively.

Examination of the data herein clearly indicates that EV21 influences ALV-J viremia and antibody production in white leghorn chickens. How EV21 may influence ALV-J infection in meat type birds is unknown. However, caution is warranted in using only serological assays to detect ALV-J infection because of the high frequency of the development of tolerance that would result in false negatives test results.

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CHAPTER 4

Tissue Tropism of Avian Leukosis Virus Subgroup J in White Leghorn Chickens

I. **Abstract**

Distribution of ALV-J in various tissues of embryonally inoculated white leghorn chickens with strain ADOL Hc1 of ALV-J was studied. At 2 and 6 weeks of age, various tissues including thymus, spleen and bursa of Fabricius from infected and phosphate buffered saline inoculated controls chickens were tested for the presence of ALV-J by immunohistochemistry using a monoclonal antibody to the envelope glycoprotein (gp85) of ALV-J. Formalin-fixed tissues from infected chickens exhibited a reddish staining indicating the presence of ALV-J in tissue specific cells as well as other widely distributed cells such as endothelial cells and smooth muscle cells. Tissues expressing gp85 included adrenal gland, bursa of Fabricius, gonads, heart, kidney, liver, bone marrow, nerve, pancreas, proventriculus, spleen and thymus at both 2 and 6 weeks of age. This distribution is different than previously reported by Arshad *et al* (1997) which may be due to the type of chickens used (Brown leghorn versus white leghorn), the antigenic differences between the European prototype and the U. S. prototype of ALV-J, or sensitivity of antibody. Bursal transformation can be documented with ALV-J using the methyl green pyronine staining procedure. However, it does not occur with the same frequency as RAV-1, ALV-A prototype virus, in the highly susceptible chicken cross $15I_5$ X 7_1. Although myelocytomatosis is the main tumor manifestation, bursal follicle transformation that results in LL can occur with ALV-J infection.

II. **Introduction**

The tissue tropism of ALVs has been documented by many different researchers over the years employing various methods of detection (Di Stefano and Dougherty 1969; Di Stefano *et al.* 1973; Spencer and Gilka 1982; Gilka and Spencer 1984; Spencer *et al.* 1984; Robinson *et al.* 1993). These previous reports dealt with ALVs that would cause LL regardless of subgroup. In many tissues varying degrees of antigen was detected and it was noted that RAV-1, a subgroup A ALV virus, was detected in bone marrow and bursal tissues more than in spleen and thymus tissue (Baba and Humphries 1986). A report by Brown and Robinson (1988) determined that RAV-1 had high tropism for bursa of Fabricius and muscle versus RAV-2, a subgroup B ALV, which had high tropism for bursa and thymus (Brown and Robinson 1988). Other reports examined site specific replication of ALVs. Macrophages harvested from the yolk sac of chicken embryos that are resistant to subgroup E infection were found to be resistant to subgroups A and D but susceptible to subgroups B and C (Gazzolo *et al.* 1974; Gazzolo *et al.* 1975). Gazzolo *et al* (1979) also determined that ALV can be present in macrophages up to three years while avian sarcoma viruses cannot. Robinson *et al* (1993) determined that RAV-1 proviral DNA can integrate in various tissues but the various tissue express gag and envelope proteins in different amounts.

Avian leukosis virus subgroup J (ALV-J) was first described in the early 1990s (Payne *et al.* 1991) in Europe and then described in the United States during the mid 1990s (Fadly and Smith 1997). The predominate tumor described in the literature and in the field is myelocytomatosis (Payne *et al.* 1991; Payne *et al.* 1992; Fadly and Smith 1997; Fadly 1998; Fadly and Smith 1999). Recently, Arshad *et al* (1997) described the

tissue tropism of HPRS-103 using *gag* expression of p27 by immunohistochemistry. Examination of previous reports on tissue tropism of subgroups A, B and J indicate that there are different levels of expression depending on the subgroup. Due to immunological differences noted between HRPS-103 and ADOL-Hc1 (Fadly and Smith 1997), tissue tropism of ADOL-Hc1 by immunohistochemistry was studied in white leghorn chickens that are highly susceptible to ALV induced tumor formation, using a monoclonal antibody against gp85 of ADOL-Hc1.

Results from Chapter 2 with regards to bursal follicle transformation demonstrate that ALV-J can cause transformation. To more accurately assess the transformation abilities of the virus, bursal follicle transformation induced by ADOL-Hc1 was studied in white leghorn chickens that are highly susceptible to ALV induced tumor formation and compared with RAV-1, an ALV-A virus.

III. **Methods and Materials**

A. **Chickens:** The highly susceptible cross $15I_5$ X 7_1 chickens from the Avian Disease and Oncology Laboratory, East Lansing, MI, were used: Sixty 7-day-old embryos for the tissue tropism experiment and 50 day-old chicks for the bursal follicle transformation experiment. The breeder flocks for this cross are free from many avian pathogens, including ALV, as determined by routine serological surveys. Birds were house by treatment group in Horsfall isolation units up to 10 weeks and given food and water *ad libitum*.

B. **Viruses:** Strain ADOL-Hc1, the U. S. prototype, of ALV-J and RAV-1, the prototype for ALV subgroup A, were used. The virus titer for ADOL-Hc1 was determined to be 10^5 infectious units (IU)/ml. The virus titer for RAV-1 was determined to be 10^5 IU/ml. Each treatment group was given 10^4 IU/bird of the assigned virus.

C. **Experimental Design:** Tissue tropism experiment: Two groups of 30, 7-day-old embryos were incubated in separate units. One group was inoculated with ADOL-Hc1 via the yolk sac with a 30g ½ inch needle and the other group served as controls and was inoculated with sterile phospate buffered saline (PBS) (0.1ml) injected via the yolk sac with a 30g ½ inch needle. All chicks were sampled for viremia at hatch. At 2 and 6 weeks of age, whole blood samples for virus isolation assays were collected. Ten birds were randomly chosen from each group and euthanized at each time period. Tissues harvested included adrenal glands, kidney, pancreas, spleen, heart, proventriculus, skeletal muscle, bone marrow, thymus, bursa of Fabricius, gonads and sciatic nerve. Tissues were fixed in 10% neutral buffered formalin over night and transferred to 60% methanol until processed for immunohistochemistry.

Bursal follicle transformation experiment: Fifty, day old chicks were randomly separated into three groups. One group (19 chicks) was inoculated with ADOL-Hc1, one group (19 chicks) with RAV-1 and one group served as uninoculated controls (12 chicks). All inoculations were intraperitoneal (IP) using a 30g ½ inch needle. At 4 and 10 weeks of age blood samples were collected for virologic assays. Bursal transformation was determined by MGP staining. At 4 and 10 weeks of age, 5 chickens were randomly chosen from each group and euthanized. Bursal tissue was harvested and plica separated

and placed flat in cassettes, usually 2 to 3 cassettes per bird. Fixation occurred in 4% paraformaldehyde in PBS. Tissues were embedded in paraffin, sectioned and stained with MGP stain (Siccardi and Burmester 1970; Carson 1990) and examine histologically for evidence of transformation.

D. **Virological and serological assays:** Virus isolation (VI) and VN were conducted on samples at each sampling period using line 0 (C/E) CEF as directed in experimental design. Briefly, VI was performed using 100 microliters of plasma added to 4% calf serum Leib McCoy media containing penicillin, streptomycin and amphotericin B plus 0.04IU of heparin per plate and CEFs in suspension. Media was changed the following day to 1% calf serum Leib McCoy media containing the above antibiotics and fungicide. Cultures were allowed to grow for 7- 9 days. Plates were frozen and thawed twice (Fadly and Witter 1998). Cell lysates from CEF cultures was tested by the gs antigen p27 ELISA (Smith *et al.* 1979). VN assays were performed according to procedures by Fadly and Witter (1998). Briefly, test samples were diluted 1:5 with Leib McCoy media without serum and heat inactivated for 30 minutes at 56° C. Dilute virus stocks of RAV-1 or ADOL-Hc1 were added such that each plate of a 96 well microtiter plate received 500-1000 viral particles/ml. Equal volumes of test samples were added to the microtiter plates and incubated for 45 minutes. Appropriate positive and negative control samples were included. Cells were added ($5X10^4$ cells/well) in 4% calf serum Leib McCoy containing antibiotics and fungicide, and incubated 7-9 days at 37° C. Cultures were frozen and thawed twice. The cell lysates were tested for the presence of p27 by ELISA.

VN were performed separately with both RAV-1 and ADOL-Hc1 for indication of accidental cross infection in the bursal transformation experiment.

E. **Immunohistochemistry:** Tissue sections were removed from 60% methanol and embedded in paraffin and sectioned at 4μm. The monoclonal antibody used was developed by A. J. Cui (Cui, Avian Dis, in press) against gp85 of ALV-J in mice. The following procedure was used for staining the tissues. Slides were deparaffinized, hydrated with distilled water and placed in tris buffered saline (TBS) buffer for 5 minutes. Next, 3% H_2O_2 in methanol was applied for 60 minutes to block endogenous peroxidases. Slides were rinsed in running tap water for 5 minutes, placed in TBS plus Tween 20 for 5 minutes and placed on an automatic stainer (LEICA St5050 Stainer) with 2, 2 minutes rinses in TBS plus Tween 20 between the following steps. Super Block, a non-species specific protein blocking agent, (Scytek, Logan, UT) was applied to the slides for 5 minutes, followed by application of a blocking agent for endogenous avidin/biotin for 5 minutes. The primary antibody was diluted at 1:200 in normal antibody diluent (Scytek) (from concentrate) and applied by hand and incubated for 60 minutes. A Vector (Burlingame, CA) biotinylated anti-mouse/anti-rabbit (in horse) antibody diluted at 1:136 in normal antibody diluent for 30 minutes was applied followed by application of a Vector Ready to Use (R.T.U.) ABC (avidin-biotin complex) for 30 minutes. The final step in the procedure was application of Vector Nova Red for 15 minutes. The slides were removed from the automatic stainer and were counterstained with Lerner 2 Hematoxylin for 1 minute, then placed in running water, with 1% Glacial acetic acid blue for 2 minutes. Slides were dehydrated, cleared and coverslipped with a

synthetic mounting media. Several negative controls included a system control that had normal horse sera diluted at 1:28 instead of primary antibody for 60 minutes on a section of infected tissues, uninfected tissues treated with primary antibody, and uninfected tissues were treated with normal horse sera. All controls were included each time a new set of slides was placed on the automatic slide stainer. Tissues were examined microscopically for red pigment within the cell cytoplasm.

F. **Scoring of Tissues:** Tissue tropism experiment: Tissue sections were scored for the presence or absence of gp85 and number of positive cells within a tissue. Tissue sections were scored by counting the tissue specific cells (kidney tubules, pancreatic cells, etc) that were stained. Widely distributed cells such as connective tissue, smooth muscle components in the gastrointestinal tract, endothelial cells, etc. were not counted. Each tissue on a slide was examined at 40X and in four randomly chosen fields and tissue specific cells were counted. The average number of cells of the four fields was determined and a positive cell staining average was given to the tissue for that bird. The averages were combined for all tissue samples and divided by the respective number of tissues scored to obtain an overall average score for each tissue. Presence of gp85 positive cells specific to a certain tissue were scored as none (no staining), mild (1-25 positive cells), moderate, (26-75 positive cells) and marked (76 and up positive cells).

Bursal transformation scoring: All sections of bursal plica were examined microscopically. Evidence of transformation included increased pyroniphilia as compared to surrounding follicles and/or normal architecture was disrupted such that the

cortex and medulla were indistinguishable from each other. If one follicle was positive for transformation, the tissue was considered positive.

IV. **Results:**

A. **Tissue tropism experiment:** The distribution of gp85 in tissue specific cells is summarized in Table 4.1. Skeletal muscle taken from the superficial pectoral muscle was not scored because of the high background staining. All other tissue examined were scored. Tissues with the highest average number of cells staining for antigen expression at 2 weeks of age were proventriculus (glandular epithelial cells) with 91 cells, spleen (reticuloendothelial cells and lymphocytes) with 79 cells, and adrenal glands (cortical and medullary) with 74 cells. Bone marrow had the least amount of cells staining for the antigen expression with an average of 16 cells. There were no tissues in the "none" category, 1 in the mild category, 10 in the moderate category and 2 in the marked category. Tissues with the highest average number of cells staining for antigen at 6 weeks of age were adrenal gland (139 cells), proventriculus (137 cells), pancreas (125 acinar and islets of Langerhans cells), kidney (121 distal tubule epithelial cells), and spleen (116 cells). Bone marrow had the least amount of cells staining for the antigen with an average of 36. There were no tissues into the "none" and mild categories, 6 in the moderate category, and 6 in the marked category. Figures 4.1-4.13 illustrate various tissues with positive staining along with the lack of staining in uninfected tissues. Images in this chapter are presented in color. Results of VI were as follows: At 2 weeks of age, 20/20 ADOL Hc1 inoculated group tested positive and 0/20 were positive in the control group.

At 6 weeks of age, 9/9 ADOL-Hc1 inoculated group, tested positive and 0/10 were positive in the control group.

Table 4.1 Distribution of gp85 expression* in $15I_5$ X 7_1 Chickens Inoculated as Embryos with ALV-J.

Tissue	2 weeks	6 weeks	Tissue	2 weeks	6 weeks
Adrenal gland	7/8(74)	9/9(139)	Muscle	nt	nt
Bursa	6/9(47)	8/9(73)	Nerve	7/10(53)	9/9(68)
Gonad	7/8(73)	9/9(67)	Pancreas	9/10(62)	9/9(125)
Heart	9/10(57)	9/9(83)	Proventriculus	10/10(91)	9/9(137)
Kidney	9/10(69)	9/9(121)	Spleen	8/10(79)	9/9(116)
Liver	8/9(46)	9/9(46)	Thymus	9/10(30)	9/9(60)
Bone marrow	5/8(16)	6/6(36)			

* No. birds positive/No. birds examined (average number of tissue specific cells).
Nt not tested due to high background staining.

B. **Bursal transformation experiment:** The results for serological and bursal transformation experiment are summarized in Tables 4.2 and 4.3. There was no evidence of cross infection between the RAV-1 and ADOL-Hc1 groups or infection of the control group. Transformation was seen in only the virus inoculated groups. At 4 weeks of age, the RAV-1 infected group had a 40% transformation rate while the ADOL-Hc1 infected group had a 20% transformation rate. At 10 weeks of age, the RAV-1infected group had 100% transformation while the ADOL-Hc1infected group had 20%.

Table 4.2: Development of ALV-J or ALV-A induced viremia and antibody in chickens of a highly susceptible cross to ALV induced tumor formation.

	4 weeks			10 weeks		
Group	**VI +**	**AbJ+**	**AbRAV-1+**	**VI +**	**AbJ+**	**AbRAV-1+**
Controls	0/12*	0/12	0/12	0/7	0/7	0/7
RAV-1	19/19	0/19	0/19	2/13	0/13	11/13
ADOL-Hc1	18/18	0/18	0/18	13/13	6/13	0/13

AbJ+: Antibody to J
AbRav-1+: Antibody to RAV-1
*No+/No tested

Table 4.3: Bursal follicle transformation induced by ALV-A or ALV-J in a highly susceptible cross of chickens to ALV induced tumor formation.

Group	**4 weeks**	**10 weeks**
Control	0/5*	0/5
RAV-1	2/5	5/5
ADOL-Hc1	1/5	1/5

*No+/No tested

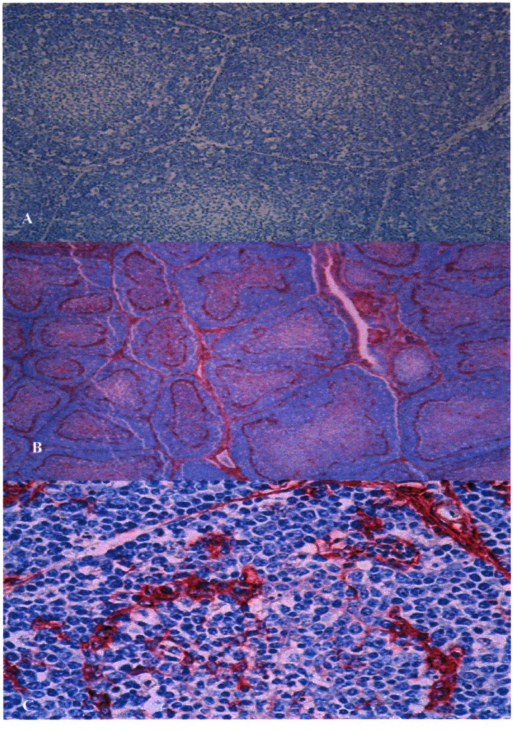

Figure 4.1: Immunohistochemistry on bursa of Fabricius sections. A. Uninfected bird. 20X. B. Infected bird. Overall distribution of staining. 4X. C. Infected bird. Staining at intersection of cortex and medulla of bursal follicle. B cells and macrophages are staining positive. 40X.

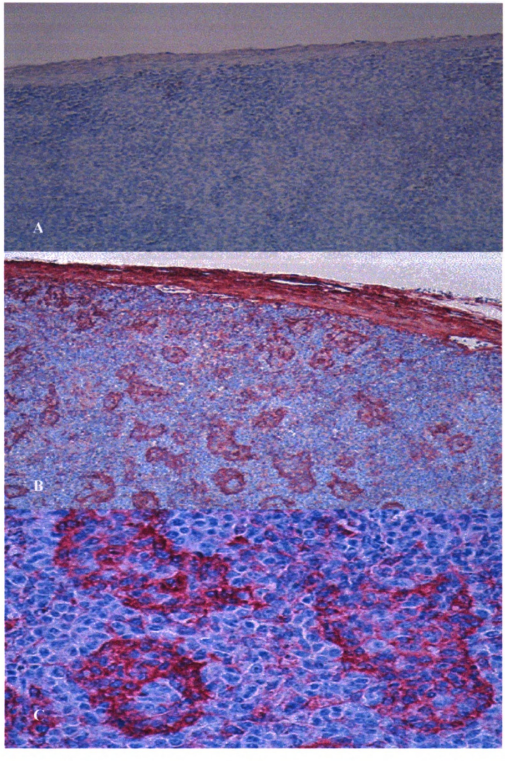

Figure 4.2: Immunohistochemistry on spleen sections. A. Uninfected bird. 10X B. Infected bird, overall distribution of staining.10X C. Infected bird, staining of reticuloendothelial cells.40X

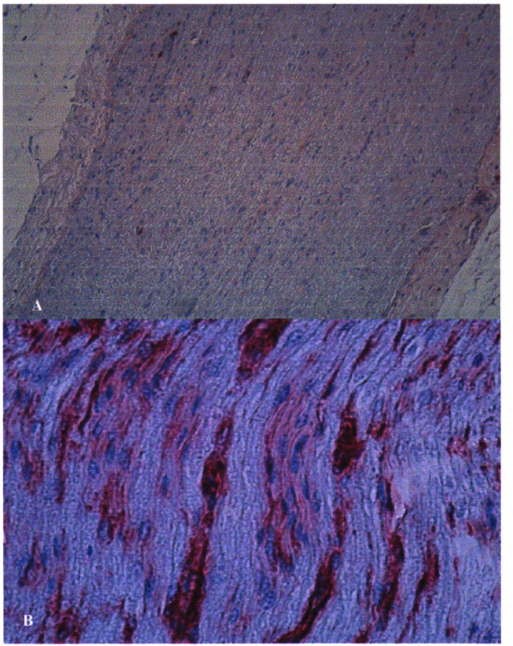

Figure 4.4: Immunohistochemistry on nerve sections. A. Uninfected sciatic nerve. 20X. B. Infected sciatic nerve section. Staining present in axons and surrounding the nuclei. 40X.

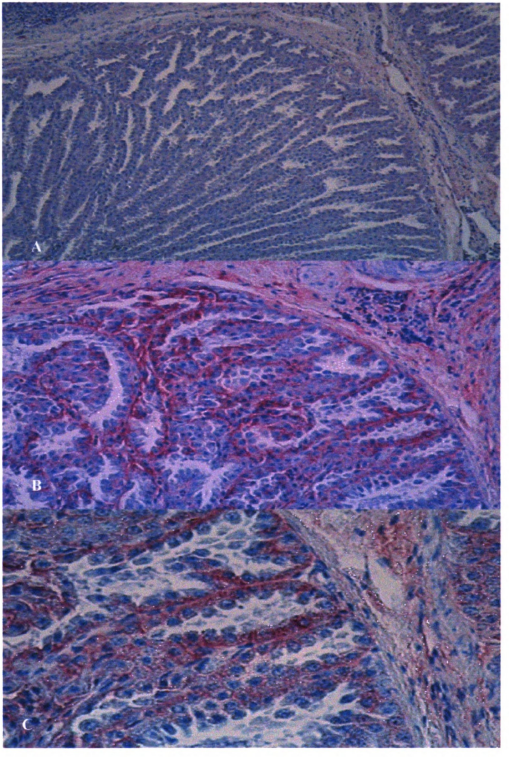

Figure 4.5: Immunohistochemistry on proventriculus sections. A. Uninfected proventriculus. 10X. B. Infected proventriculus. Staining present in glandular epithelial cells. 20X. C. Infected proventriculus. Stain present in cytoplasm of glandular epithelium. 40X.

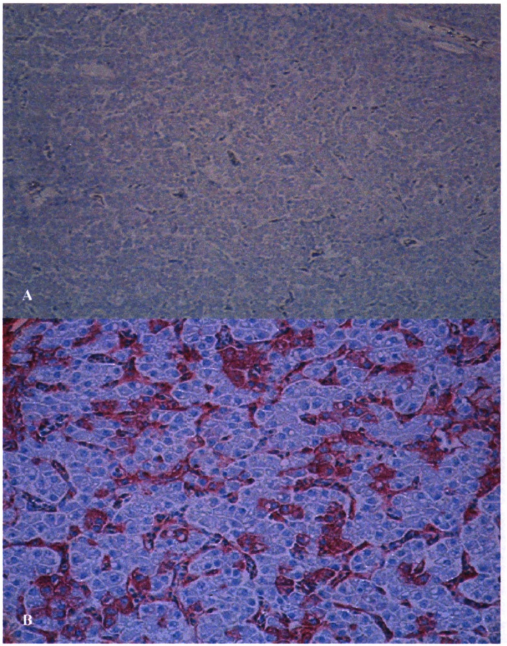

Figure 4.6: Immunohistochemistry on liver sections. A. Uninfected liver section. 10X. B. Infected liver section. Individual hepatocytes are staining positively. Positive staining occurring in sinusoids consisting of red blood cells and possibly Kupffer cells. 20X.

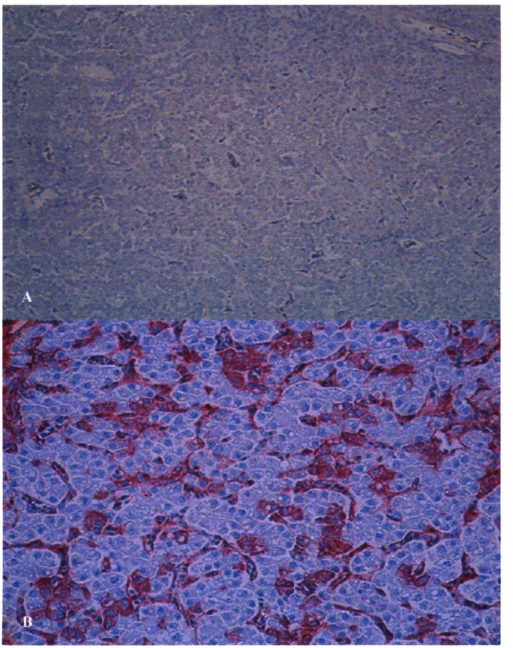

Figure 4.6: Immunohistochemistry on liver sections. A. Uninfected liver section. 10X. B. Infected liver section. Individual hepatocytes are staining positively. Positive staining occurring in sinusoids consisting of red blood cells and possibly Kupffer cells. 20X.

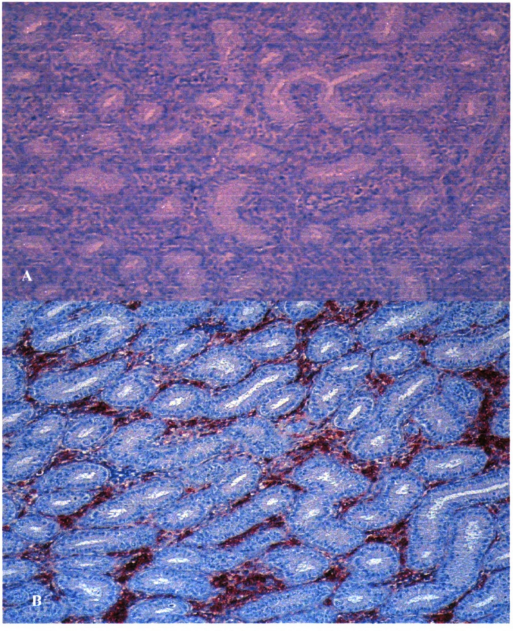

Figure 4.7: Immunohistochemistry on testis sections. A. Uninfected testis section. 20X. B. Infected testis section. Staining is present in the interstitial cells. 20X.

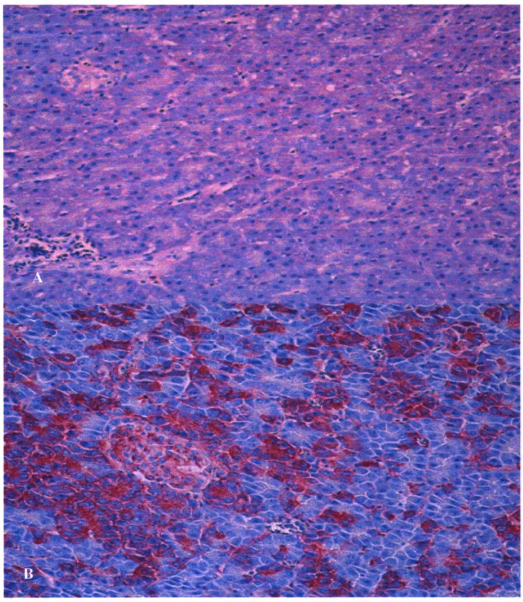

Figure 4.8: Immunohistochemistry on pancreas sections. A. Uninfected pancreas section. 10X. B. Infected pancreas section. Staining is present in both acinar cells and islets of Langerhans. 20X.

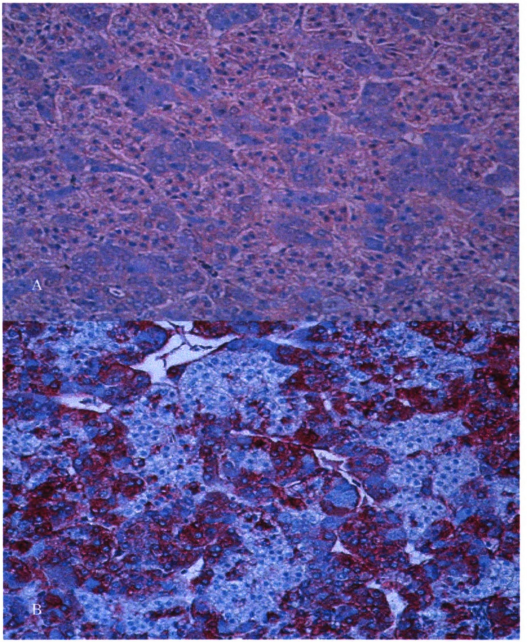

Figure 4.9: Immunohistochemistry on adrenal gland sections. A. Uninfected adrenal gland section. 20X. B. Infected adrenal gland section. Staining is present in both cortical and medullary tissue. 20X.

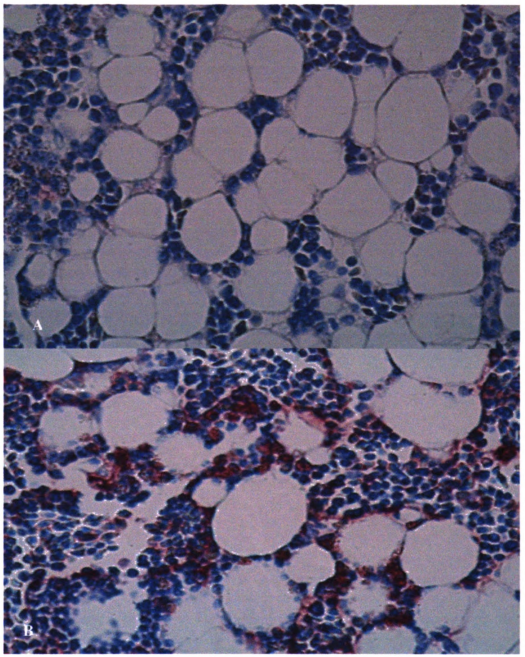

Figure 4.10: Immunohistochemistry on bone marrow sections. A. Uninfected bone marrow section. 40X. B. Infected bone marrow section. Staining in erythrocytic cell lines and granulocytic cell lines. 40X.

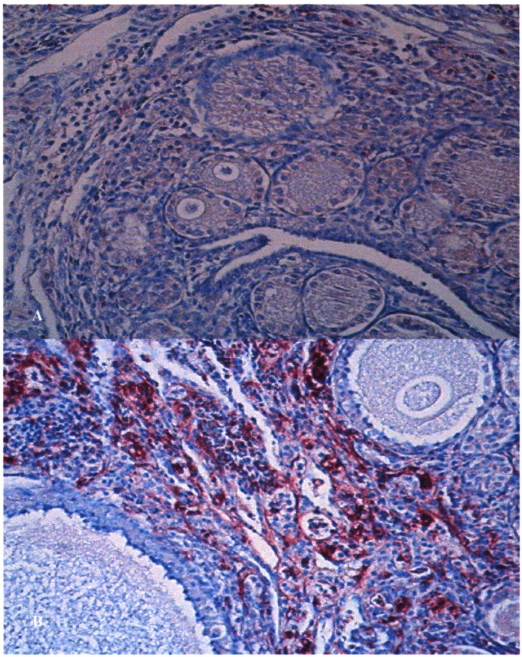

Figure 4.11: Immunohistochemistry on ovary sections. A. Uninfected ovary section. 40X. B. Infected ovary section. Stromal cells staining along with endothelial cells. 40X.

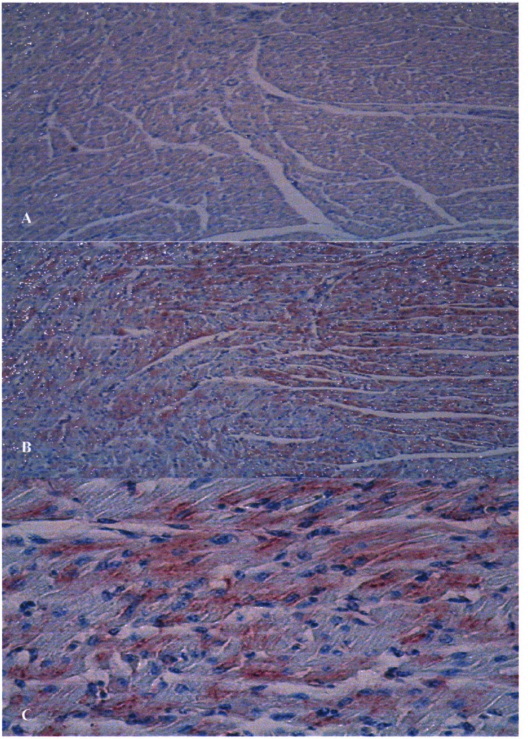

Figure 4.12: Immunohistochemistry on heart sections. A. Uninfected heart section. 10X. B. Infected heart section. 10X. C. Infected heart section. Myofibers are staining positive. 40X.

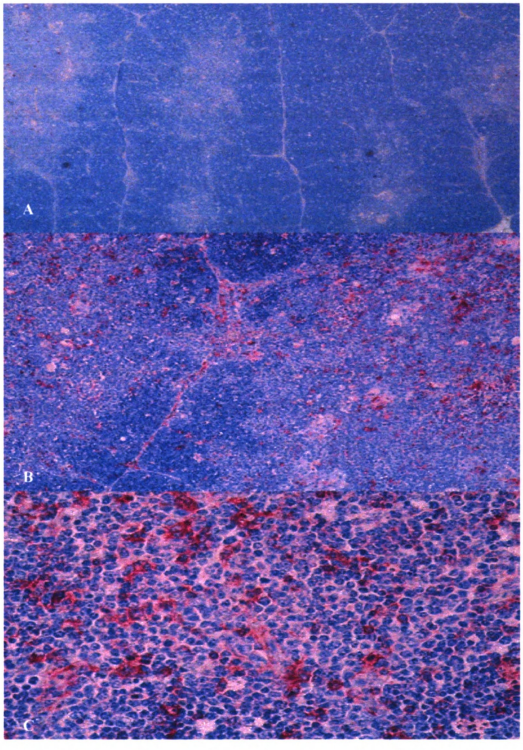

Figure 4.13: Immunohistochemistry on thymus sections. A. Uninfected thymus. 10X. B. Infected thymus. Most staining occurs in the medulla. Some staining present in cortical cells. 20X. C. Infected thymus. Cortical lymphoid cells staining. 40X.

V. **Discussion**

Tissue tropism for avian leukosis virus has been previously studied and first reported in1967 (Dougherty and Di Stefano 1967). Dougherty and Di Stefano (1967), using a subgroup A virus (ALV-F42) reported that viral budding could be seen by electron microscopy in all three embryonal germ layers and in all tissues examined except nervous tissue in congenitally infected chickens. Although they were able to detect virus in the brain by the COFAL method, it was determined by careful examination of various nervous tissue samples that the source of the virus was blood and was not virus replicating in the cells of nervous tissues.

Other researchers have used immunohistochemical staining techniques to study ALV distribution in tissue in order to gain insight about pathogenesis of the infection (Dougherty *et al.* 1972; Di Stefano *et al.* 1973; Spencer and Gilka 1982; Spencer *et al.* 1983; Gilka and Spencer 1984; Spencer *et al.* 1984; Gilka and Spencer 1985; Gilka and Spencer 1987; Arshad *et al.* 1997). All of these reports used an antibody directed against the gs antigen (p27) for detection of ALV. Using the p27 antibody allows for detection of all subgroups of ALV, including endogenous ALVs. Because p27 antibody can detect endogenous ALVs, some caution must be used when interpreting results from chickens that have endogenous virus loci that could express the p27 protein. Careful examination of control birds and/or uninfected tissues must be carried out to determine how much endogenous p27 can be detected as compared to the infected tissues. Using p27 does have advantages in that it will detect all subgroups of ALV and therefore only one antibody is needed for diagnostic purposes.

Other methods for detecting ALV include using Southern Blot analysis (Baba and Humphries 1986) and *in situ* hybridization (Arshad *et al.* 1999). Both of these methods use DNA as the basis of detection by using various probes. Southern Blot analysis can determine if there is proviral genomes in the tissue but does not indicate specifically which cells are positive because of the DNA extraction procedures. *In situ* hybridization allows for the detection of either DNA or RNA in fixed tissues so that specific cell types can be identified as containing the DNA or RNA in question.

Results presented in Table 4.1 demonstrated that a monoclonal antibody to gp85 can be successfully used to detect ALV-J in various tissues without the concern of detection of endogenous viral antigen. Detection of endogenous viral antigen was a concern because of the high number of endogenous viral loci in the genetic cross of chickens used in this study. Using only a p27 based antibody could possibly detect both ALV-J and ALV-E and make it difficult to determine what is background staining (endogenous p27) and what is exogenous p27 staining.

Previous work by Arshad *et al* (1997) evaluated the distribution of ALV-J gs antigen in tissues of various lines of chickens including a Brown Leghorn line, line 21 (a meat-type chicken), and four white leghorn lines (0, 15I, 6_1, and N). Tissues with the highest expression of ALV antigen in tissue specific cells were adrenal gland, heart, kidney, proventriculus, and pancreas. These same tissues were the same highest tissue specific staining tissues reported herein except heart which was replaced by spleen in this report. Prior work by others also noted that these tissues contained large amounts of ALV antigen (Dougherty and Di Stefano 1967; Di Stefano and Dougherty 1969; Di Stefano *et al.* 1973; Spencer *et al.* 1984). However, sciatic nerve tissue tested positive

for antigen quite often which has been infrequently reported before (Arshad *et al.* 1997) or not reported at all in previous reports where different detection techniques were used (Dougherty and Di Stefano 1967; Dougherty *et al.* 1972; Di Stefano *et al.* 1973).

Interestingly, some differences were observed between the embryo inoculated birds in Arshad's work and in this study. Bursal expression of ALV-J in this study was seen in most bursal sections examined with a pattern of distribution of more intense staining of gp85 at the cortical-medullary junction of the follicles with much less staining of the medulla in some follicles. Previous work only demonstrated bursal staining in the medulla. Another difference between Arshad's work and this study was staining of sciatic nerve tissue and bone marrow. In the current study, staining was detected in the bone marrow which had the least number of cells staining positive. Staining was detected in both red and white blood cell lineages. Arshad *et al* (1997) suggested that the reason for his inability to detect *gs* antigen in the bone marrow was due to the low sensitivity. Another possible explanation could have been due to the location in the cells where the various proteins are expressed. GAG proteins are responsible for the physical structure of the virion and are assembled in the cytosol near the cell membrane (Coffin 1992; Coffin 1996). Env proteins, mainly gp85, are expressed on the cell surface membrane where budding occurs (Coffin 1992; Coffin 1996). This difference in location of these viral proteins may explain why antibody to gp85 may react when antibodies to p27 may not.

Other possible explanations for the differences between this study and Arshad's may be due to the genetics of the chickens used for the experiments. Examination of the results from the leghorn chickens inoculated as embryos revealed that certain tissues

(gonads, liver, and pancreas) were not consistently positive for ALV-J in brown leghorns or white leghorn lines. In this study, the majority of tissues examined were positive at 2 weeks and all tissues except one bursa of Fabricius section was positive at 6 weeks. To infect a cell, the retrovirus uses a cell surface receptor that normally is expressed by the cell. In the case of subgroup A, the receptor is known to be related to the human low-density lipoprotein receptor or LDLR (Bates *et al.* 1993; Young *et al.* 1993). The receptor for ALV-J is unknown. It may be possible that in the genetic cross chosen for this experiment, the ALV-J receptor is expressed in greater abundance than in the white leghorn lines used by Arshad, thereby increasing the amount of antigen detected.

Previous work demonstrated the ability of ALV-J to transform bursal follicles in four different lines of chickens but the design of the study did not allow a direct comparison to ALV-A (see Chapter 2). Using the same genetic lines of chickens allowed for better evaluation of the ability of ALV-J to transform B cells and thus, bursal follicles, as compared to ALV-A. In this study, a clear difference in the rate of bursal follicle transformation between ALV-J and ALV-A was demonstrated; 20% for ALV-J infected group and 100% for ALV-A infected group at 10 weeks of age, in a line of chickens that is highly susceptible to ALV-induced tumors

Previous publications on ALV tissue tropism for various subgroups reveals that a variety of methods can be used for detection of antigens and that some differences can be noted in the overall distribution of the virus among subgroups A, B and now J. New information regarding detection of viral antigen in bursal follicles and the ability of ALV-J to cause follicle transformation are presented herein begins to explain the outcome of ALV-J infection in experimentally infected white leghorns (see Chapter 2). Previous

work where white leghorns were experimentally infected with ALV-J did not produce LL (Payne *et al.* 1991; Payne *et al.* 1992). The differences seen suggest that both host and viral factors are important in the outcome of infection and in tumor development.

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CHAPTER 5

Conclusions and Future Directions

Avian leukosis is an old disease of poultry dating back to its first description in 1865 by Roloff. Termed lymphoid leukosis in 1961, the disease causes decreased egg production, lower performance, and tumors of mainly B cell origin. There are six subgroups of leukosis viruses that affect chickens and four that affect other avian species. The most recently described of the six subgroups affecting chickens is subgroup J. ALV-J infection causes myelocytomatosis in meat-type chickens. Reports of infection of egg laying chickens have occurred in North America and Europe. Because of the possible threat to the egg laying industry, the overall goal for this research was to determine the pathogenicity and the pathogenesis of ALV-J in white leghorn chickens. The objectives were: 1) Compare pathogenicity of ALV-J in various lines of white Leghorn chickens; 2) Determine the effects of endogenous virus 21 on the pathogenesis of ALV-J in white leghorns; and 3) Determine the tissue tropism of ALV-J and the susceptibility of the B cell to ALV-J induced transformation in white leghorns.

OBJECTIVE 1. The most significant findings for objective 1 were that white leghorn chickens can develop LL when infected with ALV-J as embryos or at day of hatch, the genetics of the chicken will influence how the bird responds to infection after hatch, and Line 6_3 exhibits resistance to tumor formation. Previous research by Payne and others (Payne *et al.* 1991; Payne *et al.* 1992) did not report the development of LL with infection of leghorn chickens with ALV-J. This report demonstrates that LL can be an end result of ALV-J infection in egg-laying chickens. This is an important finding for the industry because producers may believe that they have a subgroup A or B infection if LL tumors develop, when in fact, it may be subgroup J. Serological tests performed on these

flocks would indicate a lack of antibody response to ALV-A or B. A second serological test for ALV-J antibodies should be included to determine exposure of the flock to ALV-J.

The next significant finding is that the genetic constitution of the bird influences the outcome of infection with ALV-J after hatch. Line 0 chickens, which lack endogenous virus loci, were able to seroconvert very quickly with ALV-J infection, as compared to a subgroup A infection. The other three lines were not able to clear the viremia despite the presence of neutralizing antibodies at thirty weeks of age. These other lines have various endogenous viral loci in their genomes, which traditionally delays the antibody response to ALV-A or –B. However, they eventually do seroconvert by 30 weeks of age. This was not the case with ALV-J infection after hatch.

The final finding is that Line 6_3 exhibits resistance to tumor formation when infected with ALV-J. Line 6_3 is known to be resistant to tumor formation when infected with subgroup A (Bacon *et al.* 2000). Approximately 0.5% of Line 6_3 chickens will develop LL when infected with ALV-A (Crittenden *et al.* 1964; Crittenden *et al.* 1972). In this study, Line 6_3 chickens only developed 1 tumor and it was classified as LL. Bursal transformation results indicated again that only 1 bird had evidence of transformation. The lack of tumor formation in Line 6 following ALV-J infection is important information for researchers in planning future experiments with ALV-J.

OBJECTIVE 2. The most significant results gained from this objective was that EV21 influences the serological outcome of ALV-J infection after hatch. The poultry industry uses early and late feathering phenotypes to sex chicks at hatch instead of vent sexing for

some genetic lines. EV21 is associated with the K gene and is responsible for the late feathering phenotype. The cross of chickens used in this study had exactly the same genetic information in each group except for the presence or absence of EV21. The mating was conducted in such a manner to cause both males and females to be early feathering or late feathering, thereby removing the factor of sex in the outcome of infection. It is well documented that females are more susceptible to tumor formation than males with ALV infection. The serological results demonstrated that an unusually high percentage of slow feathering EV21+ chickens remained viremic tolerant for the entire experiment (54%). Previous research by Smith and Fadly (Smith and Fadly 1988; Fadly and Smith 1997) reported a delayed immune response in chickens harboring EV21 when infected with ALV-A after hatch. However, the percentage of viremic tolerant birds at the end of the experiments was small. The unusually high percentage of viremic tolerant chickens in this study would escape detection using serological basis tests only for flock monitoring and would serve as a source of congenitally infected progeny. The elimination of vertical transmission between hens and progeny is the most important aspect in eradication programs for leukosis. Viremic tolerant birds would cause significant delays in eradication programs.

OBJECTIVE 3. The most significant finding for this objective was that some tissues determined to be positive for ALV-J had not been previously reported as susceptible to ALV-J infection. Previous reports that determined tissue tropism of ALV-J report variable to no detectable levels of p27 antigen in peripheral nerve, spleen, thymus, and bone marrow in a variety of different chicken lines (Arshad *et al.* 1997). This study

demonstrated ALV-J antigen in all tissues examined, including peripheral nerve, spleen, thymus and bone marrow. By six weeks of age, all but one section of tissue examined was positive for tissue specific staining of gp85 antigen. Traditionally, immunohistochemistry has used a monoclonal antibody to p27, the group specific antigen of ALVs. Using p27 permits detection of all subgroups and can be used for diagnostic purposes. However, the possibility of detecting endogenous virus is present because some subgroup E loci express p27. The genetic cross of chickens used in this study has a high endogenous viral loci load, which could have interfered with detection of ALV-J using a p27 based assay. Using a monoclonal antibody to gp85 that is subgroup specific removes the possible detection of endogenous virus. The tissues that had not been previously reported as containing ALV-J in white leghorns were detected using the gp85 monoclonal antibody and included peripheral nerve, spleen, thymus and bone marrow. Using the gp85 antibody may increase the sensitivity of the detection method compared to using the antibody to p27.

FUTURE DIRECTIONS

From the results gained from these studies, additional questions were raised about ALV-J infection in egg laying chickens. One question was, how is the virus escaping the immune system in those chickens with endogenous virus loci, causing the animals to become viremic tolerant instead of mounting a delayed immune response? Experiments designed to look at the interactions of endogenous virus and ALV-J could answer this question. By examining the MHC class I and II expression from chickens with ev loci infected with ALV-J versus line 0 chickens which lack ev loci, may give some indication

of the interactions of exogenous and endogenous viral proteins and host proteins that allow viremia to continue. Work by Stedman *et al* (2000) demonstrated that the phagocytic functions of macrophages and heterophils are not decreased and lymphocytic functions are not impaired in broilers infected with ALV-J. Therefore, the virus is apparently not immunosuppressive and does not reduce the ability of the bird to respond to infection with other pathogens. Functional assays on white leghorn white blood cells may reveal a difference and help explain the increased viremic tolerance seen with white leghorns with *ev* loci.

Another question arose from the Line 6 tumor results. To confirm the tumor resistance of Line 6_3 chickens seen in objective 1, a separate experiment using Line 6_3 chickens infected as embryos and at day-of-hatch with more birds is necessary. With only 10 at risk chickens remaining at the end of 30 weeks, the conclusion that Line 6_3 birds are resistant to ALV-J induced tumor formation is tenuous. An experiment similar to that in objective 1 more at risk birds would be necessary.

One other question that could be addressed is the effect of B haplotype on ALV-J infection in both broilers and layers. The B haplotype is well characterized in leghorn chickens. Recent work by Bacon (yet unpublished data) examined the effect of B haplotype in aged hens and their ability to mount an antibody response. There is some evidence that B21 homozygotes have higher neutralizing antibody titers compared to B2 or B5 homozygotes. By examining the effect of B haplotypes in day-of-hatch inoculated chicks, information regarding antibody response and tumor incidence can further support Bacon's findings. Additionally, examination of B haplotype in broiler chickens, which is not well characterized, may provide new information on the effect of genotype on disease

outcome, and may allow breeder production companies to consider such information in their genetic selection process.

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