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**Study of Subgroup J Avian Leukosis Virus Persistence in Meat Type Chickens**

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# STUDY OF SUBGROUP J AVIAN LEUKOSIS VIRUS PERSISTENCE IN MEAT-TYPE CHICKENS

By

Arun Kumar Reddy Pandiri

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## ABSTRACT

**Study of Subgroup J Avian Leukosis Virus Persistence in Meat Type Chickens**

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Subgroup J Avian Leukosis Virus (ALV J) is economically important to the poultry industry since it causes a variety of neoplastic and non-neoplastic syndromes primarily in meat-type chickens. A series of experiments were conducted to better understand ALV J-induced viral persistence, neutralizing antibody (NAb) response, and oncogenicity in meat-type chickens.

The aim of the first objective was to study the effect of viral strain (ADOL Hc1, ADOL 4817, and ADOL 6803) and dose (100 and 10000 $TCID^{50}$), and age at infection ($5^{th}$ day of embryonation and day of hatch) on ALV J persistence and oncogenicity in meat-type chickens. The results demonstrated a high incidence (83-100%) of ALV J persistence in all treatment groups and a large percentage (up to 75%) of viremic chickens with concurrent NAbs against the inoculated parental virus (V+A+). Viral strain and dose, and age at inoculation influenced the incidence of NAb response but only viral strain had an effect on the ability of NAb to clear the infection.

The aim of the second objective was to confirm the high incidence of V+A+ infection status in meat-type chickens infected at hatch with an ALV J molecular clone ADOL pR5-4 as demonstrated in the first objective with ALV J field isolates. In addition, autologous and heterologous virus neutralization (VN) was done to test for the emergence of NAb escape variants in persistently V+A+ meat-type chickens. The results

demonstrated a high incidence (88%) of V+A+ infection status in meat-type chickens. All V+A+ chickens failed to neutralize autologous viruses on at least 2 out of 4 to 6 sampling intervals demonstrating the emergence of NAb escape variants. Hence, this study demonstrated that NAb escape variants play a role in ALV J persistence although the heterogeneity of viral population in field isolates may also be a factor.

The goal of the third objective was to study the effect of porcine Adrenocorticotrophin (ACTH)-induced stress on chickens that had cleared viremia with an efficient NAb response. The results demonstrated that only meat-type chickens that were exposed to ALV J at hatch but not at 32 weeks of age had reverted to viremia and cloacal shedding (33%) 10 days post ACTH-induced stress.

The aim of the fourth objective was to detect ALV J gp85 antigen by immunohistochemistry (IHC) as well as provirus by polymerase chain reaction in tissues from meat-type and ADOL line 0 chickens with various infection status. The results demonstrated that ALV J proviral DNA could be demonstrated in both viremic as well as seroconverted non-viremic chickens whereas gp85 expression was restricted to chickens exhibiting overt viremia in the presence or absence of antibody response.

The aim of the fifth objective was to characterize ALV J-induced histiocytic proliferative lesions by histochemistry and IHC. The results demonstrated that these lesions only appear in persistently viremic chickens infected at hatch and are comprised of myeloid cells with histiocytic differentiation.

Thus, these research studies have added new information on ALV J viral persistence and may aid the poultry industry in controlling ALV J infection.

I am pleased to dedicate this work

to

my mother Mrs. P. Varalakshmi and my father Mr. P. Bhiksham Reddy

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*Images in this dissertation are presented in color.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5ED | 5th day of embryonation |
| ACTH | adrenocorticotropic hormone |
| ADOL | Avian Disease and Oncology Laboratory |
| AEV | avian erythroblastosis virus |
| ALV | avian leukosis virus |
| ALV A | subgroup A avian leukosis virus |
| ALV B | subgroup B avian leukosis virus |
| ALV C | subgroup C avian leukosis virus |
| ALV E | subgroup E avian leukosis virus |
| ALV J | subgroup J avian leukosis virus |
| AMV | avian myeloblastosis virus |
| ASVs | avian sarcoma viruses |
| BCG | bacillus Calmette Guerin |
| BSL-2 | biosecurity level 2 |
| C/E | CEFs that support replication of all exogenous ALVs |
| CEFs | chicken embryo fibroblasts |
| CIAV | chicken infectious anemia virus |
| CMI | cell mediated immunity |
| COFAL | complement fixation test for avian leukosis virus |
| CTL | cytotoxic lymphocyte |
| DAB | diaminobenzidine |
| DLV | defective leukemia viruses |
| DNA | deoxyribonucleic acid |
| DOH | day of hatch |
| EB | erythroblastosis |
| ELISA | enzyme linked immunosorbent assay |
| env | envelope protein |
| ERVs | endogenous retroviruses |
| FS | fibrosarcoma |
| gsa | group specific antigen p27 |
| H&E | hematoxylin and eosin |
| HA | hemangioma |
| HI | humoral immunity |
| HS | histiocytic sarcomatosis |
| IBDV | infectious bursal disease virus |
| IBV | infectious bronchitis virus |
| IHC | immunohistochemistry |
| KEDTA | potassium ethylene diamino tetra acetic acid, anticoagulant |

| | |
|---|---|
| L/S | leukosis sarcoma group |
| LL | lymphoid leukosis |
| LTRs | long terminal repeat |
| MB | myeloblastosis |
| MDV | Marek's disease virus |
| MH | multicentric histiocytosis |
| MHC II | major histocompatibility complex class II |
| ML | myeloid leukosis |
| mL | milliliter |
| Λμ | microliter |
| NAb | neutralizing antibody |
| NDV | Newcastle disease virus |
| NK | natural killer |
| NP | non producer |
| ntV+A- | non tolerized viral persistence especially inoculated at hatch |
| PBL | peripheral blood leukocyte |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PM | phenotypic mixing |
| RAV0 | Rous-associated virus 0 |
| RAV1 | Rous-associated virus 1 |
| REV | reticuloendotheliosis virus |
| RIF | resistance inducing factor |
| RMS | rhabdomyosarcoma |
| RNA | ribonucleic acid |
| RPRL | Regional Poultry Research Laboratory |
| RSV | Rous sarcoma virus |
| RT | renal tumor |
| TCID50 | tissue culture infectious dose 50 |
| tV+A- | tolerized viral persistence especially inoculated *in ovo* |
| tv-a | tumor virus-a |
| tv-b | tumor virus-b |
| tv-c | tumor virus-c |
| USDA | United States Department of agriculture |
| V | viremia |
| VI | virus isolation |
| VN | virus neutralization |

# CHAPTER 1

## Introduction and Literature review

## INTRODUCTION

Avian leukosis viruses (ALVs) are responsible for the leukosis/sarcoma (L/S) group of diseases in chickens that comprise a variety of transmissible benign and malignant neoplasms in chickens. L/S diseases primarily include lymphoid leukosis, and myelocytomatosis followed by renal tumors, hemangiomas, erythroblastosis and some uncommon to rare sarcomas and carcinomas.

ALVs are economically important poultry pathogens. In addition to loss of chickens due to tumor mortality (1-20%), ALVs are also responsible for production losses that include decrease in egg production and quality, non-uniform flocks, and high feed conversion ratio. In the USA during late 1990s, ALV J caused early mortality of broiler breeder hens (1.5% per week in excess of normal mortality), and resulted in a severe shortage of hatching eggs. At its peak prevalence, ALV J threatened the economic viability of the meat-type poultry industry (Fadly and Payne, 2003). This was soon controlled by the broiler-breeder industry by efficient diagnosis and eradication of infected flocks with support from the USDA-ARS-Avian Disease and Oncology Laboratory (ADOL, East Lansing, MI), the Institute of Animal Health (IAH, Compton, UK), and several university research centers in the USA and other countries. However, complete eradication of ALV J is very difficult to achieve due to increased levels of viral persistence in meat-type chicken flocks and also due to lack of accurate diagnostic methods. Almost all the meat-type breeders are subjected to constant ALV testing and the ALV-exposed chickens are culled from the flocks. Some flocks are designated as

"tested negative", since they are ALV-negative for successive generations before becoming ALV-positive (McKay and Rosales, 2000). As with other retroviruses, ALV J is capable of persisting at very low levels in the host and the routinely used diagnostic methods may not always detect the virus. Hence, the real prevalence of ALV J may be actually higher than what is reported.

The studies in this thesis mainly focus on factors influencing ALV J persistence in meat-type chickens by evaluating a combination of factors such as age at exposure, host immunity and genetics, viral strain and dose, and neutralizing antibody (NAb) escape variants. The following hypotheses were tested:

1) ALV J strain (ADOL Hc1, ADOL 6803, and ADOL 4817), inoculum dose (10,000 $TCID^{50}$ and 100 $TCID^{50}$), and age at inoculation ($5^{th}$ day of embryonation (5 ED) or on day of hatch (DOH)) influence viral persistence, neutralizing antibody response, oncogenicity and mortality in commercial meat-type chickens.

2) a. Chickens infected with an ALV J molecular clone suffer in a high incidence of V+A+ infection profile as was observed by infection with ALV J field isolates.
   b. The emergence of antibody escape variants contributes to the V+A+ infection profile in ALV J infected chickens.

3) a. Adult meat-type chickens that have cleared Subgroup J Avian Leukosis viremia with efficient neutralizing antibody responses can revert to viremia when subjected to stress.

b. ALV J-induced tolerance can be modified by adoptive transfer of age-matched, MHC-matched splenocytes.

4) Neutralizing antibodies against ALV J influence tissue distribution of the virus (viral antigen and provirus) in 32 week old commercial meat-type and ADOL Line 0 chickens.

5) a. The incidence of histiocytic sarcoma-like lesions in chickens is influenced by chicken strain (host genetics), age at inoculation, and viral strain and dose.

b. The HS-like lesions are comprised of myeloid cells with histiocytic differentiation.

## LITERATURE REVIEW

## ETIOLOGY

**Classification.** ALVs are the type species of the genus alpharetrovirus that belongs to the *retroviridae* family. All members of the retroviridae family share similar replication machinery. ALVs have similar physical, chemical and molecular characteristics and also share common group specific antigen p27 (gsa).

ALVs are classified into numerous categories based on several virological, immunological and pathological criteria. ALVs in chickens are classified into 6 subgroups A, B, C, D, E and J, based on envelope glycoprotein (env) that determines antigenicity, viral interference patterns between members of the same and different subgroups, and host range in chicken embryo fibroblasts (CEFs) belonging to distinct genetic lines. ALVs are also classified into 'types' based on the antigenic variation of viruses within the subgroup. Antisera raised against members of the same subgroup cross-neutralize each other to varying degrees but generally do not cross-neutralize a virus belonging to a different subgroup with the exception of antisera against ALV B that partially interacts with ALVs D and E (Payne, 1992). Antisera raised against a particular viral type tend to neutralize the homologous virus more strongly than heterologous viruses within the same subgroup, indicating a mixture of several viral types with different neutralizing epitopes within each ALV subgroup. ALVs B and J appear to be more heterogeneous than other ALV subgroups. ALVs isolated from tumors and passaged a few times in susceptible chickens are often referred to as 'strains'. Also

viruses within each ALV subgroup are classified into several strains based on the type of pathology induced in various tissues of the host i.e. most of the strains are subgroup-pathotype combinations. However, each ALV strain often contains mixtures of viruses (Payne, 1987). The oncogenic spectrum of these viruses is diverse and is generally characteristic for each viral strain.

Based on the rate of tumor induction in susceptible chickens ALVs are divided into 2 classes *viz.* acutely transforming viruses and slowly transforming viruses. Acutely transforming viruses (also called defective leukemia viruses (DLVs)) induce various leukemias and sarcomas in susceptible hosts (chickens or cell culture) within a few days or weeks since they carry viral oncogenes in their genomes. The *v-onc* generally determines the target cell that is transformed and the type of neoplasm produced. These viruses are generally replication-defective (*rd*) mutants lacking genes required for replication, so the helper leukosis viruses present in the viral mixture provide the missing replication function *in trans* for the *rd* DLVs. Acutely transforming viruses can only be isolated through either *in vivo* systems or certain transformation susceptible *in vitro* cell culture systems like bone marrow or peripheral blood leukocyte (PBL) cell cultures. Examples of acutely transforming viruses include Rous sarcoma virus (RSV), MC29, 966 and MH2. Generally, slowly transforming viruses induce various leukoses, leukemias, and sarcomas only after a long period following infection. They induce transformation by promoter insertion or related mechanisms where the cellular oncogenes are activated to induce transformation of certain cell types. Slowly transforming viruses are replication competent and do not require the assistance of helper leukosis viruses for

replication. Examples of slowly transforming viruses include Rous associated virus (RAV) RAV-1 (Subgroup A ALV), RAV-2 (Subgroup B ALV), RAV-49 (Subgroup C ALV), RAV-50 (Subgroup D ALV), and ADOL-Hc1 (Subgroup J ALV).

ALVs are also classified as exogenous or endogenous based on their nature of replication and transmission. Exogenous ALVs are free infectious viral particles that spread through both vertical and horizontal modes of transmission. Endogenous ALVs include incomplete to complete viral particles. They are inherited in a Mendelian fashion and are responsible for genetic transmission of the complete (*ev*2, *ev*7, *ev*10, *ev*11, *ev*12, *ev*14, *ev*18 and *ev*21) or incomplete (*ev*3, ev6 and *ev*9) viral particles. Since some of the endogenous viral loci encode a complete infectious virus (ALV Subgroup E), they can also be transmitted through both vertical and horizontal routes in addition to genetic transmission.

Chickens harbor endogenous retrovirus-like sequences (ERVs) in their genomes. All the ERVs are inherited in a Mendelian fashion. There are 4 families of ERVs in chickens. The first family of ERVs consists of CR1 (Chicken Repeat 1) that is a short interspersed repetitive DNA sequence belonging to the non-long terminal repeat class of retrotransposons. There are about 7000 to 20,000 repeats per haploid genome (Crittenden, 1991). They have conserved 3' ends and variable 5' truncations. The CR1s are identified in several avian and reptilian species, demonstrating their presence before the divergence of chickens and reptiles (Crittenden, 1991).

The second family of ERVs in chickens includes the ART-CH (avian retrotransposon from the chicken genome) that belongs to a class of defective retrotransposons whose replication strategy requires the use of helper viruses. There are approximately 50 copies per haploid genome and each copy is composed of functional LTRs and short regions of homology to the ALV-related gene sequences (Nikiforov and Gudkov, 1994).

The third family of ERVs include the well known endogenous viral (*ev*) loci consisting of complete or defective proviral DNA sequences of subgroup E leukosis virus integrated in the genome (Sacco *et al.*, 2000). There are over 30 *ev* loci documented in different strains of chickens and it is proposed that more exist (Sabour *et al.*, 1992). In meat type chickens, on an average about 5 *ev* loci exist in each bird (Rovigatti and Astrin, 1983). Various *ev* loci are responsible for the production of defective viral particles (*ev*3, *ev*6 and *ev*9) or complete ALVs (*ev*2, *ev*7, *ev*10, *ev*11, *ev*12, *ev*14, *ev*18 and *ev*21). Certain *ev* loci like *ev*1, *ev*4, *ev*5, *ev*8, and *ev*17 can also be transcriptionally silent with no detectable viral product. The *ev*21 locus is tightly linked to the dominant sex-linked gene *K* on the Z chromosome and regulates slow feathering that seems to render White Leghorn chickens more susceptible to ALV infections than other stocks (Bacon *et al.*, 1988); (Smith and Fadly, 1988). Many *ev* loci are also associated with decreased productivity, immunity and disease resistance to varying extents (Crittenden, 1991). Endogenous viruses rarely cause tumors apparently due to the weak promoter activity of their long terminal repeats (LTRs) (Crittenden *et al.*, 1979; Fadly and Payne, 2003; Motta *et al.*, 1975). The *ev* loci have been shown to be nonessential for survival of the chicken

as is evident from the existence of line 0 chickens that are devoid of any *ev* loci (Astrin *et al.*, 1979); (Crittenden and Fadly, 1985). The *ev* loci are considered to represent recent germ line integrations into the chicken genome because of their low copy numbers, segregation in the population and restricted distribution in various species of *Gallus* (Sacco *et al.*, 2000).

The fourth family of the ERVs consists of EAV-0 (EAV-HP or ev/J) that was discovered in line 0 chickens that are free of the *ev* loci. It is a heterogeneous group consisting of highly diverged retroviral elements (Sacco *et al.*, 2000). EAV-0 elements show a typical proviral structure with deletions in the *env* region (Boyce-Jacino *et al.*, 1989). There is 95-97% sequence homology between EAV-0 and ALV J *env* gene (Benson *et al.*, 1998; Smith *et al.*, 1999). Microarray studies demonstrated that infection of CEFs by RB1B strain of Marek's disease virus (MDV) induces ALV J (EAV-0) envelope messenger ribonucleic acid (mRNA) expression (Morgan *et al.*, 2001). However, no protein expression of EAV-0 elements was reported to date (Sacco *et al.*, 2001). A recently sequenced EAV-0 element ev/J 4.1 Rb when pseudotyped with murine leukemia virions exhibited a complete reciprocal interference with exogenous ALV J demonstrating the sharing of the same receptor(s) by both exogenous ALV J and EAV-0 element ev/J 4.1 Rb (Denesvre *et al.*, 2003). If this phenomenon occurs *in vivo*, it might have a significant value in ALV J epizootiology. The EAV-0 elements are phylogenetically older than the *ev* loci, since they are present in other species of *Gallus* unlike the *ev* loci.

**Virus structure and composition.** ALVs like all the retroviruses have similar structure and replication strategies. Previously ALVs were classified as type C retroviruses due to their morphology during budding of the virus from the host cell membrane. ALVs are approximately 80-120 nm in diameter, with an electron dense core of 35-45 nm. The outer membrane has 7 nm long projections with a 6 nm knob like structure at the tip (Beard, 1963). ALVs have a buoyant density of 1.15-1.17 g/cm$^3$ in sucrose characteristic of the avian type C viruses (Bauer, 1974). The sedimentation rate of the particles is typically about 60S. The virions are also susceptible to heat, detergent and formaldehyde (Fadly and Payne, 2003).

The avian type C retroviral particles are composed of 30-35% lipid, 60-65% protein (of which 5-7% is glycoprotein), 2.2% RNA and small amounts of DNA of host cell origin. Viral lipids (mainly phospholipids) are of cellular origin and have a bilayered structure similar to the host cell membrane from which the virion envelope was derived (Bauer, 1974; Bolognesi, 1974). The viral proteins are derived from four genes "5' *gag/pro-pol-env* 3'" present in the retroviral genome. The *gag* gene encodes 4 non-glycosylated proteins *viz.* capsid protein (CA-p27) which is the major group specific antigen (gsa), matrix protein (MA-p19) which lines the inner surface of the virion envelope, an additional gag protein (p10) located between MA and CA, and the nucleocapsid protein (NC-p12) which is a basic protein associated with genomic RNA. The *pro* gene lies between the *gag- pol* genes and forms protease (PR-15). The *pol* gene encodes the enzymes, reverse transcriptase (RT-p68) and integrase (IN-p32). The *env*

gene encodes 2 glycoproteins *viz.* the major envelope protein (SU-gp85) and the transmembrane protein (TM-gp37) (Coffin, 1992).

The ALV genome is a dimer of linear, positive-sense, single stranded RNA, with each monomer 7.8 kb in size. Since the viral RNA genome is generated by the host transcriptional machinery, it exhibits many features of a normal mRNA *viz.* capping at $5^{l}$ end, and poly(A) sequence at $3^{l}$ end. The ALV RNA genome is organized as a single stranded RNA consisting of a $5^{l}$ cap, $3^{l}$ poly(A) tail, 2 repeated regions (R-20nt) on either ends (one immediately after the $5^{l}$ cap and one immediately before the $3^{l}$ poly(A) tail), and a unique $5^{l}$ sequence (U5-80nt) lying downstream of $5^{l}$ R that contains one of the *att* sites required for proviral integration. The U5 region is followed by a primer binding site (pbs), an 18-nt sequence that hybridizes to the host tRNA and is the initiation site for the minus-strand DNA synthesis. Downstream of the pbs contains some major encapsidation signals for the viral RNA called the Psi element ($\Psi$). Following the $\Psi$ element are the genes coding for structural proteins i.e. *gag/pro-pol-env.* Subsequent to the structural genes lies a short poly purine tract (ppt), a run of at least nine A and G residues. The ppt is the initiation site for the plus-strand DNA. Downstream of the ppt is the unique $3^{l}$ sequence (U3-200nt) that contains a number of *cis*-acting elements for viral gene expression and one of the *att* sites required for proviral DNA integration. The viral RNA sediments at 60-70S and 4-5S representing the diploid viral genome and the host tRNA respectively. The host tRNA acts as the primer for the DNA polymerase during transcription of viral RNA to DNA (Coffin, 1992; Goff, 2001).

Exogenous ALVs namely ALV A, B, C and D share high sequence homology with ALV J in the *gag/pro* and *pol* regions (96-97%) but not in the *env* region. The *env* gene of ALV J share a high sequence homology with the *env* region of EAV-HP (97%), ev/J (95%), and EAV-E51 (75%) (Benson *et al.*, 1998; Smith *et al.*, 1999). ALV J has a redundant transmembrane region insert (rTM) 3' to the gp37 region of the *env* gene that shares a higher sequence homology (97%) with the TM region of other exogenous ALVs than with the TM region of ALV J. Downstream of the rTM, the 3' noncoding region of ALV J contains a single copy of the direct repeat (dr1) element, also found as a single copy in ALVs but as two copies flanking the *src* gene in avian sarcoma viruses. Downstream of dr1 is a copy of the E element (xsr) previously found only in replication-competent RSVs, but not in naturally occurring replication competent ALVs (Bai *et al.*, 1995). Based on the above observations, the emergence of the novel ALV subgroup J may be attributed to either a single or multiple recombination events between the endogenous and exogenous ALVs (Venugopal, 1999).

**Virus replication.** Adsorption of the virus to the host cell membrane is nonspecific but penetration of the virus into the host cells is mediated by the specific interaction of host gene-encoded receptors on cell membrane with the virion envelope glycoproteins (SU and TM) unique to each of the various subgroups. ALV receptors are mapped to the chicken genome at *tv*(tumor virus)-*a* (ALV-A), *tv-b* (ALV-B, -D, -E), and *tv-c* (ALV-C) loci. Susceptibility is always dominant over resistance, implying that they directly encode the receptors. The *tv-a* and *tv-c* loci are genetically linked, although they code for independent receptors (Payne and Pani, 1971). The *tv-b* locus consists of multiple alleles

encoding receptors for both ALV B and E (Crittenden and Motta, 1975), however, the existence of an independent locus *tv-e* coding for ALV E receptor is also indicated (Pani, 1976). Preinfection of cells with ALV B or D prevents superinfection by ALV E and this interference is not reciprocal (Weiss, 1982). However, subgroups B and D exhibit reciprocal interference, indicating they recognize the same receptor (Duff and Vogt, 1969). Thus ALV B, D, and E recognize closely related receptors. The above description is valid only for chicken cells since turkey or quail embryo fibroblasts are resistant to ALV B and D but still susceptible to ALV E (Vogt, 1977). Thus, these receptors are not identical among galliform species. ALV J receptor appears to be different from ALV A, B, C, D and E receptors since ALV A receptor-variant viruses could enter ALV J infected cells but not ALV A to E infected cells. This is also evident since ALV A to E envelope glycoproteins are homologous and highly related while the ALV J glycoprotein is very different (Melder *et al.*, 2003).

After receptor mediated interaction with the host cell, the virus penetrates the host cell membrane. The outer lipid layer of the virion fuses with the host cellular membrane within the endosomes and the virion core is released into the cytoplasm. The plus-strand viral RNA is reverse transcribed into a minus-strand viral DNA by reverse transcriptase (RT) resulting in RNA:DNA hybrid. RNase-H activity of RT cleaves the RNA from the hybrid and later a plus-strand DNA complimentary to the minus-strand DNA is formed with the help of DNA polymerase activity of RT giving rise to DNA duplexes flanked by long terminal repeats (LTRs) on both the ends. The LTRs consist of repeated sequences (U3-R-U5) derived from terminal regions of viral RNA. The LTRs contain promoter and

enhancer sequences that control transcription of viral DNA into RNA in the host nucleus. The full length double-stranded DNA migrates into the nucleus and forms either a linear or closed circular molecules. The closed circular DNA with one or two LTRs are dead end products and only the linear duplex DNA is integrated into the host cell DNA at multiple often random sites by the viral enzyme integrase. The integration of the proviral DNA marks the end of the early phase of the retroviral life cycle that is driven by viral enzymes and the beginning of the late phase that is mediated by host enzymes (Goff, 2001). Transcription of the viral RNA on proviral DNA template is dependent upon host cell RNA polymerase II. The resulting viral RNA transcripts are exported back into the cytoplasm. Here they are packaged as genomic RNA into virions or form messenger RNA (mRNA) that is translated into several polyproteins i.e. the resulting mRNA associate with polyribosomes and are translated into various polyproteins like Pr180*gag-prt-pol*, Pr76*gag-prt* and gPr92*env*. These precursors are cleaved by viral and cellular proteases to yield the viral proteins and glycoproteins respectively (Luciw and Leung, 1992). The viral proteins localize directly at the plasma membrane of the host cell without any apparent cytoplasmic intermediate forming crescent-shaped structures that define avian type C morphology. The resultant immature virion with a large, open spherical core matures into a centrally located condensed form with small surface projections and exits the host cell by budding (Coffin, 1992).

## EPIZOOTIOLOGY

**Incidence and Distribution.** ALVs are ubiquitous in commercial poultry operations in spite of extensive ALV eradication programs practiced by many primary breeders.

Unless the stock is genetically resistant to infection or free of ALV through successful ALV eradication programs, most flocks are exposed to ALV by sexual maturity (Fadly and Payne, 2003).

ALV A is the most frequently isolated exogenous ALV from commercial poultry operations followed by, to a much lesser extent, ALV B (Calnek, 1968). ALVs C and D are extremely rare in the USA but are reported commonly in Finland along with Subgroups A and B (Sandelin and Estola, 1974). Subgroups A, B, C and D predominantly induce lymphoid leukosis in addition to the less commonly occurring leukoses such as erythroblastosis, myeloblastosis and myelocytomatosis along with some non-leukotic tumors like hemangiomas, nephroblastomas, osteopetrosis, and histiocytic sarcomas (Purchase, 1987); (Fadly and Payne, 2003; Perek, 1960) . Subgroup E ALVs like RAV-0 have little to no oncogenicity in chickens presumably due to their weak LTR promoter activity (Crittenden *et al.*, 1979; Fadly and Payne, 2003; Motta *et al.*, 1975).

Genetic recombination between endogenous ALV and exogenous ALV can lead to oncogenic mutants due to incorporation of exogenous viral LTRs into endogenous viruses (Crittenden *et al.*, 1980; Tsichlis and Coffin, 1980; Weiss *et al.*, 1973). High incidence rates of recombination between exogenous viruses belonging to different subgroups are also reported (Gingerich *et al.*, 2002; Lupiani *et al.*, 2000).

Generally, the incidence of ALV A-induced lymphoid leukosis (LL) is about 1-2% but it can reach levels of up to 20% (Fadly and Payne, 2003). Extensive losses

(>25%) due to LL are rare. The incidence of LL depends on several factors such as genetics of the chicken, incidence of infectious bursal disease (IBD), and serotype 2 MDV (SB1) vaccination. The incidence of LL inversely correlates with the incidence of IBD due to the destruction of the target cells by IBD virus (Purchase and Cheville, 1975). Vaccination with serotype 2 MDV (SB-1) is known to enhance LL induction following ALV exposure at hatch (Bacon *et al.*, 1989; Fadly and Witter, 1993), reticuloendotheliosis virus (REV)-induced bursal lymphomas (Aly *et al.*, 1996) and spontaneous lymphoma incidence in chickens free of exogenous ALVs (Salter *et al.*, 1999).

In the late 1980s during routine screening of chickens in ALV eradication program in England, Payne and coworkers isolated Subgroup J ALV that induces myelocytomatosis specifically in meat-type chickens (Payne *et al.*, 1991). Soon after that ALV J was isolated across the globe possibly due to the existence of very few meat-type breeder companies and the spread of their franchises all over the world, and also due to exchange of genetic material between poultry companies. Before ALV J discovery, the incidence of myelocytomatosis was uncommon to rare in commercial poultry operations. The incidence of ALV J strain HPRS-103 induced myelocytomatosis in meat-type chickens was about 27% (Payne *et al.*, 1992). Moreover, losses in commercial broiler breeders were found to be 1.5% per week in excess of normal mortality (Fadly and Smith, 1999). Subgroup J ALV was responsible for early mortality of broiler breeder hens and thereby causing a severe shortage of hatching eggs. At its peak prevalence, ALV J threatened the economic viability of the meat-type poultry industry (Fadly and Payne,

2003). Many broiler-breeder companies were able to significantly control the virus spread with the help of government and university research centers. However, complete ALV J eradication is a very ambitious goal since several companies report sporadic ALV J isolation from broiler-breeder flocks supposedly free from ALV J. In addition to problems with ALV J eradication, mixed infections of chicken anemia virus, MDV, ALV J and other poultry pathogens were also reported (Cui *et al.*, 2003).

Myelocytomatosis are very common in ALV J infections, however the incidence of neoplastic conditions like erythroblastosis, nephroblastomas, hemangiomas, histiocytic sarcomatosis (HS) and connective tissue tumors is common to uncommon and are seen either alone or along with other neoplastic conditions.

**Infection profiles.** Rubin classified ALV infection status in chickens into 4 simple categories *viz.* no viremia and no neutralizing antibody (Nab) (V-A-), with viremia and no NAb (V+A-), no viremia and with NAb response (V-A+), and with viremia and with NAb (V+A+) (Rubin *et al.*, 1962). Genetically susceptible chickens in ALV-free flocks and those genetically resistant to certain ALV subgroups fall into V-A- category. Genetically susceptible chickens in infected flocks fall into the other three remaining categories depending on age at infection, dose and strain of ALV exposure, presence or absence of endogenous ALVs, and host genetics. Almost all *in ovo* infected chickens and a majority of chickens infected early (1-2 weeks) in life, are immunologically tolerant and remain persistently viremic with no NAb production i.e. V+A-. Some of the neonatally infected V+A- chickens eventually seroconvert and produce efficient NAbs

and clear the viremia i.e. V-A+. In general, most of the chickens that develop NAbs and clear viremia (V-A+) are infected horizontally. However in certain infections, a significant percentage of chickens exist with V+A+ status i.e. the chicken is unable to clear the infection in spite of efficient NAb response against the parent virus and result in viral persistence (Fadly and Payne, 2003).

Viremic hens shed large amounts of virus and gsa (p27) into the albumen of their eggs and this directly correlated with the rate of congenital transmission (Spencer *et al.*, 1976). Hence infected hens were classified into shedders and non-shedders based on the detection of infectious virus or gsa in egg albumen or vaginal swabs. The importance of the use of vaginal swabs in studying congenital ALV transmission was recognized by several workers (Payne *et al.*, 1979; Spencer *et al.*, 1976). Later, this classification was extended to both male and female chickens of all ages by testing for infectious virus in the cloacal swabs (Crittenden and Smith, 1984). Hence ALV shedding along with viremia and NAb against the inoculated parental virus data are used by several workers to describe the infection status of chickens exposed to ALV i.e. V$\pm$, A$\pm$, and S$\pm$.

Witter and coworkers classified ALV J infection status in chickens into 5 different profiles based on viremia, cloacal shedding and NAb. This classification helped in delineating V+A- class of ALV infection status into persistent or transient viremia based on longitudinal sampling for a period of 62 weeks (Witter *et al.*, 2000). In the current study, classification of ALV J infection status is based on viral persistence with and without NAb after longitudinal sampling for a period of 32 weeks.

**Antigenic variation.** Antigenic variation in ALV can be divided into 2 broad types based on the host cell receptors *viz.* subgroup-specific when the viruses use different host cell receptors and type-specific when the viruses use the same receptor. Subgroup specific and type specific antigenic variation is reported in ALV infections (Fadly and Smith, 1999; Venugopal *et al.*, 1998) . Generally subgroup specific variation leads to formation of a novel ALV subgroup (Fadly and Payne, 2003). Type specific antigenic variation is supposedly much higher in ALV J infections than in other ALV subgroups (Silva *et al.*, 2000; Venugopal *et al.*, 1998). Mutations in the viral genome can lead to epitope deletion, failure of antigen processing, loss of major histocompatibility complex (MHC) class I binding, impaired recognition by the T cell receptor and as a result viral persistence is commonly observed.

Longitudinal studies of ALV J infection revealed a significant incidence of V+A+ infection profile in chickens i.e. the chickens are simultaneously positive for both viremia as well as NAb against the parental virus (Witter *et al.*, 2000). The routine NAb test involves incubation of the inoculated parent virus with the antisera collected during various sampling intervals (Fadly and Witter, 1998). Thus, the V+A+ infection profile might be due to the emergence of novel antibody-escape variants that are not neutralized by antibodies against the parent virus. Most likely, the parent virus suffered mutations in the *env* gene and gave rise to the variants that are responsible for the V+A+ infection profile. There is little data about the *env* sequence variation of ALV J isolates with unique neutralization profiles or V+A+ infection profiles. Sequencing of ALV J isolates

with V+A+ infection profile could provide information about domains in the *env* region that code for antigenic epitopes.

As discussed earlier the *env* gene is composed of glycoproteins gp85 (SU) and gp37 (TM) and sequence variations in these regions affect both virus-host cell receptor interactions as well as NAb responses. The gp85 interacts with the host receptor and the gp37 anchors gp85 to the membrane and is directly involved in the fusion of viral and host membranes. The gp85 is composed of 5 hypervariable clusters designated *vr1-vr2-hr1-hr2-vr3* and gp37 is composed of fusion peptide and membrane anchor domains. Clusters *hr1* and *hr2* are responsible for receptor binding specificity as well as host range determination in all the ALV subgroups. The *vr3* domain plays a role in the specificity of receptor recognition but not receptor binding affinity. However, *vr1* and *vr2* do not appear to be essential for receptor specificity or binding affinity (Dorner *et al.*, 1985). Generally, gp85 is more variable than gp37. Venugopal and coworkers have demonstrated that *hr2* and *vr3* regions of ALV J *env* gene showed a high sequence variation along with very high NS/S (nonsynonymous/synonymous) ratios and suggested that these regions could be a target for immune selection (Venugopal *et al.*, 1998). Silva and colleagues also showed regions of high sequence variability in ALV J *env* gene and designated them as hypervariable regions 1-4 since the *env* region of ALV J is very different from ALV A and B (Silva *et al.*, 2000).

Commercial antibody enzyme linked immunoassay (ELISA) kits are widely used by the poultry industry to identify and cull the chickens exposed to ALV. However, the high level of antigenic variation of ALV due to hypervariability of the *env* gene questions

the reliability of the commercially available antibody-based ELISA kits. Polymerase chain reaction (PCR) based ALV-monitoring of the flocks is also not entirely reliable since the primers can not be specific and generic at the same time. So constant monitoring of the specificity and sensitivity of all ALV diagnostic methods is essential for a successful ALV eradication program. The hypervariability of the *env* gene also hinders efforts to design vaccines against ALV infections. None of the novel ALV vaccines designed and tested by several labs across the world provided satisfactory protection.

**Transmission.** The three modes of natural transmission of ALV include horizontal transmission, congenital transmission, and genetic transmission. Exogenous ALVs and complete infectious endogenous ALVs like RAV-0 are transmitted horizontally from bird to bird through feces, saliva, skin and fomites (Burmester and Gentry, 1954; Crittenden *et al.*, 1987; Spencer *et al.*, 1977).

The efficiency of horizontal transmission and shedding of RAV-0 is supposed to be less than exogenous viruses (Payne, 1987; Robinson and Eisenman, 1984). Virus shed by congenitally infected chicks is important in early horizontal transmission and leads to high levels of disease in the flock (Fadly and Payne, 2003; Weyl and Dougherty, 1977). Hence, both horizontal and vertical transmission play vital roles in ALV transmission i.e. vertical transmission is responsible for maintaining the infection between generations and horizontal transmission is responsible for maintaining high levels of infection within the flock, thereby increasing the possibility of vertical transmission.

The efficiency of congenital transmission of the virus mainly depends on the ALV infection status and age of the dam. Dams with V+A- status transmit ALV to the progeny at relatively high levels. However, the rate of congenital transmission by V-A+ dams is intermittent and at lower proportions. These dams generally have a low NAb titer. Older dams (2-3 years) shed virus into eggs less consistently and at lower levels than younger dams (<18 months) (Burmester and Waters, 1956). Shedding of the virus into egg albumen and subsequent infection of the embryo in the oviduct is known to occur in all the dams congenitally transmitting the virus (Tsukamoto *et al.*, 1991). Ultrastructural studies have demonstrated a high degree of virus replication in the magnum of the oviduct (Distefano and Dougherty, 1966). Several workers have presented evidence that only about one-half to one-eighth of the embryos with virus in the albumen actually hatched infected (Payne *et al.*, 1982; Spencer *et al.*, 1977; Tsukamoto *et al.*, 1992). The efficiency of congenital transmission of ALV depends not only on the frequency and amount of virus shed in the albumen but also on the presence of NAbs in the yolk as well as thermo sensitivity of the virus (Fadly and Payne, 2003). Congenital transmission has also been demonstrated in the absence of detectable shedding of p27 antigen (Ignjatovic, 1990). In line K28 hens, congenital transmission of RAV-0 appears to be inhibited due to a heavier p27 protein than exogenous ALV (Bhown *et al.*, 1980; Robinson and Eisenman, 1984). However, this effect was not replicated in other chicken lines like semi-congenic 15B dams since RAV-0 was detected in albumen by ELISA (Crittenden and Smith, 1984).

The role played by the sire in ALV congenital transmission is at best equivocal in spite of some experimental evidence. Ultrastructural data demonstrated ALV budding from almost all the sire's reproductive tissues except the germinal cells, thereby implying the absence of sire's role in vertical ALV transmission (Distefano and Dougherty, 1966, 1968). RAV-1 inoculated directly into the testis could not infect spermatozoa or genetically transmit ALV through semen (Afanassieff *et al.*, 1996). Most workers believe that the sire venereally transmits the virus to the dam and does not directly influence the rate of congenital transmission (Smith and Fadly, 1994). Semen production was not reduced in ALV-shedding males but significant associations of ALV shedding with higher incidence of abnormal spermatozoa and reduced fertility were found in some chicken populations but not in others (Segura *et al.*, 1988). However, in spite of some anecdotal evidence, ALV J infection does not influence the quality and quantity of semen production (Hudson *et al.*, 2002).

Genetic transmission of endogenous ALV occurs through both egg and sperm. The *ev* loci responsible for the genetic transmission of complete endogenous ALV include *ev*2, *ev*7, *ev*10, *ev*11, *ev*12, *ev*14, *ev*18 and *ev*21. However, other *ev* loci such as *ev*3, ev6 and *ev*9 give rise to genetically defective incomplete virions. Once the chicks hatch viremic with infectious endogenous ALV, they spread horizontally like exogenous ALVs.

Embryos infected *in ovo* with ALV serve as an excellent source of horizontal transmission to hatch- and pen-mates since they tend to support high levels of virus

replication and shed the virus at hatch through saliva and meconium (Burmester, 1956). Chickens infected *in ovo* or during the first 2 weeks of their life tend to be persistently viremic and more likely to develop tumors (Burmester *et al.*, 1960). Susceptibility to tumor development is genetically regulated in spite of being susceptible to ALV infection (Crittenden *et al.*, 1972). In general, the rate of tumor development is independent of immune responses against the virus. However, viremic-tolerant (V+A-) chickens are more likely to develop tumors than seroconverted (V-A+) chickens (Rubin *et al.*, 1961).

## PATHOBIOLOGY

Members of L/S group consist of ALVs, DLVs and avian sarcoma viruses (ASVs) that are capable of inducing oncogenic and non-oncogenic pathological spectra. Under optimal dose and route of exposure ALVs, DLVs and ASVs are commonly associated with leukoses, leukemias and sarcomas, respectively (Purchase, 1987). ASVs carry *src* oncogene and induce tumors more rapidly than ALVs. Also, ASVs are capable of transformation of chicken embryo fibroblasts unlike ALVs and DLVs, and this forms the basis for *in vitro* ASV diagnosis as well as for ALV subgroup identification. Unlike ALVs ad DLVs, ASVs induce tumors in a variety of hosts including mammals. DLVs unlike ALVs are capable of rapid transformation of hematopoietic cells and this property is the basis for *in vitro* transformation assays for DLVs like avian erythroblastosis virus (AEV), avian myeloblastosis virus (AMV), MC29, and strain 966. Hence in broad terms, the target cells for ALVs and DLVs are hematopoietic cells and the target cells for ASVs are fibroblasts (Purchase, 1987).

Pathology induced by members of L/S group is determined by several factors such as subgroup and strain of virus, dose of virus, route of exposure, sex of host, age of host at exposure, genotype of host, environmental factors and stress. Generally, the predominant tumor induced by an ALV strain is unique for that strain but there can be considerable variation under field conditions.

Viral subgroup plays a major role in the incidence and type of tumor pathology. In general, all ALVs except subgroup J predominantly induce tumors of lymphoid lineage whereas subgroup J mainly induces tumors of myeloid lineage. Traditionally, it was believed that ALV subgroups *per se* have no effect on the incidence of LL since ALV subgroup is an attribute of viral envelope and tumor incidence depends on viral LTRs (Payne, 1987). However, later evidence demonstrates the role of ALV envelope on the type of tumor induced in the host (Brown and Robinson, 1988; Chesters *et al.*, 2002; Jaffredo *et al.*, 1993). Viral strains within an ALV subgroup have varying capacities to induce LL, myeloid leukosis (ML) or other tumor types.

The inoculated viral dose greatly influences the incubation period for tumor formation as well as the type of tumor produced. Experimental studies by Burmester and coworkers have demonstrated that high doses of certain ALV A strains predominantly induce erythroblastosis with a short incubation period of 2 to 3 months whereas low doses predominantly induce LL with a relatively longer incubation period of 5 to 9 months (Burmester *et al.*, 1959). Under field conditions, ALV exposure is generally at low doses and hence the reason for high incidence of LL. However, congenitally infected

chickens have high viral loads but still the incidence of LL is more common than leukemias (Payne, 1987).

Traditionally, the nomenclature of most viral strains reflects the predominant tumor induced at high doses *viz.* RSV (sarcomas), AMV (myeloblastosis), AEV (erythroblastosis). However, these viral strains when administered at low doses predominantly induce LL (Purchase, 1987). This phenomenon may be explained by the following 2 hypotheses: 1) viral threshold to infect and transform erythroblasts might be higher than that is required to infect and transform B lymphocytes. Chickens succumb to erythroblastosis before they can develop LL. 2) slowly transforming ALVs may mutate into DLVs in the host and induce leukemias. High ALV doses in the host may provide far more opportunities for mutation into DLVs than low doses. Similar data indicating dose-pathology relationship for ALV J strains is not indicated in the scientific literature.

In general, the route of exposure determines the effective viral dose and the type of target cell exposed to the virus. ALV inoculation either through subcutaneous or intramuscular route predominantly induces sarcomas and LL but when inoculated through intravenous route predominantly induces erythroblastosis and myeloblastosis (Fredrickson *et al.*, 1965). Differences in pathology due to route of exposure for ALV J infection have not been reported.

Sex of the host has no effect on ALV infection but it is relatively important in the incidence of certain tumors. Female and castrated male chickens are more susceptible to

lymphoid leukosis than intact male chickens, suggesting a protective nature of testosterone due to regression of the target cells (Burmester and Nelson, 1945; Cooper *et al.*, 1968). Apparently, males seem to be more susceptible to osteopetrosis than females but the reasons are not known (Smith, 1987a). Incidence of ALV J induced myelocytomatosis is independent of the sex of the host. Sex also has no effect on the incidence of several types of leukemias and sarcomas.

Age of the host at exposure markedly influences both the course of infection and disease incidence (Piraino *et al.*, 1963; Rubin *et al.*, 1962; Rubin and Vogt, 1962). Younger hosts are more susceptible to viral persistence and tumor mortality than older hosts. Resistance to tumor development (type I resistance) is attained earlier when viral exposure is by natural routes than when it is through parenteral routes (Burmester *et al.*, 1959). However, this may be due to low effective viral dose though natural fecal-oral exposure than through parenteral exposure. Resistance to infection (type II resistance) develops more slowly than type I resistance (Purchase, 1987). As discussed, the age effect may not be true in all cases but certainly applies for some ALV strains like RPL12 (Burmester *et al.*, 1960). Chickens infected with ALV J during the first 2 weeks of life suffer a high level of viral persistence and eventual tumor mortality than chickens infected later in life (Witter *et al.*, 2000).

Host genotype is critical for both type I (cell transformation) and type II (cell infection) resistance. Chickens belonging to line 6 are resistant to tumor formation where as line 7 are susceptible to tumor formation. Genetic resistance to viral infection depends

on the absence of specific viral receptors present on the host cells and inability of the virus to penetrate the host cells. Inheritance pattern of the virus receptors is of simple Mendelian type (Crittenden, 1975). Host cells can be resistant to infection by one or several members of ALV subgroup *viz.* C/A cells are resistant to ALV A infection, C/AB cells are resistant to ALV A & B infection, and C/0 cells are susceptible to all exogenous ALV subgroups identified to date. The frequency of alleles encoding cellular resistance to infection by ALV, DLV or ASV vary greatly among commercial chicken lines (Crittenden and Motta, 1969; Motta *et al.*, 1973). To date, no chicken genetically resistant to ALV J has been identified in spite of intense screening by numerous research laboratories. Type I resistance depends on several alleles in addition to some of the factors discussed above (Purchase, 1987).

Environmental factors greatly influence the incidence of certain tumor types *viz.* factors that deplete bursa of Fabricius reduce the incidence of LL but may increase the incidence of osteopetrosis. Several managemental factors like manual vent sexing, lack of biosecurity that increase the rate of exposure tend to influence tumor incidence. Environmental factors that induce stress negatively influence the host immunity and as a result increase tumor incidence (Fadly *et al.*, 1989).

Members of the L/S group cause a variety of non-neoplastic conditions in addition to several neoplastic diseases in chickens. High dose of ALV exposure in susceptible chickens at early ages cause varying degrees of stunted growth along with anemia, hepatitis, ascites, immunosuppression, hypothyroidism and obesity (Carter and Smith,

1983; Crittenden *et al.*, 1984; Smith *et al.*, 1975; Smith and Van Eldik, 1978). Some of the conditions mentioned above are typically caused only by certain ALV strains whereas other conditions are more generic. The non-neoplastic conditions also include reduced feed efficiency, reduced growth rate, stunting and non-uniform flock size, late sexual maturity in hens, delayed age of first egg, small clutch size, small non-uniform eggs with thin shells, reduced fertility, reduced hatchability, and increase in non-neoplastic mortality (Fadly and Payne, 2003; Gavora, 1987). The quality and fertility but not quantity of semen is reduced in ALV shedding roosters (Segura *et al.*, 1988). However, ALV J does not influence either the quality or quantity of semen production (Hudson *et al.*, 2002). Inflammatory non-neoplastic lesions mimicking neoplastic lesions due to virus-host immune interaction might manifest in the form of gross or microscopic lesions consisting of discrete foci or larger diffuse accumulation of lymphoblasts and lymphocytes (Purchase, 1987). Hepatic microscopic lesions described as 'lymphomyeloid hyperplasia' have been described in natural and experimental ALV J infections due to host immune responses against the virus (Venugopal *et al.*, 2000). Some of these inflammatory non-neoplastic lesions such as lymphocytic thyroiditis may be related to clinical signs of hypothyroidism and stunting and obesity syndrome observed especially in the case of ALV C RAV-7 infection (Carter *et al.*, 1983). Congenital ALV J infection supposedly causes body weight depression in broiler chickens due to decreased serum thyroxine T4 levels (Brown *et al.*, 2000). Abnormal feather development slightly different from REV-induced "nakanuke" was recently reported to be associated with congenital ALV J infection in broiler chickens (Landman

*et al.*, 2001). However, these non-neoplastic conditions are not observed in all ALV J congenital infections.

**Tumors induced by the L/S group.** The various neoplastic diseases induced by members of the L/S group depend on the site of integration of the virus in the host's genome in case of ALVs, on the type of oncogene carried by the virus in case of DLVs and also on ALV env glycoprotein. The commonly occurring ALV-related neoplasms in chickens are as follows:

**Lymphoid leukosis (LL).** LL is typically seen in chickens only after 14 weeks of age and generally peaks at sexual maturity. Due to the long incubation period, LL does not pose a significant problem in production broilers, but can be a severe problem for broiler breeders and layers. Hyperplasia or neoplastic transformation of individual bursal follicles can be observed as early as 4 weeks in chickens infected *in ovo* or at hatch by methyl green pyronine staining. By 7 weeks, these chickens have one or more abnormal bursal follicles (Cooper *et al.*, 1968) but most of the hyperplastic/neoplastic follicles regress due to anti-tumor immune response. After a relatively long incubation period, the B cells of the transformed follicle massively proliferate and metastasize to different viscera. The long incubation period is a property of the target B cells and is independent of the host's maturational physiology (Fadly *et al.*, 1981). Southern blotting analyses of the tumors from different tissues have demonstrated the clonal nature of the tumors.

Chickens succumbing to LL present gross or microscopic lesions in the bursa of Fabricius. In some LL cases, gross bursal lesions are not readily evident but careful microscopic examination generally reveals bursal involvement (Cooper *et al.*, 1968). The target cell for transformation is IgM expressing bursal stem cells (Purchase and Gilmour, 1975). Thus ablation of the target cell either by chemical (Purchase and Gilmour, 1975), hormonal (Burmester, 1969; Romero and Frank, 1977), surgical (Peterson *et al.*, 1966) or infectious (Purchase and Cheville, 1975) means prevents LL in susceptible chickens. The transformation of the susceptible bursal cells is initiated after the viral promoters present in the LTRs of the provirus activate the host cellular oncogene c-myc. Several host genes such as c-myc and c-bic play different roles in the induction of LL. The oncogenes encode nuclear transcription factors responsible for turning on or off various genes responsible for cell replication (Fadly and Payne, 2003).

Clinical signs are non-specific and include inappetance, emaciation, pale or cyanotic comb, and enlarged abdomen. Death is generally due to organ dysfunction. Gross neoplastic lesions are presented in a diffuse, miliary, nodular, or a combination of these forms (Purchase, 1987). Gross neoplastic lesions in LL almost always involve liver and to a certain degree spleen, kidney, lung, heart, digestive tract, mesentery and other viscera. Microscopically, the neoplastic lesions appear as coalescing foci that compress rather than infiltrate the parenchyma of the affected organ. Tumor foci consist of aggregates of large lymphoblasts with a slightly basophilic cytoplasm and a large vesicular nucleus enclosing clumped chromatin with one or more acidophilic nucleoli (Fadly and Payne, 2003). In uncommon to rare instances, small reactive lymphocytes

infiltrate the morphologically more or less uniform neoplastic B cells giving the appearance of MDV-induced lymphomas, thereby complicating diagnosis. ALVs of subgroup A and B preferentially transform cells of lymphoid lineage and are principally responsible for LL, in contrast to ALV J that preferentially transforms cells of myeloid lineage. Subgroup J ALVs are seldom associated with LL (Payne, 2000). However, Williams recently presented evidence of bursal transformation with methyl green pyronine staining in ALV J infected white leghorn chickens (Williams *et al.*, 2004).

**Erythroblastosis.** Natural cases of erythroblastosis in chickens are usually manifested between 12-24 weeks of age. However, the incubation period depends not only on virus strain, dose, route of exposure, but also on age at exposure and host genotype. This neoplastic condition is not limited to any specific ALV subgroup. The incidence of erythroblastosis in slowly transforming ALV infections is dependent on the integration of the proviral LTR promoter regions near the host cellular oncogene c-*erb*B (encodes epidermal growth factor receptors). However, acutely transforming ALVs possess the viral oncogene v-*erb*B that is capable of inducing erythroblastosis with a relatively short incubation period (Fadly and Payne, 2003). The target cells for transformation are the erythroblasts present in the bone marrow sinusoids as well as in the extra-medullary erythropoietic foci (Purchase, 1987). Once the erythroblasts are transformed, further differentiation is arrested and the cells rapidly multiply and metastasize causing erythroblastic leukemia as well as solid tumors in the viscera.

Clinical signs are nonspecific and are similar to lymphoid leukosis but with a higher incidence of anemia. Gross lesions include diffusely enlarged liver and spleen which are soft and cherry red to mahogany in color due to intravascular or intrasinusoidal infiltration of neoplastic erythroblasts (Fadly and Payne, 2003). Diffuse petechial hemorrhages are usually seen in various visceral organs. The bone marrow is replaced by proliferating erythroblasts and is semisolid to liquid in consistency. The transformed erythroblasts infiltrate rather than compress the parenchymal cells. The hepatic sinusoids are expanded and engorged with the transformed erythroblasts. The erythroblasts have a large amount of basophilic cytoplasm with a characteristic perinuclear halo and a large round nucleus with very fine chromatin and one or two nucleoli (Purchase, 1987). Erythroblasts are irregular in shape often with pseudopodia and also carry unique diagnostic physiologic markers such as hemoglobin, and chicken erythrocyte-specific histone H5 (Fadly and Payne, 2003). The incidence of erythroblastosis in chickens infected with ALV J is variable but can be high in chickens experimentally infected with ALV J DLVs like strain 966 (Venugopal *et al.*, 2000).

**Myeloblastosis.** This condition occurs rarely in the field and generally manifests prior to 24 weeks of age (Purchase, 1987). However, DLVs like E26 and BAI-A can cause myeloblastosis within 10 days (Fadly and Payne, 2003). The target organ is the bone marrow and the v-*myb* gene (functions like a nuclear transcription factor) of the virus is responsible for transformation of the target cell. Further differentiation of the transformed cell is arrested and they proliferate in the bone marrow and metastasize to different parts of the body especially the liver, spleen and kidney (Purchase, 1987).

Clinical signs are similar to erythroblastosis but the disease course is longer. There is severe leukemia, with myeloblasts comprising 75% of peripheral blood cells and forming a thick "buffy" coat accompanied by anemia and thrombocytopenia (Payne, 1992). Gross lesions include massive enlargement of the liver, mottling of the visceral organs and replacement of the bone marrow by solid, yellowish-gray tumor nodules (Purchase, 1987). The affected organs show severe intravascular and extravascular infiltration, and proliferation by myeloblasts and promyelocytes. The tumor cells compress and replace the affected organ's parenchyma unlike erythroblastosis where the neoplastic cells infiltrate without actually compressing and replacing the parenchyma (Fadly and Payne, 2003). Myeloblasts are large cells with a slightly basophilic cytoplasm and a large nucleus with fine chromatin network with 1-4 faintly acidophilic nucleoli. They can be distinguished from myelocytes which have many large acidophilic granules. ALVs of subgroups A, B, and J are associated with myeloblastosis.

**Myelocytomatosis.** Most naturally occurring cases of myelocytomatosis are usually seen in immature chickens (Purchase, 1987). The incubation period is of varying lengths and is generally longer than erythroblastosis and myeloblastosis but shorter than LL. Several viral strains like MC29, CMII, HPRS-103, and ADOL Hc1 preferentially induce myelocytomatosis in susceptible hosts. HPRS-103-induced myelocytomatosis has a latent period of about 9-20 weeks (Payne, 1998). The target organ is the bone marrow and the c-myc host gene plays a major role in the pathogenesis (Chesters *et al.*, 2001; Fadly and Payne, 2003). DLVs containing *v-myc* in the form of a gag-myc fusion protein

causes myelocytomas within 4-6 weeks in susceptible hosts (Chesters *et al.*, 2001). The myelocytes arise from the bone marrow stem cell, proliferate and infiltrate the bone marrow sinusoids. Tumors are generally found in the bones, liver, spleen, ovary, and thymus.

Clinical signs include bony protuberances on the skull, ribs and other periosteal surfaces. There are no pathognomonic clinical signs. Gross lesions are characterized by moderate to massive enlargement of the liver and myelocytomas of the skull, sternal and pelvic bones (Mladenov *et al.*, 1967). Tumors consist of myelocytes with cytoplasm rich in spherical acidophilic granules and a large eccentrically placed vesicular nucleus with distinct nucleolus, and are similar to normal myelocytes present in the bone marrow (Fadly and Payne, 2003). Myeloblasts and myelocytes have myeloid markers such as adherence and phagocytic capacity, Fc receptors, and macrophage- and granuolocyte-specific cell surface markers (Fadly and Payne, 2003). The cellular populations in the blood are generally aleukemic unless the infection is complicated by erythroblastosis. Myelocytomatosis is generally considered a pathognomonic lesion for ALV J infection in meat-type chickens (Payne, 1998)

**Renal tumors.** Renal tumors observed in chickens fall into 2 main types *viz.* nephroblastoma, a mixed tumor arising from embryonic cell rests in the kidney, and adenoma/adenocarcinoma, an epithelial tumor also arising from the cell rests. The average age for the appearance of renal tumors is 6-24 weeks with most being detected at the time of necropsy (Purchase, 1987). Nephroblastomas can be induced experimentally

by strains 1911 (Payne *et al.*, 1993) and bureau of animal industry (BAI)-A strain (Mladenov *et al.*, 1967). Renal adenomas are induced by strains MC29 (Mladenov *et al.*, 1967) and HPRS-103, 17, 705, 966 (Payne *et al.*, 1993). The c-*fos* gene is implicated as a target oncogene for ALV-induced nephroblastomas but is not found to be consistent (Collart *et al.*, 1990). The tumors vary in size and structure depending on the virus strain, cells affected, age of the host and incubation period. In nephroblastomas, after virus infection and transformation, the epithelial cells differentiate into glomeruli, tubules or keratinized epithelium while mesenchymal cells differentiate into sarcomas, cartilage and bone. In case of renal adenomas/adenocarcinomas, only the epithelial differentiation is observed (Mladenov *et al.*, 1967). The renal tumors have not been observed to metastasize in chickens (Campbell, 1969). Renal tumors are frequently observed in chickens infected by ALV J virus in the field (Payne, 1998).

**Multicentric histiocytosis (MH) / Histiocytic sarcomatosis (HS).** A low incidence of proliferative histiocytic lesions consisting of macrophages, dendritic cells and lymphocytes designated as HS is frequently reported in broilers but not in White leghorns infected with ALV J. Detection of gsa by immunohistochemistry and ALV-J env RNA by *in-situ* hybridization is not a marked feature of these lesions but can be observed sporadically (Arshad *et al.*, 1997). However, similar lesions are also described as MH since it was not determined if the lesions represented a true neoplastic response or a marked hyperplastic response. Sporadic isolation of ALV was possible only in a few cases of MH (Hafner and Goodwin, 2003). There is no definite link between ALV and this lesion due to paucity of sufficient scientific data to draw any conclusions.

Gross lesions typically include nodules in the spleen, liver and kidneys. HS is capable of wide distribution. The affected chickens are pale and stunted when compared with hatchmates. Microscopic lesions in the spleen include marked expansion of periarteriolar lymphoid sheaths by macrophage-like cells with elongated oval or fusiform or bizarre-shaped nuclei. Liver and kidney also expressed similar microscopic lesions. Immunohistochemical and ultrastructural studies have demonstrated that the splenic nodules consisted of a predominance of cells of monocyte/macrophage lineage, and CD4- and CD8-positive lymphocytes (Arshad *et al.*, 1997). However, routine diagnosis is generally done by examination of H&E-stained microscopic sections.

**Connective tissue tumors.** The connective tissue tumors include many benign and malignant neoplasms with viral etiology similar to fibromas, fibrosarcoma, myxomas, myxosarcoma, osteomas, osteogenic sarcoma, and chondrosarcomas (Fadly and Payne, 2003). Most isolates of ALV from the field are multipotent and are capable of inducing a variety of tumors. The incubation period for induction of these tumors depends on numerous variables associated with virus strain, helper viruses, host genotype and environmental conditions.

**Other tumors.** Various other rare neoplasms associated with ALV- related viruses are mesothelioma, endothelioma, hepatocarcinoma, thecoma, granulosa cell tumor, hemangioma, hemangiosarcoma, pancreatic adenocarcinoma, squamous cell carcinoma, rhabdomyosarcoma, and unclassified leukemias (Fadly and Payne, 2003).

## IMMUNOLOGY

Fundamental research on avian retroviral immunology was done mainly on early models of virus-induced oncogenicity *viz.* RSV and DLVs. Rapid transformation ability of these viruses and compartmentalization of the avian immune system made them an outstanding model in the study of cell mediated immunity (CMI) as well as humoral immunity (HI) in virus-induced tumorigenesis. In addition to tumor-immunology, avian retroviruses also provide a good model to study viral-immunology, especially in the areas of viral persistence, tolerance, endogenous retroviral elements and retroviral vectors.

**Viral persistence and tolerance.** ALVs are an excellent model for studying viral persistence and tolerance. Chickens infected early in life either congenitally or neonatally, often develop immunological tolerance and viral persistence with the majority of them succumbing to tumors (Rubin *et al.*, 1962). Qualtiere and Meyers established that congenital ALV-infection indeed causes true immunological tolerance by demonstrating the absence of NAb responses against ALV, antigen-antibody complexes and host IgG deposits in the kidneys of chickens congenitally infected with ALV (Qualtiere and Meyers, 1979). There were also no differences in ALV-induced viremia titers of bursectomized chickens compared to non-bursectomized chickens (Qualtiere and Meyers, 1979). Hence tolerance is defined by a complete absence of host immunological response against the virus whereas viral persistence is defined as the presence of viremia regardless of the status of host immune system. ALV-induced tolerance is strain and subgroup specific i.e. tolerant chickens inoculated with other strains of the same subgroup produce NAbs against the inoculated viral strain but not the strain that induced

tolerance (Meyers, 1976). Generally, chickens infected later in life (>4 weeks) develop a transient viremia and a life long persisting NAb response capable of clearing the circulating ALV. However, the presence of NAb response does not guarantee sterilizing immunity since a low level of persistent ALV infection is established in lymphocytes and macrophages (de Boer *et al.*, 1981; Gazzolo *et al.*, 1979).

In addition to age related tolerance induction, endogenous retroviruses are also known to induce varying degrees of immunological tolerance and viral persistence of exogenous ALVs. Chickens positive for *ev* loci are defined by the retroviral proteins expressed by chicken embryo fibroblasts. Depending on the stage of maturation, thymic and bursal cells of *ev*+ chickens express varying amounts of retroviral proteins on their surface (Ewert *et al.*, 1984; Ewert and Halpern, 1982). The bursal cells are tolerized during the pre-B to B -cell transition since the interaction of even a very low concentration of antigen with membrane-bound immunoglobulin induces a state of clonal anergy (Nossal, 1989). The B cells in later stages of maturation can also suffer from clonal anergy, but usually require higher antigen concentrations. The T cells are susceptible to tolerance induction at even lower doses than B cells. Due to the above reasons, the immature lymphocytes are usually tolerized due to the prolonged contact with the common endogenous and exogenous viral proteins (Wainberg and Halpern, 1987).

Chickens tolerized to ALV E RAV-0 can induce "partial tolerance" to ALV -A and -B i.e. chickens inoculated *in ovo* with endogenous ALV RAV-0 and challenged with

exogenous ALV at hatch had prolonged exogenous ALV viremia and shedding, delayed NAb response against the exogenous ALV and increased tumor incidence (Crittenden *et al.*, 1987). This partial tolerance may be due to the sharing of the epitopes between exogenous and endogenous ALV. Later it was demonstrated that chickens carrying *ev-6* loci that codes for endogenous envelope protein were also partially tolerized to ALV A and showed prolonged viremia, delayed onset of NAb and increased tumor incidence (Smith *et al.*, 1991). Also line $6_3$ chickens were easily tolerized to ALV J infection and failed to produce NAbs unlike line 0 chickens that are devoid of *ev* loci (Williams *et al.*, 2000). Several *ev* loci in chickens interact synergistically to induce tolerance to exogenous ALVs (Kuhnlein *et al.*, 1993; Smith *et al.*, 1990). The above factors have a great commercial value since the sex-linked slow feathering gene K was initially selected by several breeders to reduce chick-sexing costs but the K gene is tightly linked to *ev*21 loci that codes for a complete infectious endogenous virus strain EV21 (Bacon *et al.*, 1988). Congenital transmission of EV21 from slow feathering dams to rapid-feathering female progeny increased viral persistence and decreased immune responses against exogenous ALV infections (Smith and Fadly, 1988). In addition to compromised humoral immune responses, the cell-mediated immune responses against exogenous ALVs are also down-regulated in chickens' that are tolerized to endogenous ALVs (Hunt *et al.*, 1995).

Induction of viral persistence/tolerance also depends on the type of ALV strain within each subgroup and dose of inoculation. White leghorn chickens inoculated with 4 different ALV-A strains *in ovo* and at hatch, demonstrated that the viral strain influences

the level of viral persistence and immunological tolerance. Among ALV A strains tested, RPL-40 induces greater levels of viral persistence than RPL -41, -42 and RAV-1 (Fadly *et al.*, 1987). In chickens inoculated *in ovo*, viral dose has a direct correlation with tolerant viremia levels and tumor incidence (Fadly *et al.*, 1987).

Horizontal transmission of ALV J is known to be more efficient than other ALV subgroups. However, the rate of vertical transmission of ALV J is very similar to other ALV subgroups i.e. erratic and unpredictable. Recent evidence indicates that the level of viral persistence and tolerant viremia induced by ALV J is much higher than that observed with infection with ALV A (Koch *et al.*, 2000). However, experimental data regarding the effect of viral strain, and dose of inoculation on the level of ALV J viral persistence, immunological tolerance, and tumor incidence is lacking.

**Humoral immunity (HI).** The NAb responses are directed against gp85 and gp37 glycoproteins present on the surface of the virus particle and are highly specific for each subgroup and sometimes to strains within each subgroup (Meyers, 1976; Qualtiere and Meyers, 1983; Vogt and Ishizaki, 1965). Antibodies are also produced against other viral structural proteins such as gsa (p27), but these do not appear to play any role in virus neutralization. Chickens with LL generally carry high levels of IgM that does not play any role in protection against the virus (Cooper *et al.*, 1974; Smith *et al.*, 1980). Significant increase in IgG levels was also observed early in some ALV infections, but there was no correlation with the NAb response (Banes and Smith, 1977; Qualtiere and Meyers, 1976, 1979).

Generally NAbs persist for life in the chicken and is passed on to the chicks as maternal antibodies through the egg. Maternal antibodies against ALV delay the onset of infection, reduce the level of ALV-induced viremia and shedding, and eventually reduce tumor-mortality (Burmester, 1955; Fadly, 1988; Witter *et al.*, 1966). Hence NAbs are very efficacious and vital for protection against ALV. Tolerant viremic chickens are likely to have greater tumor incidence than seroconverted chickens but there is no correlation between the development of NAb responses and tumorigenesis i.e. seroconverted chickens are as susceptible to tumor mortality as chickens with tolerant viremia.

**Cell-mediated immunity (CMI).** Most of the early ALV cellular immunology research was based on cell-mediated responses against RSV. This is mainly due to the rapid transformation ability of RSV, and the distinct immunophenotype of progressor or regressor chicken lines (Gyles and Brown, 1971). Many laboratories demonstrated that neonatal thymectomy consistently enhanced tumor progression in chickens and quail (Cotter *et al.*, 1976; Wainberg *et al.*, 1979; Yamanouchi *et al.*, 1971). Avian sarcomas were frequently infiltrated with mostly lymphocytes but in chickens that were neonatally thymectomized prior to RSV inoculation, very little lymphocytic infiltration was observed in the tumor tissue (Cotter *et al.*, 1976; Yamanouchi *et al.*, 1971). Electron microscopic examination of the RSV-induced sarcoma revealed the presence of lymphocytes with polar accumulation of organelles at the point of contact with tumor cells in regressing tumors but not progressing tumors. Moreover, regressing sarcomas

consistently had lymphocytic infiltration unlike progressing sarcomas. This demonstrated that presumably T cells are important in CMI against ASV-induced tumors (Perry *et al.*, 1978; Wainberg *et al.*, 1979). However, the effector cells were not positively identified until the advent of immunologic marker studies (Schat, 1987).

Initial studies of anti-tumor CMI did not take the MHC into account until it was demonstrated that autologous splenocytes from chickens infected with RSVs were able to lyse the tumor cells better than allogeneic splenocytes (Wainberg and Halpern, 1987). The first clear evidence of MHC class I-restricted CMI was provided with REV-infected targets and effector cells. The REV-transformed target cells were $B^{13}B^{13}$ and $B^{6}B^{6}$ haplotypes and the effector cells were isolated from chickens with $B^{13}B^{13}$ and $B^{6}B^{6}$ haplotypes respectively. Hence in a chromium release assay, significant killing was observed when the target and effector cells were syngeneic (Maccubbin and Schierman, 1986). Later, the chicken MHC class I amino acid residues forming serologic epitopes as well as residues important in antigen presentation to ALV-induced cytotoxic T lymphocytes (CTL) were identified by workers at ADOL, East Lansing, MI (Fulton *et al.*, 1995). CTL responses in ALV infections were studied by using LSCC-RP9 cells transfected with an ALV vector system RCASBP expressing MHC chicken class I (B-F) cDNA coding for either MHC $B^{21}$ or $B^{13}$ as target cells (Thacker *et al.*, 1995). The effector cells were derived from either line $15I_5$ or line 0 B congenic chickens expressing either MHC $B^{13}B^{13}$ or $B^{21}B^{21}$ haplotypes that are infected with ALV A. A CTL response that is virus- and MHC- specific was demonstrated 10 days post challenge (Thacker *et al.*, 1995). Chickens with $B^{21}B^{21}$ haplotype demonstrated greater CTL response against

ALV A than chickens with $B^{13}B^{13}$ haplotype. $B^{13}B^{13}$ haplotype chickens were unable to regress RSV-induced sarcomas unlike chickens with $B^{21}B^{21}$ haplotype (Bacon *et al.*, 1981). The extent of actual protection offered by CTL responses against ALV and RSV-induced viremia and tumors is not really clear. Also the role played by MHC and CTL response against ALV J infection is not clear.

In addition to T cells, natural killer (NK) cells and macrophages were also implicated in anti-tumor immunity. Chicken amniotic fluid contains α-fetoprotein that induces suppressor cells against NK cells. Japanese quail inoculated with amniotic fluid and challenged with RSV, suffered higher incidence of progressing sarcomas than controls (Yamada and Hayami, 1981, 1983). Furthermore, it was observed that tumor cells from regressor tumors but not progressor tumors were susceptible to the cytolytic activity of NK cells and resistant to specific cell-mediated cytolysis. Addition of autologous virus to the *in vitro* reaction inhibited the cytotoxicity of specific-immune effector cells but not NK cells. This indicated that the NK cell tumor cell lysis is immunologically mediated and is directed against the tumor cells and not the tumor virus (Wainberg *et al.*, 1987).

Depending on the site of injection of Bacillus Calmette Guerin (BCG) antigen followed by RSV challenge in chickens results in 2 separate phenomena *viz.* tumor regression and progression. Repeated injection of BCG into wing web 7 days prior to inoculation of RSV at the same site in the wing web causes a remarkable stimulatory effect on tumor growth (Wainberg and Israel, 1978). This was contrary to expectations

since BCG adjuvant is known to be immunostimulatory and support the host in regressing tumors. BCG inoculation leads to granuloma formation and when RSV was inoculated at the same site, macrophages in the granuloma serve as target cells that supported multiplication and dissemination of the virus. These BCG-enhanced tumors had higher percentage of macrophages than in tumors induced by RSV alone (Wainberg *et al.*, 1983). However, BCG does help in regressing tumors provided the sites of inoculation of BCG and RSV are different. The protective nature of macrophages against sarcomas was demonstrated in 2 congenic chicken lines line 6.6-2 ($B^2B^2$) and line 6.15-5 ($B^5B^5$) that are regressors and progressors respectively. Macrophages isolated from Sephadex® induced intra-abdominal exudate from progressor and regressor chicken lines were used in cytotoxic assays against tumor cell lines LSCC-RP9 and MDCC-CU14. Macrophages from the regressor chicken line 6.6-2 ($B^2B^2$) were significantly more tumoricidal than macrophages from line 6.15-5 ($B^5B^5$) (Qureshi and Taylor, 1993). However, the exact role played by macrophages in immunity against neoplastic cells is not entirely clear.

**Immunosuppression.** Dohms and Saif defined immunosuppression in very generic terms as "a state of temporary or permanent dysfunction of the immune responses resulting from an insult to the immune system and leading to increased susceptibility to disease". Immunosuppression is defined and identified by several criteria *viz.* morphometric changes in central and peripheral lymphoid tissues, changes in concentration of different immunoglobulins and complement factors, changes in the

functional efficiency of immune responses, and immunosuppressive responses to other pathogens or antigens (Dohms and Saif, 1984).

ALV subgroup B causes immunosuppression in chickens more than other ALV subgroups. Involution of the bursa of Fabricius, thymus and spleen is most commonly observed in the ALV B MAV-2(0) infection that causes high levels of osteopetrosis (Smith, 1987b; Smith and Van Eldik, 1978). RAV-1 infection of chicks lacking ev3 locus leads to severe atrophy of lymphoid organs (Crittenden *et al.*, 1982). Chickens infected *in ovo* with ALV A (ALVF42) and ALV B (MAV-2(0)) showed hypergammaglobulinemia (IgG) that does not correlate with NAb responses (Banes and Smith, 1977; Qualtiere and Meyers, 1976).

Assays measuring changes in the functional activity of immune responses include measuring proliferation of lymphocytes exposed to different mitogens as well as measuring vaccination responses *in vivo*. Subgroup B viruses inhibit proliferation of lymphocytes when exposed to lectins like phytohemagglutinin and concanavalin A (Price and Smith, 1982; Smith and Van Eldik, 1978). This effect was abrogated when macrophage-like adherent cells from uninfected chickens were added to lymphocytes from infected chickens. Hence, MAV-2(O) induced-immunosuppression may be due to interference with an accessory function of macrophage-like adherent cells (Price and Smith, 1982). Therefore, immunosuppression induced by ALVs B and C might be due to the tropism of these viruses for macrophages and reduction of its accessory functions (Gazzolo *et al.*, 1975; Rup *et al.*, 1982; Schat, 1987). ALV A viruses, except for some

strains like ALVF42, generally do not cause suppression of mitogen-induced proliferation of lymphocytes (Meyers, 1976).

The ability of chickens infected *in ovo* with ALV B MAV-2(0) severely limits the production of antibody against certain antigens like sheep red blood cells (SRBC), *Brucella abortus*, and human gammaglobulin. Similarly, these infected chickens also have depressed delayed hypersensitivity reaction against human gammaglobulin antigen. However, chickens infected with MAV-2(0) within 48 hours after hatch showed few signs of immunosuppression (Hirota *et al.*, 1980; Smith, 1987b). Other ALV subgroups, with the exception of subgroup B, are generally not associated with immunosuppression (Fadly *et al.*, 1982); (Smith, 1987b).

To date, unequivocal data demonstrating ALV J-induced immunosuppression is still lacking. Landman and coworkers failed to demonstrate ALV J-induced immunosuppression by a variety of assays including delayed-type hypersensitivity with keyhole limpet haemocyanin, natural killer (NK) cell activity, the production of nitric oxide free radicals by macrophages, antibody response against Newcastle disease (NDV) and IBDV vaccines, and automated total and differential leukocyte counts (Landman *et al.*, 2002). Stedman and colleagues failed to prove ALV J-induced heterophil dysfunction in chickens infected with *Staphylococcus aureus* (Stedman *et al.*, 2001). However, some workers described decreased protection against infectious bronchitis virus (IBV) and NDV vaccination followed by challenge in ALV-J infected chickens compared to uninfected hatchmates (Spackman *et al.*, 2003). Hence, sometimes

anecdotal evidence from the field incriminates ALV A- or J-induced immunosuppression for vaccination failures but there is no unambiguous experimental evidence to prove it.

Chickens infected with ALVs as well as other pathogens show an altered immune response. Chickens infected with subgroup A and infectious bursal disease virus (IBDV) showed higher ALV shedding compared with controls infected with ALV A alone (Fadly *et al.*, 1985). Recent reports from China of mixed infections of ALV J with chicken anemia virus (CIAV), reticuloendotheliosis virus (REV) and Marek's disease virus (MDV) deserve further studies (Cui *et al.*, 2003). Also detailed studies evaluating the effect of ALV J on the chicken's immune system is warranted to study interactions with other vaccines and pathogens.

## CONTROL AND ERADICATION

**Efforts to control through immunization.** Rispens and colleagues demonstrated the importance of maternal antibodies in preventing congenital transmission by inoculating specific-pathogen-free chickens with ALV at 8 weeks (Rispens *et al.*, 1976). The use of a replicating retrovirus as a vaccine is not a practical or a viable solution. This led to the development of sub-unit vaccines containing the glycoprotein gp85 expressed in baculovirus expression vectors (Noteborn *et al.*, 1990) and in fowlpox expression systems (Lee *et al.*, 1998; Nazerian and Yanagida, 1995). The efficacy of ALV sub-unit vaccines was not satisfactory probably due to incomplete glycosylation of gp85. Attempts were also made to induce an efficient immune response by inoculating chickens with primary chicken embryo fibroblasts (CEFs) or quail QT6 cells infected with an

avian erythroblastosis virus-based vector, carrying the Rous-associated virus 1 (RAV-1) env gene (gp85 and gp37) substituted for the v-erbA oncogene (Chebloune *et al.*, 1991). Similar attempts to induce immunity against ALV J by inoculating chickens with C/J cells that express ALV J- specific glycoproteins were also not satisfactory (Hunt *et al.*, 1999). So the search for vaccines capable of eliciting an efficient immune response against ALV is still in progress.

**Prevention and Control.** Control of ALV by live or attenuated vaccines is not a viable option due to retroviral integration into the vaccinee's genome. Moreover, attenuation of ALV resulted in destroying the antigenicity of the virus (Burmester, 1952). Attempts were made to immunize chickens at 8 weeks or later, so that the maternal antibody can be passed on to the progeny. However, this was a very risky proposition due to the potential of congenital transmission. Recombinant live virus vaccines incorporating endogenous *gag-pol* genes and exogenous *env* gene and LTRs provided reasonable protection with less shedding (Robinson *et al.*, 1985). Endogenous ALVs and recombinant ALVs containing endogenous gag proteins were excreted in subgroup E susceptible chickens at RPRL, East Lansing, Michigan and in several lines of meat-type chickens in Australia (Crittenden and Smith, 1984; Ignjatovic, 1986). In spite of some of the benefits of recombinant live vaccines, there is a potential danger of emergence of viral mutants with greater pathogenicity and also problems associated with viral persistence. Recombinant sub-unit vaccines comprised of the immunogenic gp85 proteins seem to induce some degree of protection. The use of subunit vaccines may induce some tolerizing effect on specific immune response against exogenous ALV infection. Crittenden and colleagues

have demonstrated the tolerizing effect of endogenous envelope proteins and reduction in production of NAbs against exogenous ALV (Crittenden *et al.*, 1984). So, the recombinant ALVs expressing exogenous gp85 proteins might hold some promise but still a considerable amount of work needs to be done before these vaccines can be licensed and used for controlling ALV in commercial poultry operations (Fadly and Payne, 2003). Some attempts were made to reduce lymphoid leukosis incidence by incorporating androgen analogue mibolerone in the diet of chickens but this approach is not practical for industry-wide ALV control measures since it reduces only LL but does not affect ALV infection (de Boer, 1987; Romero and Frank, 1977).

Genetic selection for resistance against ALV infection is also possible. Two levels of genetic resistance to ALV infection are identified i.e. genetic resistance at cellular level to virus infection controlled by single dominant alleles for susceptibility, and genetic resistance to tumor development controlled by multiple alleles that are not ALV subgroup specific (Crittenden, 1975). Genes for cellular resistance are more prevalent in meat-type chickens than in layer chickens (Crittenden and Motta, 1969). Control of ALV by genetic selection is difficult to achieve and can sometimes be counter productive, since the selection has to be for recessive genes and a majority of poultry breeding is based on crosses between several lines (Crittenden, 1983). In practice, emphasis is placed on selection for resistance to the predominating A subgroup virus and sometimes to B subgroup. At least one broiler breeder company has genetically selected chickens resistant to ALV A (McKay and Rosales, 2000). In spite of testing a number of chicken lines, none was found to be genetically resistant to ALV J infection. The *env*

gene of ALV J shares close homology with endogenous retroviral elements and the genetics of numerous endogenous loci in broiler breeder chicken strains are not completely documented. The genetic impact of eradicating chickens positive for ALV J may be considerable, especially if eradication is linked to some production traits that are not currently apparent (McKay, 1998). Since the tolerant viremic chickens are always culled, a rapid genetic change of the already limited broiler breeder stocks may occur especially if it has any genetic basis for the disease. There is a possibility of developing ALV resistant breeder stock by transgenesis but it will require a substantial capital investment and years of development (Crittenden, 1991).

To date, the only viable option for controlling ALV is by accurate diagnosis and culling of the infected birds from the primary breeding flocks. Waters and Prickett obtained the first ALV-free flock (Line $15^{I}$) at ADOL (formerly known as Regional Poultry Research Laboratory), by careful selection and isolation of families with low incidence of the disease. However, it is not clear if the above flock was free of LL or Marek's disease. ALV control procedures aimed at interrupting vertical transmission became possible only after Rubin's discovery of resistance-inducing factor (RIF) test that is based on interference of RSV multiplication in ALV-infected chicken embryo fibroblasts (Rubin, 1960). Until the early 1970s only the RIF test and complement fixation test for avian leukosis (COFAL) were available for detection of ALV (Rubin, 1960; Sarma *et al.*, 1964). RIF test and COFAL techniques were cumbersome and were applied only to experimental flocks and specific-pathogen free flocks used for vaccine production. With the discovery of non-producer cell activation (NP) test and phenotypic

mixing (PM) test, ALV eradication procedures were applied to broader range of flocks (Okazaki *et al.*, 1975; Rispens *et al.*, 1970). However, the development of direct complement fixation test (CFT) for gsa detection in egg albumen by Spencer *et al.* made routine testing of commercial flocks feasible (Spencer and Gilka, 1982). The p27 antigen ELISA by Smith *et al.* had greater sensitivity in diagnosis of ALV than CFT (Smith *et al.*, 1979). However, this technique increased false-positives due to gsa of endogenous origin (Clark and Dougherty, 1980; de Boer *et al.*, 1983; Smith *et al.*, 1979). The problem of false positives was later eliminated by culturing the virus in C/E cells that lack receptors for ALV E.

ALV eradication programs were routinely practiced by the poultry industry only after the discovery of negative effects of ALV on production traits in addition to tumor mortality (Okazaki *et al.*, 1979; de Boer, 1987; Gavora, 1987). ALV eradication programs are successful only when vertical transmission from dam to progeny is broken and early horizontal transmission between pen mates is eliminated i.e. eradication protocols involved accurate ALV diagnosis in hens, hatching eggs, embryos and chicks (Fadly and Payne, 2003). Several test materials including vaginal and cloacal swabs, albumen, embryo extracts, meconium, feathers, plasma, and buffy coats were used for ALV diagnosis. However, vaginal swabs and plasma are the most commonly used test samples. ALV-free flocks are established by hatching, rearing and reproducing ALV-free chickens in total isolation. This is only possible when hens from ALV-positive primary breeding stock are culled by constant testing for viremia, antibody, shedding, and albumen gsa. Selection of hens depends on ALV -antibody, -viremia and -shedding

status. The dams selected for producing hatching eggs included immune non-shedders, nonimmune non-shedders, or non-viremic hens irregardless of exposure history (Hughes *et al.*, 1963; Zander *et al.*, 1975); (Fadly and Payne, 2003). Successful ALV eradication requires accurate ALV diagnosis techniques, testing sufficient number of samples, and greater frequency of sampling and testing.

Fertile embryos from ALV-free hens, tested negative by examination of vaginal swabs and albumen, should be hatched in isolation in small groups. Avoidance of vent sexing and MDV vaccination with separate needles and rearing on wire cages reduces ALV infection. Small group rearing and isolation from other groups prevents horizontal transmission between groups. Testing with more than one diagnostic technique facilitates detection of ALV-infected chicks. Elimination of the entire lot from which ALV-positive chicks were isolated is a very efficient method of selecting groups of chickens for producing future generations. Early horizontal transmission occurs from pen mates in addition to transmission by fomites, and other equipment related to incubation, hatching, and brooding. Hence the farm equipment should be thoroughly fumigated and cleaned with detergent. Once an ALV-free group is obtained, it should be reared in total isolation following biosecurity procedures.

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# CHAPTER 2

## Factors influencing Subgroup J avian leukosis virus-induced viral persistence, antibody response, oncogenicity and mortality in commercial meat-type chickens

**ABSTRACT**

This study was conducted to evaluate the effect of viral strain and dose, and age at inoculation on inducing Subgroup J avian leukosis virus (ALV J) persistence, neutralizing antibody (NAb) response, tumor incidence and mortality. Commercial meat-type chickens were inoculated on the 5$^{th}$ day of embryonation (5 ED) or on day of hatch (DOH) with two doses (10,000 TCID$^{50}$ and 100 TCID$^{50}$) of one of three ALV J strains (ADOL Hc1, ADOL 6803, and ADOL 4817). The chickens were examined for ALV J viremia (V), and NAb against the inoculated parental virus (A) on 1, 3, 7, 11, 15, 19, 23, 27 and 32 weeks post hatch. Gross and microscopic examination for ALV J-induced pathology was also performed.

High incidence (83-100%) of ALV J persistence was observed when chickens were infected either at 5 ED or DOH, regardless of the viral strain or dose of viral inoculum. Development of NAb did not guarantee viremia-free status as a high percentage (up to 75%) of chickens had concurrent viremia and antibody (V+A+). The incidence of antibody response was influenced by viral strain and dose, and age at inoculation. Viral strain had an effect not only on the incidence of antibody response but also on the ability of antibody to clear the infection. Chickens infected with ADOL 6803 had the highest incidence of antibodies but antibodies against ADOL Hc1 were comparatively more successful in clearing the viremia. NAb responses were greatest in chickens inoculated with 100 TCID$^{50}$ and in chickens inoculated at DOH. ALV J-induced mortality, tumor incidence, and tumor spectrum were influenced by viral strain and dose, age at inoculation and antibody response. The data demonstrated that ALV J

viral strain and dose, and age at infection influence viral persistence, antibody response, oncogenicity and mortality in commercial meat-type chickens.

## INTRODUCTION

Subgroup J Avian Leukosis Virus (ALV J) causes a variety of neoplasms mainly of myeloid lineage along with a varied incidence of renal neoplasms, erythroblastosis, histiocytic sarcomas, hemangiomas and connective tissue neoplasms (Fadly and Payne, 2003). ALV J, in addition to tumor mortality, also causes several non-neoplastic conditions such as depression in body weight and increased feed conversion ratios resulting in production losses to the poultry producer (Fadly and Payne, 2003). ALV J infection is largely controlled by identification and eradication of chickens positive for antibody or viremia depending on the eradication protocols followed by the breeder company. Overall, the broiler-breeder industry has been successful in controlling ALV J to a large extent except for the infrequent incidence of ALV J viremia or antibodies in some "ALV J-free" flocks. As with other retroviruses, ALV J is capable of persisting at very low levels in the host and the routinely used diagnostic methods may not always detect the virus. Hence, the real prevalence of ALV J may be actually higher than what is reported. Recent reports of ALV J- or its natural recombinant (ALV B/J)- induced myelocytomatosis incidence in commercial white leghorn egg-laying flocks in USA as well as in China are of concern (Gingerich *et al.*, 2002; Xu *et al.*, 2004).

The ALV J infection profile and transmission pattern in meat-type chickens is comparable to that of other exogenous ALV infections (Payne *et al.*, 1991, 1992; Rubin

*et al.*, 1962; Witter *et al.*, 2000) . Similar to other exogenous avian leukosis viruses (ALVs), ALV J infection in chickens is described in terms viremia (V) and neutralizing antibody (NAb) response against the inoculated parental virus (A), and it results in one of the following profiles *viz.* viremic with no NAb (V+A-), viremic with concurrent NAb (V+A+), and loss of viremia with efficient NAb (V-A+). Viral persistence is defined as continued existence of the virus in the host. Most persistently infected chickens are tolerized and never develop a NAb response (V+A-). However, chickens with viral persistence may develop a NAb response against the infecting virus and the virus persists in the host in face of a patent immune response. Hence, regardless of the antibody status, chickens with V+A- and V+A+ infection profile are considered to be persistently viremic.

The efficiency of ALV J vertical transmission is similar to that of other exogenous ALVs (Payne *et al.*, 1991; Witter *et al.*, 2000). However, the rate of ALV J horizontal transmission is more efficient than other exogenous ALVs and results in higher levels of viral persistence (Fadly and Smith, 1999; Koch *et al.*, 2000; Pandiri, 2000). Also, ALV J infection is unique compared to that of other exogenous ALVs since meat-type chickens infected with ALV J during the first two weeks after hatch generally have high levels of viral persistence (Fadly and Smith, 1999; Witter and Fadly, 2001). Anecdotal field evidence and experimental findings suggests that there is a waxing and waning level of ALV J viremia (Witter *et al.*, 2000). This might be responsible for the occasional failures in conventional exogenous ALV eradication protocols that are demonstrated by sporadic incidence of ALV J positive cases in "ALV J-free" flocks. As

a result, the term "ALV J-tested negative" may be more accurate than "ALV J-free" flocks (McKay and Rosales, 2000). However, this again leads to quality control issues, since "ALV J-tested" status depends on the sensitivity and validity of ALV J diagnostic tests used.

Several studies have demonstrated the effect of viral strain and dose and age at infection on ALV A viral persistence, antibody response, and oncogenicity (Burmester *et al.*, 1959; de Boer *et al.*, 1981; Fadly *et al.*, 1987; Payne, 1987). There is a paucity of data in current literature demonstrating the effect of the above factors on ALV J persistence and oncogenicity. Thus, the objective of the current study was to better define and understand the effect of ALV J strain and dose, and age at inoculation on viral persistence, NAb response, oncogenicity and mortality in commercial meat-type chickens.

## MATERIALS AND METHODS

**Chickens and housing.** Meat-type chicken embryos were obtained from a major commercial breeder. The chickens were inoculated either at the $5^{th}$ day of embryonation (5 ED) or at day of hatch (DOH). All the viable chicken embryos were incubated and hatched in isolation at the USDA-ARS Avian Disease and Oncology Laboratory (ADOL) at East Lansing, MI. At hatch, all the chickens were wing-banded and housed in their respective biosecurity level - 2 (BSL-2) floor pens. All chickens inoculated with one type of ALV J strain were housed in one self-contained BSL-2 floor pen. Chickens receiving a low dose (100 $TCID^{50}$) were separated from chickens receiving a high dose

(10,000 $TCID^{50}$) by a 3 foot impervious barrier at the base and a wire meshing on top reaching the roof. Forty five uninoculated negative control chickens free of all exogenous ALVs were housed in a separate BSL-2 floor pen. Feed was restricted by alternate day feeding after 6 weeks to limit the growth of the meat-type chickens as recommended by breeder.

**Viruses.** Three ALV J viral strains (ADOL Hc1, ADOL 6803, and ADOL 4817) were selected based on geographic origin and nucleotide sequence differences. All the ALV J strains were isolated from separate meat-type chicken farms across the United States. The earliest ALV J viral strain isolated in the United States is ADOL Hc1 and it is considered as the American prototype ALV J strain (Fadly and Smith, 1999). In addition, two other ALV J field strains ADOL 6803 and ADOL 4817 were isolated from farms with a high incidence of myelocytomatosis (Fadly and Smith, 1999). All three viral strains had significant nucleotide sequence differences in the env protein (Silva *et al.*, 2000). The viruses were propagated in ADOL line 0 secondary chicken embryo fibroblasts (CEFs) that support all ALVs except for the endogenous viruses. The virus titers were determined by limiting dilution in tissue culture. The titers varied from $10^{5.5}$ to $10^{6.5}$ infectious units per milliliter ($TCID^{50}$/ml).

**Experimental design.** In this study, all chickens were monitored for ALV J-induced viremia and NAb response throughout the experiment. All chickens infected either *in ovo* or at hatch with one type of ALV J strain were housed in one self-contained BL-2 floor pen under negative pressure with 2 compartments depending on the doses (100

$TCID^{50}$ or 10,000 $TCID^{50}$) received. All *in ovo* inoculations were done at 5 ED via yolk sac route. Inoculations at DOH were done via intra abdominal route. All chickens were bled at 1, 3, 7, 11, 15, 19, 23, 27 and 32 weeks post hatch to test for viremia and NAb. Blood was collected in syringes coated with KEDTA and spun at 1500 rpm for 30 minutes to separate the plasma. All samples were placed on melting ice immediately after collection and assayed fresh or stored at -70° C until assayed. Plasma samples for virus micro-neutralization were diluted at 1:5 in sera-free LM media and heat inactivated at 56° C for 30 minutes. All chickens that died during the experimental period of 30 weeks and those that were euthanized at termination of the study were necropsied and examined for gross and microscopic lesions induced by ALV J.

**Virus isolation (VI).** Plasma samples collected during each sampling period were tested for viremia by VI. VI was done according to the procedures described earlier (Fadly and Witter, 1998). Briefly, about 100 µL of undiluted plasma was added to $0.18 \times 10^6$ secondary ADOL line 0 CEFs suspended in 4% calf serum (CS) Leibowitz's L-15-McCoy's 5A tissue culture medium [1:1] (LM) containing penicillin, streptomycin, amphotericin B and 0.004 IU heparin in 24 well tissue culture plates. On the following day, the 4% CS LM media was replaced with 1% CS LM media. The plates were incubated in 4% $CO_2$ at 37° C for 7-9 days before the cells were completely lysed with 50 µL of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and two alternate cycles of freezing at -70° C and thawing at 37° C. About 100 µL of the cell lysate was used to test for p27 group-specific antigen (gsa) by enzyme-linked immunosorbent assay (ELISA) (Smith *et al.*, 1979). The p27 gsa ELISA was carried out using rabbit anti-p27 polyclonal

antibody coated immunolon® plates (Dynatech, Chantilly, VA), rabbit anti-p27 antibody conjugated to horse-radish peroxidase (SPAFAS, Storrs, CT) and TMB substrate (3, 3′, 5, 5′-tetramethyl benzidine) (BD Biosciences Pharmingen, San Diego, CA). The plate was read at an absorbance of 630 nm using a MRX microplate reader (Dynex, Chantilly, VA).

**Virus micro-neutralization (VN).** Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens. VN assays were performed as described earlier (Fadly and Witter, 1998). In précis, the plasma samples were diluted 1:5 in serum-free LM media and incubated at 56° C for 30 minutes to denature the complement factors. About 500 - 1000 ALV J viral particles (viral stocks used to inoculate the chickens) in 50 µL LM media are incubated with 50 µL of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 minutes at 37° C and 4% $CO_2$. After the incubation, about $1x10^5$ cells in 150 µL of 4% CS LM media were pipetted into each of the 96 wells and incubated in 4% $CO_2$ at 37° C for 7-9 days. At the end of incubation, the cell cultures were completely lysed with 20 µL of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and were subjected to two alternate cycles of freezing at -70° C and thawing at 37° C. The cell lysates were tested for p27 gsa ELISA as described earlier (Smith *et al.*, 1979). Samples that had a chromogenic reading of 1 or negative on the p27 gsa ELISA read-out was considered to be positive for NAb against ALV J and samples with a chromogenic reading of $\geq 2$ on the p27 gsa ELISA read-out was considered to be negative for NAb against ALV J.

**Pathology.** All chickens necropsied were examined for gross or microscopic lesions induced by ALV J. Tissues from grossly detected tumors were fixed in 10% neutral buffered formalin for microscopic evaluation. All tissues were processed, sectioned and stained with hematoxylin and eosin (H&E).

**Data analysis.** Individual chicken viremia (V) and NAb (A) data from all the samplings was arbitrarily summarized in Figure 2.1 into three categories *viz.* V+A-, V+A+, and V-A+. These categories are defined as follows: V+A- = chickens that were consistently positive for viremia and did not develop any NAb response against the inoculated virus; V+A+ = chickens that remained viremic at the end of the study and were concurrently positive for viremia and NAb response on at least one occasion; V-A+ = chickens that have successfully seroconverted with loss of viremia and development of NAb by the time the study was terminated. Review of entire viremia and NAb data from multiple samplings for each bird, provides a précis of the course of ALV J infection in that particular chicken throughout the experimental period. Moreover, this method of classification gave precise indication of the relationship between viremia and NAb data from different sampling intervals for each chicken. Chickens that developed NAb against the inoculated parental virus did not necessarily clear the viremia (V+A+). Therefore chickens of categories V+A+ and V+A- were considered persistently viremic. The first sampling in the study was done one week post hatch due to logistical reasons and only chickens that are positive for viremia at that time are included in the data analysis since inoculated but uninfected chickens could skew the data aimed at studying viral persistence.

Statistical analysis was performed by testing for significance of differences in percentages by chi square test using Statistica® (Statsoft, Tulsa, OK). Statistical significance was assumed at the 0.05-0.08 level of probability.

## RESULTS

**Inoculation *vs.* Infection (Table 2.1).** Comparison of inoculation *versus* infection in chickens infected at 5 ED is represented in table 2.1. Distinguishing infected chickens from uninfected chickens is important since the study was aimed at studying viral persistence. Thirty commercial meat-type chicken embryos were inoculated at 5 ED with one type of ALV J viral strain at a dose of 100 $TCID^{50}$ or 10,000 $TCID^{50}$. The hatchability ranged from 40-67% depending on the inoculated ALV J strain. Of the inoculated chicken embryos that had hatched, the incidence of infection ranged from 45-100%. Comparison of inoculation *versus* infection in DOH infected chickens is also represented in table 2.1. Thirty commercial meat-type chickens were inoculated on DOH with one type of ALV J strain either at 100 $TCID^{50}$ or 10,000 $TCID^{50}$ dosages. The incidence of infection ranged from 25-79%.

**Factors affecting ALV J infection (Table 2.2).** Based on viremia and NAb against the inoculated parental virus, three infection profiles *viz.* V+A-, V+A+, V-A+, were categorized as described in materials and methods (Figure 2.1). Persistently viremic chickens include categories V+A+, and V+A- that remained viremic regardless of the host immune response. The percentage of persistently viremic chickens was high in all

the groups (83-100%). Development of NAb did not guarantee a viremia-free status as represented by V+A+ category (0-75%). The V+A- category represents tolerantly viremic chickens that constituted a majority in almost all the groups (25-100%). Table 2.2 demonstrates the effect of viral strain, inoculum dose and age at infection on ALV J viral persistence.

*Effect of strain on ALV J infection (Table 2.2 & 2.3).* The strain had minimal effect on the levels of viral persistence as very few chickens could clear the infection (0-17%). However, strain had a statistically significant effect on the NAb response in some groups. Chickens infected with ADOL 6803 developed NAb at a greater frequency (up to 75%) than chickens infected with ADOL Hc1 (up to 34%) and ADOL 4817 (up to 33%). However, the ability to clear infection in chickens infected with ADOL 6803 (6%) was lower than in chickens infected with ADOL Hc1 (17%). The incidence of NAb response was the lowest in chickens infected with ADOL 4817 and viremia clearance was not observed in any case. The effect of strain was more obvious and statistically significant in chickens inoculated with 100 $TCID^{50}$ at 5 ED and in chickens inoculated with 10,000 $TCID^{50}$ at DOH.

*Effect of age on ALV J infection (Table 2.2 & 2.4).* A high incidence of viral persistence was observed at both 5 ED (90-100%) and DOH (83-100%) infections. No significant differences were found between the two groups. The effect of age on NAb response was minor but the incidence of NAb response against the infecting virus or the ability to clear viremia appeared to be higher in chickens inoculated at DOH than in

chickens inoculated at 5 ED. The effect of age at infection on NAb response was more obvious in chickens inoculated with ADOL 6803. The incidence of NAb response in chickens infected with ADOL 6803 at 100 $TCID^{50}$ at 5 ED was higher (75%) than in chickens infected at hatch (28%) but the later case cleared viremia in more chickens (0% *vs.* 6%).

*Effect of dose on ALV J infection (Table 2.2 & 2.5).* Dose of viral inoculum had no effect on viral persistence. High incidence of viral persistence was observed at both 100 $TCID^{50}$ (83-100%) and 10,000 $TCID^{50}$ (90-100%) infections. The frequency of NAb response as well as the ability to clear viremia was higher in chickens inoculated at 100 $TCID^{50}$ (10-75%) than in chickens inoculated at 10,000 $TCID^{50}$ (0-26%). However, the effect of dose was statistically significant only on the incidence of NAb response but not the ability to clear the viremia.

**Factors affecting ALV J-induced oncogenicity and mortality (Table 2.6 and 2.7).**

*Effect of ALV J strain (Table 2.6).* There was a slight ALV J strain effect on the incidence of tumors and mortality. Chickens infected with ADOL 4817 had slightly lower tumor incidence (50-67%) than ADOL Hc1 (53-100%) and ADOL 6803 (44-100%). Also, chickens infected with ADOL 4817 had lower incidence of mortality (50-75%) than ADOL Hc1 (68-82 %) and ADOL 6803 (67-100%). ALV J strain seems to have an effect on the tumor spectrum. Chickens infected with ADOL 4817 had a greater variety of tumors than chickens infected with ADOL Hc1 and ADOL 6803. ADOL Hc1 induced myelocytomatosis, renal tumors, and histiocytic sarcomas. In addition to these

tumors, ADOL 6803 induced erythroblastosis and hemangiomas. Chickens infected with ADOL 4817 had the greatest tumor spectrum consisting of fibrosarcomas and rhabdomyosarcomas, as well as the above mentioned tumors.

*Effect of age (Table 2.6).* The incidence of mortality was higher in chickens infected at 5 ED than in chickens infected at DOH. Age at infection seems to influence ALV J-induced tumor spectrum since only DOH infected chickens developed histiocytic sarcomas unlike chickens infected at 5 ED. Age at infection did not have any apparent effect on tumor incidence and mean death time in days.

*Effect of dose (Table 2.6).* Chickens infected with 100 $TCID^{50}$ had higher mean death times than chickens infected with 10,000 $TCID^{50}$. Dose did not seem to have any apparent effect on the incidence of tumors, tumor spectrum or mortality.

*Effect of ALV J infection profiles (Table 2.7).* Chickens categorized as V+A- had the highest incidence of tumors (71%) and mortality (73%). These chickens also had the widest tumor spectrum and the shortest mean death time (133 days). The presence of antibody reduced the incidence of tumors and mortality, narrowed the tumor spectrum, and prolonged mean death time. The beneficial effect of antibody was more evident in groups that were able to clear viremia.

## DISCUSSION

This study demonstrated a very high incidence of ALV J persistence (V+A+ and V+A-) in meat-type chickens regardless of the viral strain and dose, and age at infection. These findings are in contrast to previous reports on ALV A infections (Fadly and Payne, 2003). Usually chickens infected with ALV A after hatch tend to develop transient viremia followed by an efficient NAb response. Generally, the NAb response against ALV A is able to prevent reappearance of viremia. In contrast, this study demonstrated that the presence of NAb does not guarantee immunity against ALV J and provide a viremia-free status. The incidence of concurrent viremia and NAb against the inoculated parental virus (V+A+) in chickens varied from 0-75%, depending mainly on strain of ALV J, followed by age and dose. This study demonstrated a high incidence of the V+A+ category in meat-type chickens infected with different ALV J viral strains. Witter and coworkers have also reported a high incidence of V+A+ category in meat-type chickens infected with ALV J (Witter *et al.*, 2000). Routine NAb testing is performed by *in vitro* VN between the plasma/serum samples collected during several intervals and the virus stock used to infect the chickens. Thus, a possible explanation for the V+A+ status is that virus might have mutated and escaped the NAb response that is directed against the parent virus. It is a common strategy used by RNA viruses to escape from the host humoral or cell-mediated immune response. NAb escape variants have been commonly reported in cases of lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV) infections (Ciurea *et al.*, 2000; Richman et al., 2003). The role of NAb escape variants in the high incidence of V+A+ category in ALV J infections is studied as a part of other objectives in this work.

Although, viral strain and dose, and age at infection did not influence the incidence of viral persistence (V+A-, V+A+), they had an effect on the NAb response. The results demonstrated that meat-type chickens inoculated with a low dose (100 $TCID^{50}$) or at DOH responded slightly better than chickens inoculated with a high dose (10,000 $TCID^{50}$) or at 5 ED. These results are similar to that produced by ALV A infections in white leghorn chickens (Fadly *et al.*, 1987). The viral strain had an influence not only on the incidence of antibody response but also on the ability to clear the viremia. Chickens infected with ADOL 6803 developed NAb at a greater frequency than chickens infected with ADOL Hc1 and ADOL 4817. However, chickens infected with ADOL 6803 were unable to clear the infection and were categorized as V+A+ category. In contrast, more chickens infected with ADOL Hc1 produced NAb that was able to clear the viremia. Similar results were reported in White leghorn chickens infected with RAV-1 that induced a better NAb response than field strains RPL-40, RPL-41 and RPL-42 (Fadly *et al.*, 1987). This finding was not surprising since it has been suggested that ADOL Hc1 similar to RAV-1 and other ALVs maintained in the laboratory are known to induce a better NAb response than field strains (Crittenden *et al.*, 1984; Fadly *et al.*, 1987).

Conventionally ALV infection has been described by the presence or absence of ALV viremia, shedding and NAb from single or multiple samplings (Rubin *et al.*, 1962). This method of data presentation did not clearly demonstrate ALV persistence from data that was obtained over several sampling intervals. Witter and coworkers defined ALV

infection profile based on frequency and consistency of viremia and cloacal shedding data obtained from 20 samplings over a period of 60 weeks. ALV J infection was divided into 5 categories *viz. con* (consistent), *int A & B* (intermittent), *tra* (transient) and *neg* (negative) (Witter *et al.*, 2000). This classification gave a précis of viremia throughout the ALV infection study and is a good aid for evaluating the pattern of ALV persistence. In the current study, the ALV J NAb data was also taken into consideration along with the viremia data to account for cases of tolerant viremia as well as viral persistence. The entire ALV infection history of each chicken obtained from 9 samplings over a period of 32 weeks was summarized into three categories (V+A-, V+A+, and V-A+). This method of classification helped to comprehensively analyze the association between viremia and NAb data of each individual chicken. This allowed for clear demonstration of the high incidence of viral persistence in the presence of antibody response (V+A+) or absence of NAb response against the inoculated parental virus (V+A- or tolerant viremic). It also helped to determine the effect of ALV J strain and dose, and age at infection on ALV J viral persistence and antibody response.

The first sampling in the study was done one week post hatch due to logistical reasons and only chickens that were positive for viremia at that time were included in the data analysis since inoculated but uninfected chickens could skew the data aimed at studying viral persistence and NAb response. Comparison of inoculation *versus* infection in both 5 ED and DOH inoculated chickens was important since not all inoculated chickens become infected due to a variety of reasons such as loss of viral titer due to the thermolabile nature of virus, low dose of viral inoculum, including improper inoculation

technique or due to errors or limitations of the diagnostic methods. Two groups especially ADOL 6803 inoculated at 5 ED and ADOL 4817 inoculated at DOH with the lower dosage of 100 $TCID^{50}$ had a very low incidence of infection. The possible reasons may be similar to the ones discussed above. The hatchability after infection with the ALV J strains ranged from 40-67% but the factors responsible for this variation were not analyzed.

Viral strain had a clear effect on mortality and tumor spectrum. ADOL 4817 induced less mortality than ADOL Hc1 and ADOL 6803. On the other hand, the tumor spectrum induced by ADOL 4817 was more diverse than that induced by ADOL 6803 and ADOL Hc1. The effect of ALV A viral strains on tumor spectrum has also been reported but the underlying mechanisms are not well understood and deserve further studies (Fadly *et al.*, 1987). Dose of viral inoculum had an effect on the mean death time since chickens inoculated with 100 $TCID^{50}$ tended to live longer than chickens inoculated with 10,000 $TCID^{50}$. Dose did not have any apparent effect on tumor spectrum in contrast to the earlier reports with ALV A infections (Purchase, 1987). The inoculated viral dose greatly influences the incubation period for tumor formation as well as the type of tumor produced. Experimental studies by Burmester *et al*, have demonstrated that high doses of certain ALV A strains predominantly induce erythroblastosis with a short incubation period of 2 to 3 months whereas low doses predominantly induce LL with a relatively longer incubation period of 5 to 9 months (Burmester *et al.*, 1959). Under field conditions, ALV exposure is generally at low doses and hence the reason for high incidence of LL. Also congenitally infected chickens have high viral loads but still the

incidence of LL is more common than leukemias (Payne, 1987). Therefore the effect of ALV dose on tumor spectrum is still unclear.

Age at infection seemed to affect the incidence of mortality since chickens infected at 5 ED had higher mortality than chickens infected at DOH. Interestingly, only chickens infected at DOH exhibited histiocytic sarcomas. Further evaluation of these lesions will be done in later experiments.

NAb response had a major effect on mortality (incidence and mean death time) and oncogenicity (incidence and tumor spectrum). Presence of antibodies even in cases unable to clear viremia reduced the pathogenicity of the virus. However, the beneficial effects were more evident when the NAb was able to clear the viremia.

This study demonstrated that early ALV J infections (5 ED or DOH) result in high incidence of viral persistence (V+A+ and V+A-). In most cases, the NAb responses against the inoculated virus were insufficient to clear the viremia. Factors like ALV J strain and dose, and age at infection affect the NAb response but not viral persistence. In addition, this work demonstrated that mortality and oncogenicity induced by ALV J are influenced by viral strain, viral dose and age at infection. Hence, this study provided novel information about the epidemiology of ALV J in meat-type chickens that could help in devising better methods of eradication and control.

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**Table 2.1:** Efficiency of ALV J infection as tested at one week post-hatch in commercial meat-type chickens following inoculation at 5 ED or at DOH.

| Virus[1] | Age at inoculation[2] | Dose[3] | # Inoculated | # Hatched[4] | % Infected[5] |
|---|---|---|---|---|---|
| **ADOL Hc1** | 5 ED | 100 | 30 | 15 | 67 |
| **ADOL Hc1** | 5 ED | 10,000 | 30 | 19 | 63 |
| **ADOL 6803** | 5 ED | 100 | 30 | 12 | 45 |
| **ADOL 6803** | 5 ED | 10,000 | 30 | 20 | 100 |
| **ADOL 4817** | 5 ED | 100 | 30 | 15 | 93 |
| **ADOL 4817** | 5 ED | 10,000 | 30 | 15 | 93 |
| **ADOL Hc1** | DOH | 100 | 29 | NA | 79 |
| **ADOL Hc1** | DOH | 10,000 | 29 | NA | 79 |
| **ADOL 6803** | DOH | 100 | 30 | NA | 62 |
| **ADOL 6803** | DOH | 10,000 | 30 | NA | 68 |
| **ADOL 4817** | DOH | 100 | 30 | NA | 25 |
| **ADOL 4817** | DOH | 10,000 | 30 | NA | 64 |

[1] Three ALV J strains (ADOL Hc1, ADOL 6803 and ADOL 4817) were used in this study.
[2] Meat type chickens were inoculated at 5 days of embryonation (5ED) or at day of hatch (DOH).
[3] Virus was inoculated with one of two doses (100 or 10,000 $TCID^{50}$).
[4] Number of chickens that hatched after *in ovo* inoculation. NA = Not available.
[5] Percentage of infected chickens at one week of age measured by viremia.

**Table 2.2.** Effect of viral strain and dose, and age at inoculation on ALV J infection profile, viral persistence and neutralizing antibody response (NAb) against the inoculated parental virus.

| Virus[2] | Age[3] | Dose[4] | # of Chicken | Infection profile (%)[1] | | | Viral Persistence[5] | NAb[6] |
|---|---|---|---|---|---|---|---|---|
| | | | | V+A+ | V+A- | V-A+ | | |
| ADOL Hc1 | 5ED | 100 | 10 | 0 | 90 | 10 | 90 | 10 |
| ADOL Hc1 | 5ED | 10,000 | 12 | 0 | 100 | 0 | 100 | 0 |
| ADOL Hc1 | DOH | 100 | 23 | 17 | 66 | 17 | 83 | 34 |
| ADOL Hc1 | DOH | 10,000 | 22 | 4 | 86 | 10 | 90 | 14 |
| ADOL 6803 | 5ED | 100 | 4 | 75 | 25 | 0 | 100 | 75 |
| ADOL 6803 | 5ED | 10,000 | 17 | 6 | 94 | 0 | 100 | 6 |
| ADOL 6803 | DOH | 100 | 18 | 22 | 72 | 6 | 94 | 28 |
| ADOL 6803 | DOH | 10,000 | 19 | 26 | 74 | 0 | 100 | 26 |
| ADOL 4817 | 5ED | 100 | 13 | 15 | 85 | 0 | 100 | 15 |
| ADOL 4817 | 5ED | 10,000 | 12 | 8 | 92 | 0 | 100 | 8 |
| ADOL 4817 | DOH | 100 | 6 | 33 | 67 | 0 | 100 | 33 |
| ADOL 4817 | DOH | 10,000 | 18 | 6 | 94 | 0 | 100 | 6 |

[1] Viremia (V) and NAb (A) data were classified into three ALV J infection profiles (V+A+, V+A-, V-A+) and expressed as percentages.
[2] Three ALV J strains (ADOL Hc1, ADOL 6803 and ADOL 4817) were used in this study.
[3] Meat type chickens were inoculated at 5 days of embryonation (5ED) or at day of hatch (DOH).
[4] Virus was inoculated with one of two doses (100 or 10,000 $TCID^{50}$).
[5] Percentage of infected chickens that are positive for viremia.
[6] Percentage of infected chickens that are positive for neutralizing antibody against the inoculated virus.

**Table 2.3.** Effect of ALV J strain on ALV J infection profile, viral persistence and neutralizing antibody (NAb) response against the inoculated parental virus[1]

| Comparison between viral strain groups | Within age groups | Within dose groups | Infection profiles[2] | | | Viral Persistence | NAb |
|---|---|---|---|---|---|---|---|
| | | | V+A+ | V+A- | V-A+ | | |
| ADOL Hc1, ADOL 6803 and ADOL 4817 | 5 ED | 100 | **[3] | ** | NS | NS | ** |
| | 5 ED | 10,000 | NS | NS | NS | NS | NS |
| | DOH | 100 | NS | NS | NS | NS | NS |
| | DOH | 10,000 | ** | ** | * | NS | ** |

[1] Effect of ALV J strain on infection profile, viral persistence and NAb was studied by comparing three different strains (ADOL Hc1, ADOL 6803 and ADOL 4817) within groups of age and dose.
[2] Viremia (V) and NAb (A) data were classified into three ALV J infection profiles (V+A+, V+A-, V-A+).
[3] Differences in percentages were calculated by chi square test. ** = Level of statistical significance < 0.05, * = Level of statistical significance is 0.05 - 0.08, NS = No statistically significant difference.

**Table 2.4.** Effect of age at infection on ALV J infection profile, viral persistence and neutralizing antibody (NAb) response against the inoculated parental virus [1]

| Comparison between age groups | Within viral strain groups | Within dose groups | Infection profiles[2] | | | Viral Persistence | NAb |
|---|---|---|---|---|---|---|---|
| | | | V+A+ | V+A- | V-A+ | | |
| 5 ED and DOH | ADOL Hc1 | 100 | *[3] | * | NS | NS | NS |
| | ADOL Hc1 | 10,000 | NS | NS | NS | NS | NS |
| | ADOL 6803 | 100 | ** | ** | NS | NS | * |
| | ADOL 6803 | 10,000 | ** | ** | NS | NS | NS |
| | ADOL 4817 | 100 | NS | NS | NS | NS | NS |
| | ADOL 4817 | 10,000 | NS | NS | NS | NS | NS |

[1] Effect of age at inoculation on infection profile, viral persistence and NAb was studied by comparing two groups of age (5ED and DOH) within groups of viral strain and dose.
[2] Viremia (V) and NAb (A) data were classified into three ALV J infection profiles (V+A+, V+A-, V-A+).
[3] Differences in percentages were calculated by chi square test. ** = Level of statistical significance < 0.05, * = Level of statistical significance is 0.05 - 0.08, NS = No statistically significant difference.

**Table 2.5.** Effect of viral dose on ALV J infection profile, viral persistence and neutralizing antibody (NAb) response against the inoculated parental virus[1]

| Comparison between dose groups | Within viral strain groups | Within age groups | Infection profiles[2] | | | Viral Persistence | NAb |
|---|---|---|---|---|---|---|---|
| | | | V+A+ | V+A- | V-A+ | | |
| 100 and 10,000 $TCID^{50}$ | ADOL Hc1 | 5 ED | NS[3] | NS | NS | NS | NS |
| | ADOL Hc1 | DOH | * | ** | NS | NS | * |
| | ADOL 6803 | 5 ED | ** | ** | NS | NS | ** |
| | ADOL 6803 | DOH | NS | NS | NS | NS | NS |
| | ADOL 4817 | 5 ED | NS | NS | NS | NS | NS |
| | ADOL 4817 | DOH | ** | ** | NS | NS | * |

[1] Effect of viral dose on infection profile, viral persistence and NAb was studied by comparing two groups of viral dose (100 and 10,000 $TCID^{50}$) within groups of viral strain and age at inoculation.
[2] Viremia (V) and NAb (A) data were classified into three ALV J infection profiles (V+A+, V+A-, V-A+).
[3] Differences in percentages were calculated by chi square test. ** = Level of statistical significance < 0.05, * = Level of statistical significance is 0.05 - 0.08, NS = No statistically significant difference.

**Table 2.6.** Effect of viral strain and dose, and age at inoculation on ALV J-induced mortality, tumor incidence, and tumor spectrum

| Virus[1] | Age[2] | Dose[3] | # of Chickens | % Mortality | Death mean in days | % Tumor | Tumor spectrum[4] |
|---|---|---|---|---|---|---|---|
| ADOL Hc1 | 5 ED | 100 | 9 | 78 | 146 | 100 | ML, MB, RT |
| ADOL Hc1 | 5 ED | 10,000 | 11 | 82 | 111 | 91 | ML |
| ADOL Hc1 | DOH | 100 | 19 | 68 | 143 | 53 | ML, HS |
| ADOL Hc1 | DOH | 10,000 | 19 | 74 | 133 | 84 | ML, HS |
| ADOL 6803 | 5 ED | 100 | 4 | 100 | 182 | 100 | ML, EB, HA |
| ADOL 6803 | 5 ED | 10,000 | 17 | 82 | 148 | 82 | ML, EB, HA, RT |
| ADOL 6803 | DOH | 100 | 17 | 82 | 142 | 71 | ML, EB, HA, HS |
| ADOL 6803 | DOH | 10,000 | 18 | 67 | 104 | 44 | ML, HS, HA |
| ADOL 4817 | 5 ED | 100 | 12 | 75 | 94 | 50 | ML, FS |
| ADOL 4817 | 5 ED | 10,000 | 11 | 55 | 146 | 64 | ML, MB, EB, FS, RT, RMS |
| ADOL 4817 | DOH | 100 | 6 | 50 | 146 | 67 | ML, RT, EB, HS |
| ADOL 4817 | DOH | 10,000 | 16 | 56 | 127 | 63 | ML, EB, HA, RT, HS |

[1] Three ALV J strains (ADOL Hc1, ADOL 6803 and ADOL 4817) were used in this study.
[2] Meat type chickens were inoculated at 5 days of embryonation (5ED) or at day of hatch (DOH).
[3] Virus was inoculated with one of two doses (100 or 10,000 $TCID^{50}$).
[4] Type of tumors diagnosed by gross and microscopic pathology. ML = Myelocytomatosis, MB = Myeloblastosis, RT = Renal Tumors, HS = Histiocytic Sarcoma, EB = Erythroblastosis, HA = Hemangioma, FS = Fibrosarcoma, and RMS = Rhabdomyosarcoma.

**Table 2.7.** Effect of ALV J infection profile on ALV J-induced mortality, tumor incidence and tumor spectrum.

| Infection profile[1] | # of Chickens | % Mortality | Death mean in days | % Tumor | Tumor Spectrum[3] |
|---|---|---|---|---|---|
| V+A- | 127 | 73[a, 2] | 133 | 71[a] | ML, RT, MB, HA, HS, EB, FS, RMS |
| V+A+ | 21 | 62[a] | 159 | 48[b] | ML, EB, HA, HS |
| V-A+ | 3 | 33[a] | 195 | 33[ab] | HA |

[1] Viremia (V) and neutralizing antibody (A) data were classified into three ALV J infection profiles (V+A+, V+A-, V-A+).
[2] Alphabetic superscripts indicate statistically significant differences at $p < 0.05$ level
[3] Type of tumors diagnosed by gross and microscopic pathology. ML = Myelocytomatosis, MB = Myeloblastosis, RT = Renal Tumors, HS = Histiocytic Sarcoma, EB = Erythroblastosis, HA = Hemangioma, FS = Fibrosarcoma, and RMS = Rhabdomyosarcoma.

**A. Category V+A-**

| #[1] | B1[2] | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | Profile[4] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | + | + | + | + | + | + | + | V |
| | - | - | - | - | - | - | - | - | - | NAb |
| 2 | + | + | + | + | + | + | + | ND[3] | ND | V |
| | - | - | - | - | - | - | - | ND | ND | NAb |
| 3 | + | + | + | + | + | + | + | + | ND | V |
| | - | - | - | - | - | - | - | - | ND | NAb |
| 4 | + | + | + | + | + | + | ND | ND | ND | V |
| | - | - | - | - | - | - | ND | ND | ND | NAb |

**B. Category V-A+**

| #[1] | B1[2] | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | Profile[4] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | + | - | - | + | - | - | - | V |
| | - | - | - | - | + | + | + | + | + | NAb |
| 2 | + | + | + | - | + | + | - | - | - | V |
| | - | - | - | + | - | + | + | + | + | NAb |
| 3 | + | + | + | + | - | - | + | - | ND[3] | V |
| | - | - | - | - | + | + | + | + | ND | NAb |
| 4 | - | + | + | + | - | - | + | + | - | V |
| | - | - | - | + | + | - | - | + | + | NAb |

**C. Category V+A+**

| #[1] | B1[2] | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | Profile[4] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | + | + | + | + | + | + | + | + | V |
| | - | - | - | - | - | + | + | + | ND[3] | NAb |
| 2 | + | + | + | + | + | + | + | + | ND | V |
| | - | - | - | - | + | + | + | + | + | NAb |
| 3 | - | + | + | + | + | + | + | + | + | V |
| | - | - | - | - | + | + | + | + | + | NAb |
| 4 | + | + | + | + | + | + | + | + | + | V |
| | - | - | - | + | - | - | - | + | - | NAb |

**Figure 2.1.** Examples of classification of ALV J infection profile into 3 categories based on the viremia (V) and neutralizing antibody (NAb) against the parental virus (A) data obtained through the 9 sampling intervals over a period of 32 weeks starting from one week post hatch. A: Category V+A- refers to chickens with persistent viremia without any NAb against the inoculated virus. B: Category V-A+ refers to chickens that cleared viremia by the end of the study with efficient NAb against the inoculated virus. C: Category V+A+ refers to chickens with concurrent viremia and NAb against the inoculated virus during at least one sampling. 1 = chicken serial number, 2 = sampling intervals, 3 = no data due to death before study termination, 4 = infection profile in terms of viremia (V) and neutralizing antibody (NAb) against the parental virus.

# CHAPTER 3

## Emergence of Subgroup J avian leukosis virus neutralizing antibody escape variants in meat-type chickens infected with virus at hatch

**ABSTRACT**

Infection of commercial meat-type chickens at hatch with field isolates of Subgroup J avian leukosis virus (ALV J) can result in a high incidence of chickens with persistent viremia even in the presence of neutralizing antibodies (NAb) against the inoculated parental virus (V+A+). The purpose of this study was to confirm the high incidence of V+A+ profile in chickens inoculated at hatch with an ALV J molecular clone (ADOL pR5-4). Fifty meat-type chickens and 100 ADOL line 0 chickens were infected with ADOL pR5-4 at hatch and 22 meat-type chickens were added as sentinels (contact exposed). Chickens were sampled for viremia and NAb on 1, 8, 16, 24, 28, and 32 weeks post inoculation before the study was terminated at 32 weeks post hatch. In addition, the emergence of NAb escape variants was evaluated by sequential autologous virus neutralization (VN) (between virus and antibody from the same sampling) and heterologous VN (between virus and antibody from preceding and succeeding samplings). Five chickens infected with ALV J field isolates from previous studies and 10 chickens infected with ADOL pR5-4 that were persistently V+A+ were evaluated for the presence of NAb escape variants. The results demonstrated that infection of meat-type chickens at hatch with ADOL pR5-4 resulted in 88% of chickens with V+A+ infection profile in contrast to 23% and 4% in the sentinel meat-type chickens and ADOL line 0 chickens, respectively. All 15 V+A+ chickens, inoculated either with ALV J field isolates or ADOL pR5-4, failed to neutralize autologous viruses demonstrating the emergence of NAb escape variants. However, most of these resilient autologous viruses were neutralized by antibodies at later sampling intervals. The incidence of autologous neutralization antibodies were lower in meat-type chickens infected with ALV J field

isolates (15%) than ADOL pR5-4 (36%). Data from this study demonstrated that NAb escape variants might play a role in ALV J persistence.

## INTRODUCTION

Subgroup J Avian Leukosis Virus (ALV J) causes a variety of neoplastic and non-neoplastic conditions primarily in meat-type chickens and is responsible for severe losses to the poultry industry. ALV J is largely controlled by identification and eradication of chickens positive for antibody or viremia depending on the eradication protocols followed by the breeder. As with other retroviruses, ALV J is capable of persisting at very low levels in the host and the routinely used diagnostic methods may not always detect the virus. Hence, the real prevalence of ALV J may be actually higher than what has been reported.

The ALV J infection profile and transmission pattern in meat-type chickens is comparable to that of other exogenous ALV infections (Payne *et al.*, 1991, 1992; Rubin *et al.*, 1962; Witter *et al.*, 2000). In general, chickens infected *in ovo* with ALV result in tolerant viremia with no apparent immune response against the inoculated virus (V+A-) and chickens infected after hatch may clear viremia by producing an efficient neutralizing antibody (NAb) response against the inoculated virus (V-A+). However, meat-type chickens infected with ALV J during the first two weeks after hatch generally have high levels of viral persistence in the absence (V+A-) or presence of NAb (V+A+) (Fadly and Smith, 1999; Pandiri, 2005a; Witter and Fadly, 2001). The continued viral persistence that is often observed in chickens that develop neutralizing antibodies against the

inoculated virus (V+A+), might indicate the presence of viral strains different from the inoculated viral strain or NAb escape variants.

The role of NAb escape variants in viral persistence has been demonstrated in other viruses, especially equine infectious anemia virus (EIAV) (Kono *et al.*, 1973), visna virus (Narayan *et al.*, 1977), human immunodeficiency virus (HIV) (Weiss *et al.*, 1986), lymphocytic choriomeningitis virus (LCMV) (Ciurea *et al.*, 2000), and foot and mouth disease virus (FMDV) (Mateu *et al.*, 1994). The relevance of NAb escape variants in ALV J-induced persistence has not been evaluated.

The objective of this work was to confirm the high incidence of V+A+ infection profile in meat-type chickens infected at hatch with an ALV J molecular clone ADOL pR5-4 as demonstrated in previous experiments with ALV J field isolates. Another objective of this work was to demonstrate NAb escape variants in V+A+ chickens infected with ALV J field isolates as well as ADOL pR5-4 by evaluating sequential autologous and heterologous NAb responses.

## MATERIALS AND METHODS

**Chickens.** Commercial meat-type chickens and ADOL Line 0 chickens (Crittenden and Fadly, 1985) were used. The chickens were free from other avian pathogens as tested by routine diagnostic protocols. Chickens were housed in floor pens maintained as isolation units under biosecurity level-2 containment for 32 weeks. Line 0 chickens were provided

feed and water *ad libitum* but feed was restricted for the commercial meat-type chickens to limit excess body weight gain as recommended by the breeder.

**Viruses.** Three ALV J field isolates (ADOL Hc1, ADOL 6803, and ADOL 4817) were selected based on geographic origin and nucleotide sequence differences (Fadly and Smith, 1999; Silva *et al.*, 2000). All the ALV J strains were isolated from separate meat-type chicken farms across the United States. All the three viral strains had significant nucleotide sequence differences in the *env* protein (Silva *et al.*, 2000). In addition, a molecularly cloned ALV J, ADOL pR5-4 derived from a field ALV J strain ADOL R5-4 (Lupiani *et al.*, 2003) was used in this study. ADOL pR5-4 was demonstrated to have similar biological characteristics as other ALV J strains (Lupiani *et al.*, 2003). All viruses were propagated in ADOL line 0 C/E secondary chicken embryo fibroblasts (CEFs) that support propagation of all ALVs except the endogenous viruses. The virus titers were determined by limiting dilution in tissue culture. The titers varied from $10^{5.5}$ to $10^{6.5}$ $TCID^{50}$ per milliliter.

**Experimental design and sample selection.** Fifty meat-type chickens and 100 ADOL line 0 chickens were intra-abdominally inoculated with 1,000 $TCID^{50}$ADOL pR5-4. In addition, 22 meat-type chickens were housed as sentinels. The chickens were bled on 1, 8, 16, 24, 28 and 32 weeks post inoculation. At sampling, 3-5 ml of blood was collected in syringes coated with KEDTA and spun at 1500 rpm for 30 minutes to separate the plasma. All samples were placed on melting ice immediately after collection and assayed fresh or stored at -70° C until assayed.

To evaluate NAb escape variants, 5 chickens from a previous experiment (Pandiri, 2005a) that were inoculated with ALV J field isolates (ADOL Hc1, ADOL 4817, and ADOL 6803) and 10 chickens inoculated with ADOL pR5-4 were selected. All 15 chickens that were selected had persistent V+A+ infection profile on the last 4-6 sampling intervals.

**Virus isolation (VI).** Plasma samples collected during each sampling period were tested for viremia by virus isolation. Samples were tested according to the procedures described earlier (Fadly and Witter, 1998). Briefly, about 100 µL of undiluted plasma was added to 0.18 x $10^6$ CEFs suspended in 4% calf serum (CS) Leibowitz's L-15-McCoy's 5A tissue culture medium [1:1] (LM) containing penicillin, streptomycin, amphotericin B and 0.004 IU heparin in 24 well tissue culture plates. On the following day, the 4% CS LM media was replaced with 1% CS LM media. The plates were incubated in 4% $CO_2$ at 37° C for 7-9 days before the cells were completely lysed with 50 µL of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and two alternate cycles of freezing at -70° C and thawing at 37° C. About 100 µL of the cell lysate was used to test for p27 group-specific antigen (gsa) by enzyme-linked immunosorbent assay (ELISA) (Smith *et al.*, 1979). The p27 gsa ELISA was carried out using rabbit anti-p27 polyclonal antibody coated immunolon® plates (Dynatech, Chantilly, VA), rabbit anti-p27 antibody conjugated to horse-radish peroxidase (SPAFAS, Storrs, CT) and TMB substrate (3, 3', 5, 5'-tetramethyl benzidine) (BD Biosciences Pharmingen, San Diego, CA). The plate was read at an absorbance of 630 nm using a MRX microplate reader (Dynex, Chantilly, VA).

**Virus micro-neutralization (VN).** Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens. VN assays were performed as described earlier (Fadly and Witter, 1998). In précis, the plasma samples were diluted 1:5 in serum-free LM media and incubated at 56° C for 30 minutes to denature the complement factors. About 500 – 1,000 ALV J viral particles in 50 µL LM media are incubated with 50 µL of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 minutes in 4% $CO_2$ at 37° C. After the incubation, about $1x10^5$ cells in 150 µL of 4% CS LM media were pipetted into each of the 96 wells and incubated at 37° C and 4% $CO_2$ for 7-9 days. At the end of incubation, the cell cultures were completely lysed with 20 µL of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and were subjected to two alternate cycles of freezing at -70° C and thawing at 37° C. The cell lysates were tested for p27 gsa ELISA as described earlier (Smith *et al.*, 1979). Samples that had a chromogenic reading of 1 or negative on the p27 gsa ELISA read-out was considered to be positive for NAb against ALV J and samples with a chromogenic reading of $\geq 2$ on the p27 gsa ELISA read-out was considered to be negative for NAb against ALV J.

**Design of VN assays.** The V+A+ infection profile is based on serum NAb isolated at various sampling intervals against the inoculated parental virus. Sequential plasma samples from each chicken with consistent V+A+ infection profile were inoculated into line 0 C/E CEFs. The virus stocks were prepared and titrated by limiting dilution in tissue culture. The plasma samples were diluted 1:2, 1:5, 1:10, and 1:20 in serum-free

LM media and heat-denatured at 56° C for 30 minutes. Each of these virus stocks and plasma samples (antibodies) were subjected to duplicate autologous VN (between virus and antibody from the same sampling) or heterologous VN (between virus and antibody from preceding and succeeding samplings). A VN matrix was constructed based on autologous and heterologous VN patterns.

**Statistics.** Statistical analysis was performed by testing for significance of differences in percentages by chi square test using Statistica® (Statsoft, Tulsa, OK). Statistical significance was assumed at less than 0.05 level of probability.

## RESULTS

**ALV J infection profile in chickens infected with ADOL pR5-4 (Table 3.1).** The incidence of V+A+ ALV J infection profile in commercial meat-type chickens inoculated at hatch with ADOL pR5-4 and in contact-exposed meat-type chickens was 88% and 23%, respectively; compared with 4% in line 0 chickens inoculated at hatch. None of the meat-type chickens were able to clear viremia even though 88% of the chickens had NAbs against the inoculated parental virus. In contrast, 77% of contact-exposed meat-type chickens and 93% of ADOL line 0 chickens were able to clear viremia by producing an efficient NAb response.

**NAb responses against autologous viruses (Table 3.2).** The following results are from samples collected from meat-type chickens during the last 4-6 sampling intervals before the study was terminated at 32 weeks post hatch. Antibodies isolated at all sampling

intervals were able to neutralize the inoculated parental virus. However, three out of five persistent V+A+ meat-type chickens inoculated with ALV J field isolates (ADOL Hc1, ADOL 4817 and ADOL 6803) were unable to neutralize autologous viruses at any of the sampling intervals. The remaining two chickens failed to neutralize autologous viruses in most of the sampling intervals (2 or 3 out of 4).

The efficacy of autologous neutralization was slightly higher in chickens infected with ADOL pR5-4 than in chickens infected with ALV J field isolates. Autologous antibodies against ALV J field isolates were able to neutralize in 3 out of 20 cases at 1:5 dilution whereas autologous antibodies against ALV J molecular clone ADOL pR5-4 were able to neutralize in 33 out of 55 cases even at 1:20 dilution. However, all 10 chickens inoculated with ADOL pR5-4 were unable to neutralize autologous viruses on at least two sampling intervals. In addition, most of the chickens (8 out of 10) could not neutralize autologous viruses on more than 50% of the sampling intervals and one chicken was unable to neutralize autologous viruses at any sampling interval.

**Neutralization matrix (Figure 3.1).** Various patterns of neutralization matrices were observed and examples are illustrated in Figure 3.1. As indicated above, most of the chickens were unable to neutralize autologous viruses. However, these viruses were neutralized within the next one or two sampling intervals in all cases. Some differences were found in heterologous VN of viruses with antibodies from earlier samplings. In some cases, viruses could not be neutralized by antibodies from earlier sampling intervals (Fig A, D), while in others viruses were neutralized by earlier antibodies (Fig B, C).

## DISCUSSION

A high incidence of V+A+ infectious profile was demonstrated in commercial meat-type chickens inoculated with ALV J molecular clone ADOL pR5-4 as demonstrated in previous experiments with ALV J field isolates (ADOL Hc1, ADOL 6803, and ADOL 4817) (Pandiri, 2005a). Also, as reported after infection with ALV J field isolates, the incidence of V+A+ profile in meat-type sentinel chickens as well as ADOL line 0 chickens infected with ADOL pR5-4 was very low, since these chickens were able to clear ALV J viremia by producing an efficient NAb response. These results demonstrated that the molecularly cloned ALV J used in the current study to inoculate meat-type chickens at hatch is capable of inducing viral persistence similar to ALV J field isolates.

Results from this study are first to demonstrate the presence of NAb escape variants in avian retrovirus infections. Most viruses isolated from persistently viremic chickens with antibodies capable of neutralizing the inoculated parental virus (V+A+) were not neutralized by autologous antibodies. In chickens inoculated with ALV J field isolates, the heterogeneity of the viral inoculum seems to have also contributed to these results since the ability to neutralize autologous viruses was lower than in chickens inoculated with the molecular clone ADOL pR5-4. However, the emergence of NAb escape variants can be confirmed since they were also detected in every meat-type chicken inoculated with the molecular clone ADOL pR5-4 that has a very low heterogeneity. The relevance of NAb escape variants in viral persistence has been

demonstrated in other retroviruses such as EIAV (Kono *et al.*, 1973), Visna virus (Narayan *et al.*, 1977), LCMV (Ciurea *et al.*, 2000), HIV (Weiss *et al.*, 1986) and FMDV (Mateu *et al.*, 1994).

The high incidence of V+A+ infection profile in chickens infected with ALV J is poorly understood. Age at infection, chicken and virus strain, and stress-induced immunosuppression have been shown to be major factors (Fadly and Payne, 2003; Pandiri, 2005b). In this study, the presence of NAb escape variants demonstrates that they also play an important role in the high incidence of V+A+ infection profile.

Analyses of the VN matrices provided relevant information regarding autologous and heterologous NAb. The complexity of the NAb response was demonstrated since the VN matrices did not have the same profile even though all chickens were V+A+ at every sampling interval. Bachman *et al* studied in detail the factors influencing VN (Bachman *et al.*, 1997). Differences in *in vitro* and *in vivo* VN might play a major role since antibody titers, virus titers and efficiency of VN may be different in both conditions. There may be more than one NAb escape variant in each virus isolate and the existing antibodies may not neutralize all the virus serotypes. The immunogenicity of each NAb escape variant might differ in addition to the efficiency of the host immune response. Finally, all the experiments were of short duration (32 weeks) and it is possible that there is an overlapping presence of NAb escape variants and the NAb for each of them. Results might vary if the sampling intervals were of longer duration as described in human studies (Richman *et al.*, 2003).

Routine testing in ALV J infection experiments involves virus isolation from plasma as well as testing for NAb against the inoculated (parental) virus at different sampling intervals. This work has demonstrated that routine description of ALV J antibody (A+ or A-) is not entirely accurate since the NAb response is always against the inoculated virus but not the autologous virus. As discussed earlier this does give some information about the immune response of the chicken but miss important information on the emergence of variant viruses. On the other hand, testing for sequential autologous antibody response is cumbersome since this involves virus isolation from plasma obtained from several sampling intervals, preparation of viral stocks, and biological titration in tissue culture by limiting dilution.

The emergence of NAb escape variants in chickens with V+A+ profile has been demonstrated based on autologous and heterologous VN matrices. Due to lack of neutralizing monoclonal antibodies against ALV J, the VN matrices do not allow us to detect variation in neutralizing epitopes. Sequencing the variant viruses might demonstrate mutations in the *env* gene that are responsible for the neutralization phenotype. In addition, targeted mutagenesis of ADOL pR5-4 and inoculation into meat-type chickens may provide additional information on the role of escape variants in ALV J persistence as well as neutralization epitopes in the virus.

Thus, this study has shown that infection of meat-type chickens with an ALV J molecular clone results in high levels of V+A+ infection profile that can be attributed to

the emergence of NAb escape variants. In addition, our results revealed the limitations on the current description on ALV infection profile. This information expands the current knowledge on ALV J persistence and it will aid in better monitoring of ALV J infections.

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**Table 3.1.** Infection profile of chickens infected with ALV J molecular clone ADOL pR5-4

| Lot # | Chicken strain | Inoculation Dose ($TCID^{50}$) | ALV J infection profile[1] V+A- (%) | V+A+ (%) | V-A+ (%) | Chickens # |
|---|---|---|---|---|---|---|
| 1 | Meat-type | 1,000 | $12^{a,2}$ | $88^{a}$ | $0^{a}$ | 50 |
| 2 | Meat-type | Contacts | $0^{b}$ | $23^{b}$ | $77^{b}$ | 22 |
| 3 | ADOL line 0 | 1,000 | $3^{b}$ | $4^{c}$ | $93^{c}$ | 100 |

[1] virus isolation (V); neutralizing antibody against the inoculated parental virus (A) V+A- = chickens that were consistently positive for viremia and did not develop any neutralizing antibody; V+A+ = chickens that remained viremic at the end of the study and were concurrently positive for viremia and neutralizing antibody response on at least one occasion; V-A+ = chickens that have successfully seroconverted with loss of viremia and developed neutralizing antibodies by the time the study was terminated.
[2] Alphabetic superscripts indicate statistically significant differences at $p < 0.05$ level

**Table 3.2.** Heterologous and autologous virus neutralization profile of commercial meat-type chickens after ALV J infection

| Experiment[1] | Chicken | Virus | V+A+ incidence[2] | Autologous VN[3] |
|---|---|---|---|---|
| #1 | 1 | ADOL Hc1 | 4/4 | 0/4 |
| #1 | 2 | ADOL Hc1 | 4/4 | 2/4 |
| #1 | 3 | ADOL 4817 | 4/4 | 1/4 |
| #1 | 4 | ADOL 4817 | 4/4 | 0/4 |
| #1 | 5 | ADOL 6803 | 4/4 | 0/4 |
| #2 | 6 | ADOL pR5-4 | 6/6 | 2/6 |
| #2 | 7 | ADOL pR5-4 | 5/5 | 2/5 |
| #2 | 8 | ADOL pR5-4 | 5/5 | 2/5 |
| #2 | 9 | ADOL pR5-4 | 6/6 | 4/6 |
| #2 | 10 | ADOL pR5-4 | 5/5 | 0/5 |
| #2 | 11 | ADOL pR5-4 | 5/5 | 3/5 |
| #2 | 12 | ADOL pR5-4 | 6/6 | 1/6 |
| #2 | 13 | ADOL pR5-4 | 5/5 | 2/5 |
| #2 | 14 | ADOL pR5-4 | 6/6 | 2/6 |
| #2 | 15 | ADOL pR5-4 | 6/6 | 2/6 |

[1] Commercial meat-type chickens were infected with either ALV J field isolates (ADOL Hc1, ADOL 4817, ADOL 6803) or ALV J molecular clone ADOL pR5-4.
[2] Heterologous virus neutralization between the sample antibody and the inoculated parental virus. Number of sampling intervals positive for heterologous neutralization/total number of sampling intervals for each chicken
[3] Autologous virus neutralization between antibody and virus from the same sampling interval. Number of sampling intervals positive for autologous neutralization/total number of sampling intervals for each chicken

| A. | A1[3] | A2[3] | A3[3] | A4[3] | A5[3] |
|---|---|---|---|---|---|
| V0[1] | + | + | + | + | + |
| V1[2] | - | + | + | + | + |
| V2[2] | - | - | + | + | + |
| V3[2] | - | - | - | + | + |
| V4[2] | - | - | - | - | + |
| V5[2] | - | - | - | - | - |

| B. | A1[3] | A2[3] | A3[3] | A4[3] | A5[3] |
|---|---|---|---|---|---|
| V0[1] | + | + | + | + | + |
| V1[2] | - | + | + | + | + |
| V2[2] | - | - | + | + | + |
| V3[2] | - | + | - | + | + |
| V4[2] | + | + | - | - | + |
| V5[2] | - | - | - | - | - |

| C. | A1[3] | A2[3] | A3[3] | A4[3] | A5[3] |
|---|---|---|---|---|---|
| V0[1] | + | + | + | + | + |
| V1[2] | + | + | + | + | + |
| V2[2] | + | - | + | + | + |
| V3[2] | + | - | - | + | + |
| V4[2] | + | + | + | - | + |
| V5[2] | - | + | - | + | - |

| D. | A1[3] | A2[3] | A3[3] | A4[3] | A5[3] |
|---|---|---|---|---|---|
| V0[1] | + | + | + | + | + |
| V1[2] | + | + | + | + | + |
| V2[2] | - | - | - | + | + |
| V3[2] | - | - | - | - | + |
| V4[2] | - | - | - | - | + |
| V5[2] | + | - | + | + | - |

**Figure 3.1.** Examples of different patterns of virus neutralization (VN) matrix.
A. autologous VN was absent in all cases but emergent viruses were neutralized by later antibodies. None of the virus isolates were neutralized by antibodies from earlier samplings. B. autologous VN was absent in all cases but emergent viruses were neutralized by later antibodies. 2 virus isolates were neutralized by antibodies from earlier samplings. C. Except for the first virus autologous VN was absent in all cases but emergent viruses were neutralized by later antibodies. All virus isolates were neutralized by antibodies from earlier samplings. D. Except for the first virus autologous VN was absent in all cases but emergent viruses were neutralized by later antibodies. Only one virus isolate was neutralized by antibodies from earlier samplings.
[1] parental virus that was used to inoculate the chickens
[2] viruses (V1-V5) isolated at various sampling intervals (8, 16, 24, 28, 32 weeks PH)
[3] antibodies (A1-A5) isolated at various sampling intervals (8, 16, 24, 28, 32 weeks PH)

# CHAPTER 4

## Studies on subgroup J avian leukosis virus (ALV J) persistence following induction of stress in adult seroconverted meat-type chickens and adoptive transfer of naïve splenocytes in ALV J tolerized chickens

## ABSTRACT

Chickens infected with Subgroup J avian leukosis virus (ALV J) early in life develop viremia followed by a neutralizing antibody (NAb) response that may or may not be able to clear viremia. Occasionally, chickens that do clear viremia by developing an efficient NAb response revert to viremia and the factors responsible for this are not clear. In this study, it was hypothesized that stress can cause seroconverted viremia-free chickens to revert to viremia. Adult male commercial meat-type chickens that were exposed to ALV J at hatch and have cleared viremia, when subjected to chronic stress induced by porcine adrenocorticotrophin (ACTH), reverted to viremia and cloacal shedding (33%). The chickens reverted to viremia and cloacal shedding 10 days post ACTH-induced stress. However chickens that were contact exposed to ALV J at 32 weeks of age and have since seroconverted failed to revert to viremia when subjected to similar chronic stress. Stress did not increase the susceptibility of adult meat-type chickens to ALV J infection by contact exposure. There was no effect of stress on the incidence of NAb response in all the seroconverted chickens in this study.

Attempts to abrogate or modify ALV J-induced tolerance in ADOL's o.p. 13 and o.p.21 chickens by adoptive transfer of splenocytes from naïve donors were unsuccessful.

## INTRODUCTION

Subgroup J avian leukosis virus (ALV J) infection and tumors have varying degrees of prevalence across the world affecting mainly meat-type chicken flocks (Fadly and Smith, 1999; Payne *et al.*, 1991; Payne *et al.*, 1992) and sporadically some egg-laying flocks

(Gingerich *et al.*, 2002; Xu *et al.*, 2004). These viruses induce a high degree of viral persistence along with tumors of myeloid lineage and production problems (Fadly and Payne, 2003). ALV J is primarily controlled by identification and eradication of chickens positive for antibody or viremia depending on the protocols followed by the breeder. On the whole, the broiler-breeder industry has been successful in controlling ALV J incidence in their flocks to a large extent except for the sporadic incidence of ALV J viremia or antibodies in flocks that previously tested negative for ALV J (McKay and Rosales, 2000). Anecdotal field evidence and experimental findings suggest that there is a waxing and waning level of ALV J viremia in the affected meat-type chicken flocks (Pandiri, 2005; Witter *et al.*, 2000). As with other retroviruses, ALV J is capable of persisting at very low levels in the host and the routinely used diagnostic methods may not always detect the virus. This may be responsible for the occasional failures in conventional exogenous ALV eradication programs. Hence, the real prevalence of ALV J may be actually higher than what is reported.

There are several factors that influence ALV persistence in chickens. Chickens infected with ALV either *in ovo* or at hatch generally are persistently viremic compared to chickens infected later in life. In addition, ALV-induced viremia and shedding also depends on the type of viral strain, viral dose, prevalence of endogenous retroviruses in the genome (Crittenden *et al.*, 1982; Crittenden *et al.*, 1987; Crittenden *et al.*, 1984; Fadly *et al.*, 1987; Pandiri, 2005; Williams *et al.*, 2004), as well as immunosuppressive conditions due to a variety of factors like stress, chemical-induced immunosuppression and viral infections (Cui *et al.*, 2003; Fadly *et al.*, 1987; Fadly *et al.*, 1985; Kim et al.,

2003, 2004; Todorov and Yakimov, 1967). The above mentioned factors consequently increase ALV vertical transmission and severely undermine the ALV eradication protocols.

Diminution of immune responses due to stress in chickens is well documented in literature. Stress activates the pituitary-hypothalamus axis and stimulates the production of adrenocorticotropic hormone (ACTH) that in turn activates the adrenal gland to produce corticosterone (Ben Nathan *et al.*, 1976; Freeman and Manning, 1978). The resulting increased plasma corticosterone has negative pleotrophic effects on the immune system *viz.*, lymphocytopenia leading to increased heterophil : lymphocyte ratios (Davison *et al.*, 1983), reduction in cell mediated immune response (Regnier and Kelley, 1981) as well as antibody response (Gross and Siegel, 1973). Interactions between stress and neoplastic poultry infections have been described (Fadly *et al.*, 1989; Powell and Davison, 1986). Corticosterone treatment of vaccinated chickens increased the incidence of Marek's disease (Powell and Davison, 1986). Administration of corticosterone in maternal antibody negative one week old Brown leghorn chickens infected with ALV A at hatch significantly increased cloacal shedding of ALV (Fadly *et al.*, 1989). However, increased corticosterone levels in 2 week old chickens as well as adult Brown leghorn hens did not influence the incidence of ALV viremia or shedding (Fadly *et al.*, 1989). The effect of stressors on ALV J infection in commercial meat-type chickens is not known. The current study aims to evaluate the effect of ACTH-induced stress on adult male seroconverted chickens that were exposed to ALV J at hatch or at 32 weeks of age.

Chickens infected *in ovo* with ALV J almost always develop tolerant viremia that is characterized by a total lack of immune response against the tolerizing virus. However, chickens that are infected with ALV J at hatch or during the first 2 weeks of life can develop a persistent ALV infection that is characterized by continued existence of the virus in the host that is capable of eliciting an immune response against the virus. Efforts to abrogate or modify persistent viremia in the face of a patent immune response or tolerant viremia were made by several workers. Efforts to reduce persistent viral infections by adoptive transfer of syngeneic splenocytes were successful in many viral infections like lymphocytic choriomeningitis virus (LCMV) (Jamieson *et al.*, 1991) and infectious bronchitis viral infection (IBV) (Seo *et al.*, 2000). However, early attempts to abrogate tolerance to ALVs by challenging White Leghorn chickens infected *in ovo* with subgroup A avian leukosis virus ALV-F42 with serologically related strains of ALV A were not successful. The chickens tolerant to ALV-F42 were able to develop NAbs against the serologically related ALV A viruses but not against the tolerizing virus ALV-F42 (Meyers, 1976). In the current study, the possibility of abrogating tolerance induced by ALV J in o.p. 13 and o.p. 21 chickens by adoptively transferring splenocytes from age-matched, major histocompatibility complex (MHC)-matched ALV J-naïve donor chickens was tested.

Hence the objective of the current study was to study ALV J persistence following alterations in the host immune system. In the first experiment, the host immune response was compromised by ACTH-induced stress and the possibility of seroconverted chickens reverting to viremia and shedding was studied. In the second

experiment, ALV J-tolerized host's immunity was supplemented by adoptive transfer of age- and MHC -matched splenocytes and the ability to overcome ALV J-induced tolerance were evaluated.

## MATERIALS AND METHODS

**Chickens.** In experiment #1, adult male 52-week-old commercial meat-type chickens saved from previous chicken experiments were used (Pandiri, 2005). These chickens were categorized into 2 groups based on their age at exposure to ALV J and subsequent seroconversion status. Group one included ALV J seroconverted chickens that were inoculated at hatch with ALV J strain ADOL Hc1 and group two included age-matched naïve chickens that were contact-exposed at 32 weeks of age to ADOL Hc1. In addition, one chicken that was infected at hatch with ADOL Hc1 and was shedding virus was also reared in the same pen along with chickens from groups one and two. All the chickens were reared together from 32 weeks to 52 weeks of age in a floor pen that was used in the previous experiment and was managed as an isolation unit with biosecurity level-2 containment (BSL-2). At 52 weeks of age, all chickens in group one remained viremia-free with efficient NAb against ADOL Hc1 (V-A+ (DOH)). However, during this time, 6/14 (43%) chickens were infected with ALV J and subsequently seroconverted (V-A+ (32 weeks)) and 6/14 (57%) chickens remained viremia-free without any NAb (V-A-(32 weeks)) (Figure 4.2).

In experiment #2, o.p. 13 and o.p. 21 chickens with Line 0 background (Crittenden *et al.*, 1984; Hunt, 2003) were used. These chickens with homozygous B21 and B13

background were produced by crossing heterozygous B21B13 parents. The MHC background was tested by using a hemagglutination test (Fulton *et al.*, 1996).

**Virus.** Strain ADOL Hc1 of ALV J, the United States prototype (Fadly and Smith, 1999) was used in both the experiments. The viral inoculum had a titer of $1x10^5$ $TCID_{50}$.

**Experimental design.** In experiment #1, 6 V-A+ (DOH) chickens, 6 V-A+ (32 Weeks) chickens, and 8 V-A- (32 Weeks) chickens were subjected to chronic stress to study its effect on ALV J persistence. Stress was artificially induced by treating the chickens with porcine ACTH (ACTH 1039, Corticotropin A, Sigma Aldrich Fine Chemicals, St. Louis, MO) dissolved in 0.85% saline solution. Porcine ACTH has been shown to increase plasma corticosterone levels in chickens (Puvadolpirod and Thaxton, 2000). ALZET® osmotic pumps (Model 2ML2, Alza Corp, Mountain View, CA) were used to maintain a constant administration of baseline ACTH at a dosage of 12 IU/kg BW/day. These osmotic pumps were designed to deliver ACTH continuously at the rate of 5.0 µL/h for 14 consecutive days. Osmotic pumps loaded with 2 mL of porcine ACTH solution were surgically implanted at the dorsal aspect of the base of the neck. The surgical area was sterilized with 70% ethanol and was locally anesthetized by percutaneous injection of 0.2 mL of lidocaine-HCl (The Butler Company, Columbus, OH, 43228). A 1 cm incision was made in the skin and the ACTH loaded osmotic pump was inserted subcutaneously with the delivery port of the capsule entering first. Lastly, the incision was closed using a couple of surgical staples and a dab of pine tar was applied on the surgical site to discourage cannibalism. Control chickens were not handled and did not receive any treatment. All the chickens were sampled (blood and cloacal swabs) for viremia, cloacal

shedding, and Nab against the parental virus; corticosterone levels were determined at day 0 and at days 3, 7, 10, 14 after ACTH administration. The experiment was terminated at 14 days after ACTH administration (54 week old) and the surviving chickens were euthanized and necropsied for ALV J-induced tumors (Figure 4.2).

In experiment #2, Line o.p. chickens of haplotypes B21B21 and B13B13 were ALV J-tolerized by administering ADOL Hc1 *via* the yolk sac route on the 5$^{th}$ day of embryonation. Twelve o.p. B21B21 and 10 o.p. B13B13 ALV J-tolerized chickens received 10 x $10^6$ viable splenocytes from age-matched, MHC-matched ALV J-naïve donors at 8 weeks of age. Spleens were harvested aseptically from age-matched, MHC-matched donor chickens of similar genetic background. The spleens were mashed in a tissue grinder with wire-mesh filters with a glass pestle and single cell suspensions were prepared in antibiotic-rich LM media. The single cell splenocyte suspensions were counted and diluted to 10 x$10^6$ live cells/mL. Each chicken received 1 ml of the splenocyte suspension *via* jugular vein. All the chickens were bled at 10 days, 4 weeks and 8 weeks post treatment for testing ALV J viral titers as well as for NAbs against ADOL Hc1. The study was terminated at 8 weeks post treatment.

**Assays.** The chicken plasma samples were used to test for ALV J-induced viremia, NAb against the inoculated parental virus and corticosterone levels; cloacal swabs were used for testing ALV J shedding.

**Virological and serological assays.** Virus isolation (VI) and virus neutralization (VN) assays were done as described earlier (Fadly and Witter, 1998). In brief, VI was done by inoculating plasma on C/E CEFs and performing a p27 group specific antigen (gsa) ELISA on the cell culture lysates after 7-9 days of incubation as described earlier (Smith *et al.*, 1979). Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens as previously described (Fadly and Witter, 1998). In précis, about 500 - 1000 ALV J viral particles were incubated with equal volume of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 minutes at 37 °C. After incubation, C/E CEFs were added to this mixture and incubated for 7-9 days. After incubation, cell culture lysates were tested for p27 gsa by ELISA as described earlier (Smith *et al.*, 1979). Samples that had a chromogenic reading of 1 or negative on the p27 gsa ELISA read-out was considered to be positive for NAb against ALV J and samples with a chromogenic reading of $\geq 2$ on the p27 gsa ELISA read-out was considered to be negative for NAb against ALV J.

**Quantitative corticosterone ELISA.** To prove the efficacy of ACTH administration, quantitative corticosterone ELISA test was performed on duplicate 100 µL plasma samples before and 3 and 7 days post ACTH treatment. This assay was performed by using a Corticosterone Correlate-EIA™ immunoassay kit following the manufacturer's guidelines (Assay Design Inc., Ann Arbor, MI).

**Data analysis.** Statistical analysis was performed by testing for significance of differences in percentages by chi square test using Statistica® (Statsoft, Tulsa, OK). Statistical significance was assumed at the 0.05 level of probability.

## RESULTS

**Response to ACTH treatment.** Chickens treated with porcine ACTH had a statistically significant increase in plasma corticosterone levels by 3 or 7 days. Most of the chickens reached their peak plasma corticosterone levels at day 3 post ACTH treatment. The efficacy of porcine-ACTH treatment was demonstrated by establishing a ratio that compares the corticosterone levels of each chicken at 3 and 7 days post treatment with corticosterone levels before ACTH treatment. There was almost 1 log difference in plasma corticosterone levels between treated and untreated groups. Figure 4.1 demonstrates statistically significant differences at 3 and 7 days post-ACTH treatment corticosterone ratios between the control and treatment groups.

**Effect of ACTH treatment on ALV J viremia, shedding and NAb response.** At 10 days post-ACTH treatment 2/6 of V-A+ (DOH) chickens (33%) reverted to viremia as well as cloacal shedding. The reverted viremia status continued onto 14 days when the study was terminated. However, none of the V-A+ (32 Weeks) chickens reverted to viremia or cloacal shedding following ACTH treatment. V-A- (32 Weeks) chickens did not become susceptible to ADOL Hc1 post-ACTH treatment. No change in the NAb status was noted in any group of chickens, regardless of ACTH treatment (Table 4.1).

**Abrogation of ALV J-induced tolerance by transfer of naïve splenocytes.** All ADOL Hc1 tolerized chickens had a viremia titer of 6-7 x $10^6$ $TCID^{50}$/ml of plasma. After adoptive transfer of splenocytes from age-matched, MHC-matched donors, the viremia titers in chickens of both B21B21 and B13B13 haplotypes remained unaffected when tested at 10 days, 4 weeks and 8 weeks post treatment (Table 4.2).

## DISCUSSION

The results observed in this study indicate that a chronic increase in circulating plasma corticosterone levels resulted in reversion to viremia as well as cloacal shedding in 33% of V-A+ chickens that have been exposed to virus at hatch. However, V-A+ chickens that have been exposed to virus at 32 weeks did not revert to viremia or cloacal shedding after ACTH-treatment. The reason for only the V-A+ (DOH) chickens reverting to viremia upon stress is not known. ALV J infection at an early age might have caused the virus to sequester in tissues that escaped immune response. Also, it is possible that early ALV J infection might have a negative effect on the immature immune system of the chicken. However, there is no experimental evidence to support the above hypotheses.

Fadly *et al.* conducted the only comparable work involving ALV and corticosterone-induced stress (Fadly *et al.*, 1989). They studied the effect of stress on ALV A viremia and shedding in Brown Leghorn chickens. The current results differ from previous work since only the effect of stress on ALV A infection in chickens younger than two weeks of age was demonstrated in that work (Fadly *et al.*, 1989). In the current study, the effect of stress on ALV J persistence was observed even at 52 week of

age. The longer duration (14 days versus 7 days) of stress, method of stress induction (ACTH versus corticosterone), higher corticosterone levels (10 fold increase versus 3 fold increase) in the current study together with differences in chicken strain (meat type versus Brown Leghorn), and ALV viral subgroup (ALV J versus ALV A) may account for differences in results of the current study compared to previous studies. In addition, the experimental design of Fadly *et al.*, was different from this study where stress induction and ALV inoculation were done simultaneously to study the effect of stress on ALV A shedding (Fadly *et al.*, 1989).

This is the first study demonstrating the reversion to viremia as well as shedding post ALV J seroconversion in adult meat-type chickens that were exposed to the chronic effects of ACTH-induced stress. As indicated in materials and methods (Figure 4.2), one chicken shedding the virus was able to infect 43% of the naïve chickens by contact exposure. Hence, the consequence of missing a single chicken that is shedding virus during an ALV eradication procedure can have devastating consequences on the breeder companies aiming to produce a flock free of ALV. Also, in the epidemiology and control of ALVs, the ALV infection status of the male is as important as of the female since a viremic male is capable of venereal transmission of the virus and thereby contributing to the vertical transmission of the virus through the dam (Smith and Fadly, 1994). Hence it is important to thoroughly test for the viremia and antibody status of the male breeders also since floor raised breeders are subjected to stress during mating. Selection of males that have consistently tested-negative for viremia and antibody is very important in ALV eradication protocols. Also every effort should be made to reduce stress in the chickens

at each level of poultry operations to minimize immunosuppression and thereby reduce its negative effects on flock health.

Attempts by several research groups to abrogate or modify persistent viremia or tolerant viremia were successful to varying degrees. Efforts to reduce persistent viral infections by adoptive transfer of syngeneic splenocytes were successful in many viral infections such as LCMV (Jamieson *et al.*, 1991) and IBV (Seo *et al.*, 2000). Also, infusion of anti-sera rich in NAbs or monoclonal NAbs tends to decrease HIV infections (Mascola *et al.*, 1999). Early attempts to abrogate tolerance to ALVs by challenging White Leghorn chickens infected *in ovo* with subgroup A avian leukosis virus ALV-F42 with serologically related strains of ALV A were not successful. The chickens tolerant to ALV-F42 were able to develop NAb against the serologically related ALV A viruses but not against the tolerizing virus ALV-F42 (Meyers, 1976). In the current study, attempts to abrogate or modify ALV J-induced tolerance by adoptively transferring age- and MHC-matched splenocytes from ALV J-naïve donors were not successful. Seo *et al* demonstrated that adoptive transfer of syngeneic immune splenocytes could reduce viral load and clinical signs associated with IBV whereas syngeneic splenocytes from naïve chickens could not modify IBV infection (Seo *et al.*, 2000). The results might have been different if splenocytes from donors that are immunized against ALV J were used. The absence of an assay to demonstrate the efficacy of splenocyte transfer treatment made it difficult to evaluate the efficacy of treatment. Multiple splenocyte treatment was not a viable option since the inbred chickens could succumb to the stress of treatment.

Thus, this study has demonstrated that treatment of chickens with ACTH can cause reversion of viremia and cloacal shedding in ALV J seroconverted adult male chickens that had been exposed to virus at day of hatch but not in chickens that were contact-exposed at 32 weeks of age. Attempts to abrogate ALV J tolerance in this study were unsuccessful. Adoptive transfer experiments might have value as a research tool for ALV J immunological research.

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**Table 4.1.** Effect of ACTH treatment on ALV J viremia (V), shedding (S), and NAb (A) in meat-type chickens exposed to ADOL Hc1 either at hatch or at 32 weeks of age.

| | Viremia | | Cloacal Shedding | | Neutralizing Antibody | |
|---|---|---|---|---|---|---|
| **Category**[1] | Pre-stress (%) | Post-stress (%) | Pre-stress (%) | Post-stress (%) | Pre-stress (%) | Post-stress (%) |
| **V-A+ (DOH)** | 0/6 (0) | 2/6 (33) | 0/6 (0) | 2/6 (33) | 6/6 (100) | 6/6 (100) |
| **V-A+ (32 Wks)** | 0/6 (0) | 0/6 (0) | 0/6 (0) | 0/6 (0) | 6/6 (100) | 6/6 (100) |
| **V-A- (32 Wks)** | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) |
| **V-A+ Controls** | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 3/3 (100) | 3/3 (100) |

[1] No statistically significant differences (< 0.05) were noted among groups, regardless of treatment.

**Table 4.2.** Viral titers ($TCID^{50}$) of ALV J tolerized chickens before and after adoptive transfer of splenocytes from naïve age-matched, MHC-matched donors.

| Wing Band #[1] | Haplotype | Pre Rx $TCID^{50}$ | DAY 10 $TCID^{50}$ | 4th week $TCID^{50}$ | 8th week $TCID^{50}$ |
|---|---|---|---|---|---|
| **6397** | B21 | 6.68 | 6.5 | 6.5 | 6.83 |
| **6385** | B21 | 6.5 | 6.32 | 6.5 | Dead |
| **6396** | B21 | 7.5 | 7.17 | 7.5 | 6.83 |
| **6374** | B21 | 6.5 | 6.5 | 6.83 | Dead |
| **6393** | B21 | 6.83 | 6.5 | 6.38 | 6.5 |
| **6388** | B21 | 7.17 | Dead | NA | NA |
| **6395** | B21 | 7.5 | 7.17 | 7.5 | Dead |
| **6386** | B21 | 7.5 | Dead | NA | NA |
| **6379** | B21 | 7.5 | Dead | NA | NA |
| **6378** | B21 | 6.3 | 6.5 | 6.38 | Dead |
| ***6383*** | *B21* | *7.17* | *7.17* | *7.5* | *Dead* |
| ***6390*** | *B21* | *7.5* | *7.5* | *7.17* | *Dead* |
| ***6389*** | *B21* | *6.5* | *6.68* | *Dead* | *NA* |
| **6361** | B13 | 6.38 | 6.38 | 6.5 | 6.32 |
| **6371** | B13 | 6.83 | 6.5 | 7.17 | 6.38 |
| **6362** | B13 | 6.83 | 6.5 | 6.63 | Dead |
| **6367** | B13 | 6.63 | 6.38 | 6.83 | Dead |
| **6370** | B13 | 6.83 | Dead | NA | NA |
| **6365** | B13 | 6.83 | Dead | NA | NA |
| ***6369*** | *B13* | *6.32* | *6.5* | *Dead* | *NA* |
| ***6373*** | *B13* | *6.63* | *6.83* | *7.17* | *Dead* |

[1] No significant differences were noted among groups, regardless of B haplotype and shaded rows in *italics* include no treatment controls.

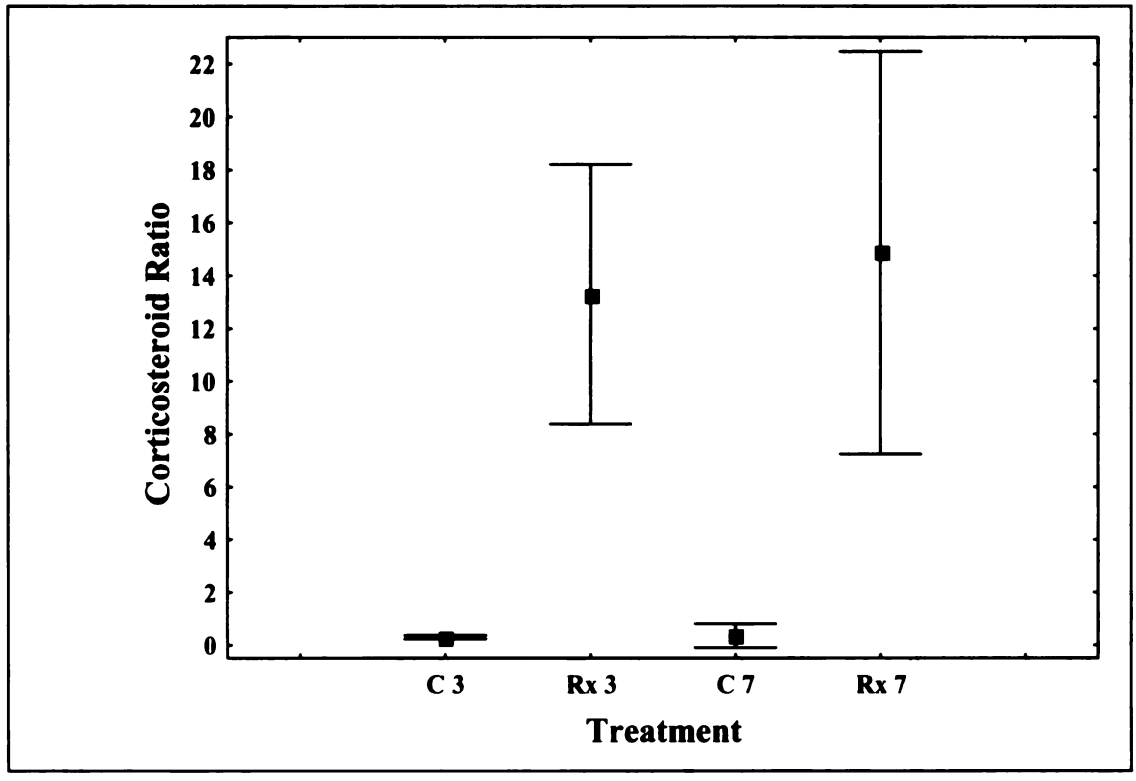


**Figure 4.1.** Differences in corticosterone ratios in control as well as treated chickens on days 3 and 7 post-ACTH treatment were statistically significant at 0.05 level of probability. C3 or C7 = ratio of corticosterone in untreated controls on day 0 and day3/day 7, Rx3 or Rx7 = ratio of corticosterone treated chickens on day 0 and day3/day 7 post treatment. The standard error of mean is shown as vertical bars.

| | | | Weeks post exposure to ALV J | | | | | | | | | Contact exposure | | Days post ACTH treatment | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment group | #[1] | Inf Pro[2] | 1 | 3 | 7 | 11 | 15 | 19 | 23 | 27 | 32 | 32 - 52 weeks PH | | 0 | 3 | 7 | 10 | 14 |
| V-A+ (DOH) | 6 | V | + | + | + | - | - | - | - | - | - | - | - | - | - | - | + | + |
| | | Ab | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| V-A+ (32 Wks) | 6 | V | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| | | Ab | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + |
| V-A- (32 Wks) | 8 | V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | | Ab | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| V-A+ Controls | 3 | V | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| | | Ab | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + |

**Figure 4.2** Example of experimental design as well as the result of ACTH-induced stress in various treatment groups. Nine meat-type chickens that were inoculated with ADOL Hc1 at day of hatch (DOH) and have seroconverted (V-A+ (DOH)) and one viremic chicken (V+A+) that was exposed at DOH were housed with 14 naïve hatchmates at 32 weeks of age for 20 weeks. During this time, 6/14 (43%) chickens became infected and subsequently seroconverted (V-A+ (32 Weeks)) whereas 8/14 (57%) chickens did not become infected (V-A- (32 Weeks)). At 52 weeks of age, all the chickens received porcine ACTH through ALZET® osmotic pumps and were subsequently monitored for corticosterone levels, viremia (V), cloacal shedding (S) and NAb (A). Only 2/6 (33%) of V-A+ (DOH) treatment group reverted to viremia post stress and none of the chickens in other treatment groups were influenced by ACTH-induced stress. There was no influence of stress on antibody response in all treatment groups.

[1] number of chickens in each treatment group;

[2] Infection profile viremia (V) and antibody (Ab)

# CHAPTER 5

## Distribution of viral antigen gp85 and provirus in tissues from commercial meat-type and ADOL Line 0 chickens with different subgroup J Avian Leukosis Virus infection profiles

**ABSTRACT**

After apparent clearance of Subgroup J avian leukosis virus (ALV J), the virus appears to persist in the chicken for a long time with no signs of overt viremia. Infrequently viremia reappears in chickens that are apparently healthy or immunosuppressed. The location of the virus in seroconverted chickens is not known. The objective of this study was to determine whether viral envelope antigen gp85 (by immunohistochemistry) as well as provirus (by polymerase chain reaction) can be detected in various tissues from 32 week old commercial meat-type and ADOL Line 0 chickens with different infection profile: 1) seroconverted with loss of viremia (V-A+); 2) chickens exposed *in ovo* that are tolerized viremia positive and neutralizing antibody (NAb) negative (tV+A-); 3) chickens exposed after hatch that are non tolerized V+A- (ntV+A-); and 4) viremia positive NAb positive (V+A+). Expression of gp85 in viremic chickens (tV+A-, ntV+A-, V+A+) was found in adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, spleen, thymus and in ALV J-induced tumors. Sciatic nerve was positive for gp85 in the tV+A- chicken but not in ntV+A-, V+A+ or V-A+ chickens. In addition, minor differences in the pattern of gp85 expression in lymphocytes were found between tV+A- chicken and viremic chickens of other groups (ntV+A- or V+A+). Expression of gp85 was absent in the tissues from V-A+ meat-type or Line 0 chickens. Hence, there was a direct correlation between viremia and tissue distribution of gp85 regardless of the NAb response and strain of chickens since only viremic chickens (tV+A-, ntV+A-, V+A+) but not non-viremic seroconverted chickens (V-A+) expressed gp85 in tissues. However, ALV J proviral DNA was demonstrated in majority of the tissues tested from viremic (tV+A-, ntV+A-, V+A+) and non-viremic chickens (V-A+). The data suggest that gp85

expression was restricted to chickens exhibiting overt viremia in the presence (V+A+) or absence of a NAb response (tV+A-, ntV+A-) but not in seroconverted non-viremic chickens (V-A+). In contrast, ALV J proviral DNA was demonstrated in various tissues of both viremic as well as seroconverted non-viremic chickens.

## INTRODUCTION

Chickens of both meat-type and egg laying strains when infected *in ovo* with subgroup J avian leukosis virus (ALV J) develop tolerant viremia and lack neutralizing antibodies (Nabs) against the virus. In contrast, most egg- and some meat-type chickens, when infected at hatch or early in life, develop efficient NAbs that eliminate viremia for prolonged periods of time. A unique characteristic of meat-type chickens is that ALV J infection at hatch or early in life induce persistent viremias even in the presence of NAbs against the inoculated parental virus (V+A+) (Pandiri, 2005a; Witter *et al.*, 2000). This has also been demonstrated in other ALV subgroups and hosts. Geryk *et al.* demonstrated the reappearance of viremia in ducks infected with ALV C in the presence of NAb (Geryk *et al.*, 1996). This situation is similar to bonafide persistent viruses such as lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), hepatitis B and C, foot and mouth disease virus (FMDV) and other RNA viruses (Ahmed *et al.*, 1997). In addition, recent work in our laboratory demonstrated that adult seroconverted male chickens free of viremia and cloacal shedding for a prolonged period of time reverted to viremia and cloacal shedding when subjected to adrenocorticotropin-induced stress (Pandiri, 2005c). However, the localization of ALV J antigen or its provirus in the tissues of adult seroconverted chickens is poorly understood.

Several studies on ALV tissue tropism were conducted to demonstrate the presence of virus in various tissues of the chicken by using electron microscopy (Di Stefano and Dougherty, 1969; Di Stefano *et al.*, 1973; Dougherty and Di Stefano, 1967; Gilka and Spencer, 1985); immunohistochemistry (Arshad *et al.*, 1997; Dougherty *et al.*, 1972; Gharaibeh *et al.*, 2001; Gilka and Spencer, 1984; Williams *et al.*, 2004); and molecular methods (Arshad *et al.*, 1999; Robinson *et al.*, 1993; Stedman *et al.*, 2001). All of the above studies demonstrated ALV protein or nucleic acid in chickens that were essentially viremic. The chickens were inoculated either *in ovo* or at day of hatch and the sampling mostly coincided with the viremic phase of the virus. Rarely, sampling was done in chickens that have seroconverted with no overt viremia (Arshad *et al.*, 1997). Arshad *et al.*, monitored ALV J serology in their tissue tropism experiments, but no attempts were made to relate the effect of ALV J viremia and NAb status history on distribution of viral antigens. Hence, the objective of this study was to demonstrate viral env antigen gp85 as well as provirus in various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus) from 32 week old commercial meat-type and ADOL Line 0 chickens with different infectious profiles:1) viremic chickens tolerized *in ovo* with no NAb on any sampling (tV+A-); 2) viremic chickens infected at hatch with NAb on one or two samplings (ntV+A-); 3) viremic chickens infected at hatch with concurrent NAb on most occasions (V+A+); 4) non-viremic chickens infected at hatch with good NAb response (V-A+).

## MATERIALS AND METHODS

**Chickens.** Commercial meat-type chickens and ADOL Line 0 chickens (Crittenden and Fadly, 1985) were used in this study. The chickens were free from other avian pathogens as tested by routine diagnostic protocols and were housed in floor pens maintained as isolation units under biosecurity level-2 (BL-2) containment for 32 weeks. Line 0 chickens were provided feed and water *ad libitum* but feed was restricted for commercial meat-type chickens to limit excess body weight gain as recommended by the breeder.

**Viruses.** Strain ADOL Hc1, the U.S. prototype of ALV J (Fadly and Smith, 1999) and a molecularly cloned ALV J ADOL R5-4 derived from a field ALV J strain R5-4 (Lupiani *et al.*, 2003) were used in this study. ADOL R5-4 was demonstrated to have similar biological characteristics as ADOL Hc1 (Lupiani *et al.*, 2003). Viruses were propagated and titrated in line 0 C/E chicken embryo fibroblasts (CEFs).

**Experimental design.** Both Line 0 and commercial meat-type chickens were inoculated with ADOL R5-4 at day of hatch (DOH) and housed in separate floor pens. In addition, one Line 0 chicken was tolerized *in ovo* with ADOL Hc1 and housed in a Horsfall-Bauer isolator. Chickens were sampled for viremia and NAb response at 8, 12, 16, 20, 24 and 32 weeks post hatch. The viremia and NAb data from the above samplings were summarized as follows: 1) tV+A-; 2) ntV+A-; 3) V+A+; and 4) V-A+. Six Line 0 (tV+A-, and V-A+) and 18 commercial meat-type (ntV+A-, V+A+ and V-A+) chickens with defined infection profiles were selected from a total population of 150 chickens (75 for each chicken strain) for this study. Tissues collected for immunohistochemistry

(IHC) and polymerase chain reaction (PCR) assays included adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus. Tissues were embedded in Tissue-Tek® O.C.T compound (Sakura Finetek USA, Inc. Torrance, CA) and snap frozen in liquid nitrogen for IHC studies. Genomic DNA was isolated from all the above tissues for PCR.

**Virological and serological assays.** Virus isolation (VI) and virus neutralization (VN) assays were done as described earlier (Fadly and Witter, 1998). Briefly, VI was done by inoculating plasma on C/E CEFs and performing a p27 gsa -ELISA on the cell culture lysates after 7-9 days of incubation as described earlier (Smith *et al.*, 1979). Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens. In précis, about 500 - 1000 ALV J viral particles were incubated with equal volume of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 minutes at 37 °C. After incubation, C/E CEFs were added to this mixture and incubated for 7-9 days. After incubation, cell culture lysates were tested for p27 gsa by ELISA as described earlier (Smith *et al.*, 1979).

**Pathology.** All chickens necropsied were examined for tumors by gross and microscopic methods. Tissues from tumors were fixed in 10 % neutral buffered formalin for microscopic evaluation. All tissues were processed, sectioned and stained with hematoxylin and eosin.

**Polymerase Chain Reaction (PCR).** Genomic DNA was extracted from different tissues using Puregene™ DNA Isolation Kit (Gentra System Inc, Minneapolis, MN), and amplified with oligonucleotide primers specific for the *env* gene of ALV J. The PCR was conducted using 4 separate oligonucleotide primers pairs (6J/Smith2, F5/Smith2, H5/R11, H5/H7) amplifying the *env* gene of ALV J provirus (Silva *et al.*, 2000; Smith *et al.*, 1998). The sequence of the oligonucleotide primer pairs were as follows: 6J 5'- CTT GCT GCC ATC GAG AGG TTA CT - 3', F5 5'- GGT ATT TTC TTG ATT TGT GGG G - 3', Smith2 5'- AGT TGT CAG GGA ATC GAC - 3', H5 5'-GGA TGA GGT GAC TAA GAA AG - 3', R11 5'- TGG GGG TGG GAA GGG AGG GT - 3', and H7 5'-CGA ACC AAA GGT AAC ACA CG - 3'. Reactions were conducted in 0.25 ml eppendorf tubes free of DNA and RNA. The final volume of each reaction was 25 µl. The amount of template in each reaction was 50 ng of DNA in 1 µl volume. The master mix consisted of 3.5 µl 10 x PCR buffer (10 mM Tris-Hcl (pH 8.3 at 25 °C), 500 mM KCl, and 15 mM $MgCl_2$), 1.5 µl (12.5 pM) of forward primer (6J, F5, H5), 1.5 µl (12.5 pM) of reverse primer (Smith2, R11), 0.2 µl (100 mM) of dNTPs, 0.125 µl of Taq polymerase, and 18.2 µl of water. The PCR conditions for 6J/Smith2 or F5/Smith 2 oligonucleotide primer sets were 95 °C for 3 minutes, 95 °C for 1 minute, 57 °C for 1 minute, 72 °C for 2 minutes, go to step 2 for 29 times, 72 °C for 5 minutes, and 4 °C hold. The PCR conditions for oligonucleotide primers H5/R11 were similar to 6J-Smith2 or F5-Smith2 except for a lower extension time of 30 seconds. A 'touch down' PCR was performed using oligonucleotide primers H5/H7 following the published protocols (Smith *et al.*, 1998). The PCR amplified products were run on a 1% agarose gel. The product sizes for 6J/Smith2, F5/Smith2, H5/R11 and H5/H7 are 2.3 kb, 1.5 kb, 445 b, 545 b respectively.

**Immunohistochemistry (IHC).** A modified avidin-biotin peroxidase complex method (Hsu *et al.*, 1981) using the Vectastain® ABC kit (Vector Laboratories; Burlingame, CA) was performed. Briefly, 5 μM frozen sections were cut in a cryostat, were mounted on clean poly-L-Lysine (Sigma Diagnostic, ST. Louis, MO) coated glass slides and vacuum dried overnight at room temperature. The dried cryosections were fixed in acetone for 45 minutes at room temperature, air dried, and stained or stored at -70 °C until further processing. Samples were hydrated for 15 minutes in isotonic phosphate buffered saline (PBS), pH=7.4. Endogenous biotin was blocked using an Avidin-Biotin blocking kit (Vector Laboratories Inc, Burlingame, CA) following manufacturer's instructions. Sections were pre-incubated for 20 min. with normal blocking serum to decrease nonspecific background staining due to secondary antibody. Between the remaining steps slides were washed three times for 5 minutes each in PBS.

Samples were incubated at room temperature with G2-3 monoclonal antibody (Qin *et al.*, 2001) that is specific for the gp85 protein of ALV J in a 1:500 concentration for 30 minutes followed by incubation at room temperature with the biotinylated secondary antibody for 30 minutes. The sections were incubated for 30 minutes with an avidin-biotin-peroxidase complex. After the PBS wash, the immunohistochemical reaction was visualized following incubation with a solution of hydrogen peroxide and 3, 3′ diaminobenzidine (DAB) kit Vector® SK-4100 (Vector Laboratories; Burlingame, CA) for 7 min. All sections were lightly counterstained with Gill's hematoxylin #2, dehydrated and mounted in Refrax mounting medium (Anatech Ltd., Battlecreek, MI).

**Scoring of tissues.** The slides were read without the knowledge of infection profile to avoid bias. The gp85 staining was scored as 0 (no positive cells), 1 (a few scattered positive cells), 2 (moderate number of positive cells), and 3 (large number of positive cells). Mean tissue scores were calculated for each tissue within each group of chickens.

## RESULTS

**Viral antigen distribution in various tissues from chickens with different ALV J infection status.** Results of gp85 viral antigen expression in various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus) from chickens with different ALV J infection profiles are summarized in table 5.1. Most ntV+A- and V+A+ chickens tested positive for gp85 expression with a score of 1.2 to 3.0 in all tissues, with the exception of sciatic nerve. There were no obvious differences in mean tissue scores between ntV+A- (1.2 to 3.0) and V+A+ (1.3 to 2.3) groups. However, the tolerant viremic chicken (tV+A-) showed high level expression of gp85 in all tissues (3.0); sciatic nerve had a score of 1.0. In contrast to viremic chickens, no gp85 expression was observed in any tissue from both meat-type and Line 0 chickens classed as V-A+ chickens.

Distribution of gp85 expression in each tissue collected from ntV+A-, V+A+ and tV+A- chickens is shown in table 5.2. Since no major differences in the pattern of gp85 distribution were observed between tissues collected from ntV+A- and V+A+ chickens, the results are presented together. However, distribution of gp85 expression in the tissues

from the tV+A- chicken differed from that of ntV+A- and V+A+ chickens. Only tV+A- chicken had expression of gp85 in sciatic nerve (nerve fibers and endothelial cells), and lymphocytes (thymus, spleen and lymphoid aggregates in proventriculus, kidney and liver) (figure 5.1).

Several chickens with different ALV J infection profiles developed tumors *viz.* nephroblastoma (one V+A+ chicken), myelocytoma (two V+A+ chickens and one tV+A- chicken) and hemangiopericytoma (one ntV+A- chicken). In all cases, tumor cells demonstrated a very strong gp85 expression (3.0) (figure 5.2).

**Distribution of proviral DNA in various tissues isolated from chickens with different ALV J infection profile.** The results of these studies demonstrated the presence of provirus in the genomic DNA from various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus) from meat-type and Line 0 chickens classed as tV+A-, ntV+A-, V+A+, and V-A+ are summarized in table 5.3. Primer sets 6J/Smith2 and F5/Smith2 amplifying *env* sequences yielded almost identical PCR results. All tissues collected from viremic chickens (tV+A-, ntV+A-, and V+A+) consistently demonstrated ALV J proviral DNA. However, no ALV J proviral DNA was detected in any of the tissues collected from V-A+ meat-type or Line 0 chickens. There were no differences in tissue distribution of ALV J provirus in any of the tissues tested from viremic chickens (tV+A-, ntV+A-, and V+A+). Results obtained using H5/R11 or H5/H7 primer sets amplifying *env* sequences yielded similar results for tissues collected from viremic tV+A-, ntV+A-, and V+A+ meat-type or Line 0

chickens. However, H5/R11 PCR results for tissues from V-A+ meat-type or Line 0 chickens were not clear due to spurious amplification and no conclusive results could be obtained in spite of performing a PCR under "touch down " conditions. PCR results using H5/H7 oligonucleotide primers on tissues collected from V-A+ meat-type or Line 0 chickens were unambiguous with no spurious amplification but the amplified PCR product was faint. The PCR amplified product in viremic chickens (V+A-, ntV+A- and V+A+) yielded a bright band upon UV-transillumination unlike the PCR-amplified product from seroconverted chickens (V-A+) that yielded a faint band. This differential pattern, in the intensity of the PCR-amplified product upon UV-transillumination, between viremic and seroconverted chickens was fairly consistent. The pattern of tissue distribution of ALV J proviral DNA was dissimilar in seroconverted viremia-free meat-type and Line 0 chickens. Tissues from V-A+ Line 0 chickens demonstrated proviral DNA more frequently than V-A+ meat type chickens. All the tumor tissues tested in the study were positive for ALV J proviral DNA with any of the above primer sets (data not presented).

## DISCUSSION

Results demonstrated that the distribution of gp85 was directly correlated to the viremia status of the chicken, regardless of the NAb status. High levels of gp85 expression was found in viremic chickens (tV+A-, ntV+A- and V+A+), but not in non-viremic seroconverted chickens (V-A+). However, proviral DNA was observed in majority of the tissues collected from viremic chickens as well as non-viremic seroconverted chickens although the latter had apparently lower proviral DNA levels. The tissue distribution of

ALV J antigen was in agreement with previous studies (Arshad *et al.*, 1997; Dougherty and Di Stefano, 1967; Gharaibeh *et al.*, 2001; Stedman *et al.*, 2001; Williams *et al.*, 2004). All previously reported studies used tissues from viremic chickens and had extensive distribution of gp85, similar to tV+A-, ntV+A- and V+A+ chickens used in the current study. In addition, this study shows that gp85 and ALV J proviral distribution was similar in viremic chickens with (V+A+) or without NAbs (ntV+A-).

Minor differences were found in the pattern of gp85 expression between tV+A- and non-tolerized viremic chickens (ntV+A-, V+A+). The tV+A- chicken had positive staining in the nerve fibers and endothelial cells in the sciatic nerve, and lymphoid aggregates in several tissues. This might be explained by the total lack of immune response against ALV J in tolerized chickens or it might be due to differences in viral strain used to inoculate the tV+A- chicken in this study. The latter hypothesis is very unlikely since ADOL R5-4 and ADOL Hc1 have been shown to have very similar biological properties (Lupiani *et al.*, 2003). Also, gp85 expression was consistently detected in sciatic nerve, and lymphocytes as in other studies involving tolerized chickens that had been inoculated with different ALV J strains (Arshad *et al.*, 1997; Stedman *et al.*, 2001; Williams *et al.*, 2004).

The presence of NAb did not influence the gp85 or the proviral DNA distribution in viremic meat-type chickens (V+A+). The V+A+ category was included only for meat-type chickens since this infection pattern is very common in this type of chickens but occurs very rarely in Line 0 chickens (Mays *et al.*, 2005; Pandiri, 2005b; Witter *et al.*,

2000). Previous data from ALV J infection in Line 0 and meat-type chickens always demonstrated the ability of Line 0 chickens to clear viremia with an efficient NAb response better than meat-type chickens (Mays *et al.*, 2005). The high incidence of V+A+ in meat-type chickens following infection at hatch may be due to the emergence of ALV J NAb escape variants that were not neutralized by the circulating antibodies against the inoculated parent virus (Pandiri, 2005b). Besides of the role of humoral immune response in the high incidence of V+A+ in meat-type chickens, the exact role of other aspects of the immune system or the impact of chicken's endogenous viruses needs to be determined.

Our previous studies demonstrated that ALV J seroconverted chickens could revert to viremia when subjected to ACTH-induced stress (Pandiri, 2005c). One of the main objectives of the current study was to elucidate the distribution of virus in chickens that have successfully cleared ALV J viremia with a consistent NAb response (V-A+). We were able to demonstrate ALV J provirus in both meat-type and Line 0 chickens that had cleared viremia (V-A+) using oligonucleotide primer set H5/H7 but not 6J/Smith2, F5/Smith2 or H5/R11. However, the pattern of proviral distribution was variable in tissues from both V-A+ meat-type and Line 0 chickens. The differences in the intensity of the PCR-amplified product upon UV-transillumination between viremic and seroconverted chickens were fairly consistent. This may be due to the very low ALV J proviral copy number in the tissues of seroconverted chickens. This can also be attributed to the limitations in the sensitivity of our testing system. The oligonucleotide primer set H5/H7 was able to amplify a single defined product unlike the products of

other primer sets like F5/Smith2 or H5/R11 where multiple nonspecific bands were observed especially in meat-type chickens. This study reveals the limitation of the current molecular techniques and demonstrates the importance of using more sensitive and specific methods to detect ALV J provirus in V-A+ chickens.

Infection by retroviruses like ALV J involves reverse transcription and genomic integration of the virus in proviral DNA form. In this study, proviral DNA sequences but not gp85 expression was observed in seroconverted non-viremic chickens (V-A+) implying a latent ALV J infection in these chickens. Apparently, under some known (immunosuppression) (Pandiri, 2005c) and unknown conditions, the ALV J seroconverted non-viremic chickens are able to revert to viremia. This may be due to switching from latent to productive infection that is characterized by transcription of proviral DNA into viral mRNA and consequently into an infectious viral particle (Ahmed *et al.*, 1997). The factors responsible for switching the latent infection into a productive infection were not determined.

High expression of viral antigen (gp85) in ALV J-induced tumors was demonstrated in this study. This finding contrasts with previous work by Arshad *et al.* since they did not demonstrate viral antigen (p27 gsa) expression in neoplastic tissues (Arshad *et al.*, 1997). This may be due to technical factors since they used paraffin sections instead of cryosections. Also, Arshad *et al.* studied the expression of ALV p27 gsa rather than env antigen gp85. Finally, differences in the viral strain in both the studies might have contributed to the discrepancy.

To the knowledge of the authors, this is the first study that demonstrated tissue specific expression of ALV J antigen as well as proviral DNA in chickens in the context of various infectious profiles. This work expands the current knowledge of ALV J distribution in meat-type and Line 0 chickens with different infection profiles. Results demonstrated a direct correlation between viremia and tissue distribution of gp85, regardless of the NAb response and strain of chickens. Provirus could be detected in seroconverted viremia-free chickens but the pattern of distribution was dissimilar in meat-type and Line 0 chickens. In conclusion, ALV J proviral DNA was demonstrated in both viremic as well as seroconverted non-viremic chickens where as gp85 expression was restricted to chickens exhibiting overt viremia in the presence or absence of NAb response.

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**Table 5.1.** Distribution of ALV J antigen gp85 as demonstrated by immunohistochemistry using 6-23 monoclonal antibody.

| Inf. Status[1] | #[2] | Adr[3] | BM | Gon | Hrt | Kid | Liv | Lng | Pnc | Prv | Nrv | Spl | Thy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Broiler ntV+A-** | 5 | 5/5[4] | 2/5 | 3/5 | 5/5 | 5/5 | 4/5 | 3/5 | 5/5 | 5/5 | 0/5 | 5/5 | 5/5 |
| | | 2.4[5] | 1.5 | 1.3 | 1.8 | 2.2 | 1.0 | 2.0 | 2.4 | 3.0 | 0 | 1.4 | 1.2 |
| **Broiler V+A+** | 8 | 7/7 | 5/7 | 7/8 | 5/8 | 8/8 | 7/8 | 7/8 | 8/8 | 8/8 | 0/8 | 8/8 | 6/8 |
| | | 1.9 | 2.2 | 1.6 | 1.5 | 2.3 | 1.6 | 1.4 | 2.0 | 2.0 | 0 | 1.8 | 1.3 |
| **Broiler V-A+** | 5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Line 0 V-A+** | 5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Line 0 tV+A-** | 1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 |
| | | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 1.0 | 3.0 | 3.0 |

[1] viremia and neutralizing antibody (NAb) data from the above samplings were summarized as follows: tV+A- = viremic chickens tolerized *in ovo* with no NAb on any sampling; ntV+A- = viremic chickens infected at hatch with NAb on one or two samplings; V+A+ = viremic chickens infected at hatch with concurrent NAb on most occasions; V-A+ = non-viremic chickens infected at hatch with good NAb response.

[2] number of chickens for each group

[3] tissues tested for the presence of gp85 viral antigen by IHC included adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus.

[4] number of positive tissues per total number of tissues tested

[5] mean tissues scores. The lowest is 0 and the maximum is 3.0

**Table 5.2.** Cell specific ALV J gp85 expression in various tissues from chickens classed as V+A+ & ntV+A-, and tV+A-

| Tissue | V+A+ & V+A- (Non tolerized)[1] | V+A- (Tolerized)[1] |
|---|---|---|
| **Adrenal gland** | chromaffin cells, a few interrenal cells | chromaffin cells, a few interrenal cells, lymphoid aggregates |
| **Bone marrow** | scattered distribution, ML[2] | scattered distribution, ML |
| **Gonad** | | |
| **Testis** | Leydig cells | NA[3] |
| **Ovary** | follicular epithelium, stromal cells | follicular epithelium, stromal cells |
| **Heart** | myofibers, endothelial cells | myofibers, endothelial cells |
| **Kidney** | glomeruli, tubules | glomeruli, tubules, lymphocytic infiltration |
| **Liver** | hepatic sinusoidal network, biliary ducts | hepatic sinusoidal network, biliary ducts |
| **Lung** | scattered distribution | wide distribution |
| **Pancreas** | acinar cells | acinar cells, lymphoid aggregates |
| **Proventriculus** | surface and glandular epithelium, muscle | surface and glandular epithelium, muscle, lymphoid aggregates |
| **Sciatic nerve** | None | endothelial cells, nerve fibres |
| **Spleen** | MNPC[4], endothelial cells | MNPC, endothelial cells, germinal centers and other scattered lymphocytes |
| **Thymus** | MNPC, endothelial cells | MNPC, endothelial cells, scattered cortical and medullary lymphocytes |

[1] The viremia and neutralizing antibody (NAb) data from the above samplings were summarized as follows: tV+A- = viremic chickens tolerized *in ovo* with no NAb on any sampling; ntV+A- = viremic chickens infected at hatch with NAb on one or two samplings; V+A+ = viremic chickens infected at hatch with concurrent NAb on most occasions; V-A+ = non-viremic chickens infected at hatch with good NAb response.
[2] myelocytoma
[3] not available
[4] mononuclear phagocytic cells

**Table 5.3.** Distribution of ALV J proviral DNA as demonstrated by polymerase chain reaction using H5/H7 oligonucleotide primer set[1].

| Inf. Status[2] | #[3] | Adr[4] | BM | Gon | Hrt | Kid | Liv | Lng | Pnc | Prv | Nrv | Spl | Thy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Broiler ntV+A-** | 5 | 5/5[5] | 5/5 | 3/3 | 4/4 | 5/5 | 5/5 | 3/3 | 5/5 | 5/5 | 2/4 | 3/3 | 5/5 |
| **Broiler V+A+** | 8 | 5/6 | 6/7 | 6/6 | 8/8 | 8/8 | 7/8 | 6/7 | 8/8 | 8/8 | 6/7 | 6/7 | 6/7 |
| **Broiler V-A+** | 4 | 3/4 | 1/4 | 0/4 | 1/4 | 0/4 | 0/4 | 1/4 | 2/4 | 2/4 | 2/4 | 2/4 | 1/3 |
| **Line 0 V-A+** | 5 | 2/5 | 2/4 | 1/5 | 5/5 | 4/5 | 2/5 | 4/5 | 3/5 | 2/5 | 0/5 | 4/5 | 2/4 |
| **Line 0 tV+A-** | 1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 |

[1] 4 different oligonucleotide primer sets were used in this study. Primer sets 6J/Smith2 and F5/Smith2 were able to amplify proviral DNA from only viremic chickens but were unable to detect provirus from non-viremic seroconverted chickens. Though H5/R11 and H5/H7 primer sets were able to detect provirus in all infected chickens with or without overt viremia but only H5/H7 gave unambiguous PCR results without any spurious amplification like H5/R11.

[2] The viremia and neutralizing antibody (NAb) data from the above samplings were summarized as follows: tV+A- = viremic chickens tolerized *in ovo* with no NAb on any sampling; ntV+A- = viremic chickens infected at hatch with NAb on one or two samplings; V+A+ = viremic chickens infected at hatch with concurrent NAb on most occasions; V-A+ = non-viremic chickens infected at hatch with good neutralizing NAb response.

[3] number of chickens for each group

[4] Tissues tested for the presence of ALV J proviral DNA by PCR included adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus.

[5] number of positive tissues per total number of tissues tested

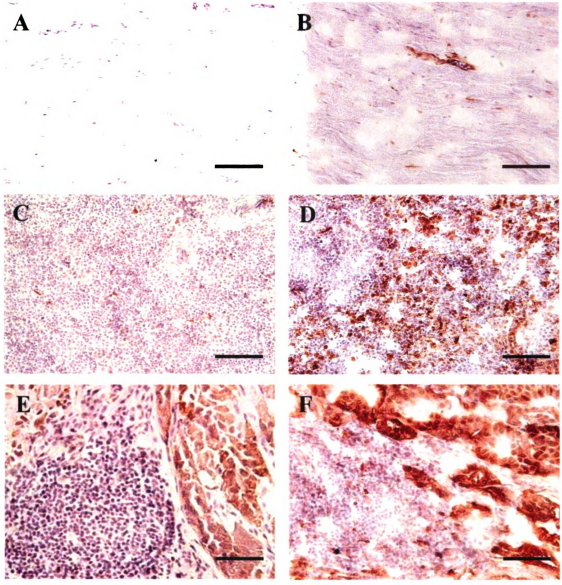


**Figure 5.1.** Distribution of gp85 in various tissues from 32 week old chickens with ntV+A- (A, C, E), and tV+A- (B, D, F) ALV J infectious status. Sections were stained with an ALV J gp85 specific monoclonal antibody using a modified avidin-biotin peroxidase complex method (Vectastain® ABC kit) and lightly counter stained with Gill's #2 hematoxylin. (A) Nerve section from a ntV+A- chicken with no gp85 expression. Bar = 120 μM (B) Nerve section from a tV+A- chicken with gp85 expression in endothelial cells, and nerve fibers. Bar = 120 μM (C) Spleen from a ntV+A- chicken with scattered reticular cells with gp85 expression. Bar = 120 μM (D) Spleen from a tV+A- chicken with gp85 expression in macrophages, endothelial cells, and lymphocytes. Bar = 120 μM (E) Proventriculus from a ntV+A- chicken with strong gp85 expression in glandular epithelium but no staining in lymphoid aggregates. Bar = 100 μM (F) Proventriculus from a tV+A- chicken with strong gp85 expression in glandular epithelium and lymphoid aggregates. Bar = 100 μM.

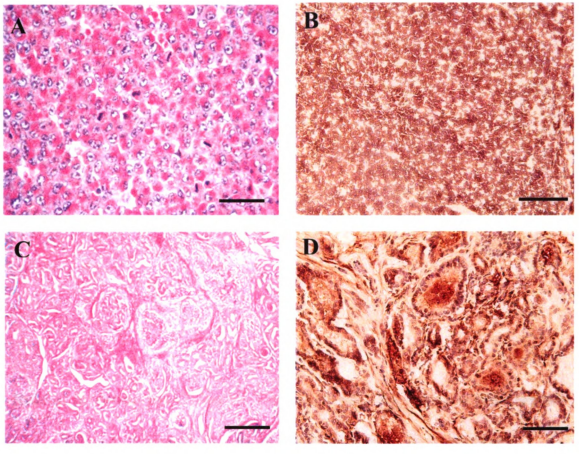


**Figure 5.2.** Hematoxylin and eosin staining, and gp85 staining in ALV J induced tumors. Sections were stained with an ALV J gp85 specific monoclonal antibody using a modified avidin-biotin peroxidase complex method (Vectastain® ABC kit) and lightly counter stained with Gill's #2 hematoxylin. (A). ALV J-induced myelocytoma characterized by myelocytes with abundant mitotic figures indicating the malignant nature of the tumor. Bar = 50 μM (B). gp85 staining in bone marrow invaded by neoplastic myelocytes. Bar = 200 μM (C). ALV J-induced nephroblastoma characterized by primitive nephrons. Bar = 100 μM (D). gp85 staining in ALV J-induced nephroblastoma. Bar = 100 μM.

# CHAPTER 6

## Role of infection profile on the frequency of subgroup J avian leukosis virus-induced histiocytic sarcomas in meat type chickens

**ABSTRACT**

Histiocytic proliferative lesions diagnosed as multicentric histiocytosis (MH) have been reported with increasing frequency at poultry processing plants within the last 15 years. Subgroup J avian leukosis virus (ALV J) has been implicated in some cases and the lesion has been characterized as histiocytic sarcomatosis (HS). The role played by viral and host factors in the pathogenesis of HS is poorly understood. A retrospective study was done to study the epidemiology and pathogenesis of ALV J-induced HS by using samples from previous experiments. The effect of ALV J strain and dose, and age at infection on the incidence of HS was evaluated, retrospectively, in a study involving 374 meat-type chickens. Also, the effect of genetic line on the incidence of HS-like lesions was evaluated in an experiment involving 75 meat-type chickens and 100 ADOL Line 0 chickens. There was no effect of strain or dose of ALV J on the incidence of HS since only chickens inoculated with ALV J at hatch but not *in ovo* developed this lesion. HS was observed in meat-type chickens but not in ADOL Line 0 chickens and the incidence was up to 4%. Chickens that developed HS were persistently viremic and developed little or no neutralizing antibodies (NAb) against the inoculated virus. HS was consistently observed in spleen along with frequent involvement of liver and kidney. Microscopically, the proliferating histiocytes were polyhedral to spindle-shaped cells with abundant eosinophilic cytoplasm and anisokaryosis with various levels of invasiveness. Foamy macrophages exhibiting erythrophagocytosis and rare multinucleate giant cells were observed within the lesions. Tumor cells stained markedly positive for ChL5 (myelomonocytic cells and activated T cells), K55 (common leukocyte antigen CD45), and CIa (major histocompatibility complex II (MHC II)), slightly positive for K1

(macrophages and thrombocytes), and negative for CB4 & CB5 (B cells), and p53. In addition, some lesions had infiltration of minor proportion of cells positive for CT3 (CD3), CT4 (CD4), CT8 (CD8) and ALV J (6-23). Based on the above data, these histiocytic proliferations appeared to be histiocytic sarcomas of myeloid origin.

## INTRODUCTION

Histiocytic proliferative lesions have been described in chickens since 1916 (Pentimalli, 1916). The incidence of these lesions was sporadic and variable (up to 90%) and viral etiology was suspected although the exact nature of the causative agent was not identified (Campbell, 1943; Jackson, 1936; McGowan, 1928; Olson and Bullis, 1942; Pentimalli, 1916; Perek, 1960). In the early 1990s, histiocytic proliferative lesions were observed with increasing incidence in broilers condemned in poultry processing plants as "leukosis" and were described as multicentric histiocytosis (MH) (Hafner *et al.*, 1996). Hafner *et al.* could not determine the etiology of MH in this study (Hafner *et al.*, 1996). Later, Goodwin *et al.* (1999) reproduced MH in broilers as well as in specific pathogen free (SPF) leghorns by inoculating tissue homogenates collected from organs of chickens affected with MH (Goodwin *et al.*, 1999). However, the etiology of MH could not be confirmed since several avian retroviruses including ALVs and reticuloendotheliosis virus (REV) were detected.

Arshad *et al.* reported similar lesions in the context of ALV J infections from both field and experimental cases (Arshad *et al.*, 1997). They characterized these lesions as histiocytic sarcomatosis (HS) based on histochemistry, immunohistochemistry and

transmission electron microscopy. In experiments involving line 21 strain of meat-type chickens infected with ALV J at hatch, ALV J-induced HS was reported at an incidence of 1.1%. In addition, the absence of these lesions in leghorn-type chickens inoculated at hatch or *in ovo* and in line 21 strain of meat-type chickens inoculated *in ovo* was also reported (Arshad *et al.*, 1997). However, the role played by ALV J infection profile in the incidence of HS was not evaluated. Based on the available information, it remains unclear if ALV J-induced HS and the previously reported MH is the same lesion.

In our previous ALV J studies, we have also observed HS mainly in the spleen with occasional to frequent concurrent incidence in liver and kidney. These lesions were only observed in several commercial meat-type chicken strains but never in ADOL line 0 and White Rock chicken strains. The incidence of HS varied from 1.6%-6.4% depending on the meat-type chicken strain.

The objective of this study was to evaluate the role of strain and dose of ALV J, age at infection as well as chicken genetic line in the pathogenesis of HS. The role of ALV J persistence and the host immune response in inducing HS-like lesions was also analyzed. In addition, we further characterized ALV J-induced HS by using histochemistry and immunohistochemistry.

## MATERIALS AND METHODS

**Experimental design.** Information and samples used in this study were obtained from two experiments aimed at studying different aspects of ALV J persistence (Pandiri, 2005a,

b). A retrospective study was conducted to study the effect of strain and dose of ALV J, and age at inoculation on the development of ALV J-induced HS. In an experiment reported elsewhere (Pandiri, 2005a), 374 commercial meat-type chickens were inoculated at 5$^{th}$ day of embryonation (*via* Y/S route) or at day of hatch (*via* intra-abdominal route) with either 100 $TCID^{50}$ or 10,000 $TCID^{50}$ with one of the three ALV J strains (ADOL Hc1, ADOL 4817, ADOL 6803) (Fadly and Smith, 1999). Samples for histochemical and immunohistochemical characterization of HS were also obtained from this experiment. The effect of chicken strain on the development of ALV J-induced HS was studied retrospectively in an experiment that has been reported elsewhere (Pandiri, 2005b). In brief, 75 commercial meat-type chickens and 75 white leghorn line 0 chickens (Crittenden and Fadly, 1985) were infected at hatch (*via* intra-abdominal route) with 1,000 $TCID^{50}$ of ALV J molecular clone ADOL pR5-4 (Lupiani *et al.*, 2003). All chickens were housed in BL-2 containment.

Retrospective analysis of ALV J infection profile of chickens in this study were obtained from previous experiments where sampling for viremia and NAb was done on 6-9 occasions before the study was terminated at 32 weeks post hatch. At necropsy, tissues with gross pathology were collected and fixed in 10% neutral buffered formalin for histochemical evaluation. In addition, the same tissues were also embedded in Tissue-Tek® O.C.T compound (Sakura Finetek USA, Inc. Torrance, CA) and snap frozen in liquid nitrogen for immunohistochemistry studies.

**Virological and serological assays.** Virus isolation (VI) and virus neutralization (VN) assays were done as described earlier (Fadly and Witter, 1998). In brief, VI was done by inoculating plasma on C/E chicken embryo fibroblasts (CEFs) and performing a p27 gsa-ELISA on the cell culture lysates after 7-9 days of incubation as described earlier (Smith *et al.*, 1979). Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens as previously described. In précis, about 500 - 1000 ALV J viral particles were incubated with equal volume of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 minutes at 37 °C. After incubation, C/E CEFs were added to this mixture and incubated for 7-9 days. After incubation, cell culture lysates were tested for p27 gsa by ELISA as described earlier (Smith *et al.*, 1979).

**Histology.** Tissues for histopathology were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were cut at 5 µm thickness and processed through graded alcohols and xylene, and stained by hematoxylin and eosin (H&E), Giemsa, Snook's reticulin, and Van Gieson's trichrome methods.

**Immunohistochemistry.** All tissues were stained with a panel of 11 monoclonal or polyclonal antibodies. These include 6-23 (Qin *et al.*, 2001) at 1:500 dilution (ALV J gp85), 5M19 (ChL5) (Barth *et al.*, 1990) at 1:1 dilution (myelomonocytic cells and activated T lymphocytes), K55 (Chung *et al.*, 1991) at 1:10 dilution (common leukocyte antigen CD45), CIa (Ewert *et al.*, 1984) at 1: 20 dilution (MHC II), CT3 (Chen *et al.*, 1986) at 1:20 dilution (CD3), CT4 (Chan *et al.*, 1988) at 1:20 dilution (CD4), CT8 (Chan

*et al.*, 1988) at 1:1 dilution (CD8), K1 (Chung and Lillehoj, 1991) at 1:5 dilution (macrophages and thrombocytes), CB4 and CB5 (Chen and Cooper, 1987) at 1:25 dilution (B cells), and p53 (Abcam, CA) at 1:100 dilution.

A modified avidin-biotin-peroxidase complex method (Hsu *et al.*, 1981) using the Vectastain® ABC kit (Vector Laboratories; Burlingame, CA) was performed. Briefly, 5 µM frozen sections were cut in a cryotome, mounted on clean poly-L-Lysine (Sigma Diagnostic, ST. Louis, MO) coated glass slides and vacuum dried overnight at room temperature. The dried cryosections were fixed in acetone for 45 minutes at room temperature, air dried, and stained or stored at -70 °C until further processing. Samples were hydrated for 15 minutes in isotonic phosphate buffered saline (PBS), pH=7.4. Endogenous biotin was blocked using an Avidin-Biotin blocking kit (Vector Laboratories Inc, Burlingame, CA) following manufacturer's instructions. Sections were pre-incubated for 20 min. with normal blocking serum to decrease nonspecific background staining due to secondary antibody. Between the remaining steps, slides were washed three times for 5 minutes each in PBS.

All incubations were conducted at room temperature. Samples were incubated for 30 minutes with different monoclonal antibodies at appropriate dilutions as discussed above. This was followed by incubation with the biotinylated secondary antibody for 30 minutes. The sections were incubated for 30 minutes with an avidin-biotin-peroxidase complex. After the PBS wash, the immunohistochemical reaction was visualized following incubation with a solution of hydrogen peroxide and 3, 3′ diaminobenzidine

(DAB) kit Vector® SK-4100 (Vector Laboratories; Burlingame, CA) for 7 min. All sections were lightly counterstained with Gill's hematoxylin #2, dehydrated in graded alcohols and mounted in Refrax mounting medium (Anatech Ltd., Battlecreek, MI).

Specific brown DAB staining was visualized using a light microscope and the tissues were scored as 0 (no positive cells), 1 (a few scattered positive cells), 2 (moderate number of positive cells), and 3 (large number of positive cells).

## RESULTS

### Factors influencing the development of ALV J-induced HS lesions (Table 6.1).

*Effect of viral strain, dose and age at infection.* There was no effect of strain of ALV J or dose of viral inoculum on the incidence of HS since lesions of varying severity were observed in spleen, liver, and kidney in meat-type chickens inoculated with either 100 $TCID^{50}$ or 10,000 $TCID^{50}$ with one of the three ALV J strains (ADOL Hc1, ADOL 4817, and ADOL 6803). All of the HS lesions were only observed in chickens that were inoculated at day of hatch and the incidence of these lesions was 4%. None of the chickens inoculated *in ovo* had evidence of histiocytic proliferative lesions.

*Effect of chicken strain.* HS lesions were observed only in meat-type chickens but not in ADOL line 0 chickens that were inoculated with ADOL pR5-4 at day of hatch. The incidence in meat-type chickens was 1.3%.

*Effect of ALV J infection profile.* All the affected chickens had persistent viremia on almost all the sampling intervals and had very little to no NAb against the inoculated virus. The lesions were observed in chickens that had succumbed to disease starting from 11 weeks post hatch (PH) until the study was terminated at 32 weeks post hatch.

**Histopathology.** In affected chickens, the histiocytic proliferative lesions were always observed in spleen with or without concurrent incidence in the liver followed by even lower incidence in the kidney (Figure 6.1 A-D). These lesions can be described histologically as severe diffuse and/or coalescing multifocal proliferations of polyhedral to spindle-shaped histiocytes with abundant eosinophilic cytoplasm and anisokaryosis. The proliferating histiocytic cells followed a palisading or whorled or haphazard pattern of distribution (Figure 6.2 A-D). In general, the nuclei were open-faced with a nucleolus but some nuclei had condensed chromatin with no obvious nucleoli. About 1-5 normal to bizarre mitotic figures per 40X field were common in some sections. The cytoplasm was often slightly vacuolated and the cell margins were not obvious in some areas giving the appearance of faux-multinucleate giant cells. However, multinucleate giant cells due to fusion of the histiocytes were observed on rare occasions. In some lesions, the histiocytes appeared large with foamy eosinophilic cytoplasm and there was evidence of erythrophagocytosis in such cases (Figure 6.3 A-D). Reticulin staining as demonstrated by Snook's method, depicted breakdown of reticulin network leading to formation of large spaces devoid of reticulin or a haphazard reticulin distribution in the proliferative areas. The normal architecture of the parenchyma was nearly lost in many cases (Figure 6.4 A, B). Collagen as demonstrated by van Gieson's trichrome staining was minimal to

none in almost all cases. In some cases, the histiocytic proliferations were accompanied by neoplastic cells of myeloid lineage *viz.* myelocytes, and myeloid stem cells. Infiltration of variable numbers of lymphocytes, plasma cells and heterophils was observed in some histiocytic proliferative lesions (Figure 6.5 A-C). There were a few minor differences in these histiocytic proliferations based on the distribution and invasiveness. Some lesions were clearly circumscribed or mildly invasive, focal or multifocal histiocytic proliferations within the splenic parenchyma. Alternatively, other lesions included extensive multifocal to diffuse histiocytic proliferations in splenic parenchyma that were either circumscribed or very invasive, with metastasis to liver and kidney. The proliferating metastatic histiocytes were observed in the portal vessels either as solitary cells or as metastatic emboli completely blocking the vessel (Figure 6.6 A, B). In some cases, the metastatic histiocytes appeared to proliferate on the tunica intima of the portal vessels. The proliferating histiocytic foci in hepatic parenchyma were lined by sinusoidal cells, giving the appearance of metastasis through the hepatic sinusoids (Figure 6.6 C, D).

**Immunohistochemistry (Table 6.2, Figure 6.7 A-F).** The proliferating tumor cells stained strongly positive with ChL5 (myelomonocytic cells and activated T lymphocytes) (Figure 6.7 A, B), K55 (common leukocyte antigen CD45) (Figure 6.7 C, D), and CIa (MHC II) (Figure 6.7 E, F) antibodies, slightly positive with K1 (macrophage and thrombocytes), and negative with CB4/CB5 (B lymphocytes), and p53 antibodies. In addition, there was infiltration of variable proportions of cells staining positive with CT3

(CD3), CT4 (CD4) (Figure 6.5B), CT8 (CD8) (Figure 6.5C) and 6-23 (ALV J gp85) (Figure 6.8 A, B) MAbs.

## DISCUSSION

Chicken strain, age at infection and ALV J-infection profile had major effects on the development of ALV J-induced HS lesions since only meat-type chickens that were inoculated at hatch and were persistently viremic developed these lesions. There was no apparent effect of viral strain or viral dose on the incidence of HS. Similar to earlier reports, HS lesions were not observed in meat-type chickens infected *in ovo*, or in egg-laying chickens infected with ALV J at hatch or *in ovo* (Arshad *et al.*, 1997). The histiocytic proliferative lesions described in this study were morphologically similar to earlier reports of MH (Hafner *et al.*, 1996) or HS (Arshad *et al.*, 1997). In addition, Arshad *et al.* also provided evidence that the cells comprising HS lesions are histiocytes of myelomonocytic origin (Arshad *et al.*, 1997).

All the chickens with HS lesions were persistently viremic with little or no NAb response against the inoculated virus. However it appears that the chickens with HS lesions were not tolerant to ALV J since they were able to mount an immune response, albeit an inefficient one, as evident by the presence of infiltration of numerous plasma cells, lymphocytes and heterophils in the affected tissues. It has been demonstrated earlier that spleens of ALV J-infected chickens have numerous antibody producing cells that are ineffective in clearing viremia (Russell *et al.*, 1997). Hence, the continued presence of the virus in the face of a patent immune system may cause reactive

hyperplasia of splenocytes that proliferate and eventually transform. This hypothesis may be valid since histiocytic proliferative lesions have never been observed in White Leghorns, White Rocks or meat-type chickens that were exposed to ALV J *in ovo* since they develop tolerant persistent viremia. Also, HS lesions have never been observed in White leghorns, White Rocks or even meat-type chickens capable of clearing ALV J-induced viremia by mounting an efficient NAb response (Arshad *et al.*, 1997). In addition, HS lesions had very low viral antigen expression (ALV J gp85) in contrast to high gp85 expression in other tumors like myelocytomas, and nephroblastomas that are products of ALV J-induced transformation events. However, based on our results we cannot rule out that HS is a result of activation of an oncogene as in other ALV-induced tumors and further studies are warranted.

In humans and canines, histiocytic proliferative disorders were studied in greater detail mainly due to the availability of various types of immunological cell markers. The availability of such markers in avians and other non-mammalian species is limited and hence a limited capability to conduct such studies. Most of the markers used in this study are not available commercially and were gifts from several labs. Based on the immunophenotype, the proliferating cells were histiocytes of myelomonocytic origin since they were positive for ChL5 (myelomonocytic cells and activated T cells), K55 (common leukocyte antigen CD45), and CIa (MHC II) and negative for CT3 (CD3), CT4 (CD4), CT8 (CD8), B4 and B5 (B cells). Remarkably, these cells were only slightly positive for K1 antigen which is very specific for macrophages as demonstrated by a strong positive staining of HD11 and MQ-NCSU cell lines that were used as a positive

control for K1 MAb (Kaspers *et al.*, 1993; Qureshi *et al.*, 1990). These lesions may not be lymphomas with histiocytic differentiation since they were negative for CD3, CD4, CD8 and several B cell markers. In addition, the variable cellular morphology (round cell, spindle cell, foamy cytoplasm), as well as erythrophagocytosis, hint at the histiocytic nature of the tumor. The possibility of these lesions being of dendritic cell origin is remote since mature dendritic cells lack phagocytic or endocytosing ability and immature dendritic cells have limited ability to do the same (Steinman and Inaba, 1999). Hence these proliferative lesions can be diagnosed as histiocytic sarcomatosis based on their morphology, immunophenotype and invasiveness. Our results are in agreement with Arshad *et al.* and confirm the accuracy of the nomenclature HS (Arshad *et al.*, 1997).

It remains obscure if ALV J-induced HS are the same pathological entity as the MH previously reported (Goodwin *et al.*, 1999; Hafner *et al.*, 1996). There are some aspects in common between HS and MH such as consistent splenic and hepatic involvement in all cases and morphology of the tumor cells. However, several differences can be noted. MH is characterized by high incidence (12%-30%) in broilers and can be experimentally induced in leghorns (Goodwin *et al.*, 1999), unlike ALV J-induced HS that occurs at lower frequency (1.1%-6.4%) only in meat type chickens. Hafner *et al.* diagnosed the histiocytic proliferations as MH and described it as multi-system, rapidly progressive disease that simultaneously involves several organs such as spleen, liver, lung, kidney, proventriculus, gizzard, pancreas, and intestines and not the result of metastasis (Hafner *et al.*, 1996). Based on our results, ALV J-induced HS were most likely disseminated histiocytic sarcomas or histiocytic sarcomatosis originated in

spleen but not MH. Firstly, histiocytic proliferative lesions were invariably present in spleen in every case. The microscopic morphology and immunophenotype of splenic lesions were identical to hepatic and renal lesions. In addition, invasion of the blood vessels by the histiocytes, and presence of neoplastic emboli suggests the possibility of metastasis of neoplastic histiocytes from spleen to other organs and not of a multicentric origin. Another difference between MH and HS is that MH is never accompanied by myelocytic sarcomas or other myeloid tumors, however ALV J-induced HS infrequently occur in conjunction with myelocytomas or undifferentiated myeloid blast cell tumors. Since the etiology of MH is not completely elucidated it is difficult to conduct comparative studies but further research on MH might clarify this issue.

In summary, this study expands the knowledge on the factors influencing the pathogenesis of HS. Only meat-type chickens that were infected at hatch and were persistently viremic in the presence of a patent immune response developed ALV J-induced HS. The phenotype of HS tumor cells was confirmed to be histiocytes of myelomonocytic origin. In addition, several differences were observed between HS and other ALV J-induced tumors that might suggest different mechanisms of oncogenesis. Further studies are required to determine if the ALV J-induced HS lesions are the result of ALV J antigen-induced hyperplasia followed by subsequent neoplasia or the result of a direct transformation event induced by ALV J similar to myelocytomatosis or myeloid leukosis induced by ALV J.

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**Table 6.1.** The effect of age at infection, viral dose, ALV J strain, chicken strain and ALV J-infection profile (viremia and NAb) on ALV J-induced histiocytic sarcoma

| Expt. # | Chicken[1] # | Age @ Inoculation | Viral dose $TCID^{50}$ | Viral Strain | Infection profile Viremia[2] | Infection profile Nab[3] |
|---|---|---|---|---|---|---|
| 1 | 1 | DOH | 100 | ADOL Hc1 | 8/8 | 0/8 |
| 1 | 2 | DOH | 100 | ADOL Hc1 | 7/9 | 0/9 |
| 1 | 3 | DOH | 100 | ADOL Hc1 | 5/5 | 0/5 |
| 1 | 4 | DOH | 10,000 | ADOL Hc1 | 8/8 | 0/8 |
| 1 | 5 | DOH | 10,000 | ADOL Hc1 | 4/8 | 1/8 |
| 1 | 6 | DOH | 10,000 | ADOL Hc1 | 6/6 | 0/6 |
| 1 | 7 | DOH | 10,000 | ADOL 6803 | 9/9 | 0/9 |
| 1 | 8 | DOH | 10,000 | ADOL 6803 | 4/4 | 1/4 |
| 1 | 9 | DOH | 10,000 | ADOL 6803 | 8/9 | 2/9 |
| 1 | 10 | DOH | 100 | ADOL 6803 | 6/7 | 1/8 |
| 1 | 11 | DOH | 100 | ADOL 6803 | 6/6 | 1/6 |
| 1 | 12 | DOH | 100 | ADOL 4817 | 7/7 | 1/7 |
| 1 | 13 | DOH | 100 | ADOL 4817 | 8/8 | 2/8 |
| 1 | 14 | DOH | 100 | ADOL 4817 | 6/7 | 1/8 |
| 2 | 15 | DOH | 1,000 | ADOL pR5-4 | 7/7 | 2/7 |

[1] data from 15 meat-type chickens is represented in this table
[2] viremia as tested by virus isolation on several sampling intervals. The study was terminated at 32 weeks post hatch. Some chickens have fewer than 9 samplings due to mortality before study termination. Samples tested positive/total number of samplings
[3] NAb response on several sampling intervals. Samples tested positive/total number of samplings

**Table 6.2.** Immunophenotype of the ALV J-induced histiocytic sarcoma tumor cells

| Tumor # | K55[1] | ChL5[2] | CIa[3] | K1[4] | CT3[5] | CT4[6] | CT8[7] | CB4/CB5[8] | 6-23[9] | P53[10] |
|---|---|---|---|---|---|---|---|---|---|---|
| HS1 | 3+ | 3+ | 3+ | ± | 1 | 1 | 1 | - | 1 | - |
| HS2 | 3+ | 3+ | 3+ | ± | 1 | - | 1 | - | ± | - |
| HS3 | 3+ | 3+ | 3+ | ± | 1 | 1 | 1 | - | ± | - |
| HS4 | 3+ | 3+ | 3+ | ± | 1 | 1 | - | - | ± | - |
| HS5 | 3+ | 3+ | 3+ | ± | 1 | 1 | - | - | - | - |
| HS6 | 3+ | 3+ | 3+ | ± | 1 | - | - | - | 1 | - |
| ML1[11] | 3+ | 3+ | 0 | 0 | - | - | - | - | 3+ | - |
| ML2[11] | 3+ | 3+ | 0 | 0 | - | - | - | - | 3+ | - |
| NB[12] | 3+ | 3+ | 0 | 0 | - | - | - | - | 3+ | - |

[1] K55 (common leukocyte antigen CD45)
[2] ChL5 (myelomonocytic cells and activated T cells)
[3] CIa (major histocompatibility class II)
[4] K1 (macrophages and thrombocytes)
[5] CT3 (CD3 specific for all T cells)
[6] CT4 (CD4 T cells)
[7] CT8 (CD8 T cells)
[8] CB/CB5 (peripheral B cells)
[9] 6-23 (ALV J gp85 antigen)
[10] p53
[11] Myelocytoma tumor samples
[12] Nephroblastoma

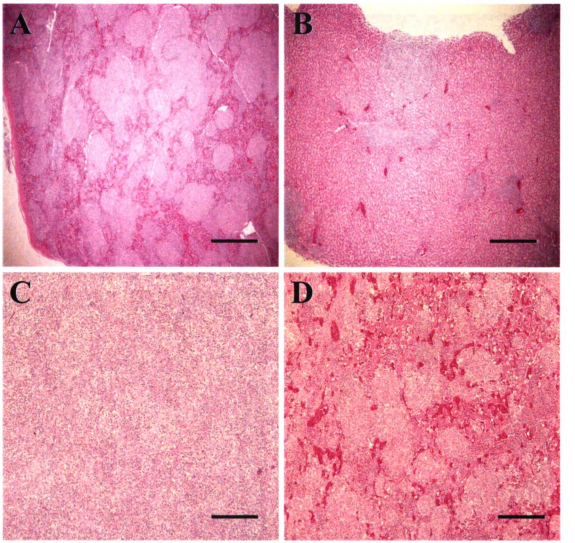


**Figure 6.1 A-D.** Hematoxylin and eosin staining of ALV J-induced histiocytic sarcomas.
A. Extensive multifocal histiocytic proliferations invading the splenic parenchyma. Bar = 400 μM
B. Scattered histiocytic proliferations in the hepatic parenchyma that had metastasized from the spleen. Sections A and B are from the same chicken. Bar = 400 μM
C. Uniform sheets of proliferating histiocytic sarcoma cells that have totally obliterated the splenic parenchyma. Bar = 200 μM
D. Extensive multifocal histiocytic proliferations that have metastasized from the spleen. Sections C and D are from the same chicken. Bar = 200 μM

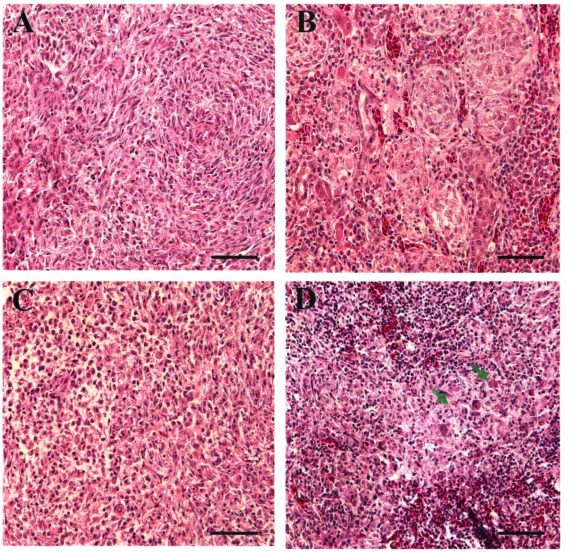


**Figure 6.2 A-D.** ALV J-induced histiocytic sarcoma tumor cells exhibiting cellular and nuclear pleomorphism with various patterns of distribution.
A. Spindle shaped proliferating histiocytes in whorled pattern of distribution in spleen. Bar = 100 μM
B. Clearly circumscribed proliferating histiocytes in liver. Bar = 100 μM
C. Polyhedral epitheliod proliferating histiocytes arranged either as uniform sheets or as tiny circumscribed pockets in spleen. Bar = 100 μM
D. Proliferating histiocytes with rare multinucleate giant cells (green arrows) in splenic parenchyma. Bar = 120 μM

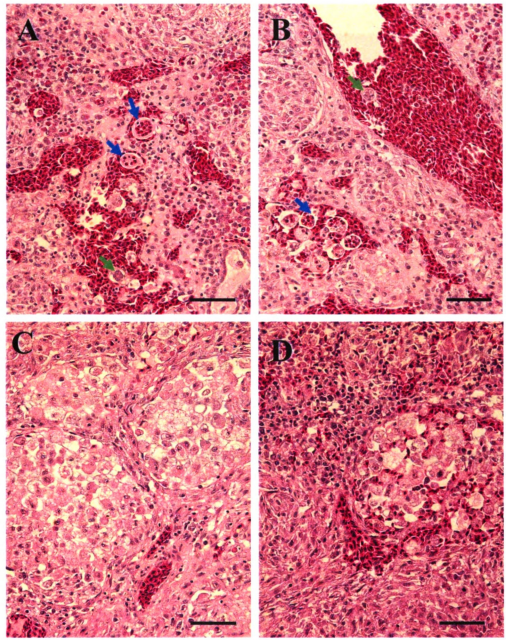


**Figure 6.3 A-D.** Erythrophagocytosis exhibited by ALV J-induced histiocytic sarcoma tumor cells with large foamy eosinophilic cytoplasm. A. Large foamy polyhedral cells actively phagocytosing erythrocytes (blue arrows). Rare multinucleate giant cells (green arrows) were also observed among the histiocytic cells. Lympho-plasmacytic cells and heterophilic infiltration can also observed Bar = 100 μM. B. Large histiocytes erythrophagocytosing (blue arrows) within the blood vessels. A polyhedral cell with multiple nuclei within the blood vessel (green arrow). Bar = 100 μM. C. Clearly circumscribed histiocytic proliferative cells with highly vacuolated eosinophilic cytoplasm. Bar = 100 μM D. Large histiocytes erythrophagocytosing (blue arrows) within the blood vessels. Bar = 100 μM

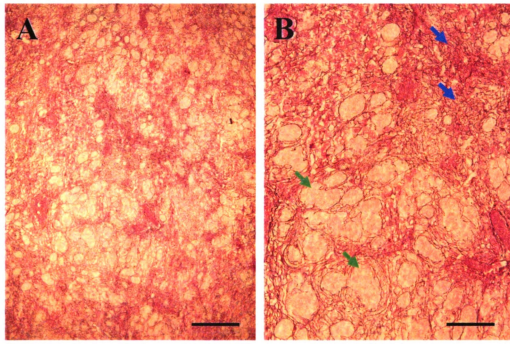


**Figure 6.4 A, B** Reticulin staining demonstrated breakdown of reticulin network and destruction of normal architecture of the hepatic parenchyma leading to formation of large spaces devoid of reticulin (green arrow) or a haphazard reticulin distribution (blue arrow) in the proliferative areas. A. Bar = 200 µM. B. Bar = 100 µM

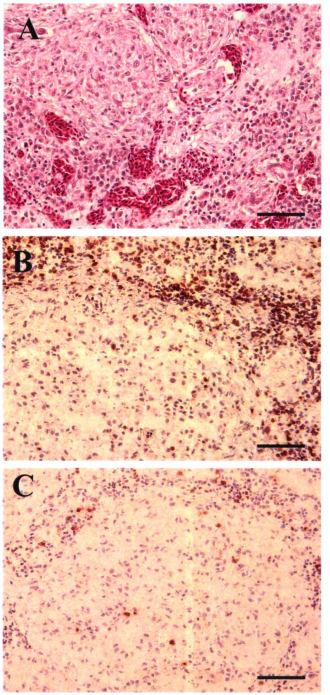


**Figure 6.5 A, B, C.** Inflammatory infiltrate in histiocytic sarcoma tumors A. Infiltration of variable numbers of lymphocytes, plasma cells and heterophils was observed in some histiocytic proliferative lesions Bar = 100 µM B and C. Infiltration of CD4 T cells (B) and CD8 T cells (C) was mainly observed on the periphery of the proliferating histiocytic nodules and there are very few scattered lymphocytes within the tumor mass. Bar = 100 µM

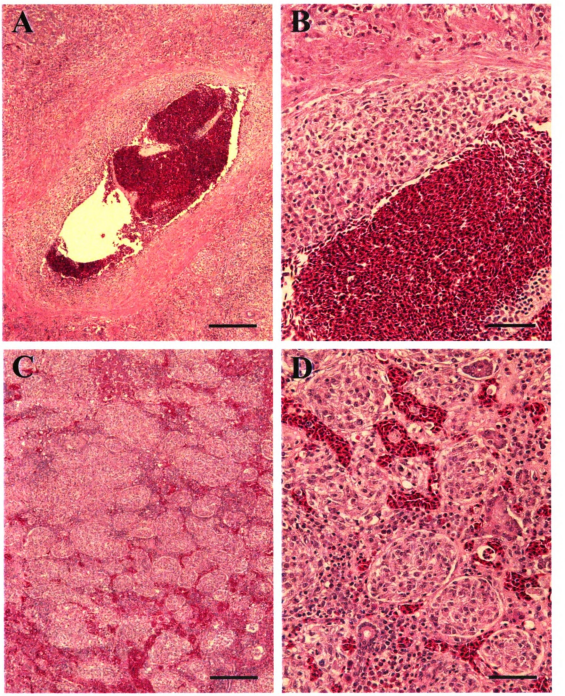


**Figure 6.6 A-D** Metastasis in ALV J-induced HS. A and B. Proliferating histiocytes were observed in the portal vessels either as solitary cells or as metastatic emboli partially blocking the vessel (Bars: A = 200 μM, B = 100 μM). C and D. The proliferating histiocytic foci in hepatic parenchyma were lined by sinusoidal cells, giving the appearance of metastasis through the hepatic sinusoids (Bars: C = 200 μM, D = 100 μM)

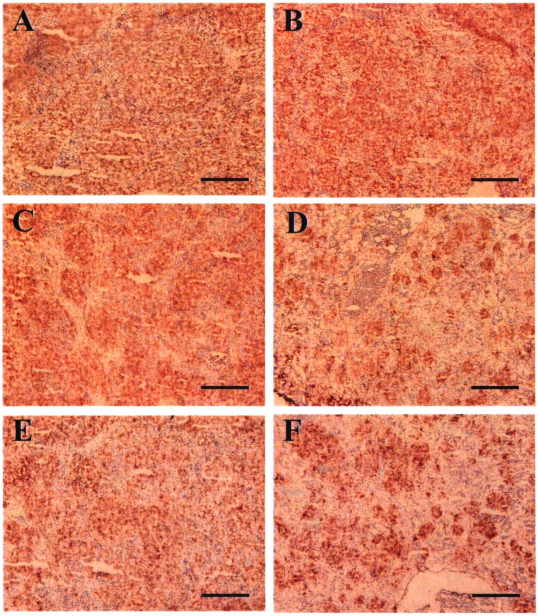


**Figure 6.7 A-F** Immunophenotype of ALV J-induced HS
A. The proliferating HS tumor cells in spleen stained strongly positive for ChL5 (myelomonocytic cells and activated T lymphocytes). Bar = 200 μM
B. HS tumor cells in liver staining strongly positive for ChL5. Bar = 200 μM
C. The proliferating HS tumor cells in spleen stained strongly positive for K55 (common leukocyte antigen CD45) Bar = 200 μM
D. HS tumor cells in liver staining strongly positive for K55. Bar = 200 μM
E. The proliferating HS tumor cells in spleen stained strongly positive for CIa (MHC II) antibody. Bar = 200 μM
F. HS tumor cells in liver staining strongly positive for CIa. Bar = 200 μM

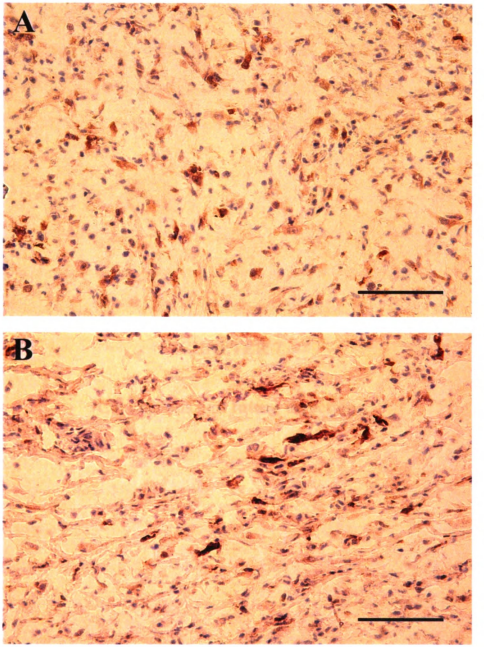


**Figure 6.8 A-D** 6-23 (ALV J gp85) staining in HS
A. Faint 6-23 staining in HS tumor cells in spleen. Bar = 100 μM
B. Faint 6-23 staining in HS tumor cells in liver. Bar = 100 μM

# CHAPTER 7

# Conclusions and future directions

The research reported in this dissertation mainly focused on studying ALV J persistence in meat-type chickens by evaluating a combination of host factors such as age at exposure, host immunity and genetics, and viral factors such as viral strain and dose, and neutralizing antibody (NAb) escape variants. In addition, the influence of ALV J infection profile on tissue tropism as well as histiocytic proliferative lesions was evaluated.

ALV J infection profile was monitored by chronological evaluation of both viremia and NAb status from 9 sampling intervals over a period of 32 weeks post hatch. This allowed identification of much higher levels of viral persistence than previously reported. As demonstrated in objective #1 (Chapter 2), a high incidence (83-100%) of viral persistence was observed in ALV J-inoculated meat-type chickens regardless of the viral strain and dose, and age at inoculation. The presence of neutralizing antibody (NAb (A)) did not alter the viremia status (V) in most cases since the NAb response was not efficacious in clearing the autologous virus. There was a high incidence (up to 75%) of chickens that were concurrently positive for V as well as A (V+A+). The incidence of NAb response against the inoculated parental virus was influenced by viral strain and dose, and age at inoculation. However, the ability of NAb to clear the infection was influenced only by viral strain. Routinely reported NAb responses in ALV infections is against ALV stocks used for inoculation but not against the autologous viruses that are isolated at the same time as serum antibody. This led to the hypotheses that the high incidence of V+A+ infection profile may be due to NAb escape variants or due to the heterogeneity of the ALV J field isolates used in this study.

In objective #2 (Chapter 3), high levels of ALV J persistence (100%) and V+A+ infection profile (88%) were also observed in meat-type chickens inoculated at hatch with an ALV J molecular clone ADOL pR5-4 that has very low heterogeneity compared to field isolates. The high incidence of V+A+ infection profile could be at least partially explained by the emergence of NAb escape variants since every chicken with V+A+ infection profile failed to neutralize autologous viruses on at least 2 occasions in 4-6 sampling intervals. The incidence of autologous NAbs was lower in meat-type chickens infected with ALV J field isolates (15%) than ADOL pR5-4 (36%). Hence, this study demonstrated that NAb escape variants may play a role in ALV J persistence although the heterogeneity of viral population in field isolates may also be a factor. Sequencing ALV J NAb escape variants as well as developing neutralizing monoclonal antibodies may provide information on the neutralization epitopes.

Diagnosis of ALV J infection is based on antibody testing by using commercially available ELISA kits. In addition, virus isolation and virus neutralization are also conducted in pedigree flocks. This study reveals the limitations of these methods since the constant antigenic variation of ALV J might interfere with the commercial ELISA test for antibody detection and can lead to false negatives. Moreover, NAbs against the inoculated parental virus do not neutralize the emergent NAb escape variants.

The role of host immune status in ALV J persistence was investigated in objective #3 (Chapter 4). Chickens that appear to have an efficient NAb response and are apparently viremia-free for a long period can revert to viremia when the immune system is

compromised. Effect of the stress on viremia was only observed in chickens that were infected with ALV J at hatch (33%) but not at 32 weeks. This might be due to higher efficiency of the immune response against the virus at 32 weeks of age than at hatch. In addition, it may also be due to increased ALV J persistence when infected at hatch than at 32 weeks of age. It was also observed that a single viremic chicken could horizontally transmit infection to 42% of contact chickens. This study demonstrates the importance of eradication of every source of infection in the flock and also of good husbandry practices in reducing stress in the farm.

Virus seems to persist in seroconverted chickens that remained free of viremia for a long period of time as demonstrated in objective #3 (chapter 4). ALV J proviral DNA was demonstrated in various tissues of both viremic as well as seroconverted non-viremic chickens (objective #4, chapter 5). However, expression of gp85 viral antigen was restricted to chickens exhibiting overt viremia in the presence (V+A+) or absence of antibody response (tV+A-, ntV+A-) but not in seroconverted non-viremic chickens (V-A+). These results confirm the persistence of ALV J infection in chickens that have cleared viremia for a long period of time and promote the practice of multiple sampling to increase the possibility to efficiently detect ALV J infection.

Detection of proviral DNA by PCR seems to be the most sensitive technique to detect ALV J infected chickens. However, this technique had several limitations. Several primers could detect provirus in chickens with overt viremia but only one primer set (H5/H7) using a touch-down PCR method was able to detect provirus in seroconverted non-viremic chickens.

This demonstrates the importance of primer design, PCR reaction conditions, and issues related to sensitivity and specificity.

ALV J-induced viral persistence also seems to influence the pathological manifestation of ALV J infection since histiocytic proliferative lesions were observed only in persistently viremic meat-type chickens that were infected at hatch with little or no NAb response. These histiocytic proliferative lesions were characterized as histiocytic sarcomas (HS) of myeloid origin with some inflammatory component. These results suggest that ALV J might have different mechanisms of transformation. Further studies are required to elucidate if the ALV J-induced HS lesions are the result of ALV J antigen-induced hyperplasia followed by subsequent neoplasia or the result of a direct oncogene-mediated transformation event induced by ALV J similar to myeloid leukosis.

The relevance of host genetics in ALV J persistence has been confirmed in this work. ADOL line 0 chickens had very low incidence of viral persistence (7%) and V+A+ infectious profile (4%) (objective #2, chapter 3). This is due to the ability of these chickens to develop efficient NAb capable of clearing viremia. Studies comparing immunological responses such as natural killer cell activity, cytotoxic T lymphocyte responses, and humoral immune responses, as well as evaluation of host endogenous viral elements may explain differences in immune responses between ADOL line 0 and meat-type chickens. In addition, none of the line 0 chickens developed HS (objective #5). Although the exact pathogenesis of HS is still obscure, the lack of HS in line 0 chickens could be a consequence of low incidence of viral persistence.

In summary, these studies expand the knowledge on factors that influence ALV J persistence and also the effects of viral persistence on the immunity and pathology of ALV J infection. The findings in these experiments will aid poultry industry in diagnosis and control of ALV J.