SAAD MAHMOUD GHARAIBEH
Avian Leukosis Virus Subgroup J in Chickens: Tissue Tropism and Effects of Antibody on Infection and Viral Mutation
(Under the Direction of THOMAS P. BROWN)

This research includes tissue tropism of avian leukosis virus subgroup J (ALV-J) in congenitally infected broiler chickens using an immunohistochemistry (IHC) technique detecting gp85 viral glycoprotein. All organs examined contained detectable antigen. The most intense staining was in the adrenal gland, heart, kidney, and proventriculus. Intense staining for viral antigen in the heart may explain the ability of ALVs to cause cardiomyopathy. Although recent investigations failed to demonstrate specific viral staining in bone marrow from infected chickens, this research shows moderate staining in myelocytic precursor cells in bone marrow. This agrees with previous work showing that cell cultures of bone marrow are susceptible to ALV-J infection, and the tendency of subgroup J to predominantly induce myeloid rather than lymphoid neoplasms. This research includes production of neutralization-resistant isolates of ALV-J. ADOL-7501 isolate of ALV-J was cloned *in vitro* by three serial terminal dilutions. The cloned virus was injected into specific pathogen free (SPF) chickens, and the antiserum produced had *in vitro* neutralizing activity against the cloned virus. The cloned virus was then serially passed three times in the presence of subneutralizing levels of this antiserum, and the resultant viral isolates were more resistant to antiserum neutralization *in vitro* than was the parent cloned virus. No nucleotide differences were detected between the *env* gene of the parent cloned virus and that of the neutralization-resistant mutants. Other possible genomic changes outside the env gene (*i.e.* in the LTR and *gag* genes) may account for these non-*env* based differences in neutralization indices. This research also includes an investigation of the protective effect of injected ALV-J antiserum in embryonated chickens eggs. In one experiment, chickens exposed to ALV-J by cohatching with virus-shedders did not suffer from body weight suppression or ALV related tumors, and the

injected ALV-J antiserum did not protect against development of viremia or increase the number of chickens subsequently developing active immunity. In a second experiment, chickens were exposed to ALV-J by injection of virus at hatch. Injection of ALV-J antiserum protected these chickens against development of ALV-J related tumors, but did not protect against virus induced body weight suppression or development of viremia, and did not increase the number of chickens developing active immunity. In the third experiment, the protective effects of injecting antiserum against ALV-J into embryonating-chicken eggs before infection as embryos were determined by evaluating viremia, transfer of passive immunity, and localization of the virus in tissues from hatched chicks. The injected antiserum prevented viremia at hatch in four out of five chicks. Localization of ALV-J in chicks that were viremic at hatch was similar to previous investigations, with intense staining for viral antigen present in adrenal gland, heart, kidney, proventriculus, and spleen. Two of the chicks that were not viremic at hatch developed viremia at one week of age and had viral tissue distribution suggesting oral exposure to the virus from their hatchmate.

INDEX WORDS: ADOL-7501, ALV-J, Antiserum, Avian leukosis virus, Chicken, Egg inoculation, Escape mutants, Immunohistochemistry, Neoplasia, Neutralization resistant, Retrovirus, Tissue distribution

# AVIAN LEUKOSIS VIRUS SUBGROUP J IN CHICKENS: TISSUE TROPISM AND EFFECTS OF ANTIBODY ON INFECTION AND VIRAL MUTATION

by

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## DEDICATION

My parents have taught me many things in my life; lessons that I have held close to my heart. With those lessons, I have gone many places, met many people, and done many things. But of all the things I have done and of all the things I am, the thing that I am most proud of is that I am the son of my parents. May the spirit of our family live on forever. I dedicate this work to my parents Mahmoud and Radieh and my siblings Tareq, Adma, Omar, and Dima.

I also dedicate this work to the ancient land of the Middle East, which have struggled and suffered throughout history. Anyone who knows the tragic history of this land is vexed by the ancient frenzied struggle on that land between men fighting for their freedom and oppressors raving to crush them. To all the people who have lived, and will live on that land.

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# CHAPTER 1

# INTRODUCTION

## Purpose of the Study

In 1991, a novel subgroup J (ALV-J) of avian leukosis virus (ALV) was isolated from meat-type chickens. This new subgroup infects domestic fowl, red jungle fowl, Sonnerat's jungle fowl, and turkeys. Clinical effects in infected chickens include body weight suppression, myeloid leukemia, other neoplasms, and reduced egg size in broiler breeders. Similar to previously recognized ALV infections, ALV-J infections occur either by horizontal transmission between older chickens or by congenital transmission from infected parent stock to their progeny. Earlier work showed that not all congenitally infected birds have the same heavy level of viremia, which might indicate that embryos are infected at different stages of development. However, in ALV-infected egg-laying hens, viral particles are abundant in the ovarian stroma, bud from cells in direct contact with oogonia and oocytes, and are in the largest concentration in the albumen-secreting glands of the magnum. This early ovarian and oviductal exposure results in natural congenital ALV infection at a very early embryonic stage. Contact infection results in transient or permanent viremia depending on age of exposure. For ALV-A, presence of circulating maternal antibodies reduces the number of chickens developing viremia, cloacal viral-shedding, tumors, and increases the number of chickens developing active immunity by 18 weeks of age.

Tissue tropism of ALV-J after experimental *in ovo* exposure at 11 days of incubation using an IHC technique to detect expression of viral group-specific antigen (*gag*) has been described. The greatest *gag* staining was observed in the adrenal gland, heart, kidney, and proventriculus at 3 and 7 weeks of age with other organs having little

or no staining. ALV-J has also been detected in tissues by localization of viral nucleic acid using *in situ* hybridization (ISH). Chickens exposed experimentally at 11 days of embryo incubation *in ovo* had the greatest detectable nucleic acid in the adrenal gland, heart, kidney, and proventriculus. This localization agrees with that previously demonstrated by IHC detection of viral *gag*. Congenitally infected chickens examined by ISH had similar staining patterns to those inoculated *in* ovo with some differences in intensity within and between organs. ALV-J infects and causes neoplasms of myeloid precursors. Although ALV-A has been found in bone marrow by electron microscopy, no ultrastructural studies have shown ALV-J in this location. Furthermore, no viral nucleic acid or protein has been demonstrated in bone marrow by ISH, or IHC, respectively. There is no IHC study describing ALV-J localization in congenitally infected chickens.

RNA viruses infect a wide variety of natural hosts and frequently escape *in vivo* pressure from induced antibodies or antiviral treatments. One mechanism for this escape is the development of new quasispecies during persistent infections. For retroviruses, the development of quasispecies is especially favored by their rapid replication and large viral copy numbers typically present in infected hosts. Furthermore, their reverse transcriptase is prone to replication errors caused by copy transitions, transversions, deletions, and insertions. These copy errors make *in vivo* occurrence of neutralization-resistant quasispecies the norm rather than the exception. Such quasispecies development occurs during natural infections with equine infectious anemia virus, visna virus, and human immunodeficiency virus (HIV).

Within subgroup J of avian leukosis virus (ALV-J), naturally occurring antigenic variations are detectable using monoclonal antibodies and are most often associated with nucleotide sequence changes in the variable regions of the viral envelope glycoprotein *env*. These variations are important because they may lead to problems using antigen-antibody based diagnostic assays. Even though the control of ALV-J has been oriented

towards elimination of infection at the parent stock level, vaccination may also be needed to finally control the disease in some situations. Efficacy of such vaccines would be negatively impacted by known existing variations. Use of inactivated vaccines may actually encourage increased development of antibody-resistant quasispecies within a vaccinated infected host, as has been shown for other retroviral infections.

Passive transfer of antibodies into chickens by intraperitoneal antiserum injection is protective against subsequent challenge with the homologous pathogen. Injection of antibodies in the yolk sac of incubating embryos sometimes results in circulating antibodies depending on the concentration of the injected antibody and has a similar protective effect in chickens. In turkeys, injection of anti-*Mycoplasma* antiserum in embryonating turkeys-eggs reduced the adverse effects of egg-transmitted *Mycoplasma*. and turkeys. Embryo inoculation can be automated making it a potentially more practical approach than inoculating of antisera into hatched chicks. Embryo inoculation can be automated making it a potentially more practical approach than inoculating of antisera into hatched chicks.

How This Study Is Original

There is no previous study using IHC staining of ALV-J specific gp85 to determine the chronologic tissue tropism after natural congenital viral infection. Furthermore, specific IHC examination of bone marrow was included to attempt detection of ALV-J after congenital viral infection. One hypothesis hypothesis that was tested was that chicken anti-ALV-J polyclonal neutralizing antibody will encourage rapid emergence of viral quasispecies with altered envelope gene sequences and resultant resistance to antibody neutralization.

This study also investigates the potential use of injecting ALV-J antiserum into embryonating chicken eggs to reduce the number of chickens developing viremia, protect against ALV-J associated body weight suppression and tumor formation and to reduce

the effects of egg transmitted ALV-J by reducing the level of viremia, and hence shedding of virus to the environment.

# CHAPTER 2

# LITERATURE REVIEW

## Retroviridae

Retroviruses continue to receive significant attention since the 1980s compared to other infectious agents (27). The *Retroviridae* is a large family of viruses that infect vertebrate and other animals. They cause many diseases including rapid and long-latency malignancies, wasting diseases, neurological disorders, and immunodeficiencies. They can also result in life long viremia with no lesions.

Retroviruses are divided into seven genera based on their genetic evolutionary relatedness (126): Avian sarcoma and leukosis virus group (ALSV), mammalian type B group, murine leukemia virus group, human T-cell leukemia-bovine leukemia virus group, D-type group, lentiviruses group, and spumaviruses group.

### Avian leukosis sarcoma viruses, exogenous avian leukosis virus

Mature ALVs are approximately 100 nm in diameter and have a condensed, round or slightly angular core of electron-dense material (127). Similar to other retroviruses, ALVs have an RNA genome and replicate via a DNA intermediate. Viral genes include *gag, pro, pol,* and *env*. *Gag* (group specific antigen) is proteolytically processed into p19 MA (matrix), p27 CA (capsid), and p12 NC (nucleocapsid). *Pol* codes for the enzymes reverse transcriptase (RT) and integrase (IN). *Pro* codes for the viral protease (PR). *Env* codes for gp85 the surface (SU) glycoprotein, and gp37 the transmembrane (TM) protein of the virion envelope.  Sarcoma viruses have an additional *onc* gene which enables such viruses to acutely cause tumors in animals and transform cell cultures. This *onc* gene usually replaces either *env* or *gag* genes rendering the virus

defective in replication on its own and it can replicate only if the host cell is also infected with a nondefective virus (helper virus that has *env* and *gag*). The genes in the proviral DNA are bracketed by two identical long terminal repeats (LTRs). LTRs are divided into U3, P, and U5. LTRs work as enhancers and promoters for viral transcription. (27, 127).

Since the initial description of "lymphosarcomatosis" in a chicken in Europe in 1868 (94), ten subgroups (subgroup A to J) of ALV have been recognized based on their host range, interference pattern, and viral envelope antigens (21, 128) with numerous isolates that are either classified into one of these subgroups or recognized as a defective isolate that does not belong to any subgroup (83). Of these 10 subgroups, A, B, C, D, E and J occur in chickens. Subgroup E is an endogenous virus of chickens and will be discussed later. Subgroups A and J have been recognized as the most frequent exogenous ALSVs in commercial poultry (23, 46).

Neutralizing epitopes are present on the viral surface, exposed as the virus enters the host cell, or are required for membrane assembly of viral particles (100). Retroviral *env* genes code for most neutralizing epitopes(27), but other genes may also code for such epitopes. The non-*env gag* protein p17 of HIV virus has 2 neutralizing epitopes (78) and antibodies to p17 neutralize HIV (72, 78, 105). Numerous p17 mutations occur in HIV infected patients (62, 63, 64, 135, 137). Neutralization by p17 *gag* induced antibodies may occur by interaction with undefined components of the host cell membrane or viral replication (78). Polyproteins coded by *gag* assemble into a network of ring-like structures producing icosahedral viral core structures (73). Certain p17 C-terminal sequences are also involved in viral penetration and uncoating (133). Antiserum against p15 of Friend murine leukemia virus, a counterpart of HIV's p17, is also neutralizing in the presence of complement indicating that p15 is a surface antigen (52). However, no non-*env* neutralizing epitopes have been described for ALV.

LTR of retroviruses are not well understood although they play a major role in replication and oncogenicity of the virus. For example, progressive adaptation to cultivation in a specific cell or microenvironment is controlled by the LTR. LTR is susceptible to mutations in retroviruses (1, 2, 3, 33, 57, 58, 61, 65, 106, 129). Such retroviral LTR alterations can increase promoter activity (89, 136), change tissue specificity (1, 2, 58, 106), and change target cell oncogenicity (61, 106). The LTRs of ALVs have been shown to change cell specificity (33, 57), pathogenicity (58), and increase Rous sarcoma virus (RSV) LTR-driven transcription 5-fold (13, 44).

**Endogenous avian leukosis viruses**

Chickens of all breeds have an endogenous virus of ALV that is integrated into the genome and inherited from generation to generation (7, 29). Four different families of endogenous retrovirus have been identified in the chicken genome (98). CR1 (chicken repeat 1) element is a short interspersed repetitive DNA element (25). The second family is the ALV subgroup E (31). It is believed that there are over 22 *ev* loci in the genome of layer chickens and more in broilers (31) with an average of 5 loci in each chicken (95). The third family is the ART-CH (avian retrotransposon from the chicken genome) with approximately 50 genomic copies (55). The last family, the EAV-0, is believed to be more ancient than ALV-E because it is present in the genome of non-chicken species in the genus *Gallus*. (17, 43, 91).

Most endogenous viruses contain stop codons in the reading frames of their genes or have truncated genes which preclude gene expression and render the virus replication defective (90). However, fully expressed infectious endogenous virus can occur and be transmitted vertically and horizontally (26, 110). Subgroup E ALV has little or no oncogenicity because of the weak promoter activity of the LTR (71).

Expression of endogenous viruses or even a portion of a protein might affect disease progression. Endogenous expression of functional envelope glycoprotein can be

at least partially protective to superinfection by a virus from a similar subgroup due receptor blockage (32, 67). Alternatively, it has been suggested that the endogenous expression of even a truncated envelope protein could induce tolerance to certain exogenous viruses and thereby result in increased susceptibility to lymphomagenesis by an infecting exogenous virus (109).

**Neoplastic transformation**

Retrovirus proviral DNA integrates into the host genome to complete its life cycle. Viruses in the ALSV group have the ability to transform host cells after this integration (126). There are multiple mechanisms that ALSVs use to induce neoplasias. The acutely-transforming replication-defective viruses carry an oncogene to accomplish this (27). Displacement of the cellular oncogene away from the regulatory sequence is a second mechanism (93). A third mechanism, is the integration of the provirus close to the cellular oncogene, mainly *c-myc*, which will be over-expressed due to the influence of the proviral LTR promoter enhancing effects (27).

**Avian leukosis virus subgroup J**

This subgroup was first reported from UK in 1991 (82). Based on its envelope properties and host-range it was classified as a new subgroup J (9, 81, 87). The first isolation of this new subgroup in the USA was in 1993 (51). ALV-J differs from other subgroups by its tendency to cause myeloid rather than lymphoid tumors, and its decreased tropism for bursal cells (5, 6, 85).

This new subgroup has a few additional genes within its 3’-UTRs (untranslated region) that have not been reported earlier in other subgroups of ALVs. There is a nonfunctional redundant transmembrane region (rTM) which is a truncated version of the gp37 (TM) region of the envelope (8). rTM is believed to originate from the exogenous virus parent of ALV-J after recombination with an endogenous virus. This recombination event will be discussed later. Downstream of rTM, there is the DR1 (direct repeat

sequence) similar to that previously identified in other sarcoma viruses (56). ALV-Js also have sequences of approximately 150-bp called E elements. E elements are found in several strains of Rous sarcoma virus (59, 103, 120) but have not been reported in other strains of ALV (11).

Sequence comparison of the *env* gene of the English prototype (HPRS-103) and other isolates of ALV-J revealed that their gp85 glycoprotein (a major determinant of subgroup specificity) showed only 40% overall similarity to that of subgroup A to E viruses (8, 9, 11, 125). This is in contrast to the A to E subgroups, which are over 85% similar to each other. ALV-J differs primarily in its hypervariable and variable regions. These determine subgroup specificity and neutralization patterns (14, 15, 38, 119). The subgroup J *env* gene includes sequences highly related to an ancient endogenous avian virus (EAV) family called E51 (16, 17, 43). This suggests that the subgroup J *env* gene may have been generated by multiple recombination events between exogenous and endogenous viruses. In 1998 Benson *et al.* (10) described a novel endogenous virus family (ev/J) with over 95% similarity to the *env* gene of ALV-J. This new discovery led to a modified hypothesis that ALV-J acquired its *env* gene from ev/J by only one recombination event (10). Similar retroviral recombinations have been described with feline leukemia virus (FeLV), where *env* gene variants were generated by recombination of exogenous virus with endogenous FeLV-related sequence (12, 24, 77, 96). In 2000, Sacco *et al.*(98) published a similar study to that of Benson *et al*. showing that *env* gene of ALV-J has over 97% similarity to EAV-HP endogenous virus which belongs to EAV family endogenous viruses. In that report, Sacco's group postulated that EAV-HP and ev/J are the same endogenous elements.

**Pathogenicity of ALV-J in meat-type chickens**

Similar to previously recognized ALV infections, ALV-J occurs after either horizontal or congenital infection from infected parent stock to their progeny (83). Congenital infection and embryonal inoculation of chickens results in a permanently tolerant viremic chicken (87) with a higher titer viremia than that present in horizontally infected chickens (97). Horizontal infection results in either permanent or transient viremia, depending on the age at infection. For ALV-A, delay of infection by maternal antibodies reduces tumor formation and increases the number of chickens developing active immunity later in life (48, 50). No similar data is available for ALV-J or other subgroups.

Clinical effects of ALV-J infection in chickens include body weight suppression in congenitally infected chickens (117). Similar to ALV-A (28) inoculation of chickens with ALV-J at 1 day of age also causes body weight suppression (unpublished observation, Gharaibeh). ALV-J also causes development of myeloid leukemia (85) and other tumors such as renal adenomas, hemangiomas, and histiocytic sarcomas (4, 84, 87). Reduced egg size of broiler breeders infected with ALV-J has also been reported (113). ALV-J is suspected to play a role in cardiomyopathy and ascites syndrome in broilers (66, 82, 115). It is also believed that ALV-J causes immunosuppression, however, heterophil and macrophage functions were not different between infected and non-infected chickens (114).

**Detection of ALV(-J)**

Like other ALVs, ALV-J can be detected by commercially available antigen-capture enzyme-linked immunosrobent assay (Ag-ELISA). This assay detects group specific antigen p27 (9, 49, 51, 83, 85, 108, 112), and will be positive if endogenous virus is expressed in chickens. The industry standard is to inoculate a sample (meconium, feather pulp, plasma, serum, cloacal and vaginal swabs) into chicken embryo fibroblasts

derived from line 0 chickens-resistant to endogenous virus (47), then test this culture using Ag-ELISA. This combined test system will be positive only if exogenous ALV is present in the sample.

Commercial antibody ELISAs (Ab-ELISAs) specific for subgroup J were made available after the *in vitro* expression of the surface glycoprotein gp85 (124). However, these Ab-ELISAs are insensitive (54) because of the high antigenic variability of the isolates (107, 125, 134).

Polymerase chain reaction (PCR), and reverse transcriptase-PCR (RT-PCR) are probably the most specific tests to detect the nucleic acid of the virus or the integrated provirus even in the absence of viremia (49, 107, 111, 134). These molecular tests can be designed to detect a specific subgroup or all subgroups depending on the primer sets used (107).

There are many other historical tests that can be used to detect ALV including resistance-inducing factor, phenotypic mixing, and complement fixation test (30, 47, 49, 76, 83, 99, 101, 112, 121, 122) which are rarely used now because they are time consuming and require unique reagents not widely available.

**Tissue tropism of ALV-J**

For ALV-infected egg-laying hens, viral particles are abundant in the ovarian stroma, bud from cells in direct contact with oogonia and oocytes (40), and are in the largest concentration in the albumen-secreting glands of the magnum. This early ovarian and oviductal exposure of embryo results in natural congenital ALV infection very early in embryonic development (35, 40).

Retroviral envelope glycoproteins in other species have been detected using immunohistochemistry (IHC) (60, 74, 102). Monoclonal and polyclonal antibodies against ALV-J *gag* or *env* glycoproteins have been produced and have broad reactivity for most ALV-J isolates examined (5, 46). These antibodies have been used to determine

the tissue tropism of ALV-J after experimental *in ovo* exposure at 11 days of incubation using an IHC technique to localize viral *gag* (5). The greatest *gag* staining was observed in the adrenal gland, heart, kidney, and proventriculus at 3 and 7 weeks of age with other organs having little or no staining.

ALV-J has also been detected in tissues by localization of viral nucleic acid using *in situ* hybridization (ISH) (6, 116). Chickens exposed experimentally at 11 days of embryo incubation *in ovo* had the greatest detectable nucleic acid in the adrenal gland, heart, kidney, and proventriculus (6). This localization agrees with that previously demonstrated by IHC detection of viral *gag* (5). Congenitally infected chickens examined by ISH had similar staining patterns to those inoculated *in ovo* with some differences in intensity within and between organs (116).

ALV-J infects and causes neoplasms of myeloid precursors (82, 86). Although ALV-A has been found in bone marrow by electron microscopy (41), no ultrastructural studies have shown ALV-J in this location. Furthermore, no viral nucleic acid or protein has been demonstrated in bone marrow by ISH (6, 116) or IHC (5), respectively. The IHC and ISH localization of the virus in the previous studies was more intense in the adrenal gland, heart, kidney, and proventriculus. This suggests high tropism of the virus to these organs. Tropism in these organs may be explained by these cells having an abundance of viral receptors, or high replication efficiency due to adaptation of long terminal repeats of the virus in enhancing and promoting replication in these organs (18). Alternatively, tissue specific endogenous factors may upregulate expression of virus at the transcriptional level (53), or simply, these cells may synthesize more protein than other cells (40).

**Induced Variability of RNA viruses, retroviruses, and ALV-J**

RNA viruses infect a wide variety of natural hosts, and frequently escape *in vivo* pressure from induced antibodies or antiviral treatments (42). One mechanism for this

escape is development of new quasispecies during persistent infections (36). Mutation rate are in the range of $10^{-3}$ to $10^{-5}$ substitutions per site per round of copying of the genome due to errors of RNA polymerase enzyme (37). For retroviruses, development of quasispecies is especially favored by their rapid replication and large viral copy numbers typically present in infected hosts (79, 80). Furthermore, their reverse transcriptase is prone to replication errors due to copy transitions, transversions, deletions, and insertions. The mutation rate of retroviruses is estimated to be $2 \times 10^{-5}$ substitutions per site per round of copying of the genome (39). These copy errors make *in vivo* occurrence of neutralization resistant retroviral quasispecies the norm rather than the exception (36). Such quasispecies development has been documented during natural infections with equine infectious anemia virus (68, 88), visna virus (104), and human immunodeficiency virus (HIV) (131, 132, 138, 139).

Within ALV-Js, naturally occurring antigenic variations are detectable using monoclonal antibodies (46), and are most often associated with nucleotide sequence changes in the variable regions of the viral envelope glycoprotein *env* (125). These variations are important because they may lead to problems using antigen-antibody based diagnostic assays (107). Even though the control of ALV-J has been oriented towards elimination of infection at the parent stock level (123), vaccination may also be needed to finally control this infection in some situations where eradication is not economically possible. Efficacy of such vaccines would be negatively impacted by known existing variations. Use of inactivated vaccines to induce neutralizing antibody may actually encourage increased development *in vivo* of antibody resistant quasispecies in vaccinated infected hosts, as has been shown for other retroviral infections (69, 70, 118).

**Effects of antibodies on ALV infection**

Direct evidence for the presence of maternal ALV antibody (MAB) and for its effects on the incidence of tumor development in the progeny of virus-infected dams was

first reported by Burmester *et al.* (19, 20, 22). Later studies by Witter *et al*. (130) showed that MAB delayed infection with virus. Rispens (92) *et al*. and de Boer *et* al. (34) showed that actively acquired antibody induced by inoculation of infectious ALV after 8 weeks of age can prevent shedding and congenital transmission to subsequent generations. Conversely, other studies showed vaccination of chickens immediately before exposure to a low-pathogenicity subgroup A ALV at 8 weeks of age did not eliminate subsequent shedding of virus (75). Most recently, maternal antibodies have been shown to delay ALV infection and to reduce the incidence of viremia, shedding, tumors, and increase the number of chickens developing active immunity by 18 weeks of age (45, 48, 50, 130).

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# CHAPTER 3

# IMMUNOHISTOCHEMICAL LOCALIZATION OF AVIAN LEUKOSIS VIRUS SUBGROUP J IN TISSUES FROM NATURALLY INFECTED CHICKENS[1]

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SUMMARY. The tissue tropism of avian leukosis virus subgroup J (ALV-J) was investigated in congenitally infected broiler chickens using an immunohistochemistry (IHC) technique detecting gp85 viral glycoprotein. All organs examined contained detectable antigen. The most intense staining was in the adrenal gland, heart, kidney, and proventriculus. Intense staining for viral antigen in the heart may explain the ability of ALVs to cause cardiomyopathy. Although recent investigations failed to demonstrate specific viral staining in bone marrow from infected chickens, we were able to show moderate staining in myelocytic precursor cells in bone marrow. This agrees with previous work showing cell cultures of bone marrow are susceptible to ALV-J infection, and the tendency of subgroup J to predominantly induce myeloid rather than lymphoid neoplasms.

Key words: Avian leukosis virus subgroup J, retrovirus, immunohistochemistry, chicken

Abbreviations: ALV: avian leukosis virus, ALV-A: ALV subgroup A, ALV-J: ALV subgroup J, ELISA: enzyme linked immunosorbent assay, *env*: envelope, *gag*: group specific antigen, IHC: immunohistochemistry, ISH: *in situ* hybridization, RT-PCR: reverse transcriptase polymerase chain reaction, SPF: specific pathogen free.

In 1991, a novel subgroup J (ALV-J) of avian leukosis virus (ALV) was isolated from meat-type chickens (21). This new subgroup infects domestic fowl, red jungle fowl, Sonnerat's jungle fowl, and turkeys (24). Clinical effects in infected chickens include body weight suppression (31), myeloid leukemia (14,22), other neoplasms (1,24), and reduced egg size in broiler breeders (29). Similar to previously recognized ALV infections, ALV-J infections occur either by horizontal transmission between older chickens or by congenital transmission from infected parent stock to their progeny. Earlier work showed that not all congenitally infected birds have the same heavy level of viremia, which might indicate that embryos are infected at different stages of development (27). However, in ALV-infected egg-laying hens, viral particles are abundant in the ovarian stroma, bud from cells in direct contact with oogonia and oocytes (10), and are in the largest concentration in the albumen-secreting glands of the magnum. This early ovarian and oviductal exposure results in natural congenital ALV infection at very early embryonic stage (8,10). This early exposure may produce a more diffuse tissue infection than that previously described for chicks exposed *in ovo* during incubation.

Retroviral envelope glycoproteins in other species have been detected using immunohistochemistry (IHC) (17,20,28). Monoclonal antibodies against ALV-J envelope glycoproteins have been produced and have broad reactivity for most ALV-J isolates examined (12). These antibodies have been used to determine the tissue tropism of ALV-J after experimental *in ovo* exposure at 11 days of incubation using an IHC technique to detect expression of viral group-specific antigen (*gag*) (2). The greatest *gag* staining was observed in the adrenal gland, heart, kidney, and proventriculus at 3 and 7 weeks of age with other organs having little or no staining.

ALV-J has also been detected in tissues by localization of viral nucleic acid using *in situ* hybridization (ISH) (3,30). Chickens exposed experimentally at 11 days of embryo incubation *in ovo* had the greatest detectable nucleic acid in the adrenal gland, heart,

kidney, and proventriculus (3). This localization agrees with that previously demonstrated by IHC detection of viral *gag* (2). Congenitally infected chickens examined by ISH had similar staining patterns to those inoculated *in* ovo with some differences in intensity within and between organs (30).

ALV-J infects and causes neoplasms of myeloid precursors (21,23). Although ALV-A has been found in bone marrow by electron microscopy (11), no ultrastructural studies have shown ALV-J in this location. Furthermore, no viral nucleic acid or protein has been demonstrated in bone marrow by ISH (3,30) or IHC (2), respectively. Specific examination of bone marrow for ALV-J specific viral protein was included in this study to attempt resolution of this apparent contradiction.

The objectives of this experiment were to use IHC staining of ALV-J specific gp85 to determine the chronologic tissue tropism after natural congenital viral infection and to compare these findings with those produced after experimental *in ovo* exposure. Furthermore, specific IHC examination of bone marrow was included to attempt detection of ALV-J after congenital viral infection.

## MATERIALS AND METHODS

**Chicken tissues.** One-day-old commercial broilers were hatched and separated as ALV negative (n=4) and ALV-J positive (n=12) and reared as described previously (31). Briefly, ALV-J positive chickens were hatched from fertile eggs laid by ALV-J positive commercial broiler breeders. Chickens negative for exogenous ALV were hatched from fertile eggs laid by commercial broiler breeders negative for exogenous ALV as determined by p27 antigen capture enzyme linked immunosorbent assay (ELISA) and ALV-J specific reverse transcriptase polymerase chain reaction (RT-PCR). At 1 day of age, broilers were individually confirmed as ALV negative or ALV-J positive using p27 antigen capture ELISA and ALV-J specific RT-PCR as previously described (31). ALV

negative (n=1) and ALV-J positive (n=3) chickens were euthanized at each of 1 day, 3 weeks, 6 weeks, or 9 weeks of age. At necropsy, samples of heart, proventriculus, kidney, liver, lung, spleen, bursa, thymus, bone marrow, peripheral nerve, brain, pancreas, duodenum, adrenal, skeletal muscle, and gonad from each chicken were fixed by immersion in 10% neutral buffered formalin for less than 36 hours and embedded in paraffin for sectioning.

**Preparation of cell culture pellets for a positive and a negative control.** ALV-J infected and uninfected secondary chicken embryo fibroblast made from line 0 embryos were paraffin embedded as previously described (33) and served as positive and negative controls, respectively. Briefly, cells were either infected (positive control) or uninfected (negative control), incubated 7 days at 37 C, scraped from the tissue culture flask and suspended in 10% neutral buffered formalin for 15 minutes, washed 2 times with normal saline, and pelleted by centrifugation for 3 minutes at 10,000 revolution per minute. Cells were then resuspended in twice the volume of the pellet in 0.5 agarose at 42 C and fixed for 2 hours in 10% neutral buffered formalin. Finally, the pellets were routinely processed for paraffin embedding and sectioning.

**Preparation of tissue samples and IHC.** All techniques were done at room temperature. Tissue sections were cut at 4 µm and mounted on charged glass slides (Superfrost / Plus, Fisher Scientific, Pittsburgh, PA). Paraffin was melted from the slides (10 minutes at 65 C) and removed by immersion in Hemo-De three times (5 minutes each time). Slides were air dried and digested with ready-to-use proteinase K (DAKO, Carpinteria, CA) for 5 minutes to expose antigenic target sites by breaking the protein cross-links caused by formalin fixation (6). IHC staining was performed in an automated stainer (Leica ST 5050, Nussloch, Germany) using a nonbiotin peroxidase kit (Dako Envision System, DAKO, Carpinteria, CA) according to the manufacturer's recommendations. The primary antibody used was a monoclonal antibody specific for the

gp85 envelope glycoprotein of ALV-J (provided by Dr. Lucy Lee, ADOL, East Lansing, MI) (12). After IHC staining, sections were counter-stained with hematoxylin, air dried, cover slipped, and examined using light microscopy. Tissues with multifocal staining of less than 30% of the cells were defined as having mild staining (+). Tissues with multifocal staining of 30-80% of the cells were defined as having moderate staining (++). Tissues with multifocal staining of greater than 80% of the cells were defined as having intense staining (+++).

## RESULTS

Sections from the infected positive control cell culture pellet had stainable antigen, whereas no staining was present in sections of the uninfected negative control cell culture pellet. All tissues from ALV-J positive chickens in all age groups had IHC stainable antigen, whereas no staining was present in tissues from ALV negative chickens. There was variability in staining intensity between tissues from ALV-J positive chickens within age groups, as well as among age groups. However, tissues stained were consistent in all ALV-J positive chickens.

The most intense staining was present in the heart, proventriculus, adrenal gland, and kidney (Fig. 3.1). Other organs had less intense staining (Table 3.1). The staining pattern was very similar to that of chickens inoculated *in ovo*. In the adrenal gland, staining was more intense in cortical cells than in medullary cells. In the heart, there was staining of the cytoplasm and plasma membrane of myofibers as well as Purkinje fibers. In the kidney, there was staining in the proximal, distal, and medullary cone tubular epithelial cells. This staining was more intense in the apical portion of these cells. There was also staining in all glomerular cells with the greatest intensity in podocytes. In the proventriculus, staining was present at the basal portion of glandular epithelial cells, smooth muscle in the tunica muscularis, and the connective tissue of the interstitium. In

the bone marrow, staining was multifocal and limited to cells with cytoplasmic granules interpreted to be myelocytic precursor cells. In the lung, staining was present in atrial and bronchiolar epithelial cells, in the adjacent connective tissue, and in cells lining air and blood capillaries. In the pancreas, there was staining at the apical portion of individual acinar epithelial cells. In the brain, there was staining of both neurons and glial cells. In the bursa of Fabricius, there was staining in medullary, cortical, and surface epithelial cells. In the liver, there was staining of hepatocyte cytoplasm adjacent to canaliculi and Kupffer's cells. In peripheral nerves, there was staining in both axons and myelin sheaths. In the gonads, there was staining in the interstitial cells in both ovaries and testes. In skeletal muscle, there was staining at the plasma membrane of myocytes. In the spleen, staining was greatest in the centers of germinal centers. In the thymus, medullary epithelial cells stained, but cortical lymphocytes did not. In other tissues, there was staining in smooth muscle and some connective tissues, but in general staining was less intense and more inconsistent than in the organs described above.

## DISCUSSION

The IHC staining present in the tissues from congenitally infected chickens used in this study was predominantly in the adrenal gland, heart, kidney, and proventriculus. This agreed with results using chickens inoculated *in ovo* (2). This suggests high tropism of the virus to these organs. Tropism in these organs may be explained by the possible abundance of viral receptors on the cells of these organs, efficiency, and adaptation of long terminal repeats of the virus in enhancing and promoting replication in these organs (7), tissue specific endogenous factors that regulated the expression of the virus at the transcriptional level (15), or simply by the ability of these cell to synthesize more protein than other cells (10). Some tissues from chickens exposed by *in ovo* inoculation were negative by IHC staining for *gag*, whereas all tissues from congenitally exposed chicken

were positive by IHC staining for gp85. In addition, the distribution and intensity of stain in the different tissues was similarly stable among age groups examined up to 9 weeks of age.

At 1 day of age, the viral antigen distribution in congenitally infected chickens was also greater than distribution at 3 weeks of age in chickens exposed during incubation. Embryos infected at the single cell stage will have all cells infected (8,9,10), and this likely accounts for more disseminated staining. Additional possible explanations exist. Reinfection during viremia may increase intracellular viral protein concentration, resulting in more stainable antigen per cell and increased detection by IHC. Second, differences in viral replication efficiency have been reported in different chicken lines (4,5). Similar differences in viral replication efficiency may exist between the commercial broilers used in our study and the inbred line 21 chickens used in previous studies. Third, differences in tissue tropism exist between isolates of ALV-J (32); and thus, the isolate of ALV-J used in the present study may differ in its tropism from that of previous studies.

*Gag* proteins are detected in more tissues than *env* proteins for ALV-A (RAV-1) (26). Retroviruses in general express slightly more protein from *gag* genes than from *env* genes (28). However, we detected the opposite in the sections we examined, with more stainable *env* protein gp85 in the sections examined than previously described for IHC staining of *gag* proteins (2). This may be because of one or more of the hypotheses mentioned above resulting in intracellular viral protein concentration in excess of the minor difference present between *gag* and *env* (18).

Recent investigations failed to demonstrate specific viral staining in bone marrow from infected chickens (2,3,30). We were able to show moderate staining in bone marrow cells with cytoplasmic granules interpreted to be myelocytic precursor cells (Table 3.1). This finding agrees with previous work showing cell cultures of bone marrow are

susceptible to ALV-J infection (23) and the tendency of subgroup J to predominantly induce myeloid rather than lymphoid neoplasms (13,32).

Ascites secondary to right ventricular failure occurs worldwide in young broiler chickens and is a significant cause of mortality in many flocks (25). Avian tumor virus-like particles were seen by electron microscopy between myocardial fibers in chickens affected with ascites (19). Furthermore, RAV-1 infection in SPF chickens can cause right sided heart failure (16). In this study, there was intense staining of the myocardium together with Purkinje fibers, this intense staining correlate with large amounts of antigen present in these tissues. This may explain the ability of ALV to cause cardiomyopathy and ascites may be by impairing the function of the cardiac muscle or its conducting system (Purkinje fibers).

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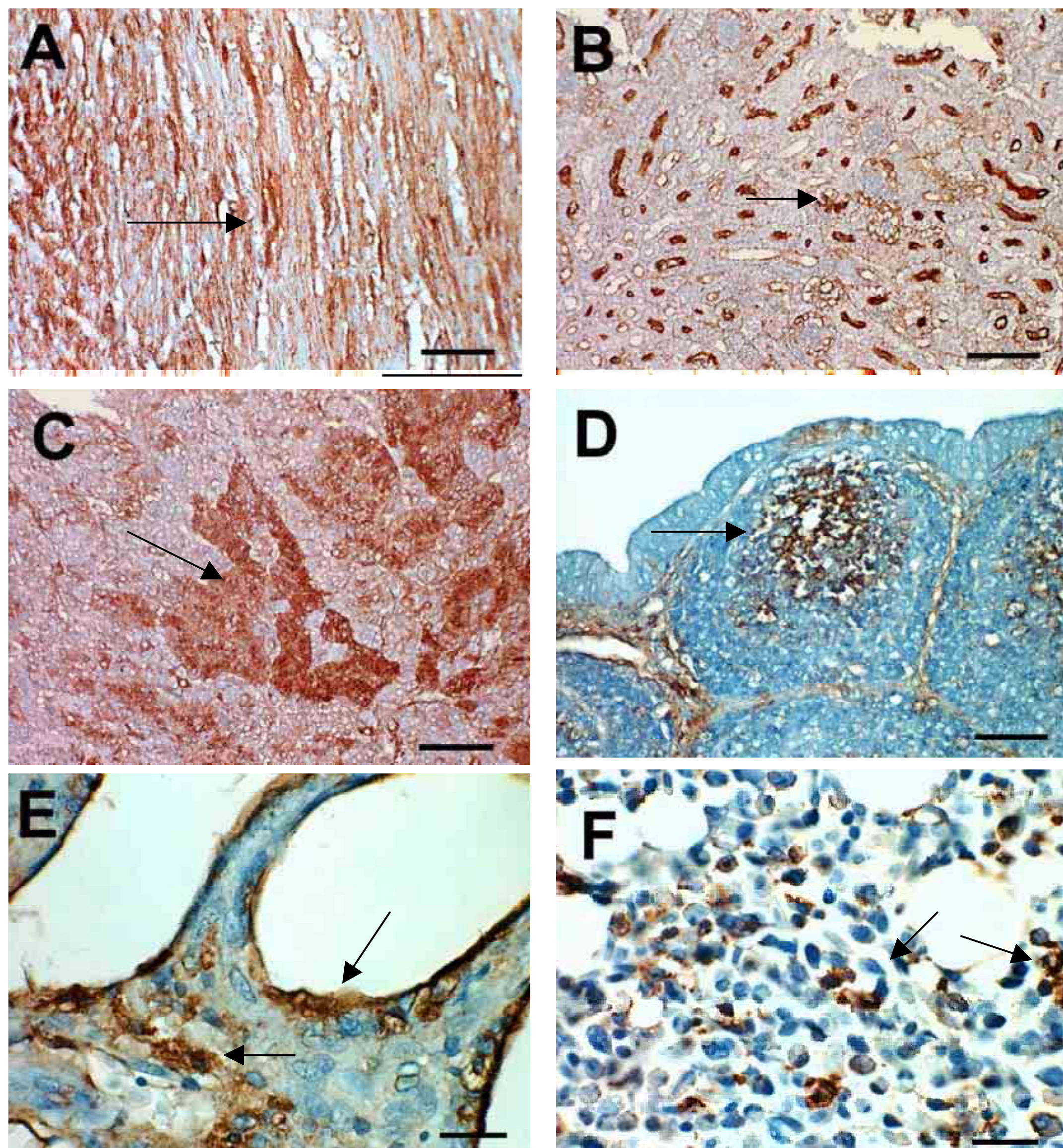
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Fig. 3.1 Photomicrographs of IHC staining for envelope glycoprotein (gp85) of ALV-J in tissues from ALV-J congenitally infected chickens. (A) Intense staining (+++) in the myocardium of a 3-week-old chicken. (B) Intense staining (+++) in the kidney of a 3-week-old chicken. (C) intense staining (+++) in the adrenal gland of a 3-week-old chicken. (D) Mild staining (+) in the bursa of a 3-week-old chicken. (E) Moderate staining (++) in the lung of a 3-week-old chicken. (F) Moderate staining (++) in the bone marrow of a 3-week-old chicken. Bars in A, B, C, and D are 100 µm and in E and F are 30 µm. Arrows are pointing examples of positively staining cells.

Table 3.1 IHC staining intensity for ALV-J antigen in congenitally infected chicken tissues at 0, 3, 6, or 9 weeks of age. [A]

| | Age (wk) | | | |
|---|---|---|---|---|
| Tissue | 0 | 3 | 6 | 9 |
| Adrenal | +++ | +++ | +++ | +++ |
| Bone marrow | ++ | ++ | ++ | ++ |
| Brain | + | + | + | + |
| Bursa | + | + | + | + |
| Duodenum | + | + | + | + |
| Heart | +++ | +++ | +++ | +++ |
| Kidney | +++ | +++ | +++ | +++ |
| Liver | + | + | + | + |
| Lung | ++ | ++ | ++ | ++ |
| Nerve | + | + | + | + |
| Ovary | + | + | + | + |
| Pancreas | ++ | ++ | ++ | + |
| Proventriculus | +++ | +++ | +++ | +++ |
| Skeletal muscle | + | + | + | + |
| Spleen | + | + | + | + |
| Thymus | + | + | + | + |
| Testes | + | + | + | + |

[A]Symbols: + = mild staining; ++ = moderate staining; +++ = intense staining.

# CHAPTER 4

# PRODUCTION OF AVIAN LEUKOSIS VIRUS SUBGROUP J (ALV-J) STRAINS RESISTANT TO POLYCLONAL ANTIBODY NEUTRALIZATION[1]

[1] Gharaibeh, S, T. Brown, M Pantin. Submitted to *Avian Diseases.*

SUMMARY. An ADOL-7501 isolate of avian leukosis virus subgroup J (ALV-J) was cloned *in vitro* by three serial terminal dilutions. The cloned virus was injected into specific pathogen free (SPF) chickens, and the antiserum produced had *in vitro* neutralizing activity against the cloned virus. The cloned virus was then serially passed three times in the presence of subneutralizing levels of this antiserum, and the resultant viral isolates were more resistant to antiserum neutralization *in vitro* than was the parent cloned virus. No nucleotide differences were detected between the *env* gene of the parent cloned virus and that of the neutralization-resistant mutants. Other possible genomic changes outside the env gene (*i.e.* in the LTR and *gag* genes) may account for these non-*env* based differences in neutralization indices.

Key Words: avian leukosis virus subgroup J, retrovirus, antiserum, neutralization-resistant, envelope gene

List of Abreviations: ALV-J: avian leukosis virus subgroup J, ALV: avian leukosis virus, CEF: chicken embryo fibroblast, CS: cloned stock, ELISA: enzyme linked immunosorbent assay, HIV: human immunodeficiency virus, LTR: long terminal repeat, NSV: negative-serum virus, PCR: polymerase chain reaction, RSV: Rous sarcoma virus, RT-PCR: reverse transcriptase polymerase chain reaction, SPF: specific pathogen free, VN: virus neutralization,

RNA viruses infect a wide variety of natural hosts and frequently escape *in vivo* pressure from induced antibodies or antiviral treatments (12). One mechanism for this escape is the development of new quasispecies during persistent infections (10). For retroviruses, the development of quasispecies is especially favored by their rapid replication and large viral copy numbers typically present in infected hosts (35,36).

Furthermore, their reverse transcriptase is prone to replication errors caused by copy transitions, transversions, deletions, and insertions. These copy errors make *in vivo* occurrence of neutralization-resistant quasispecies the norm rather than the exception (10). Such quasispecies development occurs during natural infections with equine infectious anemia virus (27,37), visna virus (41), and human immunodeficiency virus (HIV) (54,55,61,62).

Within subgroup J of avian leukosis virus (ALV-J), naturally occurring antigenic variations are detectable using monoclonal antibodies (14) and are most often associated with nucleotide sequence changes in the variable regions of the viral envelope glycoprotein *env* (51). These variations are important because they may lead to problems using antigen-antibody based diagnostic assays (44). Even though the control of ALV-J has been oriented towards elimination of infection at the parent stock level (49), vaccination may also be needed to finally control the disease in some situations. Efficacy of such vaccines would be negatively impacted by known existing variations. Use of inactivated vaccines may actually encourage increased development of antibody-resistant quasispecies within a vaccinated infected host, as has been shown for other retroviral infections (28,29,47).

The present study was designed to test the hypothesis that chicken anti-ALV-J polyclonal neutralizing antibody will encourage rapid emergence of viral quasispecies with altered envelope gene sequences and resultant resistance to antibody neutralization.

## MATERIALS AND METHODS

**Cells.** Secondary cells of chicken embryo fibroblasts (CEF) made from line 0 chicken embryos (Kestrel, Waukee, IA) were used as a culture system throughout the experiment. CEF cells from line 0 embryos are resistant to infection by endogenous avian leukosis virus (ALV-E) (C/E phenotype) (15).

**Virus and cloning.** A 3rd tissue culture passage of ADOL-7501 isolate of ALV-J was obtained (Dr. A. Fadly, ADOL, East Lansing, MI). The virus was confirmed as ALV-J using reverse transcriptase polymerase chain reaction (RT-PCR) with previously published specific primers H5 & H7 (45). This virus was cloned as previously described for equine infectious anemia virus (20) with some modifications. Briefly, secondary cells from line 0 CEF cultures were inoculated with 10-fold serial dilutions of ADOL-7501 ALV-J. After 9 days, cultures were tested for the presence of the virus by p27 antigen capture enzyme linked immunosorbent assay (ELISA). The virus obtained from the culture with the lowest concentration of the inoculum was used as inoculum for a second passage. This procedure was repeated 3 times. The resulting virus in cell culture was aliquoted as a stock to be used in this experiment. Thus, the stock virus corresponded to passage 7 of the ADOL-7501 ALV-J.

**Antisera production.** Nine specific pathogen free (SPF) chickens (SPAFAS, Preston, CT) were inoculated intramuscularly with 0.5 ml of $10^{6.5}$ tissue culture infective dose 50 ($TCID_{50}$) / ml of the cloned virus multiple times at 20, 22, 24, and 28 weeks of age. Serum was collected at 29 weeks of age one week after the last injection . Sera from chickens that had high neutralizing titer were pooled and used in all subsequent experiments.

**Generation of neutralization-resistant virus.** A technique previously used to generate HIV escape mutants was used (56). Briefly, 1 ml of culture supernatant of the cloned stock virus was incubated for 30 minutes at 37 C with 2-fold dilutions of homologous antiserum generated as described above. Virus-antiserum mixture was then added to secondary line 0 CEF cells with an antiserum concentration identical to that in the mixture. After 7 days, cell culture fluid was harvested and tested by p27 antigen capture ELISA. ELISA positive cultures with the highest antiserum concentration were

used as inoculum for a second passage. The virus was passed 3 times in the presence of the antiserum. This procedure was done in duplicate, and additional negative antiserum control samples were similarly passed.

**Serology.** A virus-neutralization (VN) test was carried out as described previously (15) with 2-fold dilution beginning at 1:5 of antiserum. Serum samples were assayed for ALV-J specific antibody using two ELISA systems (KPL, Gaithersburg, MD and IDEXX Laboratories, Westbrook, Maine).

**Polymerase chain reaction (PCR) amplification and cloning.** Aliquots of original cloned ADOL-7501 (passage 7), 2 replicates of virus after passage with antibody, and virus similarly passed with negative serum were all amplified by PCR, molecularly cloned, and sequenced. Primers were kindly provided by Dr. Guillermo Zavala (PDRC, Athens, GA). The forward primer (5'-GTG CGT GGT TAT TAT TTC C-3') annealed on the 3' of the integrase gene and upstream of the protein coding region of gp85, and the reverse primer (5'-TAT TGC TGT TTC ATC GTT A-3') annealed on the 3' long terminal repeat (LTR). PCR mixes were in a commercial kit (Titan One Tube RT-PCR System, Roche, Mannheim, Germany) and were used according to manufacturer's directions. This system uses *Pwo* DNA polymerase, which has higher fidelity than *Taq* DNA polymerase (30). The expected amplicon size was approximately 1.9 Kb. Reverse transcription was done at 50 C for 30 min. Following an initial melting step at 94 C for 5 min, cDNA was amplified during 35 cycles of 94 C for 15 seconds, 55 C for 2 min, and 74 C for 4 min. PCR amplifications were performed on the viruses passed in the presence of antiserum and the cloned stock virus. DNA from each PCR reaction was independently cloned into TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

**DNA sequencing and analysis.** Four different clones of each virus were sequenced on an ABI Model 373 or 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The first sequencing was done using M13 forward and reverse vector primers (Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA). Internal forward primer was 5'-GCG GAT TCA CCA GCA ACG AG-3' and internal reverse primer was 5'-TCA TCT TCC ACC CGT ATC TT-3'. Internal walking primers were designed and produced based on the results from this first sequencing run. Contiguous sequences were constructed using DNASTAR program (DNASTAR, Madison, WI). The correct sequence was defined as the one matching results for more than 2 viral replicas, and gp85 and gp37 were identified by comparison with published sequences for ALV-J strain Hc1 (44).

## RESULTS

**Antisera production.** After all virus injections, all sera from the 9 chickens were negative by the two Ab-ELISAs for ALV-J specific antibodies. However, the VN titers for these sera against the cloned virus ranged from 1:5 to 1:1280 with a geometric mean titer of 1:121 (Table 4.1). A pooled positive serum produced by combining positive serum from chickens 1, 3, 5, and 7 used in this experiment had a neutralizing titer of 1:640.

**Generation of neutralization-resistant virus.** The highest concentrations (lowest dilution) of the antiserum allowing the virus to grow in each passage are depicted in Table 4.2. After the 3$^{rd}$ passage, isolates from both replicates were able to grow in an antiserum dilution of 1:40 and 1:80, respectively. The virus passed in the presence of negative serum (NSV) grew in all negative serum dilutions used, including the highest concentration used (1:20).

After 3 passages in the presence of antiserum, $TCID_{50}$ for neutralization-resistant isolates 1, 2, and the NSV were $5x10^{5.5}$ / ml, $5x10^{5.5}$ / ml, and $5x10^{7.6}$ / ml, respectively. Microneutralization titration using the positive antiserum was carried out on the four viral sources after equalization of the $TCID_{50}$ of the viral inoculum. The results of the virus neutralization were 1:5, 1:10, 1:80, and 1:640 for isolates 1, 2, NSV, and cloned stock (CS), respectively.

Nucleotide sequences for the 1.9 Kb amplicons spanning the *env* genes of isolates 1, 2, and NSV were identical to each other and to the CS (ADOL-7501) in Fig 4.1 , and CS were identical to each other. Fig. 4.1 shows the amino acid sequence deduced from *env* gene sequence of cloned ADOL-7501 (Gene Bank accession # AY040857) and Hc1 (Gene Bank accession #: AF247391) isolates of ALV-J.

## DISCUSSION

The differences of $log_2$ neutralization titer between viruses 1, 2, and NSV were 4 and 3, respectively. Passage of ALV-J in the presence of the antiserum resulted in increased resistance of virus isolates 1 and 2 to neutralization by the antiserum. This difference in neutralization is larger than that previously reported in a similar experiment using respiratory syncytial virus (48). These findings suggested changes in the nucleotide sequence of the *env* gene may have occurred resulting in amino acid changes conferring different protein conformation and change of antigenic epitopes. Surprisingly, sequencing of the *env* genes of the neutralization-resistant viruses showed no such changes. Multiple possible explanations exist for this finding.

First, retroviral *env* genes code for most neutralizing epitopes, but other genes may also code for such epitopes. Neutralizing epitopes are present on the viral surface, exposed as the virus enters the host cell, or are required for membrane assembly of viral particles (40). The non-*env gag* protein p17 of HIV virus has 2 neutralizing epitopes (34)

and antibodies to p17 neutralize HIV (31,34,42). Numerous p17 mutations occur in HIV infected patients (23,24,25,58,60). Neutralization by p17 *gag* induced antibodies may occur by interaction with undefined components of the host cell membrane or viral replication (34). Polyproteins coded by *gag* assemble into a network of ring-like structures producing icosahedral viral core structures (32). Certain p17 C-terminal sequences are also involved in viral penetration and uncoating (57). Antiserum against p15 of Friend murine leukemia virus, a counterpart of HIV's p17, is also neutralizing in the presence of complement indicating that p15 is a surface antigen (16). If similar neutralizing *gag* proteins exist for ALV, changes in such epitopes may explain the absence of *env* changes in our experiment.

Second, passage of virus in the presence serum may have induced quasispecies progressively more efficient at replication in such sera. Progressive adaptation to cultivation in serum is caused by changes in the LTR, which are susceptible to mutations in retroviruses (1,2,3,9,18,19,22,26,43,52). Such retroviral LTR alterations can increase promoter activity (38,59), change tissue specificity (1,2,19,43), and change target cell oncogenicity (22,43). The LTRs of ALVs have been shown to change cell specificity (9,18), pathogenicity (19), and increase Rous sarcoma virus (RSV) LTR-driven transcription 5-fold (5,13). In our study, passage of viruses 3 times in cell culture in the presence of serum may have induced LTR mutations resulting in enhanced viral replication in the presence of such serum, effectively overcoming the partial neutralization by low levels of our polyclonal antibody.

Third, we used fibroblasts for viral passage in our work. This may have imposed a conservation pressure on receptor binding epitopes in *env* that was greater than the mutation pressure exerted by our antiserum. This would result in preferential stability of *env* gene, variation elsewhere in the viral genome, and has been previously observed in the early stages of other retroviral infections (58).

Although it has been shown that multiple antigen injections of some viruses (infectious bronchitis virus) increases cross reactivity of the antiserum between different isolates (8,17), both commercially available Ab-ELISAs did not detect the neutralizing polyclonal antibodies produced after injecting chickens with the cloned ALV-J several times. These ELISA assays use recombinant surface proteins (gp85) coded for by *env* genes of an English prototype isolate HPRS-103 (IDEXX) or an American prototype isolate HC1 (KPL). The ALV-J *env* gene has more than 95% similarity to endogenous ALVs (4,39). The cloned ALV-J (ADOL-7501) we used has more than 92% similarity to endogenous viruses EV/J (4) and EAV/HP (46). Chickens are often immunologically tolerant to their own endogenous viruses (21). Tolerance to these highly similar endogenous viruses may have prevented *in vivo* development of antibodies detectable by *env* based ELISA assays similar to previous studies (21) and may explain the results using these assays in the present experiment. Furthermore, if this was the case, antibody neutralizing pressure for mutation would then have been imposed on non-*env* epitopes as has been documented to occur with other retroviruses (16,34).

It is also possible that sufficient antigenic variations exist between the antigen coating the ELISA plates and the ADOL-7501 isolate used in our studies that prevented the detection of the antibodies to the latter. Antigenic variations of ALV-J exist, and some will not cross react with antibody to prototype strains (44,51). Recombinant baculovirus-produced gp85 coating the ELISA plates is 54 kD (50) compared with 85 kD for the naturally produced homologue. This difference is caused by altered glycosylation of viral proteins produced in insect cells that is used in commercially available ELISA systems compared with those produced in chicken cells (33). Because glycosylation can impact antigen-antibody reactivity (53), the observed lack of ELISA reactivity may have occurred because such putative glycosylation changes.

Further studies will be required to locate and determine the mechanism for induction of these antibody neutralization-resistant mutants of ALV-J.

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Table 4.1. Virus neutralization results for chickens given four injections of ALV-J at 20, 22, 24, and 28 weeks of age and bled one week after the last injection.

| Chicken # | Virus neutralization titer |
|---|---|
| 1 | 40 |
| 2 | 1280 |
| 3 | 1280 |
| 4 | 5 |
| 5 | 320 |
| 6 | 40 |
| 7 | 1280 |
| 8 | 10 |
| 9 | ND |
| GMT* | 121 |

*GMT geometric mean titer;  ND not done.

Table 4.2. The highest neutralizing antiserum concentration in the media allowing the virus to grow in each passage except for the negative serum virus (NSV) where these are the highest negative serum concentrations used.

| Virus isolate | First passage | Second passage | Third passage |
|---|---|---|---|
| 1 | 1:320 | 1:320 | 1:40 |
| 2 | 1:320 | 1:320 | 1:80 |
| NSV* | 1:80 | 1:80 | 1:20 |

* negative serum virus.

```
                                                                     
1    G V H L L Q Q P G N V W V T W A N K T G Q T D F C L S L Q S   ADOL-7501.PRO
1    . . . . . . . . . . . . . . . . . . . . R . . . . . . . . .   Hc1.PRO

31   A T S P F R T C L V G L P S Y Q L E E F R E Y T V N Y T E C   ADOL-7501.PRO
31   . . . . . . . . . I . I . Q . P . S T . E G . V T . V . A .   Hc1.PRO
                                          |---vr1----|
61   R N D T D M A T Q T A C L I Q S L N T T L P W D P Q E L D I   ADOL-7501.PRO
61   D . S A . L . N . . . . . . K A . . . . . . . . . . . . . .   Hc1.PRO
                             |-------------------------------
91   L G S R M I K N G T T R T C V T F G S V C Y K N N G S T T C   ADOL-7501.PRO
91   . . . Q . . . . . . K . . . . . . . . A . . . E D N . . V .   Hc1.PRO
                             hr1
    -----------------------------------------------------------
121  H I F D G N F N G T G G A E A E L R D F I E I W K G N D L L   ADOL-7501.PRO
121  . N . . . . . . . . . . . . . . . . . . . A K . . S D . P .   Hc1.PRO
    ---------------|                                          |--
151  I R P Y V N Q S W T M V S P I N T E S F S I S S R Y C G F T   ADOL-7501.PRO
151  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hc1.PRO
                   hr2
    ----------------------------------------|
181  S N E T R Y Y S M N T S D W C D T K G G T W S A A D R N G T   ADOL-7501.PRO
181  . . . . . . . R G . F . N . . G S . . . K . . . G Y S . . .   Hc1.PRO
                                            |------------vr3--
211  K C F S N T T G C G G N C T A E W N Y Y A Y G F T F G K Q P   ADOL-7501.PRO
211  E . S D G . A . . . . . . . . . . . . . . . . . . . . N K .   Hc1.PRO
    -----|
241  E V L W N N G T A K A L P P G I F L I C G D R A W Q G I P R   ADOL-7501.PRO
241  . I . . . . . . . . . . . . . . . . . . . . . . . . . . . S   Hc1.PRO

271  N A L G G P C Y L G Q L T M L S P N L T T W M T Y G P N I T   ADOL-7501.PRO
271  . . . . . . . . . . . . . . . . . . F . . . I . . . . . . .   Hc1.PRO

301  G H H R S R R S A S Q L S S D C S D D V Q L W S A T A R I F   ADOL-7501.PRO
301  . . R . . . . . L . R . . P . . G . E L . . . . V . . . . .   Hc1.PRO

331  A S F F A P G V A A A Q A L R E I E R L A C W S V K Q A N L   ADOL-7501.PRO
331  . . . . . . . . . . . . . . K . . . . . . . . . . . . . . .   Hc1.PRO

361  T S L I L N A M L E D T N S I R H A V L Q N R A A I D F L L   ADOL-7501.PRO
361  . . . . . . . . . . . M . . . . . . . . . . . . . . . . . .   Hc1.PRO

391  L A Q G H G C Q D V E G M C C F N L S D H S E S I H K A L Q   ADOL-7501.PRO
391  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hc1.PRO

421  A M K E H T Q K I R V E D D P I G D W F T R T F G S L G G W   ADOL-7501.PRO
421  . . . . . . E . . . . . . . . . . . . . . . . . . G . . . .   Hc1.PRO

451  L A K G V K T L L F A L L V I V C L L A I I P C I I K C F Q   ADOL-7501.PRO
451  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hc1.PRO

481  D C L S R T M Y Q F M D E R I R Y H R I R E Q L .             ADOL-7501.PRO
481  . . . . . . . . . . . . . . . . . . . . . . . . .             Hc1.PRO
```

Fig. 4.1 Amino acid sequence deduced from *env* gene sequence of cloned ADOL-7501 (ADOL-7501.PRO) and Hc1 (Hc1.PRO) (Gene Bank accession #: AF247391). Deduction of amino acid sequence and its alignment is done using DNASTAR program (DNASTAR, Madison, WI). Variable regions (vr1, hr1, hr2, and vr3) are previously described (6,7,11, and 51). Dots in the sequence of Hc1 indicate residue similar to ADOL-7501 at that position.

# CHAPTER 5

# EFFECTS OF INJECTING ALV-J ANTISERUM IN EMBRYONATING BROILER EGGS ON VIREMIA, BODY WEIGHTS, TUMOR INCIDENCE, AND ACTIVE IMMUNITY IN BROILER CHICKENS[1]

[1]Gharaibeh, S., T. Brown, and M. Pantin. To be submitted to *Avian Diseases*.

SUMMARY. The purpose of these two experiments was to determine the effect of injecting ALV-J antiserum into embryonated chicken-eggs on protection against body weight suppression, viremia, development of ALV-J related tumors, and the number of chickens developing active immunity. In the first experiment, chickens exposed to ALV-J by cohatching with virus-shedders did not suffer from body weight suppression or ALV related tumors, and the injected ALV-J antiserum did not protect against development of viremia or increase the number of chickens subsequently developing active immunity. In the second experiment, chickens were exposed to ALV-J by injection of virus at hatch. Injection of ALV-J antiserum protected these chickens against development of ALV-J related tumors, but did not protect against virus induced body weight suppression or development of viremia, and did not increase the number of chickens developing active immunity.

Key words: chicken, avian leukosis virus subgroup J, maternal antibody, tumors, viremia, body weight suppression, active immunity

Abbreviations: Ag-ELISA = antigen-capture enzyme linked immunosorbent assay; ALV-J = avian leukosis virus subgroup J; CEF = chicken-embryo fibroblast; SPF = specific-pathogen free; $TCID_{50}$ = tissue culture infective dose 50; VN = virus neutralization

The new subgroup J of avian leukosis virus (ALV) was first reported in 1991 (12). This new subgroup infects mainly chickens but also can infect other fowl species (15). ALV-J causes body weight suppression (19), myeloid leukemia (14), other neoplasms (1,15), and reduction in egg size (18). ALV-J is transmitted horizontally or vertically in chicken flocks (13).

Embryonal infection of chickens with ALV-J results in a permanently tolerant viremia (14). Contact infection results in transient or permanent viremia depending on age of exposure. For ALV-A, presence of circulating maternal antibodies reduces the number of chickens developing viremia, cloacal viral-shedding, tumors, and increases the number of chickens developing active immunity by 18 weeks of age (7,8).

Passive transfer of antibodies into chickens by intraperitoneal antiserum injection is protective against subsequent challenge with the homologous pathogen (3,9,11). Injection of antibodies in the yolk sac of incubating embryos results in circulating antibodies and has a similar protective effect in chickens (5) and turkeys (2). Embryo inoculation can be automated (16) making it a potentially more practical approach than inoculating of antisera into hatched chicks. In this study, we inoculated embryonating chicken eggs in the yolk sac with anti-ALV-J antiserum to investigate its potential protective effects against ALV-J.

## MATERIALS AND METHODS

**Virus and Serology.** ADOL-7501 isolate of ALV-J was cloned by three limiting dilutions in secondary line 0 (6) chicken embryo fibroblast (CEF) cultures. This cloned virus had a tissue culture infective dose 50 ($TCID_{50}$) of $10^{6.5}$/ ml. It was diluted 1:100 with culture medium and was used for embryo inoculations. A virus neutralization (VN) test was carried out on secondary line 0 chicken embryo fibroblast (CEF) cultures as a microneutralization assay using 100 TCID / well (6). Viremia in plasma was tested by

commercial antigen capture enzyme linked immunosorbent assay (Ag-ELISA) (IDEXX Laboratories, Westbrook, ME)

**Sera.** ALV-J antiserum was produced by injecting a group of specific pathogen free (SPF) chickens (n=9) (SPAFAS, Preston, CT) intramuscularly with 0.5 ml of $10^{6.5}$ $TCID_{50}$ of live cloned virus multiple times at 20, 22, 24, 28 weeks of age. Serum was collected at 29 weeks of age. SPF preimmune serum was collected prior to the first injection (negative serum control), and after the last injection at 29 weeks of age. All sera were heat inactivated at 56 C for one hour and filtered at 0.2 μm (Whatman Inc., Clifton, NJ). Sera from inoculated chickens with a virus-neutralizing-titer greater than 1:640 (n=3) were pooled. The neutralizing titer of this positive pool was 1:1280, while the negative serum control did not have any detectable neutralizing titer.

**Serum or Virus Inoculations.** ALV-J negative White Plymouth Rock chicken eggs (provided by Dr. David Swayne, Southeast Poultry Research Laboratory, USDA-ARS, Athens, GA) were used in all experiments. All egg inoculations were done in the yolk sac (17). Sera inoculations were performed at 5 days of incubation with 0.1 ml of either the positive pool or the negative serum control. Virus inoculations were performed at 7 days of incubation with the cloned virus described above. Shedders were produced by *in vivo* virus inoculation without sera inoculation. Chicks exposed at hatch were injected intraperitoneally with 0.1 ml undiluted cloned virus.

**Experimental Design.** Numbers of eggs in experimental treatment groups are shown in Table 5.1. *Expt. 1. Contact exposure.* Chicks hatched from eggs injected with ALV-J antiserum (n=11) or hatched from eggs injected with negative serum (n=11) were cohatched (Natureform NOM-45, Jacksonville, FL) with shedders (n=7). *Expt. 2. Parentral inoculation.* Chicks hatched from eggs injected with ALV-J antiserum (n=14) or with negative serum (n=12) were hatched in a separate hatcher free of virus and exposed by injection at hatch as described above. Negative control chickens (n=32) were

hatched and raised separately with no exposure to virus or antiserum. Chickens were wing banded and raised in floor pens and allowed unlimited access to feed and water. Chickens were bled and weighed at 0, 1, 3, 5, 7, 9, and 13 weeks of age. Necropsy was performed on chickens that died during the 13 weeks and on all the remaining chickens at the end of the 13 weeks.

## RESULTS

**Body Weight.** ***Expt. 1.*** Average body weights at 0, 1, 3, 5, 7, 9, and 13 weeks of age for experimental, shedder, and negative control chickens are shown in Table 5.2 There was no statistically significant difference between chickens that received negative or positive ALV-J antiserum and the negative-control-group. The shedder group weighed less than all other groups at all times, but was only statistically significant compared to the negative-control-group at 3 and 5 weeks of age. Due to ALV-J induced mortality only one shedder chicken survived to the end of the study, and no statistical comparison for the shedder group was done after 5 weeks. *Expt. 2.* Body weights at 0, 1, 3, 5, 7, 9, and 13 weeks of age for all chickens hatched are shown in Table 5.3. There was no statistically significant difference between chickens receiving negative or positive ALV-J antiserum ; however, both treatment groups were underweight compared to the negative-control-group from 5 weeks to the end of the experiment at 13 weeks of age (*P*-value <0.05).

**Viremia.** *Expt. 1.* Plasma Ag-ELISA results at 1, 3, 5, 7, 9, and 13 weeks of age are shown in Table 5.4. There was no statistically significant difference between chickens receiving negative or ALV-J positive antiserum. All shedders were consistently viremic at all times tested. *Expt. 2.* Ag-ELISA results at 1, 3, 5, 7, 9, and 13 weeks of age on plasma samples are shown in Table 5.5. There was no statistically significant difference between chickens receiving negative or ALV-J positive antiserum.

**Active Immunity.** ***Expt. 1.*** VN test results at 0, 1, 7, and 13 weeks of age on serum samples from chickens are shown in Table 5.6. There was no statistically significant difference between chickens receiving negative or ALV-J positive antiserum. All the shedders were viremic at all intervals, did not have detectable neutralizing titer, and thus were ALV-J tolerant. *Expt. 2.* VN test results at 0, 1, 7, and 13 weeks of age on serum samples from chickens are shown in Table 5.7. There was no statistically significant difference between chickens receiving negative or ALV-J positive antiserum.

**Tumor Incidence and Mortality.** *Expt. 1.* By the end of the experiment at 13 weeks of age, 6 of the 7 shedders had died. Three had ALV related tumors (2 myelocytomas, and one hemangiosarcoma). The other 3 chickens that died were severely decomposed at the time of the necropsy and the cause of death was not determined. None of the chickens exposed to shedders developed gross or histologically detectable tumors (Table 5.8). Histologic lesions compatible with Marek's disease were observed in two chickens hatched from eggs inoculated with negative serum and 1 chicken hatched from an egg inoculated with ALV-J positive antiserum. None of the shedders had gross or histological evidence of Marek's disease. *Expt. 2.* Chickens in treatment groups inoculated with ALV-J at hatch had tumors related to ALV in 7 of the 13 chickens hatched from eggs inoculated with negative antiserum (3 nephroblastomas, 2 hemangiosarcomas, and 2 undifferentiated spindle cell tumors) but only one of 12 hatched from eggs inoculated with ALV-J positive antiserum developed a tumor (undifferentiated spindle cell tumor ) (Table 5.8). The difference in tumor incidence between treatments was significant (*P*-value = 0.008). One chicken hatched from an egg inoculated with negative serum died after ALV-J injection after hatch and was excluded from the tumor incidence data. Five chickens from each treatment group had histologic lesions compatible with Marek's disease. The negative-control-group did not have any

gross or histologic evidence of ALV related tumors or lesions compatible with Marek's disease.

## DISCUSSION

None of the chickens hatched from eggs inoculated with ALV-J antiserum had detectable antibodies by VN test at hatch. This could be due to the dilution effect of injecting only a small volume (0.1 ml) of antiserum in each egg.

In *Expt. 1*, contact exposure to ALV-J did not cause body weight suppression in the two groups exposed to shedders. Shedder chickens (congenitally infected) had depressed body weights compared to the negative control. In *Expt. 2*, the two groups that were inoculated with ALV-J at hatch also had depressed body weights compared to the negative-control-group. The injected ALV-J antiserum in the eggs did not protect against this body weight suppression. This suppression in body weight is similar to what previously seen with infection with ALV-A at 1 day of age (4).

In *Expt. 1*, contact exposure to ALV-J did not cause ALV related tumors in any of the two groups exposed to shedders for the 13 weeks period of the experiment. In *Expt. 2*, chickens hatched from eggs injected with ALV-J antiserum and then inoculated with ALV-J at hatch had a lower incidence of avian leukosis virus related tumors compared to chickens hatched from eggs injected with negative antiserum and then inoculated with ALV-J at hatch (Table 5.8). This protective effect was present in spite of there being no antibodies detectable by VN test. This protection against tumor formation could be explained by the presence of antibody titers below the detection limit of the VN test performed. It is also possible that the inoculated virus in the abdomen came in direct contact with residual levels of antibodies leaking from the yolk sac resulting in decreased numbers of viral particles which may explain the reduction of ALV related tumors in this group.

In *Expt. 1*, three out of 22 chickens exposed to shedders developed lesions compatible with Marek's disease, while in *Expt. 2*, ten out of 25 chickens inoculated with ALV-J at hatch, developed lesions compatible with Marek's disease. This difference is statistically significant (*P*-value = 0.02). Chickens infected with ALV are more susceptible to Marek's disease (10) and the difference in incidence between the groups in the two experiments indicates that parentral injection of the virus is more pathogenic than contact exposure. None of the shedders developed lesions compatible with Marek's disease, probably because most of the chickens in this group died by 7 weeks of age. The effects of ALV-J infection were more pronounced on body weight and tumor formation in the groups inoculated with ALV-J at hatch or as embryos (shedders) than the groups that were contact-exposed to ALV-J.

The injected antiserum did not reduce the number of viremic chickens or increase the number of chickens developing active immunity by 13 weeks of age. This disagrees with previous studies with other ALV subgroups that showed protection. against these effects (7,8). However, in those previous studies maternal antibodies were passed into eggs by the hen, and detectable circulating levels of antibodies were detected in chicks after hatching.

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Table 5.1 White Plymouth Rock breeder eggs numbers in different treatments, numbers hatched, percent hatch, and the p-value between treatment groups and control group.

| Treatment | No. of eggs | Hatched | % Hatch | *P*-value |
|---|---|---|---|---|
| -S | 55 | 25 | 45 | 0.02 |
| +S | 55 | 23 | 42 | 0.008 |
| V | 29 | 7 | 24 | 0.0002 |
| neg cont | 49 | 32 | 65 | N/D |

Symbols: -S: eggs inoculated with negative serum at 5 days of incubation in the yolk sac, +S: eggs inoculated with anti-ALV-J (ADOL-7501 isolate) antiserum at 5 days of incubation in the yolk sac, V: eggs inoculated with ALV-J (ADOL-7501 isolate) at 7 days of incubation in the yolk sac, neg cont: negative control, N/D: not done.

Table 5.2 Experiment # 1. Average body weights in grams at 0, 1, 3, 5, 7, 9, and 13 weeks of chickens hatched from eggs inoculated with either negative serum (-), or ALV-J antiserum (+) and hatched with shedders (s) are compared to negative control (neg cont).

| | Age (wks) | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 0 | 1 | 3 | 5 | 7 | 9 | 13 |
| - | 40[a] (11)[b] | 79 (10) | 276 (10) | 551 (10) | 925 (10) | 1345 (10) | 2044 (10) |
| | 0.69[c] | 0.83 | 0.09 | 0.66 | 0.82 | 0.59 | 0.74 |
| + | 40 (11) | 81 (11) | 267 (11) | 525 (11) | 851 (11) | 1304 (11) | 1837 (10) |
| | 0.82 | 0.87 | 0.38 | 0.14 | 0.14 | 0.24 | 0.19 |
| S | 41 (7) | 73 (7) | 218 (7) | 445 (6) | 631 (1) | 870 (1) | 1688 (1) |
| | 0.45 | 0.77 | 0.02* | 0.0007* | N/D | N/D | N/D |
| neg cont | 40 (32) | 88 (32) | 256 (32) | 563 (32) | 916 (32) | 1383 (32) | 2000 (32) |

Symbols: [a]: average body weight in grams, [b]: number of chickens weighed, [c]: *P*-value, *: statistically significant, N/D: statistics not done.

Table 5.3 Experiment # 2. Average body weights in grams at 0, 1, 3, 5, 7, 9, and 13 weeks of chickens hatched from eggs inoculated with either negative serum (-), or ALV-J antiserum (+), and then inoculated with ALV-J at hatch. Weights are compared to negative control (neg cont) chickens.

| | Age (wks) | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 0 | 1 | 3 | 5 | 7 | 9 | 13 |
| - | 41[a] (14)[b] | 85 (13) | 257 (13) | 495 (13) | 813 (12) | 1199 (11) | 1713 (9) |
| | 0.17[c] | 0.92 | 0.89 | 0.006* | 0.02* | 0.006* | 0.03* |
| + | 40 (12) | 81 (12) | 259 (12) | 498 (12) | 832 (12) | 1229 (12) | 1673 (12) |
| | 0.54 | 0.86 | 0.76 | 0.006* | 0.04* | 0.02* | 0.005* |
| control | 40 (32) | 88 (32) | 256 (32) | 563 (32) | 916 (32) | 1383 (32) | 2000 (32) |

Symbols: [a]: average body weight in grams. [b]: number of chickens weighed. [c]: *P*-value. *: statistically significant.

Table 5.4 Experiment # 1. Ag-ELISA results at 1, 3, 5, 7, 9, and 13 weeks of age on plasma samples from chickens hatched from eggs inoculated with negative antiserum (-), or ALV-J antiserum (+), and then cohatched with shedders (S).

| | Age (wks) | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 3 | 5 | 7 | 9 | 13 |
| - | 3/9*<br>(33.3)♣ | 6/10<br>(60.0) | 7/10<br>(70.0) | 4/9<br>(44.4) | 2/9<br>(22.2) | 5/9<br>(55.6) |
| + | 4/9<br>(44.4) | 7/10<br>(70.0) | 6/10<br>(60.0) | 7/10<br>(70.0) | 2/10<br>(20.0) | 6/9<br>(66.7) |
| *P*-value | 0.32 | 0.32 | 0.32 | 0.12 | 0.45 | 0.19 |
| S | 7/7<br>(100.0) | 7/7<br>(100.0) | 6/6<br>(100.0) | 1/1<br>(100.0) | 1/1<br>(100.0) | 1/1<br>(100.0) |

Symbols: *: number of Ag-ELISA positive chickens / number of chickens tested, ♣: percentage of positive chickens

Table 5.5 Experiment # 2. Ag-ELISA results at 1, 3, 5, 7, 9, and 13 weeks of age on plasma samples from chickens hatched from eggs inoculated with negative antiserum (-), or ALV-J antiserum (+), and then inoculated with ALV-J at hatch.

| | Age (wks) | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 3 | 5 | 7 | 9 | 13 |
| - | 7/13* (53.8)♣ | 13/13 (100.0) | 13/13 (100.0) | 13/13 (100.0) | 9/10 (90.0) | 8/9 (88.9) |
| + | 4/12 (33.3) | 12/12 (100.0) | 11/12 (91.7) | 10/12 (83.3) | 10/12 (83.3) | 9/12 (75.0) |
| *P*-value | 0.14 | 1.0 | 0.15 | 0.06 | 0.32 | 0.20 |

Symbols: *: number of Ag-ELISA positive chickens / number of chickens tested, ♣: percentage of positive chickens,

Table 5.6 Experiment # 1. VN results at 0, 1, 7, and 13 weeks of age on serum samples from chickens hatched from eggs inoculated with negative antiserum (-), or ALV-J antiserum (+), and then cohatched with shedders (S).

| | Age (wks) | | | |
|---|---|---|---|---|
| Treatment | 0 | 1 | 7 | 13 |
| - | 0/11* (0.0)♣ | 0/11 (0.0) | 0/7 (0.0) | 6/10 (60.0) |
| + | 0/11 (0.0) | 0/10 (0.0) | 0/11 (0.0) | 6/10 (60.0) |
| *P*-value | N/D | N/D | N/D | 0.50 |
| S | 0/7 (0.0) | 0/7 (0.0) | 0/1 (0.0) | 0/1 (0.0) |

Symbols: *: number of chickens having detectable antibodies by VN test / number of chickens tested, ♣: percentage of chickens having detectable antibodies by VN test, N/D: not done.

Table 5.7 Experiment # 2. VN results at 0, 1, 7 and 13 weeks of age on serum samples from chickens hatched from eggs inoculated with negative antiserum (-), or ALV-J antiserum (+), and then inoculated with ALV-J at hatch.

| | Age (wks) | | | |
|---|---|---|---|---|
| Treatment | 0 | 1 | 7 | 13 |
| - | 0/14* (0.0)♣ | 0/13 (0.0) | 0/12 (0.0) | 5/9 (55.6) |
| + | 0/12 (0.0) | 0/12 (0.0) | 2/10 (20.0) | 7/12 (58.3) |
| *P*-value | N/D | N/D | 0.06 | 0.50 |

Symbols: *: number of Ag-ELISA positive chickens / number of chickens tested, ♣: percentage of positive chickens, N/D: not done.

Table 5.8 Number of chickens in each treatment group developing ALV related tumors or Marek's Disease lesions.

| ALV-J Exposure | Antiserum Treatment | Number of Chickens | Number developing ALV tumors | Number developing Marek's Disease |
|---|---|---|---|---|
| contact | - | 11 | 0 | 2 |
| contact | + | 11 | 0 | 1 |
| injection | - | 13[a] | 7 | 5 |
| injection | + | 12 | 1 | 5 |
| congenital (S) | non | 7 | 3 | 0 |
| neg control | non | 10[b] | 0 | 0 |

Symbols: contact: exposed to shedders, -: chickens hatched from eggs injected with negative serum, +: chickens hatched from eggs injected with ALV-J antiserum, injection: chickens inoculated with ALV-J at hatch, [a]: Excluding one chicken died after ALV-J injection after hatch, congenital (S): chickens hatched from eggs inoculated with ALV-J to act as shedders, [b]: Only 10 chickens were necropsied of the 32.

# CHAPTER 6

# EFFECTS OF INJECTING ALV-J ANTISERUM IN EMBRYONATING BROILER EGGS ON LOCALIZATION OF ALV-J IN THE TISSUES OF BROILER CHICKENS INFECTED *IN OVO*[1]

[1] Gharaibeh, S., and T. Brown. To be submitted to *Veterinary Pathology*.

**Abstract.** The protective effects of injecting antiserum against subgroup J avian leukosis virus (ALV-J) into embryonating-chicken eggs before infection were determined by evaluating viremia, transfer of passive immunity, and localization of the virus in tissues from hatched chicks. None of the chicks hatched from eggs inoculated with ALV-J antiserum had detectable antibodies by virus neutralization test; however, the injected antiserum prevented viremia at hatch in four out of five chicks. Localization of ALV-J in chicks that were viremic at hatch was similar to previous investigations with intense staining for viral antigen present in adrenal gland, heart, kidney, proventriculus, and spleen. Two of the chicks that were not viremic at hatch developed viremia at one week of age and had viral tissue distribution suggesting oral exposure to the virus from their hatchmate.

Key words: chicken, avian leukosis virus subgroup J, immunohistochemistry, tissue distribution, localization, passive immunity

Abbreviations: Ag-ELISA = antigen-capture enzyme linked immunosorbent assay; ALV-J = avian leukosis virus subgroup J; CEF = chicken-embryo fibroblast; IHC = immunohistochemistry; SPF = specific-pathogen free; $TCID_{50}$ = tissue culture infective dose 50; VN = virus neutralization

The first published report of the new subgroup J of avian leukosis virus (ALV) was in 1991[16]. ALV-J causes natural disease in chickens but it also can infect turkey and other birds[20]. The disease in chickens is characterized by body weight suppression[25], myeloid leukemia[18], other neoplasms[1,20], and reduction in egg size[23]. ALV-J is transmitted horizontally or vertically in chicken flocks[17].

Intraperitoneal injection of chickens with antibodies protected the injected chickens against subsequent challenge with a homologous pathogen[6,13,15]. Injection of antibodies in the yolk sac of incubating embryos had resulted in circulating antibodies and has a protective effect in chickens[9]. Injection of anti-*Mycoplasma* antiserum in embryonating turkeys-eggs reduced the adverse effects of egg-transmitted *Mycoplasma*[4]. Embryo inoculation can be automated[21] making it a potentially more practical approach than inoculation of antisera into hatched chicks.

Tissue tropism for ALV-J detected by immunohistochemistry (IHC) has been previously described in both naturally-congenitally-infected chickens[14]and in experimental-congenitally-infected chickens[2]. The viral antigen localization was observed most intensely in the adrenal gland, heart, kidney, and proventriculus in both studies. ALV-J has also been detected in tissues by localization of viral nucleic acid using *in situ* hybridization (ISH)[3,24] with similar results to that of IHC.

The objective of this study is to investigate the potential use of injecting ALV-J antiserum into embryonating chicken eggs to reduce the effects of egg transmitted ALV-J by reducing the level of viremia, and hence shedding of virus to the environment. We also investigated the potential reduction in virus spread into different tissues.

## Materials and Methods

**Virus and Serology.** ADOL-7501 isolate of ALV-J was cloned by three limiting dilutions in secondary line 0[11] chicken embryo fibroblast (CEF) cultures. This cloned

virus had a tissue culture infective dose 50 ($TCID_{50}$) of $10^{6.5}$/ ml. It was diluted 1:100 with culture medium and was used for embryo inoculations. A virus neutralization (VN) test was carried out on secondary line 0 chicken embryo fibroblast (CEF) cultures as a microneutralization assay using 100 TCID / well[11]. Viremia in plasma was tested by commercial antigen capture enzyme linked immunosorbent assay (Ag-ELISA) (IDEXX Laboratories, Westbrook, ME)

**Sera.** ALV-J antiserum was produced by injecting a group of specific pathogen free (SPF) chickens (n=9) (SPAFAS, Preston, CT) intramuscularly with 0.5 ml of $10^{6.5}$ $TCID_{50}$ of live cloned virus multiple times at 20, 22, 24, 28 weeks of age. Serum was collected at 29 weeks of age. All sera were heat inactivated at 56 C for one hour and filtered at 0.2 μm (Whatman Inc., Clifton, NJ). Sera from inoculated chickens with a virus-neutralizing-titer greater than 1:640 (n=3) were pooled. The neutralizing titer of this positive pool was 1:1280.

**Serum or Virus Inoculations.** ALV-J negative White Plymouth Rock chicken eggs (provided by Dr. David Swayne, Southeast Poultry Research Laboratory, USDA-ARS, Athens, GA) were used in this experiment. All egg inoculations were done in the yolk sac[22]. Sera inoculations were performed at 5 days of incubation with 0.1 ml of the positive pool serum. Virus inoculations were performed at 7 days of incubation with the cloned virus described above.

**Experimental Design and Tissues Collected.** Chicks (n=5) hatched from eggs inoculated with both virus and ALV-J antiserum were hatched separately, bled, and raised in a positive pressure Horsfal isolation unit. Chicks (n=5) hatched from eggs inoculated with virus alone were hatched separately, bled and raised in a separate positive pressure Horsfal isolation unit. Chicks (n=5) hatched from eggs with no treatment were hatched separately and raised in a positive pressure Horsfal isolation unit separate from any contact with ALV-J, and served as a negative control group. At one week of age all

chicks were bled and euthanized. At necropsy, samples of heart, proventriculus, gizzard, kidney, liver, lung, spleen, bursa, thymus, bone marrow, peripheral nerve, brain, pancreas, duodenum, large intestine, cecum, adrenal, skeletal muscle, and gonad from each chicken were fixed by immersion in 10% neutral buffered formalin for 24 hours and embedded in paraffin for sectioning.

**Preparation of cell culture pellets for a positive and a negative control.** ALV-J infected and uninfected secondary chicken embryo fibroblast made from line 0 embryos were paraffin embedded as previously described[27] and served as positive and negative controls, respectively. Briefly, cells were either infected (positive control) or uninfected (negative control), incubated 7 days at 37 C, scraped from the tissue culture flask and suspended in 10% neutral buffered formalin for 15 minutes, washed 2 times with normal saline, and pelleted by centrifugation for 3 minutes at 10,000 revolution per minute. Cells were then resuspended in twice the volume of the pellet in 0.5 agarose at 42 C and fixed for 2 hours in 10% neutral buffered formalin. Finally, the pellets were routinely processed for paraffin embedding and sectioning.

**Preparation of tissue samples and IHC.** All techniques were done at room temperature. Tissue sections were cut at 4 µm and mounted on charged glass slides (Superfrost / Plus, Fisher Scientific, Pittsburgh, PA). Paraffin was melted from the slides (10 minutes at 65 C) and removed by immersion in Hemo-De three times (5 minutes each time). Slides were air dried and digested with ready-to-use proteinase K (DAKO, Carpinteria, CA) for 5 minutes to expose antigenic target sites by breaking the protein cross-links caused by formalin fixation[5]. IHC staining was performed in an automated stainer (Leica ST 5050, Nussloch, Germany) using a nonbiotin peroxidase kit (Dako Envision System, DAKO, Carpinteria, CA) according to the manufacturer's recommendations. The primary antibody used was a monoclonal antibody specific for the gp85 envelope glycoprotein of ALV-J (provided by Dr. Lucy Lee, ADOL, East Lansing,

MI)[10]. After IHC staining, sections were counter-stained with hematoxylin, air dried, cover slipped, and examined using light microscopy. Tissues with multifocal staining of less than 10% of the cells were defined as having mild staining (+). Tissues with multifocal staining of 10-40% of the cells were defined as having moderate staining (++). Tissues with multifocal staining of greater than 40% of the cells were defined as having intense staining (+++).

## Results

At hatch, all the five chicks that hatched from eggs inoculated with virus only were viremic with tissue infective dose ($TCID_{50}$) ranging from $10^{5.0}$-$10^{7.4}$ / ml plasma. Only one chick out of five hatched from eggs inoculated with ALV-J antiserum then inoculated with virus was viremic with $TCID_{50}$ of $10^{5.5}$ / ml plasma. All the chicks from the negative control group were not viremic. All the chicks from all groups did not have any detectable neutralizing titer by VN test.

By one week of age all the five chicks that hatched from eggs inoculated with virus only were still viremic. Only 3 chicks out of five hatched from eggs inoculated with ALV-J antiserum then inoculated with virus were viremic. All the chicks from the negative control group were not viremic

Sections from the infected positive control cell culture pellet had stainable antigen, whereas no staining was present in sections of the uninfected negative control cell culture pellet.

All the chicks that were viremic at hatch (5 chicks that hatched from eggs inoculated with virus alone and one chick hatched from an egg inoculated with both ALV-J antiserum and virus) had very similar staining pattern. The most intense staining was present in the heart, proventriculus, gizzard, adrenal gland, kidney, and spleen. Other

organs had less intense staining (Table 6.1). The staining pattern was very similar to that in previous studies[2,14].

In the adrenal gland (Fig. 6.1), staining was more intense in cortical cells than in medullary cells. In the heart (Fig. 6.2), there was staining of the cytoplasm and plasma membrane of myofibers as well as Purkinje fibers. In the kidney (Fig. 6.3), there was staining in the proximal, distal, and medullary cone tubular epithelial cells. This staining was more intense in the apical portion of these cells. There was also staining in glomerular cells with the greatest intensity in podocytes. In the spleen (Fig. 6.4), both lymphocytes and macrophages stained and the staining was greatest in the centers of germinal centers. In the proventriculus,(Fig. 6.5) and gizzard staining was present at the basal portion of glandular epithelial cells, smooth muscle in the tunica muscularis, and the connective tissue of the interstitium. The duodenum (Fig. 6.6) and cecum had positive staining in the surface epithelium, lamina propria, and the tunica muscularis. In the bone marrow (Fig. 6.7), staining was multifocal and limited to cells with cytoplasmic granules interpreted to be myelocytic precursor cells. In the lung, staining was present in atrial and bronchiolar epithelial cells, in the adjacent connective tissue, and in cells lining air and blood capillaries. In the pancreas,(Fig. 6.8) there was staining at the apical portion of individual acinar epithelial cells. In the bursa of Fabricius (Fig. 6.9), there was staining in medullary, cortical, and surface epithelial cells. In the liver (Fig. 6.10), there was staining of hepatocyte cytoplasm adjacent to canaliculi and Kupffer's cells. In peripheral nerves, there was staining in both axons and myelin sheaths. In the gonads, there was staining in the interstitial cells in both ovaries and testes. In skeletal muscle, there was staining in the cytoplasm and at the plasma membrane of myocytes. In the thymus, medullary epithelial cells stained, but cortical lymphocytes did not. In other tissues, there was staining in smooth muscle and some connective tissues, but in general staining was less intense and more inconsistent than in the organs described above.

The four chicks that were not viremic at hatch but exposed to the viremic hatchmate in the hatcher and the Horsfal unit had very limited staining (Table 6.2) present in the muscularis of the duodenum (Fig 6.11), cecum, proventriculus, and gizzard. There was also mild staining in the subepithelium of the bursa (Fig. 6.12).

None of the chicks from the negative control group had any detectable positive staining.

## Discussion

None of the hatched chicks had detectable antibodies by VN test at hatch. This could be due to the dilution effect of injecting only a small volume (0.1 ml) of the antiserum into each egg and degradation of the antibodies in the injected serum by the time of hatch (16 days later).

Most of the chicks (4 out of 5) that hatched from eggs inoculated with both ALV-J antiserum and ALV-J were not viremic at hatch. This is because the injected antiserum probably neutralized the injected virus. The one chick that was viremic in that group may be explained by failure of the injected ALV-J antiserum to neutralize the virus may be due to an error in antiserum injection in the yolk sac of that specific egg.

The staining patterm in the chicks that were viremic at hatch agrees in general with previous localization studies[2,3,14,24]. In this experiment, as with our previous IHC localization of ALV-J in tissues from congenital infected chickens, we demonstrated specific staining of myelocytic precursor cells in bone marrow which agrees with previous work showing cell cultures of bone marrow are susceptible to ALV-J infection[19] and the tendency of subgroup J to predominantly induce myeloid rather than lymphoid neoplasms[12,26] in chickens. In this study, the spleen had an intense staining. This is different than previous studies where only mild to moderate staining was present in the

spleen. This difference could be due to differences in virus tropism, chicken lines, or the method used to infect these embryos with the virus.

By one week of age two of the chicks that were not viremic at hatch developed viremia. This is due to contact exposure with their viremic hatchmate. The only organs that were mildly positive were associated with gastrointestinal tract (proventriculus, gizzard, duodenum, cecum, and bursa) while no staining was present in lungs. This suggests fecal oral transmission of the virus to the hatch and pen-mates.

In ALV-infected egg-laying hens, viral particles are abundant in the ovarian stroma, bud from cells in direct contact with oogonia and oocytes[8], and are in the largest concentration in the albumen-secreting glands of the magnum. This early ovarian and oviductal exposure results in natural congenital ALV infection at very early embryonic stage[7,8]. Even if the injected antiserum neutralized all the circulating virus at the time of injection, the integrated provirus in the genome of embryo cells have the ability to produce more infectious particles that will probably initiate viremia again by the time of hatch. The injected antiserum did not change viral-tissue distribution in the chick that hatched with viremia. Further studies should be conducted with inoculated concentrated antibodies that will be detected in the serum of hatched chicks. That may protect or decrease the effects of the egg transmitted ALV-J and could protect the hatchmates against horizontal infection in the first few days of life.

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**Table 6.1** IHC staining intensity and number of positive organs for ALV-J antigen in chicks hatched from eggs inoculated with ALV-J only.*

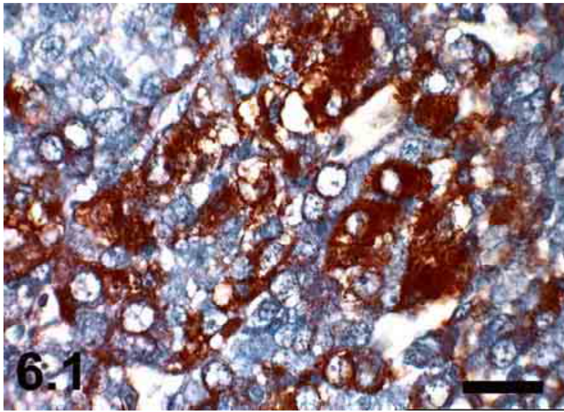
| Tissue | Stain Intensity | Number Positive / Number examined (% positive) |
|---|---|---|
| Adrenal | +++ | 5/5 (100) |
| Bone marrow | ++ | 5/5 (100) |
| Brain | + | 0/5 (0) |
| Bursa | + | 5/5 (100) |
| Duodenum and cecum | ++ | 5/5 (100) |
| Gizzard | +++ | 5/5 (100) |
| Heart | +++ | 5/5 (100) |
| Kidney | +++ | 5/5 (100) |
| Liver | ++ | 5/5 (100) |
| Lung | ++ | 5/5 (100) |
| Nerve | + | 1/5 (20) |
| Ovary | + | 2/2 (100) |
| Pancreas | ++ | 5/5 (100) |
| Proventriculus | +++ | 5/5 (100) |
| Skeletal muscle | + | 3/5 (60) |
| Spleen | +++ | 5/5 (100) |
| Thymus | + | 5/5 (100) |
| Testes | + | 2/3 (67) |

* Symbols: + = mild staining; ++ = moderate staining; +++ = intense staining.

**Table 6.2** Number of organs positive by IHC for ALV-J antigen in chicks hatched from eggs inoculated with ALV-J antiserum then inoculated with ALV-J*.

| Tissue | Number Positive / Number examined (% positive) |
|---|---|
| Adrenal | 1/5 (20) |
| Bone marrow | 1/5 (20) |
| Brain | 0/5 (0) |
| Bursa | 3/5 (60) |
| Duodenum and cecum | 5/5 (100) |
| Gizzard | 5/5 (100) |
| Heart | 1/5 (20) |
| Kidney | 1/5 (20) |
| Liver | 1/5 (20) |
| Lung | 1/5 (20) |
| Nerve | 0/5 (0) |
| Ovary | 0/1 (0) |
| Pancreas | 1/5 (20) |
| Proventriculus | 5/5 (100) |
| Skeletal muscle | 2/5 (40) |
| Spleen | 1/5 (20) |
| Thymus | 2/5 (40) |
| Testes | 1/4 (25) |

*: Stain intensity for the chick that was viremic at hatch was very similar to the intensity shown in table 6.1. Tissues showing positive staining in the other chicks had a mild staining intensity.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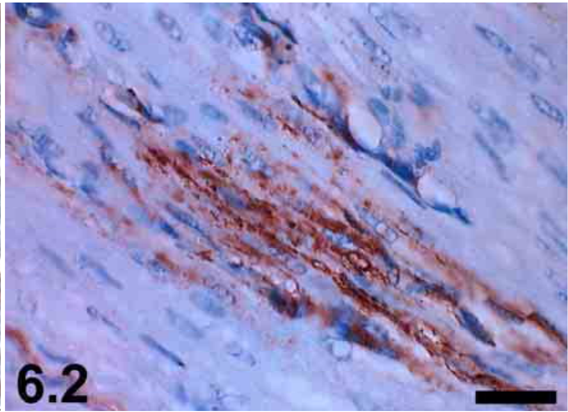
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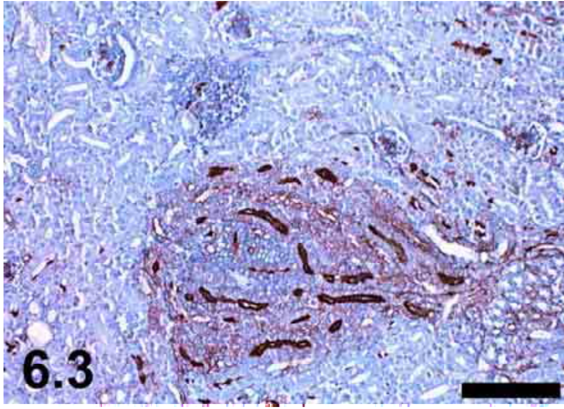
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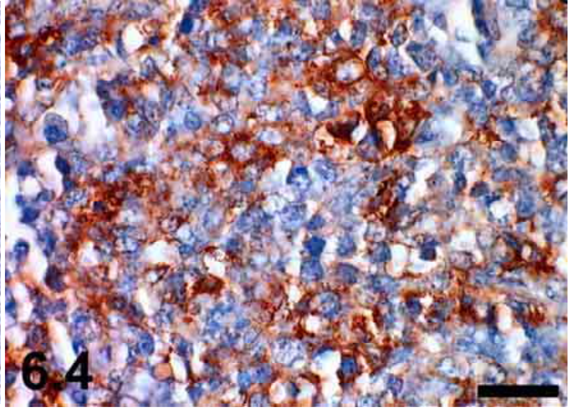
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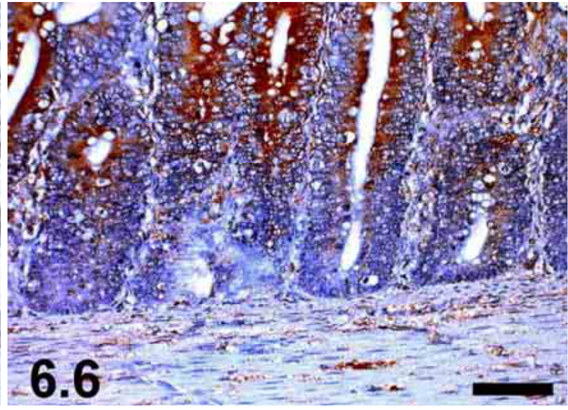
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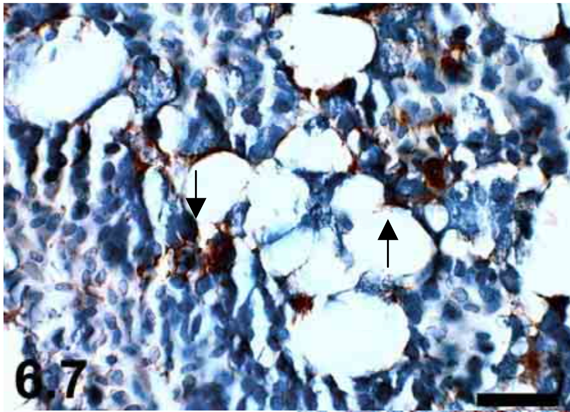
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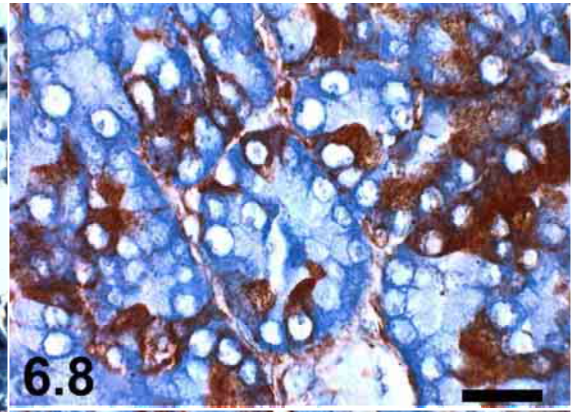
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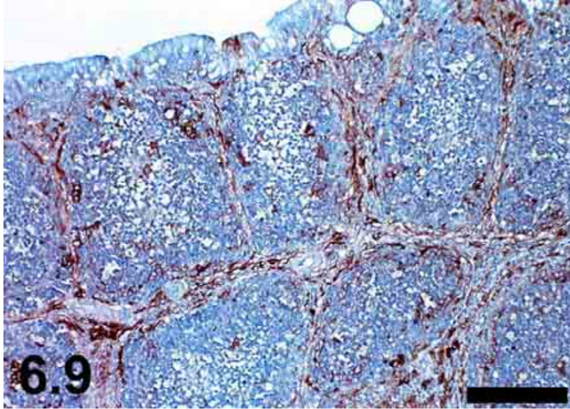
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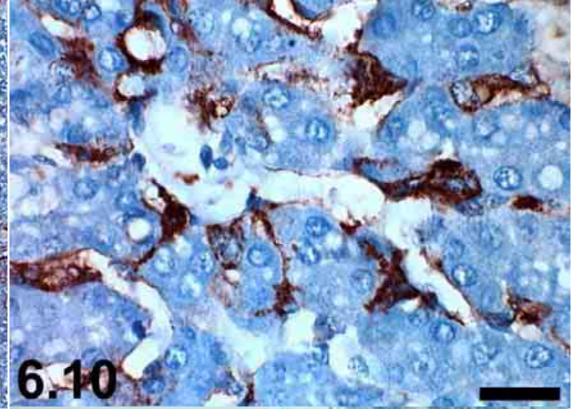
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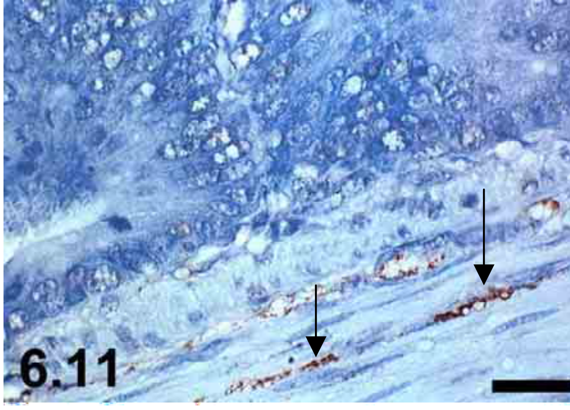
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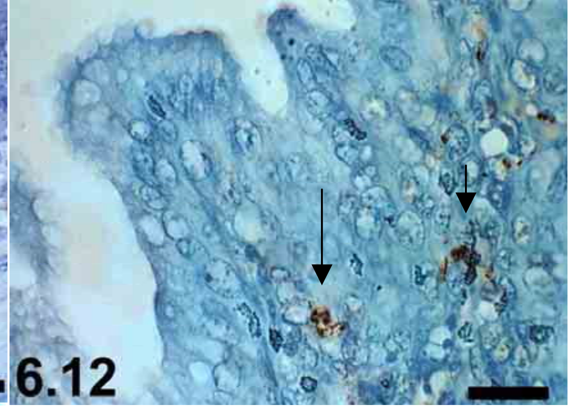
6.12

**Fig. 6.1.** Adrenal gland; chick hatched from egg inoculated with ALV-J only. Intense staining. Antigen is detected mainly in cortical cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 30 µm.

**Fig. 6.2.** Heart; chick hatched from egg inoculated with ALV-J only. Intense staining. Antigen is detected in myocardial cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 30 µm.

**Fig. 6.3.** Kidney; chick hatched from egg inoculated with ALV-J only. Intense staining. Antigen is detected in proximal, distal, and medullary cone tubular epithelial cells. Also antigen is present in glomerular cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 100 µm.

**Fig. 6.4.** Spleen; chick hatched from egg inoculated with ALV-J only. Intense staining. Antigen is detected in macrophages and lymphocytes. IHC; peroxidase method, hematoxylin counterstaining. Bar = 30 µm.

**Fig. 6.5.** Proventriculus; chick hatched from egg inoculated with ALV-J only. Intense staining. Antigen is detected at the basal portion of the glandular epithelium and in tunica muscularis. IHC; peroxidase method, hematoxylin counterstaining. Bar = 100 µm.

**Fig. 6.6.** Duodenum; chick hatched from egg inoculated with ALV-J only. Moderate staining. Antigen is detected in Surface epithelium, lamina propria, and tunica muscularis. IHC; peroxidase method, hematoxylin counterstaining. Bar = 40 µm.

**Fig. 6.7.** Bone marrow; chick hatched from egg inoculated with ALV-J only. Moderate staining. Antigen is detected in myelocytic precursor cells (arrows). IHC; peroxidase method, hematoxylin counterstaining. Arrows are pointing into examples of cells with positive staining. Bar = 30 µm.

**Fig. 6.8.** Pancreas; chick hatched from egg inoculated with ALV-J only. Moderate staining. Antigen is detected in individual acinar epithelial cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 30 μm.

**Fig. 6.9.** Bursa of Fabricius; chick hatched from egg inoculated with ALV-J only. Mild staining. Antigen is detected in medullary, cortical, and surface epithelial cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 100 μm.

**Fig. 6.10.** Liver; chick hatched from egg inoculated with ALV-J only. Moderate staining. Antigen is detected in Hepatocytes adjacent to canalculi and in Kupffer's cells. IHC; peroxidase method, hematoxylin counterstaining. Bar = 30 μm.

**Fig. 6.11.** Duodenum; chick hatched from egg inoculated with ALV-J antiserum then ALV-J. Mild staining. Antigen is detected in tunica muscularis (arrows). IHC; peroxidase method, hematoxylin counterstaining. Arrows are pointing into examples of cells with positive staining. Bar = 30 μm.

**Fig. 6.12.** Bursa of Fabricius; chick hatched from egg inoculated with ALV-J antiserum then ALV-J. Mild staining. Antigen is detected in The subepithelium (arrows). IHC; peroxidase method, hematoxylin counterstaining. Arrows are pointing into examples of cells with positive staining. Bar = 30 μm.

# CHAPTER 7

# CONCLUSIONS

The IHC staining present in the tissues from congenitally infected chickens used in this study was predominantly in the adrenal gland, heart, kidney, and proventriculus. This agreed with results using chickens inoculated *in ovo*. This suggests high tropism of the virus to these organs. Some tissues from chickens exposed by *in ovo* inoculation were negative by IHC staining for *gag*, whereas all tissues from congenitally exposed chicken were positive by IHC staining for gp85. In addition, the distribution and intensity of stain in the different tissues was similarly stable among age groups examined up to 9 weeks of age.

Recent investigations failed to demonstrate specific viral staining in bone marrow from infected chickens. We were able to show moderate staining in bone marrow cells with cytoplasmic granules interpreted to be myelocytic precursor cells. This finding agrees with previous work showing cell cultures of bone marrow are susceptible to ALV-J infection and the tendency of subgroup J to predominantly induce myeloid rather than lymphoid neoplasms. In this study, there was intense staining of the myocardium together with Purkinje fibers; this intense staining correlates with large amounts of antigen present in these tissues. This may explain the ability of ALV to cause cardiomyopathy and ascites by impairing the function of the cardiac muscle or its conducting system (Purkinje fibers).

Passage of ALV-J in the presence of the antiserum resulted in increased resistance of virus to neutralization by the antiserum. These findings suggested changes in the nucleotide sequence of the *env* gene may have occurred resulting in amino acid changes conferring different protein conformation and change of antigenic epitopes. Surprisingly,

sequencing of the *env* genes of the neutralization-resistant viruses showed no such changes. Multiple possible explanations exist for this finding.

Injecting 0.1 ml of ALV-J antiserum with a neutralizing titer of 1:1280 in the yolk sac of embryonating chickens eggs will not be detected by virus neutralization in serum of the hatched chicks and will not protect against body weight suppression, reduce the number of chickens developing viremia or increase the number of chickens developing active immunity. Contact exposure to ALV-J did not cause body weight suppression, while congenitally infected, and parentrally injected chicks had depressed body weights compared to a negative control. Contact exposure to ALV-J did not cause ALV related tumors. Chickens hatched from eggs injected with ALV-J antiserum and then inoculated with ALV-J at hatch less frequently developed tumors than did positive control chickens. Parentral injection of the virus is more pathogenic than contact exposure and parentrally infected chickens have increased susceptibility to Marek's disease.

Most of the chicks that hatched from eggs inoculated with both ALV-J antiserum and ALV-J were not viremic at hatch. This is because the injected antiserum probably neutralized the injected virus.

Contact exposure to a viremic hatchmate may result in viremia by one week of age. The only organs that were mildly positive were associated with the gastrointestinal tract (proventriculus, gizzard, duodenum, cecum, and bursa) while no staining was present in the lungs. This suggests fecal oral transmission of the virus to the hatch and pen-mates.

Further studies should be conducted to examine the effects of inoculated concentrated antibodies that will be detected in the serum of hatched chicks. These may protect or decrease the effects of egg transmitted ALV-J and may protect hatchmates against horizontal infection in the first few days of life.