AVIAN LEUKOSIS VIRUS SUBGROUP J: RT-PCR-BASED QUANTITATIVE METHODS AND THE EFFECTS OF IMMUNOSUPPRESSION ON THE PATHOGENESIS

By

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(Under the direction of Dr. Thomas P. Brown)

ABSTRACT

Avian leukosis virus subgroup J (ALV-J) infection causes significant economic loss because of increased mortality, tumor production, decreased production, and high costs for eradication. This research was performed to investigate the effects of immunosuppression on ALV-J pathogenesis. We developed RT-PCR based quantification methods for ALV-J RNA. The results of quantitative competitive RT-PCR and real time RT-PCR based on a fluorogenic probe and SYBR Green I dye were strongly correlated to the TCID$_{50}$s as determined by conventional culture methods. The new methods were also very specific, sensitive, relatively easy to perform, reproducible, and rapid compared with conventional culture methods.

The effects of the immunosuppression on ALV-J pathogenesis were determined in chickens with natural exposure to Marek’s disease virus. Suppression of B-cell and T-cell was induced by treatment of cyclophosphamide (CY) and cyclosporin A (CSP), respectively. Cyclophosphamide treatment induced significant B-lymphocyte suppression in broiler chickens throughout the experiment with transient effects on feather follicle epithelium, liver, kidney and bone marrow function. Chickens with immunosuppression of T- and B- cell function were more frequent ALV-J viremia with a higher virus titer compared to non-immunosuppressed groups. No significant difference in body weight gain was induced by ALV-J infection. While there were no neoplastic foci consistent with ALV-J infection in any of the control or CY-treated chickens, there was a nephroblastoma in a CSP treated chickens. Increased numbers of chickens in CSP treated groups had myeloid infiltrates compared to those present in control groups. This may indicate T-cell suppression increased tumor formation or preneoplastic myeloid proliferation in chickens with ALV-J infection. In immunohistochemical staining using monoclonal antibodies against ALV-J envelope glycoprotein, expression of the viral antigen was present in more chickens in the CY treated group compared to the control group at 3 weeks post-infection. With CSP treatment, the overall mean score of viral antigen expression was significantly higher compared to the control group at 10 weeks post-infection. With CY and CSP treatment, expression of viral antigen was significantly
higher at 9 and 10 weeks post-infection at the end of the experiment compared to that present in 3 and 4 weeks post-infection, respectively.

INDEX WORDS: Avian leukosis virus subgroup J, Quantitative RT-PCR, Real time RT-PCR, Cyclophosphamide, Cyclosporin, Immunosuppression, Marek’s disease virus, Immunohistochemistry
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CHAPTER 1

INTRODUCTION

Purpose of the study

Avian retroviruses have four distinct species; leukosis-sarcoma group viruses, reticuloendotheliosis viruses, lymphoproliferative disease virus of turkeys, and pheasant type C oncoviruses. In size, shape, and ultrastructural detail, the various avian leukemia sarcoma group viruses (ALSVs) are identical and similar to other C-type oncoviruses. Particle diameter is 80 – 120nm, with an average of 90nm (5). The inner, centrally located, electron-dense core is about 35 – 45nm in diameter; there is an inner membrane and an outer membrane bearing projections 7nm long with knob-like ends about 6nm in diameter (5). ALVs carry three genes: gag, which encodes for the internal structural proteins of the virion; pol, which codes for the RNA-dependent DNA polymerase; and env, which codes for the viral envelope. They are arranged in the order 5’gag-pol-env-poly(A)3’ flanked by 5’ and 3’ LTR (41).

The ALSVs of the chicken are classified into six subgroups, A-E and J, on the basis of differences in their viral envelope glycoprotein antigens, which determine most virus-serum neutralization properties, viral interference patterns, and range of infectivity in chicken and other avian cells of different phenotypes (26, 39). A virus isolated from Ring-necked pheasants belongs to subgroup F (14), an isolate from a Hungarian partridge is placed in subgroup H (15), and one from Gambel’s quail in subgroup I (38).
Viruses of subgroups A and B occur as common exogenous viruses in the field as pathogenic, infectious viruses which spread vertically and horizontally. They are oncogenic causing principally lymphoid leukemia (8, 23). Those of subgroups C and D are also exogenous, but have been reported rarely in the field (21). Subgroup E viruses include the ubiquitous endogenous leukemia viruses of low pathogenicity and usually are transmitted as viral genes in a non-infectious state (34).

In 1989, an ALV of a new envelope subgroup, designated J was isolated from commercial meat-type chickens in the UK (26). The prototype strain, HPRS-103, has been completely sequenced (3, 4). It has gag and pol genes which show high homology to these genes in ALVs of other subgroups, but an env gene whose gp85 (SU) domain shows only about 40% homology with other subgroups. The env gene shows higher (75%) homology to env-like sequences of the E51 clone of the recently discovered EAV family of endogenous avian retroviruses (7). Novel EAV-related sequences, designated EAV-HP, showing over 97% homology to that of the HPRS-103 env gene have been demonstrated in the chicken genome (4, 32). EAV-HP elements, also referred to as ev/J (6), were present in multiple copies in the genome of all the lines of chickens and the ancestral jungle fowl, but were absent in most other avian species studied (36). These findings suggest that HPRS-103 evolved by recombination between an exogenous ALV and EAV-HP env sequences to evolve as a new ALV subgroup (39).

The virus has a wide host range with chicken lines, with all of 11 lines of chickens studied being susceptible to the ALV-J infection. However, amongst 12 different species of poultry and game birds, only chickens, jungle fowl, and turkeys were susceptible (29). In the field, presence of the infection is associated with the occurrence of
myelocytomatosis, and myelocytoma, renal tumors and other rarer tumors are induced by experimental infection of chickens (27). Susceptibility to tumors varies markedly between different lines of chickens, but meat-type lines are particularly prone to tumors.

From some field or experimental cases of myelocytomatosis, ALVs can be isolated that acutely transform cultured chicken bone marrow cells or monocytes (27). HPRS-103 itself does not transform these cells in vitro. HPRS-103 lacks a viral oncogene and presumably induces myelocytomatosis by insertional mutagenesis (4). Studies on cell tropism of HPRS-103 indicate that, in contrast to subgroup A ALV, it has a tropism for cells of the myelomonocytic series, but a low tropism for bursal cells, consistent with its induction of myeloid, and not lymphoid leukosis (2).

Diagnosis of ALV-J infection and related diseases involves pathological identification of tumors and virological identification of ALV-J infection. Pathologically, myelocytomas consist of well-differentiated myelocytes, and are present on the inner sternum, vertebrae and ribs, and myelocytomatous infiltration of the liver, spleen and kidneys usually occurs, causing their enlargement (27). Occasionally blast-cell tumors and histiocytic sarcomas occur in naturally infected chickens (1).

ALV-J virus can be isolated from materials such as tumors, serum, and cloacal or vaginal swabs by culture in C/E chick embryo fibroblasts in a microculture system followed by gs-antigen ELISA testing of cell extracts for evidence of virus growth (30). Isolates are identified as subgroup J ALV by neutralization with J subgroup chicken antiserum. The ALV group specific antigen (gsa) ELISA is also used to demonstrate increased gsa levels in egg albumen and cloacal or vaginal swabs (30). ALV-J antibody in serum is identified by neutralization test of ALV-J in the microculture system (27), or
in antibody ELISA tests using plates coated with lysate of HPRS-103 infected cells or with gp85 envelope glycoprotein of HPRS-103 produced in insect cell cultures infected with a recombinant baculovirus (40).

As an adjunct to conventional tests for ALV-J, subgroup J ALV-specific PCR tests have been developed. These tests have been used to detect ALV-J proviral DNA in tumor samples and in CEF’s infected with ALV-J (35). By incorporation of a reverse transcriptase step the PCR can be used to detect viral RNA.

Chickens infected horizontally with ALV develop, after a transient viremia, subgroup specific virus-neutralizing antibodies most frequently directed against virus envelope antigens (31). In this study, birds naturally infected after hatching first developed antibodies at 9 weeks of age with a marked increase in the proportion with antibodies between 14 and 18 weeks of age, when 80% were positive. All chicks infected as embryos were permanently tolerant viremic without antibodies to HPRS-103 (25). But after HPRS-103 strain infection of one-day-old chicks, layer-type chicks always developed serum-neutralizing antibody whereas the majority of meat type chickens became viremic (27, 28).

Serum antibodies are passed by the hen to her progeny via the egg yolk and provide a passive immunity to infection which lasts 3-4 weeks, and which delays infection by ALV and reduces the incidence of tumors and of viremia and ALV shedding (12). Passively administered antibody decreased or eliminated virus replication in some tissues, and virus multiplication was prolonged in bursectomized birds (42).

Neonatal thymectomy of chickens increased the incidence of Rous sarcomas and prevented tumor regression, thus implicating T-cell mediated mechanisms in tumor
immunity (9). Tests based on the release of lymphokines or on blastogenic transformation also indicate cell-mediated immunity in regression of Rous sarcomas (10, 17).

Cyclophosphamide (CY) has been shown to eliminate B-cells and cause severe depression of humoral immune response in chickens (13, 18). CY also induces transient, but profound, T-cell deficiency and although it is restored to near normal levels within 1 to 2 weeks in CY treated chickens (11, 19). Neonatal treatment with CY significantly increased the frequency of viremic chickens in contact transmission model of ALV subgroup A (13).

Cyclosporin (CSP), a selective T-cell immune-suppressant drug, depresses cell-mediated immunity in chickens and causes prolonged skin graft survival, depressed proliferative responses in mitogen-stimulated lymphocytes and decreased wattle responses to injected antigen (16, 22). CSP prevents the synthesis of cytokines, especially interleukin-2 (IL-2) by T-cells, via blocking a late stage in the signaling pathway initiated by the T-cell receptor.

Eradication of exogenous ALV from a flock is dependent on breaking the vertical transmission of virus from dam to progeny, and prevention of reinfection (24). Eradication procedures used on a commercial scale by poultry breeding companies depend on the close associations between virus infections in hens, egg albumen, embryos, and chicks (37). Although use of antiviral vaccines to increase resistance is attractive, attempts to produce killed or attenuated vaccines have had limited or no success. This is due partially to the fact that attenuation was not caused by serial cell culture passage of ALV subgroup A field isolates and the possibility of an avirulent strain of ALV subgroup A or B occurring in field is very low (23). More recently a recombinant ALV with
characteristics of RAV-0 yet expressing subgroup A envelope glycoprotein has been produced which could have potential as a vaccine (20, 33).

Identification of the specific immune compartments involved in the progression of this disease is very important for strategies to control this disease. However, little is known about the role of the immune system against ALV-J infection.

**Objectives**

This research is designed to identify which compartment(s) of the immune system is involved in the protection against ALV-J infection and subsequent disease. In this study we tested the hypothesis that *in vivo* suppression of a specific immune compartment involved in ALV-J resistance would cause more severe consequences in ALV-J infection. Specific objectives to achieve this goal are: 1) to develop and verify PCR-based quantitation methods; 2) to establish an experimental ALV-J infection model; 3) to establish an immunosuppressed chicken model; and 4) to combine these two models and determine the effects of ALV-J infection in immunosuppressed chickens. This research will provide information about immune function and resistance to ALV-J infection. It will also be valuable in developing control methods for this disease by increasing resistance to ALV-J infection.

**REFERENCES**


CHAPTER 2
LITERATURE REVIEW

The Retroviridae

Retroviral infections are associated with many diseases, including rapid and long-latency malignancies, wasting diseases, neurological disorders, and immunodeficiencies, as well as lifelong viremia in the absence of any obvious ill effects (18). Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition, and mode of replication (19, 20). The virions are 80-100 nm in diameter, and their outer lipid envelope incorporates and displays the viral glycoproteins. The virion RNA is 7-12 kb in size, and it is linear, single stranded, non-segmented, of positive polarity. The characteristic of the family is its replicative strategy which include as essential steps reverse transcription of the virion RNA into linear double stranded DNA and the subsequent integration of this DNA into the genome of the cell (126).

Different classification schemes have been applied to retroviridae. Retroviruses are divided into three subfamilies based on pathogenicity rather than on genomic relationships- oncovirinae, lentivirinae, and spumavirinae (121). Differences in the morphology of the virion led to the classification into four morphological groups- type A to D (19, 20, 121). Currently, seven genera of retroviruses are recognized based on factors including morphology, sequence similarity, presence or absence of additional genes, reverse transcriptase cofactors, oncogenic potential, and host range; avian sarcoma
and leukemia virus group, mammalian B-type viral group, murine leukemia-related viral
group, human T-cell leukemia-ovine leukemia viral group, D-type viral group,
lentiviruses, and spumaviruses (126, 127).

The dimeric genome of the retroviruses include the following, from 5’ to 3’: the
capping group, the primer tRNA, the coding regions, the 3’ poly(A) sequence (18, 20).
Coding regions of the retroviral genome contain three domains with information for
virion proteins: gag, which directs the synthesis of internal virion proteins that form the
matrix, the capsid, and the nucleoprotein structure; pol, which contains the information
for the reverse transcriptase and integrase enzymes; env, from which are derived the
surface and transmembrane components of the viral envelope protein and pro, which
codes the virion protease (126). Based on the genomic organization, retroviruses are
divided into two categories -simple and complex. Simple retroviruses usually carry only
these elementary information, whereas complex retroviruses code for additional
regulatory nonvirion proteins derived from multiply spliced messages.

Like all other viruses, retroviral infection requires interaction between cell-surface
receptor molecule and SU protein on the virion envelope. CD4 was the first retrovirus
receptor to be identified (29, 67, 76). Mouse cells expressing human CD4 bind HIV but
are not infected by it suggesting that there may be a second human-cell-specific factor
necessary for HIV infection (65). This factor remains to be identified. Retroviruses are
highly unusual among animal viruses in the polymorphism of receptor utilization
displayed by otherwise closely related viruses. Use of different receptors results in a
distinctive host range that reflects the presence or absence of a receptor in animals of
different species. The distribution of receptor expression among cells in the body is the first (but not the only) determinant that specifies the pathogenic outcome of infection.

Reverse transcription of viral RNA genome generates a linear DNA duplex in the cytoplasm via complicated steps. Extant models for reverse transcription proposes that two specialized template switches known as strand transfer reactions or “jumps” are required to generate the retroviral DNA. Retroviral DNA synthesis is dependent on the two distinctive enzymatic activities of RT: a DNA polymerase and ribonuclease H (RNase H). Integration contributes to viral replication in two ways; stable maintenance in dividing cells, and efficient transcription of viral DNA into new copies of viral genome and mRNAs. Persistent infection may be partly due to the generation of mutant viruses in the host by genetic recombination. Recombination is a frequent event in retroviral replication and likely contributes to viral genetic diversity, allowing escape from a virologically lethal host immune response. The rate of recombination during antisense-strand synthesis is estimated to be approximately 0.8% during one replication cycle. In contrast, the rate of recombination between proviruses is estimated to be only one event per every 100,000 cell divisions.

Retroviruses were first discovered in association with cancer in chickens and subsequently tumor-inducing retroviruses were isolated from rodents, cats, cows, primates, and other animals. Retroviruses that induce malignancies belong to one of five genera: avian sarcoma leukemia viruses (ALSV); mammalian C-type viruses; B-type viruses; D-type viruses; and the HTLV-BLV group.

Most tumors induced by retroviruses that lack oncogenes involve hematopoietic cells; a few of these induce carcinomas. The oncogenic agents are replication-competent,
and extensive viral replication occurs during the long latent period that precedes typical tumor development. Retroviruses lacking viral $v$-onc genes integrate near some of the same $c$-onc genes and activate their expression; oncogene capture; insertional mutagenesis, and translocation. Retroviruses that carry $v$-onc genes induce a wide range of malignancies including sarcomas and hematopoietic cell tumors. Most of these viruses are replication-defective, and the $v$-onc-encoded protein is often the only viral product. Most classes of the proteins are divided into growth factor receptors, nonreceptor protein tyrosine kinase, adapter signaling proteins, G proteins, serine/threonine kinases, and transcription factors.

**The Avian leukosis sarcoma virus group**

In 1908, Vilhelm Ellermann and Oluf Bang showed that chicken leukosis was caused by a virus. And in 1911, Peyton Rous reported the cell free transmission of a sarcoma in chickens. These viruses constitute avian C-type virus genus, avian sarcoma/leukosis viruses (ASLV). Nine subgroups of these viruses, named A through J, representing distinct pattern of receptor usage defining host range and pathogenicity have been identified. Subgroups A to D are exogenous viruses of chickens; E is unique to endogenous viruses of chickens; and F and G are endogenous viruses of pheasants. Proteins coded for several variable regions in gp85 are responsible for defining these subgroups (34). These variable regions are involved in receptor binding and the induction of neutralizing antibody production. These and additional other variable regions in gp85 also are responsible for determining the host range.

Exogenous ALVs are transmitted by conventional means through vertical and horizontal transmission, a process that requires a fully infectious virus. In contrast
endogenous viral genes are inherited as host genes and may or may not be expressed (44). Fully infectious endogenous viruses can be transmitted congenitally, horizontally, or genetically. Endogenous ALV-21 (EV-21) is also venereally transmitted to embryos from viremic semen (112). The oviducts in hens that congenitally transmit ALV contain the highest titers of virus, compared to other visceral organs, suggesting that embryo infection is closely related with ALV produced at the oviduct but not with ALV transferred from other parts of the body (122).

Several studies showed that chickens infected with ALV after hatching transmit virus at a much lower rate than congenitally-infected, immune tolerant chickens (41, 83, 102, 129, 30). Viremia, antibody development, cloacal and albumen shedding, and incidences of the tumors were significantly lower in chicks with maternal antibody following massive exposure by a strain of ALV subgroup A at hatching (43). However, certain strains of ALV, immunosuppression, or the presence of genes for endogenous virus can increase the frequency of ALV shedding with a consequent increase in congenital transmission in chickens infected with the virus after hatching (26, 27, 28, 38, 48). Thus conditions of environmental exposure and genetic constitution of the host may influence the dynamics of viremia, antibody, and cloacal shedding in chickens infected with ALV after hatching. The incidence of regression of wing-web tumors induced by Rous sarcoma virus was shown to be dependent on the quantity of thymus tissue remaining after neonatal thymetomy in chickens of inbred line 6 (24).

The outcome of the ALV infection is highly variable within the host. ALVs are associated with a variety of neoplastic disease and non-neoplastic disease in their hosts. Commercially, ALV have been associated most frequently with B cell lymphosarcoma
originating in the cloacal bursa and metastasizing to multiple visceral organs. Myeolid origin tumors associated with subgroup J ALV infection have become prevalent recently. Other nonneoplastic manifestations of ALV infection, such as anemia, osteopetrosis, immunosuppression, cardiomyopathy, and poor growth, have also been described. Many factors are involved in the development and severity of the ALV-induced disease. These factors include strain of the virus, genetics of the host, immune status of the host (43, 45), age at exposure (40, 94), and other concurrent disease. Concurrent infectious bursal disease virus, reticuloendotheliosis virus, or Marek’s disease virus infection increased the frequently and duration of ALV viremia and shedding in experimentally infected chickens (48). Chickens infected with ALV are more susceptible to lymphoid tumor development when vaccinated with serotype 2 Marek’s disease virus (MDV) but not serotype 3 virus (turkey herpesvirus) at a young age (7, 47). One hypothesis states that vaccination may induce B cell hyperplasia in the cloacal bursa, and the proliferating cells may be more susceptible to neoplastic transformation (7). The direct relationship between the presence of MDV and the increase in ALV gene expression indicate MDV may induce trans-activation of ALV LTR (95).

Host genetic factors are perhaps the most important modulators of ALV infection. Receptor expression can vary greatly due to polymorphisms in the loci encoding ALV receptors, and major histocompatibility antigen may also affect susceptibility similar to the infection of Marek’s disease virus and Rous sarcoma virus (66). However endogenous retroviral sequences in the genome of the host are also significant but highly complex modulators of ALV infection. Endogenous retroviral sequences are widespread in the animal kingdom (58). The elements are genes of retroviral origin and are
transmitted in a Mendelian fashion. At least three families of retroviral elements have been described in chickens. First, ALVE, which comprises proviruses closely related to ALV, Second, the ART-CH elements that are avian transposon from the chicken genome (54), third, there are endogenous avian retroviruses (EAV) (35). The ALVE elements may be complete with the viral sequences gag, pol, and env flanked by long terminal repeats (LTR), or they may exhibit deletions for portions of these sequences (25, 101). Most birds carry several ALVE genes, with as many as five loci in non-inbred leghorns (1, 101) and an average of 7.3 loci in broilers (104).

Endogenous retroviruses may actually be harmful to the host. These genes are believed to impart some degree of immunologic tolerance in the host to similar exogenous retroviruses. The presence of either ev2, the endogenous viral gene encoding the complete subgroup E virus RAV-0, or ev3, the endogenous viral gene encoding group specific and envelope antigens, is associated with more frequent viremia and decreased antibody response in chickens after infections with ALV subgroup A, RAV-1 (26, 28). Similarly, in ovo infection of subgroup E virus RAV-0 also increases viremia and tumor development and decreased humoral immune response in chickens infected after hatching with RAV-1 and RAV-2 (27). The presence of endogenous viral genes ev21 or ev6 increases incidences of tumors, viremia, and virus shedding in chickens infected with the subgroup A virus RPL40 (111).

The presence of endogenous retroviruses also affects the diagnosis of ALV infection. Many methods have been described for the detection and propagation of exogenous ALV. (37). Enzyme linked immunosorbent assays (ELISA) have been developed to detect group specific ALV antigen and can be applied to a variety of samples including
feather pulp, cloacal swabs, sera, leukocytes, albumen, comb tissue, and meconium (16). Because both endogenous and exogenous ALVs share the group specific antigen (p27), direct assays based on detection of p27 cannot be used to differentiate between these two groups of viruses in samples such as serum (88). A combination of virus isolation and ELISA test is the standard procedure for differentiating between endogenous and exogenous ALVs (39). If a positive ELISA is obtained from C/O CEFs (cells susceptible to infection with exogenous and endogenous ALVs) but not C/E CEFs (cells resistant to endogenous ALVs), the sample is positive for endogenous ALV. Positive ELISA results from both C/E and C/O CEFs indicate the presence of exogenous and endogenous ALVs. RT-PCR techniques have been developed to detect and distinguish endogenous and exogenous ALV, and are more sensitive for the detection of ALV than ELISA (91).

**The Avian leukosis virus subgroup J**

In 1988, an exogenous avian leukemia virus belonging to a new subgroup for chickens was isolated from meat-type chicken lines (85). The virus of which HPRS-103 strain is the prototype, was of low oncogenicity. The novel ALV was distinct from viruses of subgroups A, B, C, D, E, and F on the basis of viral interference, neutralization, and host range patterns, indicating unique properties of the env gene and the gp85 gene product (85). Domestic fowl, red jungle fowl, Sonnerat’s jungle fowl, and turkeys are susceptible to infection by HPRS-103; Ring necked pheasant, Japanese green pheasant, golden pheasant, Japanese quail, guinea-fowl, Peking duck, Muscovy duck and goose are resistant (89). In the United States, several subgroup J avian leukemia viruses were isolated from broiler breeder and commercial broiler flocks experiencing myeloid
leukosis (46). The US type strain, ADOL-Hc1, is antigenically related, but not identical, to strain HPRS-103.

ALV-J has an overall structure of a typical slowly transforming replication-competent ALV containing LTR-leader-gag/pol-env-LTR (8). The LTR has more than 90% homology to that of other ALVs. HPRS-103 gag and pol genes are conserved (96 to 97% identity) with those of other ALVs. Two independent isolates from the United States were also shown to have a similar structure with very close sequence relationship to HPRS-103 suggesting they had a common ancestor (11). However, the sequence of \( env \) gene of the HPRS-103 is highly divergent from those of other ALV subgroups (9). The SU (gp85) surface domain of HPRS-103 has only about 40% identity to the corresponding sequences of subgroups A to E, as against 77 to 87% identity among each other. HPRS-103 \( env \) gene has over 75% identity to the \( env \) gene of E51, a member of the EAV family of endogenous retroviruses (12), suggesting that these endogenous sequences might have contributed to the origin of ALV-J \( env \). Novel EAV-related sequences, designated EAV-HP, showing over 97% homology to that of HPRS-103 \( env \) gene have been demonstrated in the chicken genome (9, 105). EAV-HP elements, also referred to as ev/J (10), were present in multiple copies in the genome of all the lines of chickens and the ancestral jungle fowl, but were absent in most other avian species studied (115). Those evidences suggest that HPRS-103 evolved by recombination between an exogenous ALV and EAV-HP \( env \) sequences to evolve as a new ALV subgroup (123).

Analysis of the sequence of the \( env \) gene from field isolates of ALV-J from England and the United States showed a close homology to HPRS-103, with the sequence identity
at the amino acid level ranging from 92.3 to 98.8 % (110, 125). Most of the sequence variations were clustered near the hr1, hr2 and vr3 regions, and the pattern of the nucleotide substitutions and high non-synonymous/synonymous ratios in these regions suggested that the changes might have resulted from selection pressure, possibly from an immune response.

ALV subgroup J induces late-onset myeloid leukemia targeting mainly the cells of the myeloid lineage (86). The myeloid tropism was further demonstrated by the ability to replicate in blood monocyte cultures (5). Myeloid tumors, consisting of accumulations of immature granulated myelocytes, are observed predominantly in the liver, spleen, gonads, kidney, thymus and bone including sternum, ribs, and vertebrae (81). Renal tumors and other sarcomas such as histiocytic sarcoma, hemangiosarcoma, mesothelioma, granulosa cell tumors, pancreatic adenocarcinoma, fibroma, and an unclassified leukemia are also observed (4, 57, 84, 86). In about 60 % of the cases of the myeloid tumors induced by HPRS-103, acute transforming variant viruses capable of rapid in vitro transformation of cultured bone marrow cells have been isolated (87). The acutely transforming virus strain 966 provirus is closely related to HPRS-103 but is defective, with the entire pol and parts of the gag and env genes replaced by a part of c-myc gene (15). Furthermore, LSTC-IAH30, a stable cell line derived from turkey monocyte cultures transformed by the 966 strain of ALV-J, expressed a 72-kDa Gag-Myc fusion protein. Based on the results, it was suggested that the induction of myeloid tumors by ALV-J occurs through mechanisms involving the activation of the c-myc oncogene (15). The incidence of tumor-associated mortality has been reported to vary widely between ALV-J infected flocks, suggesting the involvement of additional factors such as immunosuppressive
agents, concurrent infections, and vaccination against other diseases and husbandry practices in the manifestation of the disease.

Congenital infection of ALV-J is associated with significant body weight suppression, approximately 64% of that of ALV-negative flocks until 8 weeks of age (119). Eggs weighing less than 60 g from subgroup J infected broiler breeder flocks are more likely to contain subgroup J ALV detectable by PCR, suggesting a negative impact of the infection in the breeder hen on egg size (117). Some of the ALV-J isolates came from the broiler chicken flocks experiencing approximately 30% overall mortality due to ascites (77, 85). Retroviral particles were observed within the myocardium of the chickens from flocks with ascites mortality but not in the myocardium of the chickens from flocks without ascites, implying subgroup J ALV may induce a cardiomyopathy similar to some subgroup A ALV (77).

By immunohistochemical technique, the Gag expressions was observed in cells of the adrenal gland, heart, kidney, and proventriculus and especially in smooth muscle cells and connective tissue (5). After infection of 1-day-old chicks, greater tissue expression was observed in line 21 chicks, that mostly developed a tolerant viremic infection, as in opposed to Brown Leghorn chicks, that developed virus neutralizing antibodies (5). Viral RNAs can be detected by RNA in situ hybridization technique using riboprobes (6, 120). Strong positive staining for ALV-J-specific nucleic acid was present in cardiac myocytes, Purkinje fibers, vascular and pulmonary smooth muscle, renal glomeruli, distal tubules, and pituitary glands (120). The viral tropism for cardiac myocytes and Purkinje fibers may relate pathogenetically to the cardiomyopathy and congestive heart failure in
chicken flocks infected with ALV-J and the viral tropism for endocrine organs may relate to the weight suppression associated with infection (120).

Like all other exogenous ALVs, transmission of ALV-J occurs either by vertical or horizontal infection. In vertical transmission, chicks become immunologically tolerant to the virus and do not clear the virus and will remain persistently viremic shedders and are more likely to develop tumors. While the horizontal infection of other subgroups often lead to immune non-shedders, the consequences of similar infections with ALV-J can vary between egg-type and meat-type birds. Infection of egg-type birds post-hatch leads to immune non-shedders, however, similar infection of meat-type birds can result in either tolerant viremic infection or in an immune response resulting in only transient viremia (86, 87). The tolerance in some of these birds is not complete, and antibody-producing cells can be detected in their spleen, despite of the absence of serum neutralizing antibodies (103). Birds exposed to the virus at a younger age of a few weeks tend to develop tolerant viremia. The reason was postulated that the difference in the constitutive embryonic expression of EAV-HP env sequences could be an attributing factor for the induction of tolerance in these birds (10, 106, 115).

ALV-J infections in a flock can be tentatively diagnosed by pathological identification of characteristic tumors and confirmed by various virological methods. (90). ALV-J can be isolated from materials such as tumors, blood, albumen and cloacal and vaginal swabs in chick embryo (C/E) fibroblasts, which only support the growth of exogenous ALVs. Continuous, nontransformed chicken cell line, DF-1 which was derived from EV-O embryo (62, 109), can be used for propagation of the ALV-J (75). The growth of the virus in cell culture is detected by the identification of the group-
specific antigen ELISA in cell extracts. The subgroup can be confirmed by a neutralization test with subgroup J antiserum. A PCR based diagnostic technique is being widely used in the detection and identification of ALV-J infected birds (113, 114). Identification of ALV-J infected flocks can also be made by detecting ALV-J-specific antibodies in the serum by using a neutralization test or by using an antibody ELISA coated with ALV-J cell lysate or a recombinant baculovirus-derived HPRS-103 gp85 envelope glycoprotein (124).

Eradication programs applied for ALVs are essentially based on the experience with lymphoid leukosis, where the virus is primarily transmitted vertically. In vertical transmission, ALV-J behaves like other exogenous ALVs and established ALV eradication programs (116) should be effective in eradicating ALV-J infections (131). However, as the horizontal transmission of the ALV-J is more significant, the eradication programs have to be applied more intensely. An eradication scheme has been developed specially for ALV-J in commercial broiler breeder flocks (90). This program consists of testing pedigree birds at 20 weeks for group specific antigen by ELISA in the cloacal/vaginal swabs, at 22 weeks for viremia and serum antibodies, at 23 weeks for group specific antigen in albumen of the first two eggs, at 26 weeks for group specific antigen in the meconium of the hatched chicks and at 40 weeks for group specific antigen in the egg albumen (84). The hens showing positive results as well as the whole meconium positive group are removed immediately. This program has been successful in reducing the infection in commercial operations (84). Because the significance of horizontal transmission, vaccination adjunct to the eradication program should be considered. However, the widespread diversity among ALV-J strains and the lack of
knowledge on the immunogenic properties of different viral antigens, make the
development of an effective vaccine a difficult task.

**PCR-based quantitation methodologies of viral nucleic acid**

Since the development of polymerase chain reaction (PCR) amplification, one of the
major application of this method has been the highly sensitive and direct detection of
viral nucleic acids in clinical samples. The theoretical relationship between amount of
starting target sequence and amount of PCR product can be demonstrated under ideal
conditions (79). However this does not apply for most typical clinical samples. Because
of differential efficiencies and kinetics of the PCR, depending on the abundance of the
target sequence in the specimen of interest (31, 82), along with the variable presence of
various inhibitors of the PCR in typical clinical samples and exponential compounding of
minor variation in even replicate samples, the amount of product obtained from a sample
following a particular numbers of PCR cycles cannot be assumed to be an accurate
reflection of the amount of target sequence present in the starting sample (92). The assay
of limiting dilutions of samples was first proposed to obtain semiquantitative results.
Complification of internal reference templates has been used to control the tube-to-tube
variation. In this case, the major shortcoming is that different PCR templates may have
highly different thermodynamics and amplification efficiencies, thus affecting the relative
amounts of products. Additionally, a further limit of this procedure in virological
applications is that it cannot be used in the quantitative detection of extracellular template
species, such as free viruses in plasma samples.

One attempt to overcome this limit uses competitive PCR. In this technique, the
reference template sharing the primer recognition site with the target sequence is
coamplified with the target template to be amplified (17, 92). Because the templates compete for amplification, any variable affecting amplification has the same effect on both. As a consequence, the ratio of PCR products reflects the ratio between the initial amounts of the two sequences, thus allowing the precise evaluation of the amount of wild-type template. Different types of competitors have been proposed and used for quantitative PCR (17): competitors with a different length from that of the target sequence; competitors bearing a mutation that creates a new restriction site; and competitors separated by temperature gradient gel electrophoresis.

Higuchi et al constructed a system that detects PCR products as they accumulate (59, 60). This real time system includes the intercalator, ethidium bromide or SYBR® Green I dye, in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with computer-controlled CCD camera. An alternative method, fluorogenic probe based system, provides a real time method for detecting only specific amplification products. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached at each end (70). If the target sequence is present, the probe anneals downstreams of one of the primer sites and cleaved by the 5’ nuclease activity of Taq DNA polymerase as this primer is extended.

A clear correlation exists between disease progression and the presence of increasing amounts of infectious virus (55, 56), viral antigens, and virus specific nucleic acids. This correlation has been shown for human immunodeficiency virus type I (HIV-1) (3, 56, 108, 132). Quantitation methods are needed for the next generation of research on the pathogenesis of a ALV-J infection. For ALV-J, only tissue culture infective dose 50
(TCID$_{50}$) has been used to express the viral quantity for ALV-J. Because no observable cytopathic effect is produced by ALV-J infection in any culture system available, TCID$_{50}$ has been laboriously determined by serial dilution of a sample and inoculating it into C/E cells to prevent growth of endogenous virus. After 7-9 days incubation, viral growth in diluted samples is detected using an ag-ELISA for a group specific p27 antigen (42). This technique is time-consuming, laborious, expensive, and very difficult to perform in some laboratories due to lack of C/E cells.

**Immunosuppression in chickens**

In poultry production system, immunosuppressive diseases have been and remain economically important. Vaccination failure, increased condemnation and mortality, poor feed conversion, and increased morbidity and medication costs commonly results from immunosuppression. Immunosuppression has been defined as “a state of temporary or permanent dysfunction of the immune response resulting from damage to the immune system and leading to increased susceptibility to disease” (33). Numerous immunosuppressive agents affect avian and mammalian species (80) including viruses, prokaryotic and eukaryotic parasites, microbial toxins, chemicals, drugs, nutritional deficiencies (69) and various psychological or physical-environmental stressors (32).

Infectious bursal disease virus (IBDV) is of major interest because of the widespread occurrence of the infection in commercial chickens. Infection with IBDV at an early age significantly compromises the humoral and local immune responses of the chickens (107). The cellular response is also compromised but to a lesser extent and for a short period. The immunosuppression seems to be the result of a direct lysis of B cells or their precursors (63, 99). Chicken anemia virus appears to target erythroid and lymphoid
progenitor cells in the bone marrow and thymus respectively (2). Destruction of erythroid progenitors in bone marrow results in severe anemia, and depletion of granulocytes and thrombocytes. Destruction of T cells result in depletion of mature cytotoxic and helper T cells with consequent immune suppression. In Marek’s disease virus (MDV) infection, the degree of immunosuppression was determined by persistence of early cytolytic infection, atrophy of bursa of Fabricius and thymus, and histologic evidence of necrosis and atrophy in lymphoid organs (13, 14). The results suggests that it is linked to virulence and that a simple measure of atrophic changes in the bursa of Fabricius and thymus might be useful in determining the pathotype classification of new MDV isolates (14).

Syndromes caused by consumption of moderate to high amounts of mycotoxins range from acute mortality to slow growth and reduced reproductive efficiency (93). Consumption of lesser amounts of fungal toxins may result in impaired immunity and decreased resistance to infectious disease. Mycotoxin-induced immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement activity, or impaired macrophage-effector cell function (22).

Effects of subgroup J ALV infection on immune function have been extensively studied (118). Peripheral blood heterophils and peritoneal macrophages isolated from infected broilers exhibited no functional deficits in microbicidal and phagocytic ability relative to cell isolated from uninfected control chickens. The splenic mitogenic response were similar for ALV-J infected and uninfected chickens. Thymus, cloacal bursa, and spleen were histologically similar in ALV-J infected and uninfected chickens.
Cyclophosphamide is an antineoplastic agent and immunomodulator used in the treatment of tumors and autoimmune disorders. The parent compound, cyclophosphamide, _in vitro_ is neither alkylating, cytotoxic, nor immunosuppressive (53). In _vivo_, cyclophosphamide is converted by hepatic microsomal enzymes to 4-hydroxycyclophosphamide that is reversibly altered to aldophosphamide (AP) (21). Further the 4-OHCP/AP compound is either enzymatically detoxified or undergoes spontaneous degradation to phosphoramid mustard (PM) and acrolein within cells (21). This alkylating agent induces DNA cross-links – an important lesion in causing the development of point mutations and chromosome aberrations (21). Newly hatched chickens treated with cyclophosphamide are rendered irreversibly B cell deficient (71, 74). Furthermore, selective B-lymphocyte cytotoxicity is most dramatically achieved when cyclophosphamide exposure occurs during embryogenesis (36, 130). T cell can be killed or slowed in proliferation by single or multiple, high dose CP treatment in neonatal chicks, but the numbers of thymocytes can recover in two weeks (51, 74). The selective toxicity of cyclophosphamide is primarily due to its differential lymphocyte sensitivity, and not due to differential compound distribution and uptake by immune tissues, or to site-specific activation and detoxification (78). Structure-activity studies in the chick embryo revealed induction of selective B lymphocyte toxicity that was induced by cyclophosphamide analogs capable of forming DNA interstrand cross-links (130).

Cyclosporin, a selective T-cell immunosuppressant drug, depresses cell-mediated immunity in chickens, causing prolonged skin graft survival, depressed proliferative responses in mitogen-stimulated lymphocytes and decreased wattle responses to injected antigen (61). Cyclosporin prevents the synthesis of cytokines by T cells by blocking a
late stage in the signaling pathway initiated by the T-cell receptor. This especially affects
the production of interleukin-2 (IL-2), hence T cell proliferation is affected. As a
consequence IL-2 dependent functions which include T-helper activities, cytotoxicity,
natural killer cell activity and antibody dependent cell cytotoxicity would be depressed
after cyclosporin treatment (61).

Treatments of cyclophosphamide and cyclosporin have been used as a means of
inhibiting the humoral and cell-mediated immune response in order to determine the role
of T and B cells in protective responses to infectious pathogens of chickens (48, 49, 52,
96, 97, 128). (23, 50, 64, 68, 73).

Many factors affecting the pathogenesis of the ALV have been studied. Chemical or
virus-induced immunosuppression leads to increased rates of viremia and shedding of
subgroup A ALV in chickens infected with the virus after hatching (48). Cloacal
shedding, viremia, and tumor development were significantly lower in chickens with
maternal antibody following exposure to subgroup A ALV at hatching (39, 43). However, induced moulting or raised circulating corticosterone in adult hens did not
influence ALV infection or shedding. Rispens et al.(98) showed that actively acquired
antibody induced by inoculation of infectious ALV at 8 weeks of age can prevent
shedding and congenital transmission to subsequent generation. Cotter et. al (24) showed
that regression of wing-web tumors induced by Rous sarcoma virus was dependent on the
quantity of thymus tissue remaining after neonatal thymectomy. For subgroup J avian
leukosis virus, injection of subgroup J ALV antiserum protected chickens infected at
hatching 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 (unpublished observation, Gharaibeh). The immune compartment involved in protection against viremia and oncogenesis is undetermined. Understanding the effects of concurrent immune suppression on ALV-J pathogenesis is required to design effective control program for this infection.

REFERENCES


CHAPTER 3
COMPARISON AND VERIFICATION OF QUANTITATIVE COMPETITIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QC-RT-PCR) AND REAL TIME RT-PCR FOR AVIAN LEUKOSIS VIRUS SUBGROUP J

ABSTRACT

Avian leukosis virus subgroup J (ALV-J) infections cause significant economic losses because of increased mortality, tumor production, decreased production, and cost for eradication. Current quantification methods for ALV-J expressed by TCID\textsubscript{50} are difficult to determine because of the lack of cytopathic effect in cell cultures and non-specificity of currently available antigen-capture ELISA tests. In this study, we developed one-tube fluorescent probe based real time RT-PCR method for quantification of ALV-J and compared it with available quantification methods. Cell lysates with different TCID\textsubscript{50}s determined by cell culture and antigen capture ELISA (ag-ELISA) were used for one-tube real time RT-PCR using fluorogenic probe and quantitative competitive RT-PCR (QC-RT-PCR). The results of QC-RT-PCR and real time RT-PCR were highly correlated to the TCID\textsubscript{50}s determined by conventional culture methods. They were also very specific, sensitive, easy to perform, reproducible, and rapid compared with conventional methods. These RT-PCR based quantification methods of ALV-J viral RNA will be useful in virological and pathogenesis studies.

Keywords: QC-RT-PCR; real time RT-PCR; ALV-J; fluorogenic; probe; TCID\textsubscript{50}

INTRODUCTION

Avian leukosis viruses (ALVs) of chickens are classified into six subgroups, A through E, and newly identified J (Payne and Fadly, 1997). Subgroup classification is based on differences in viral envelope glycoprotein antigens, which determine virus-serum neutralization properties, viral interference patterns, and host range of \textit{in vivo} and \textit{in vitro} infectivity (Coffin, 1996). The prototype strain of avian leukosis virus subgroup J (ALV-
J), HPRS-103, was isolated from commercial meat-type chickens associated with myelocytomatosis in England (Payne et al., 1991). The strain was completely sequenced and designated a novel subgroup. It was hypothesized to be a recombinant between exogenous ALVs and the EAV family of endogenous avian retroviruses (Bai et al., 1995). The virus has a wide host range and is capable of infecting jungle fowl, turkeys, and all of 11 genetic lines of chickens studied (Payne et al., 1992). Susceptibility to tumors varies markedly between different genetic lines of chickens, but meat-type chickens are particularly prone to tumors.

ALV-J infection occurs worldwide and is ubiquitous in broiler breeders and commercial broilers. Infection causes significant economic losses in the broiler industry caused by increased mortality, tumor production, decreased weight gain, and cost for eradication (Stedman and Brown, 1999). In the field, presence of infection is associated with variable tumor types including myelocytoma, renal tumors and others (Venugopal, 1999).

Because no observable cytopathic effect is produced in vitro, TCID$_{50}$ of ALV-J is determined by serially diluting a sample, inoculating it into cells resistant to growth of endogenous viruses, incubating it for 7-9 days, and detecting group specific antigen p27 using an antigen capture ELISA (Fadly and Witter, 1998). This technique is laborious, expensive, requires cells resistant to endogenous viruses, and takes 8-10 days to complete.

Polymerase chain reaction (PCR)-based techniques have been used to detect the presence of cellular and viral genes and also have been used to quantify these genes (Foley et al., 1993; Jung et al., 2000; Killeen, 1997). Recently, real time monitoring systems of PCR amplification have been developed and are widely used for detection and quantification
of genes (Orlando et al., 1998). For diagnosis of ALV-J infection, several PCR systems have been developed and are widely used for both laboratory and field samples (Smith et al., 1998). This manuscript describes development of a new one-tube real time reverse transcription (RT)-PCR system using fluorogenic probe for ALV-J RNA quantification. This study also was performed to verify and compare this new real time RT-PCR with a previously developed quantitative competitive (QC)-RT-PCR (Kim et al., 2000) and the conventional quantitative method using cell culture systems and ag-ELISA.

**MATERIALS AND METHODS**

**Production of viral samples.** ALV-J (strain ADOL-7501, Avian Disease Diagnostic Lab, East Lansing, MI) was propagated in primary or secondary C/E chicken embryo fibroblasts (C/E CEF, Kestrel Inc., Waukee, IA, USA) grown at 37 C. After propagation, samples were collected and TCID$_{50}$s determined as follows. The stock virus was serially ten-fold diluted to $10^{-8}$ with cell culture media and each dilution of the virus was cultivated at 37 C and 5% CO$_2$ for 8 days in monolayer of C/E CEF. After freezing and thawing (3X), 100 µl of the cell lysate were used to detect p27 gs-antigen using a commercial ag-ELISA kit (IDEXX Inc, Westbrook, MA, USA). TCID$_{50}$s of the samples were calculated by the method described by Reed and Muench (Reed and Muench, 1938). Samples with $10^{6.5}$, $10^{5.5}$, and $10^{3.5}$ TCID$_{50}$s were used in the studies described below.

**RNA extraction.** Total RNAs were extracted from 250 µl of each of the three samples described above using a commercial reagent and according to manufacturer’s recommendations (Tri Reagent BD, Molecular Research Center Inc. Cincinnati, OH).
Each RNA sample was resuspended in 20 µl of diethyl pyrocarbonate (DEPC) treated water and stored at –80 C until used.

**Development of one-tube real time RT-PCR**

**Primers and probe.** Oligonucleotide primers used in this study (Table 3.1) were a forward primer (H5) and reverse primer (H7). This pair of primers was specific for ALV-J and did not detect other subgroups of ALVs (Smith et al., 1998). A probe sequence (Table 3.1) was selected within the location between primers H5 and H7 in conformity to the Perkin Elmer guidelines (Livak et al., 1995a). The probe was also designed to have a high cross reactivity with ALV-J sequences (Venugopal et al., 1998). The TaqMan™ fluorogenic probe was synthesized by Perkin-Elmer (Foster City, CA. USA). The probe was a 26 bp oligonucleotide and located 14 bp down from the 3’ end of forward primer (H5). The fluorescent reporter dye at the 5’ end of the probe was FAM (6-carboxyfluorescein), and the rhodamine quencher dye at the 3’ end was TAMRA (6-carboxytetramethylrhodamine).

**Production of control RNA.** Complementary DNA (cDNA) was amplified by RT-PCR (primers H5 and H7) from ALV-J (ADOL-7501), separated on a 1.5% agarose gel, excised, isolated, and ligated into the cloning site of the pGEM-T Easy vector system (Promega, Madison, WI, USA). High efficiency competent *Escherichia coli* strain (JM 109, Promega, Madison, WI, USA) was transformed with the recombinant plasmid and plated on Luria Broth (LB) plates containing Ampicillin (100 µg/ml), 0.5 mM IPTG, and X-gal (80 mg/ml). After 18 hours incubation at 37 C, white colonies with the plasmid were selected and propagated in the LB broth overnight at 37 C. The plasmid was purified from these cultures (Mini prep kit, Qiagen, Valencia, CA, USA). The direction
and sequence of the insert was identified by sequencing reactions using primers T7 (5’-TAATACGACTCACTATAGGG-3’) and SP6 (5’-GATTTAGGTGACACTATAG-3’). Colonies containing the recombinant plasmid with the correct insert were propagated in the LB broth and the plasmid was purified as described above (Mini prep kit, Qiagen, Valencia, CA, USA). The plasmid was digested by Nco I (Roche Molecular Biochemicals, Indianapolis, IN) for 2 hours at 37 C, and control RNA was prepared by in vitro transcription reaction using SP6 primer. A transcription reaction was performed using a commercial kit with slight modification (Dig RNA labeling kit, Boeringer Mannheim, GmbH, Germany). After the transcription reaction, RNase-free DNase was added and incubated at 37 C for 20 min. To precipitate the RNA transcripts, 2.0 µl of 2 mM EDTA pH 8.0, 2.5 µl of 4 M LiCl, and 80 µl absolute ethanol were added and left at –70 C overnight. The RNA was precipitated in a pellet by centrifugation at the speed of 12,000 g for 30 min at 4 C. The pellet was washed with 75 µl of 70% cold ethanol and precipitated in a pellet by centrifugation at 12,000 g for 30 min at 4 C. The resultant pellet was dissolved in RNase free, DEPC-treated ddH₂O. The amount of RNA in this preparation was measured using a spectrophotometer at absorbance 260 nm (Pharmacia, USA). The RNA solution was divided into aliquots and stored at –70C until used.

**Amplification kinetics of control RNA.** To determine the amplification efficiencies of control and viral RNA, real time fluorogenic RT-PCR were performed with serially ten-fold diluted control RNA and viral RNA, respectively. Standard curves for the respective RNAs were generated by plotting their threshold cycle numbers (C₇) versus their dilution factors. The slopes of these standard curves were compared with each other. Efficiencies
were defined as equal if the difference of the slopes was smaller than 0.1 (Gut et al., 1999).

**Dilution of the samples.** To standardize the amount of RNA in each sample a preliminary real time RT-PCR was performed with serial dilution of RNAs from each sample, and the resulting amplification plots were compared with the amplification plots of controls. Based on these findings, RNAs from the samples were diluted as follows: 1:100 for samples with $10^{6.5}$ TCID$_{50}$, 1:50 for samples with $10^{5.5}$ TCID$_{50}$, and 1:10 dilution for samples with $10^{3.5}$ TCID$_{50}$. 2.3.5. One-tube real time RT-PCR

The real time RT-PCR was performed in a 20 µl reaction volume containing 0.25 u/µl MultiScribe Reverse Transcriptase (Perkin Elmer, Foster City, USA), 0.025 u/µl AmpliTaq Gold DNA Polymerase (Perkin Elmer), 5.5 mM MgCl$_2$, 500 µM dNTP mix, 50 nmols of forward and reverse primers, 50 nmol of fluorogenic probe, 0.4 u/µl RNase inhibitor, and the reaction buffer recommended by manufacturer. Ten µl of each of the diluted samples and controls were amplified individually in a tube of a 96-well plate.

The RT-PCR program consisted of 42 C for 30 min, 95 C for 10 min, and 40 identical cycles of 15 sec at 95 C and 2 min at 60 C. Reverse transcription and amplification were carried out in a single tube in an ABI Prism® 7700 Sequence Detector (Perkin Elmer, Foster City, USA) without modifying or moving the samples between RT and PCR steps. Fluorescence was measured after each cycle, calculated, automatically, and graphically presented (Sequence Detection Software, Perkin Elmer, Foster City, USA). This system calculates a $C_T$ identifying the first PCR cycle with fluorescence above a base line signal. The correlation between this $C_T$ value and the starting amount of template is used to quantify the initial amount of RNA in a sample. The standard curve of $C_T$ versus starting
amount of RNA for all standards was used to determine the starting amount of RNA in a similarly treated unknown by interpolation.

**Determination of the detection limit.** Sensitivity of the real time RT-PCR was determined using serial dilution of control RNA described above. Control RNAs were serially, ten-fold diluted from 25 pg/µl to 2.5 fg/µl.

**One-tube QC-RT-PCR.** One-tube QC-RT-PCR was performed on each sample according to the methods described by Kim et al. (Kim et al., 2000). Primers (H5 and H7) were identical to those used for the real time RT-PCR described above. The sequence of the competitor RNA was identical to cDNA amplified by RT-PCR (H5/H7) from ALV-J (ADOL-7501), except there was a 176 bp internal deletion. Briefly, serially ten-fold diluted from 2.5 ng/ml to 25 fg/ml, the 456 bp competitor RNA, with the same primer binding sites but producing a different sized amplification product, were added to each RT-PCR tube. Sample RNA was added to each tube. A one-tube RT-PCR was carried out (Titan™ One-tube RT-PCR system, Boeringer Mannheim GmbH, Germany) according to manufacturer’s recommendations. The program consisted of reverse transcription at 42 C for 45 min, 95 C for 5 min, and 31 identical cycles of denaturation (94 C for 30 sec), annealing (52 C for 30 sec), and extension (72 C for 1 min). The last cycle was followed by an extra 5 minutes. Amplified products of QC-RT-PCR were loaded on a 1.5% agarose gel, and band intensities of each product were optically analyzed (Gel-doc 1000 camera system and image analysis software, Molecular analysis software, Bio-Rad Lab Inc., Hercules, CA. USA). The amount of viral RNA was calculated by determining the equivalent point in a logarithmic linear regression plot as described previously (Piatak et al., 1993).
For quantitation by QC-RT-PCR, the viral RNA from each sample should be within the range of the competitor RNA. To determine the appropriate dilution factor of each sample for QC-RT-PCR, we performed a RT-PCR reaction with 31 cycles on serial dilutions of sample RNAs and serial dilutions of competitor RNA. Band intensities of 369 bp and 545 bp sized products in each tube were compared. Then, RNAs from samples were diluted as follows; 1:1000 for samples with $10^{6.5}$ TCID$_{50}$, 1:100 for samples with $10^{5.5}$ TCID$_{50}$, and no dilution for samples with $10^{3.5}$ TCID$_{50}$.

**Ag-ELISA examination.** Prior to nucleic acid extraction, an optical density (OD) was determined for each sample (ag-ELISA, IDEXX Inc, Westbrook, MA, USA). Briefly, 100 µl of freeze-thawed cell lysates infected with ALV-J (ADOL-7501) were tested for group specific p27 antigen and an OD value was determined at 650 nm for each.

**RESULTS**

**Real time RT-PCR**

**Production of control RNA.** By insertion into the plasmid, transformation, and the *in vitro* transcription reaction, a 636 bp RNA was successfully produced, and the quantity was measured by spectrophotometer (data not shown). In this experiment, the slopes of the standard curve of control RNA and viral RNA were $-3.632$ ($R^2 = 0.985$) and $-3.55$ ($R^2 = 0.998$), respectively, showing the control RNA transcripts were reliable for ALV-J quantification.

**Real time RT-PCR.** Amplification plots and standard curves were generated after completion of the PCR reactions (Fig. 3.1). Threshold cycle numbers of the diluted samples were within the range of controls. The amount of viral RNA measured by real
time RT-PCR was plotted against the known TCID\textsubscript{50} for each each sample (Fig. 3.3). Results showed a strong positive correlation between the TCID\textsubscript{50} and the amount of viral RNA measured by real time RT-PCR. The real time RT-PCR could measure control RNA levels to 250 fg/µl, corresponding to approximately 5 \texttimes 10^5 RNA copies/µl.

**QC-RT-PCR.** Amplified products of QC-RT-PCR were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and fluorescence measured using camera and image analysis software, as described above. Strong progressive competition between the competitor RNA and viral RNA was observed in the gel (Fig. 3.2). A regression line for each sample was generated, and the amount of viral RNA was calculated as described above (Fig. 3.2). The amount of viral RNA for each sample was successfully measured by one-tube QC-RT-PCR and was plotted against the TCID\textsubscript{50} of the corresponding sample (Fig. 3.3). Results showed a strong positive correlation between the amount of viral RNA measured by QC-RT-PCR and the TCID\textsubscript{50} of that sample. Results were highly reproducible for replicate runs of the same sample.

**Ag-ELISA.** Triplicates of each sample with different TCID\textsubscript{50}s were tested by ag-ELISA, and Ods were obtained (Fig. 3.4). ODs for each sample were reproducible for each sample and were roughly correlated with TCID\textsubscript{50}s. However, the ODs of the samples with 10^{5.5} TCID\textsubscript{50} and 10^{6.5} TCID\textsubscript{50} were similar to each other.

**DISCUSSION**

Both QC-RT-PCR and real time RT-PCR used in this study highly correlated with TCID\textsubscript{50} determined by conventional cell culture and ag-ELISA methodologies (Fig. 3.3).
Both methods detected the amount of RNA in samples with different TCID₅₀s of 10⁶.5, 10⁵.5, and 10³.5. These results were reproducible in separate trials using both methods.

Real-time RT-PCR is a newly introduced technique but has been widely used for detection and quantification of genes. It has a higher sensitivity than conventional RT-PCR in well-adjusted reaction conditions. In our study, we could quantify ALV-J viral RNA and could differentiate ten-fold dilutions from each other using one-tube real-time RT-PCR with a fluorogenic probe system. The sensitivity of this system was much lower than the expected 10-100 RNA copies per sample. This was most likely because of our product size being longer than recommended, and less than perfect compatibility of primers and our probe set (Livak et al., 1995a; Livak et al., 1995b). These factors could affect the capability of polymerase to amplify the template in our reactions. However, those limitations were unavoidable in our study because the primer sets had to be specific for ALV-J, so as not to detect other ALV subgroups. Additionally, the probe had to be compatible with the primers used. As more sequence data of different ALV-J isolates are accumulated, more appropriate sets of primers and probes can be designed and the sensitivity of this assay will likely increase to maximal levels.

QC-RT-PCR was a very sensitive, accurate method and was easy to perform (Foley et al., 1993; Piatak et al., 1993). QC-RT-PCR also is appropriate system for quantification of viral RNA from plasma which has no endogenously expressed genes. QC-RT-PCR in our study was highly reproducible and the results showed strong positive correlation with the TCID₅₀s determined by conventional methods (Fig. 3.3).

Because the quantification using either of these new RT-PCR based methodologies is made by extrapolation based on a standard curve, the amount of viral RNA from the
samples should be within the range of reference RNA amounts; for example, competitor RNA in QC-RT-PCR, and the control RNA in real time RT-PCR. In our study, we determined an appropriate dilution factor by performing a preliminary RT-PCR reaction to determine these ranges with the competitor RNA and serial dilution of RNA from the samples, followed by comparing band intensities of cDNA products of each RNA. Determination of this dilution factor was also used for real time RT-PCR. Addition of excessive template caused the amplification plot of the sample to be out of the range of the control RNA, and prevented accurate quantitation.

OD values of ag-ELISA roughly reflected the TCID$_{50}$ of low and intermediate titer samples in our experiment. However, ODs could not differentiate the intermediate and high titer samples (Fig. 3.4). Ag-ELISA was not specific for ALV-J but detected a p27 group specific antigen common to all ALVs (Payne and Fadly, 1997). This conventional method of TCID$_{50}$ determination is time consuming (at least 7-10 days) and is laborious and expensive. Moreover, because of the inherent nonspecificity of ag-ELISA, specialized C/E cells are required to inhibit growth of endogenous virus (Fadly and Witter, 1998). Furthermore, the sensitivity of ag-ELISA for p27 antigen, and the ratio of p27 concentration to virion number has not been determined.

In conclusion, the results of QC-RT-PCR and real time RT-PCR were highly correlated to viral TCID$_{50}$ determined by the conventional culture method. They were also sensitive, specific, easy to perform, reproducible, and rapid compared with conventional methods. The real time RT-PCR developed in our study should be further modified to increase the sensitivity. QC-RT-PCR method for the quantitation of ALV-J viral RNA will be useful in virological and pathogenesis studies.
ACKNOWLEDGEMENTS

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REFERENCES


avian retrovirus envelope subgroup, designated J. J Gen Virol 73(Pt 11), 2995-2997.


Table 3.1
Sequences of oligonucleotide primers, probe, and position

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’ – 3’)</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5</td>
<td>GGATGAGGGTGACTAAGAAAG</td>
<td>5258 - 5277</td>
</tr>
<tr>
<td>H7</td>
<td>CGAACCAAGGTAACACACG</td>
<td>5783 - 5802</td>
</tr>
<tr>
<td>Probe</td>
<td>^aCTCTTTGCGGCCATTTCTGACTGGGc^b</td>
<td>5292 - 5317</td>
</tr>
</tbody>
</table>

*a* Positions were derived from the HPRS-103 sequence (Genebank accession No. Z46390)

^a* Reporter dye (FAM) labeled nucleotide

^b* Quencher dye (TAMRA) labeled nucleotide
Fig. 3.1. Standard curve showing threshold cycle values (Cₜ) vs. the starting quantities of standard RNAs and ALV-J RNAs from cell culture samples. The amounts of standard RNA added were from ten-fold dilutions from 25ng/ml to 250 fg/ml. ALV-J RNAs were from the samples with 1:100 diluted 10⁶.⁵ TCID₅₀, 1:50 diluted 10⁵.⁵ TCID₅₀, and 1:10 diluted 10³.⁵ TCID₅₀. The regression line is linear and Cₜ values are highly correlated with starting RNA quantity. Amounts of the ALV-J RNAs were calculated based on the standard curve.
Fig. 3.2. Ethidium bromide stained gel and a linear regression line used to generate equation for quantitative competitive RT-PCR (QC-RT-PCR) for quantitation of the $10^{5.5}$ TCID$_{50}$ sample. Lane 1 was 1 kb DNA ladder. Lanes 2-7 shows RT-PCR products using H5/H7 primers: 545 bp cDNA amplicon from viral RNA and 369 bp cDNA amplicon from competitor. These products result from the co-amplification of a 10-fold serially diluted competitor and 1ul of 1:100 diluted RNA extracted from a $10^{5.5}$ TCID$_{50}$ sample. Strong progressive competition was observed. Band intensities of each product were measured and used to generate regression line as described in materials and methods. The equation of the regression line was $y = 0.9119x - 2.5293$ with an $R^2$ value of 0.9972. The log amount of RNA of the $10^{5.5}$ TCID$_{50}$ sample was determined to be 4.7737 ($= 59.4$ ng/ml ). Similar methods were done for the quantitation of viral RNA from the samples with $10^{6.5}$ and $10^{3.5}$ TCID$_{50}$s.
Fig. 3.3. Comparison of the amounts of ALV-J determined by conventional culture method (log TCID$_{50}$) vs amounts calculated by real time RT-PCR and quantitative competitive (QC) RT-PCR (log RNA (fg)). There is a strong positive correlation between the amounts of viral RNAs and the TCID$_{50}$ of each sample. Amounts measured by real time RT-PCR were similar to those from QC-RT PCR.
Fig. 3.4. Optical densities (ODs) obtained by commercial p27 ag-ELISA (IDEXX) test for three different cell culture samples containing $10^{3.5}$, $10^{5.5}$, $10^{6.5}$ TCID$_{50}$ ADOL-7501 (ALV-J). ODs of the samples are roughly proportional to TCID$_{50}$s of the samples. However, samples with $10^{5.5}$ and $10^{6.5}$ TCID$_{50}$ could not be differentiated from each other using OD values.
CHAPTER 4

LESIONS INDUCED BY CYCLOPHOSPHAMIDE TREATMENT IN BROILER CHICKENS

1 Kim, Y., M. J. Pantin and T. P. Brown. To be submitted to Avian disease.
ABSTRACT

Cyclophosphamide (CY) is an immunosuppressant and inhibits cell division by alkylating nucleic acids. This study was performed to determine the toxic effects of CY treatment in broiler chickens. One-day-old White Plymouth Rock broiler chickens were treated with either 4mg / day CY or PBS intraperitoneally for 4 consecutive days. Mortality of the CY-treated chickens was 65.4 % (34/52), while that of the PBS-treated chickens was 10 % (5/50). CY-treated chickens were significantly smaller, and had delayed feathering and lower relative bursal weight compared to PBS treated. Histologically, the lymphocytes were markedly depleted in the spleen and bursa. Bone marrow was severely hypocellular and replaced by adipose tissue. Diffuse vacuolation of hepatocytes with individual cell necrosis was present in the liver. There was segmental necrosis of lining epithelial cells of renal tubules with eosinophilic material and necrotic detritus within the lumens. The lesions in the liver, kidney and bone marrow were transient and repaired by 2 weeks of age.

INTRODUCTION

Cyclophosphamide (CY) is an antineoplastic agent and immunomodulator used in the treatment of tumors and autoimmune disorders. In vitro, as the parent compound, CY is neither alkylating, cytotoxic, nor immunosuppressive (5). In vivo, CY is converted by hepatic microsomal enzymes to 4-hydroxycyclophosphamide (1). Selective CY toxicity to the B-cell population is due primarily to differential lymphocyte sensitivities, and not due to differential compound distribution/uptake by immune tissues, or to site specific activation /detoxification (9). Structure-activity studies
in the chick embryo showed selective B lymphocyte toxicity was induced by CY analogs capable of forming DNA interstrand cross links. Newly hatched chickens treated with CY are rendered irreversibly B cell deficient.

CY treatment has been used as a means of abrogating the humoral immune response in order to determine the role of T and B cells in protective responses to infectious pathogens (2, 10, 13). However, the morphologic toxic effects of CY treatment in neonatal chickens has not been reported. This study was designed to investigate the toxic morphologic effects of CY treatment on newly hatched SPF broiler chickens.

**MATERIALS AND METHODS**

**Chickens.** White Plymouth Rock SPF chicken eggs (Dr. David Swayne, SEPRL, USDA, Athens, GA, USA) were hatched and reared on wire-floored cages to 3 weeks of age. Feed and water were provided *ad libitum*.

**Medium and Reagent.** A preparation of CY (Cyclophosphamide monohydrate; Sigma Chemical Co., St. Louis, MO) was obtained in dry form. An aqueous solution was prepared by reconstituting 1.6 g in 40 ml (40 mg/ml) of calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and filtered through a 0.22-um syringe filter.

**CY treatment.** Groups of 52 and 50 chickens were injected intraperitoneally with either 4 mg of CY or 0.1 ml of PBS, respectively. Injections were given at 1, 2, 3, and 4 days of age as previously described (14).

**Histopathology.** To determine if CY could damage selected tissues, trachea, lung, liver, kidney, bursa of Fabricius, bone marrow, spleen, thymus, and brain were collected from animals dying during the experiment. Also chickens (n=5) were killed and
necropsied at 1, 2, and 3 weeks of age, and tissues collected. The tissues were fixed in 10% buffered formalin and routinely processed for microscopic examination.

**Statistical analysis.** Two-tailed Student $t$-test with assumption of different variance was used to analyze the data at a significance level of 0.05.

**RESULTS**

**Mortality.** The CY-treated group showed significantly higher mortality (34 out of 52 chickens, 65.4%) than that of the PBS-treated group (5 out of 50 chickens, 10%). Mortality in the PBS-treated group occurred only at 4 days of age. In the CY-treated group, 28 chickens died between 4 and 7 days of age.

**Body and bursa weight.** Body and bursal weights were measured at 1, 2, and 3 weeks of age. CY-treated chickens weighed significantly less on average than those that were PBS-treated (Fig. 4.1, $p<0.001$). As anticipated, relative bursa weight also was significantly reduced in CY-treated chickens (Fig. 4.2, $p<0.001$).

**Gross findings.** Feathering were delayed in chickens treated with CY when compared to chickens given PBS. After this delay, some CY treated chickens began developing normal feathers, while some still had impaired feather growth. All PBS treated chickens had normal feathering. The bursa of Fabricius of the CY-treated birds was grossly smaller than that of PBS treated birds.

**Histologic findings.** Microscopically, the trachea, lung, thymus, and brain had no significant morphologic alterations. Compared to the control group (Fig. 4.3), bursal lymphoid follicles were smaller and separated by thick fibroblastic connective tissue in CY-treated birds (Fig. 4.4). All of the bursal lymphoid follicles of CY-treated birds had
severe depletion of lymphocytes (Fig. 4.4). The changes in the bursa of Fabricius were persistent throughout the experiment.

Hepatocytes of CY-treated birds were swollen and hydropic (Fig. 4.5). Numerous hepatocytes had clear round vacuoles interpreted to be lipid vacuoles. Occasional necrosis of individual hepatocytes also was present. Kidneys of CY-treated birds had segmental necrosis of renal tubules (Fig. 4.6). Tubular epithelial cells in affected tubules were hypereosinophilic and the nuclei were pyknotic. Scattered renal tubules in the CY-treated birds were severely dilated and contained eosinophilic material and necrotic cell debris within their lumens. Spleens from CY-treated birds had decreased numbers of lymphoid follicles and reticular cells were more prominent (Fig. 4.8) compared to that of the control group (Fig. 4.7). The lymphoid follicles were severely depleted of lymphocytes and with severe necrosis (Fig. 4.8). The bone marrow from CY-treated birds was hypocellular and had a decreased number of both erythroid and myeloid precursor cells. Extensive areas of the bone marrow were replaced by adipose tissue. The lesions in the liver, kidney, spleen and bone marrow were repaired by 2 weeks of age, and were then indistinguishable morphologically from PBS treated birds.

**DISCUSSION**

Cyclophosphamide in its original form lacks genotoxic properties. The mutagenic activity is attained via biotransformation mediated by liver microsome monooxygenases (4). 4-hydroxycyclophosphamide (4-OHCP), originating from the parental compound by cytochrome P-450 dependent oxidation, is reversibly altered to aldophosphamide (AP). Further the 4-OHCP/AP complex is either enzymatically detoxified or undergoes
spontaneous degradation to phosphamide mustard (PM) and acrolein within cells (3). These alkylating agents induce DNA cross-links causing the development of point mutations and chromosome aberrations (3). Treatment with cyclophosphamide on incubation day 3 exhibited variable teratogenic effects including heart defects, facial clefts and limb malformations (11). The consequences of the mutagenic action of CY on the cell cycle causes a delay and excessive death of cells with unstable aberrations result in abnormal morphogenesis (11).

Neonatal chickens treated with 4 mg of CY intra-abdominally per day for the first 4 days after hatching induced permanent depression of the humoral response and temporary profound suppression of the T cell system (14). It also has reported that cyclophosphamide, when give in high doses to chicken in newly hatched period, depresses development of the humoral immune response (7, 8). The survival of the neonatal chickens treated with CY was inversely dose-dependent (7). In our study, the chickens with daily injections of cyclophosphamide 4 mg for 4 days after hatching had about 65% mortality. The majority of deaths in CY treated groups occurred within 1 week following treatment. Also the size of the CY treated chickens was significantly smaller than the PBS treated.

Cyclophosphamide can irreversibly destroy both the bursal lymphocytes and the capacity of the bursa to differentiate the system subserving the humoral immunological response (8). This form of chemical bursectomy destroys not only the bursal lymphocytes, but also the already peripheralized bursa derived cells. The localization of lymphocytes in the lymphoid organs of CY treated chickens showed severe depletion of B-lymphocytes in the bursa of Fabricius, thymus, spleen and cecal tonsils. In our
experiment, relative bursal weight was significantly lower in CY treated chickens than that of the PBS treated. Also there was destruction of bursal cell populations while changes in thymic morphology were minimal. In peripheral lymphoid organs the most consistent lesion was the lack of germinal centers with severe depletion of lymphocytes. Those changes were persistent throughout the experiment.

Feathering defects have been reported in chicken infected with reticuloendotheliosis virus (15) and subgroup J avian leukosis virus infection (6). Ratnamohan (12) reported feather lesions in neonatal chickens treated with 4 mg of cyclophosphamide for 3 consecutive days. The lesions developed 4 days after treatment and disappeared when the chickens were 4 weeks old (12). In our experiment, similar skin lesions were observed and persisted until 3 weeks of age. In addition, we found mild to moderate lesions in the kidney, liver, spleen and bone marrow. These lesions were transient and completely repaired by two weeks of age. Those changes were first reported in this study.

In summary, the persistent toxic effects of cyclophosphamide treatment in neonatal chickens were decreased body weight, bursal atrophy, and late feathering. Transient lesions were produced in the kidney, liver, spleen and bone marrow, but these regressed by 2 weeks after cessation of CY administration.

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Fig 4.1. Body weight gain of broiler chickens treated with 4 mg of cyclophosphamide at first 4 days after hatching. Body weight gain of CY-treated were significantly decreased, approximately 40% to 50% of those of PBS-treated.

* Significantly different (p <0.05)
**Fig 4.2.** Relative bursal weight of broiler chickens treated with 4 mg of cyclophosphamide at first 4 days after hatching. Relative bursal weights were significantly decreased in CY treated chickens compared to those of PBS-treated.

Relative bursal weight = \[ \frac{\text{bursal weight}}{\text{body weight}} \] X 1,000

* Significantly different (p<0.05)
Legends for Microscopic pictures (Fig. 4.3 – 4.8)

Fig. 4.3. Bursa of Fabricius (H&E) from a 3 week-old broiler chicken treated with 0.1 ml of PBS for first 4 days after hatching. Normal architectures of bursas are retained.

Fig. 4.4. Bursa of Fabricius (H&E) from a 3 week-old broiler chicken treated with 4 mg of cyclophosphamide for first 4 days after hatching. Severe lymphoid depletion and atrophy of the lymphoid follicles and proliferation of interfollicular connective tissue are observed.

Fig. 4.5. Liver (H&E) from a 1 week-old broiler chicken treated with 4 mg of cyclophosphamide for first 4 days after hatching. Diffuse vacuolar degeneration of hepatocytes with occasional individual cell necrosis is present.

Fig. 4.6. Kidney (H&E) from a 1 week-old broiler chicken treated with 4 mg of cyclophosphamide for first 4 days after hatching. Segmental necrosis of tubular epithelial cells is present and occasional dilated tubules contain eosinophilic homogenous material with necrotic cell detritus within the lumens.

Fig. 4.7. Spleen (H&E) from a 1 week-old broiler chicken treated with 0.1 ml of PBS for first 4 days after hatching. Normal architecture of the spleen is retained.

Fig. 4.8. Spleen (H&E) from a 1 week-old broiler chicken treated with 4 mg of cyclophosphamide for first 4 days after hatching. Severe depletion of lymphocytes and numerous necrotic cells are observed.
CHAPTER 5

THE EFFECTS OF CYCLOPHOSPHAMIDE TREATMENT ON THE PATHOGENESIS OF SUBGROUP J AVIAN LEUKOSIS VIRUS (ALV-J) INFECTION IN BROILER CHICKENS WITH MAREK’S DISEASE VIRUS EXPOSURE

1 Kim, Y., M. J. Pantin and T. P. Brown. To be submitted to Avian Pathology
ABSTRACT

Studies were performed to determine the effects of B-cell suppression on the pathogenesis of Subgroup J avian leukosis virus (ALV-J) in broiler chickens. Neonatal chickens were treated with cyclophosphamide (CY) or PBS, and then infected with ALV-J (ADOL-7501) at 2 weeks of age. CY treatment induced B cell specific immunosuppression throughout the experiment confirmed by decreased bursal weight, intact lymphocyte mitogenetic activity stimulated by Con A and increased relative subpopulation of CD3-positive cells as measured by flowcytometry.

Chickens in this experiment had Marek’s disease virus exposure prior to three weeks of age as determined by the presence of lymphocytic infiltration and antibody. Virus neutralizing antibody against ALV-J was first observed at 6 weeks post-infection in some of the infected chickens in the PBS group. As expected, none of the chickens from the CY group and uninfected chickens developed virus neutralizing antibody. The viremic status was measured by real time RT-PCR using SYBR green I dye. The percentage of viremic chickens was significantly higher, and more chickens had high titered viremia, in the CY treated group. No neoplastic foci consistent with ALV-J infection were observed in any of the experimental chickens. The frequency and intensity of viral antigen expression determined by immunohistochemistry was significantly higher in tissues from CY treated birds than those of PBS treated chickens at 3 weeks post-infection.

This study showed that B cell specific immunosuppression with CY treatment in chickens resulted in increase in viremia and viral antigen load in tissues.
INTRODUCTION

Cyclophosphamide (CY) is an antineoplastic and immunomodulating agent used to treat tumors and autoimmune disorders. Newly hatched chickens treated with CY are rendered irreversibly B cell deficient (Lerman & Weidanz, 1970; Linna et al., 1972). Cyclophosphamide treatments have been used to inhibit humoral immunity in order to determine its role in the pathogenesis of infectious pathogens of chickens (Arnold & Holt, 1995; Reynolds & Maraqa, 2000).

In 1988, an exogenous avian leukosis virus (ALV) belonging to a new subgroup for chickens was isolated from meat-type chicken lines and designated as subgroup J (Payne et al., 1991). Subgroup J ALV (ALV-J) induces tumors and decreased weight gain in experimentally or naturally infected chickens (Payne et al., 1997; Payne, 1998a; Stedman & Brown, 1999). Like all other exogenous ALVs, transmission of ALV-J occurs either by vertical or horizontal infection. In vertical transmission, chicks become immunologically tolerant to the virus and are persistently viremic. Those chickens will remain viremic, will shed virus, and are more likely to develop tumors. While horizontal infection with other ALV subgroups often leads to immune non-shedders, the consequences of similar infections with ALV-J can vary between egg-type and meat-type birds. Infection of egg-type birds post-hatch leads to immune non-shedders. However, similar infection of meat-type birds can result in either tolerant viremic infections or transient viremia (Payne et al., 1992; Payne et al., 1993). Since horizontal transmission of the ALV-J is more significant, eradication programs for this subgroup have to be applied more intensely (Payne, 1998b).
Mortality in flocks with ALV-J varies widely, suggesting involvement of additional factors such as immunosuppressive agents, concurrent infections, vaccination against other diseases and husbandry practices in the manifestation of the disease. This study was performed to determine the effects of suppression of humoral immunity on the pathogenesis of ALV-J infection in the broiler chickens.

MATERIALS AND METHODS

**Chickens.** White Plymouth Rock eggs (SEPRL, USDA, Athens, GA, USA) were obtained from a flock that was free of avian leukosis viruses and other common poultry diseases. Chickens were hatched and reared on wire-floored isolation units until 2 weeks of age, then transferred to plastic isolation units. Feed and water were provided *ad libitum*.

**Virus.** ADOL-7501 isolate of ALV-J (ADOL, East Lansing, MI) was cloned by three limiting dilutions in secondary line 0 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 with cell culture medium and 0.1 ml containing 10\(^{4.5}\) TCID\(_{50}\) was inoculated into chickens intraperitoneally. A virus neutralization (VN) test was carried out on secondary line 0 chicken embryo fibroblast (CEF) cultures as a microneutralization assay using 100 TCID\(_{50}\)/well (Fadly & Witter, 1998).

**Experimental design.** Chicks (n=140) were hatched from fertilized eggs (n=170). The hatched chicks were divided into a PBS treated group (n=45 chicks) and a CY treated group (n=95 chicks). The latter received one intraperitoneal injection of 4 mg CY (Cyclophosphamide monohydrate; Sigma Chemical Co., St. Louis, MO) daily for 4 days.
from the first day after hatch. For injection, CY was obtained in a dry form, and an aqueous solution was prepared by reconstituting 1.6 g in 40 ml of calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and filtering this through a 0.22-µm syringe filter. The resulting solution contained 40 mg of CY/ml. The PBS group received one intraperitoneal injection of 0.1 ml sterile CMF-PBS daily for 4 days from the first day after hatch. At 2 weeks of age, 38 chickens from each of the PBS and CY treated group were randomly selected. Groups were then subdivided into the following treatments: PBS without ALV-J (n=18), PBS + ALV-J (n=20), CY without ALV-J (n=18), CY + ALV-J (n=20). At 2 weeks of age, chickens were infected with an ALV-J isolate, ADOL-7501.

At 3 days, 1, 3, 6 and 9 weeks post-infection, all chickens were bled to test their viremia and antibody status of ALV-J. At 3 days, 1, 3, 6, and 9 weeks post-infection, three to four chickens from each of the four groups were killed by cervical dislocation and sampled for lymphocyte blastogenesis assay, flow cytometry, and histopathology. Body weights and relative bursal weights were also measured at this time using the formula [Relative bursal weight = (bursal weight / body weight) X 1000].

**Isolation of splenocytes and mitogenesis assay.** Approximately half of the spleen was harvested from chickens from each group at necropsy. Spleens were collected individually in Hank’s balanced salt solution (HBSS, Sigma, St. Louis, MO) and prepared as described previously with minor modifications (Bounous et al., 1995). Briefly, spleens were homogenized using a Tissue Tearor (Biospec Products Inc., Racine, WI) and splenocytes were resuspended in HBSS-CMF with 1% fetal bovine serum (FBS). Splenocytes were centrifuged over 3 ml Histopaque 1077 (Sigma, St. Louis, MO) for 30 minutes at 400 g. The recovered mononuclear cell fraction was washed and resuspended as described previously at 2.67 X 10⁷ cells per ml (Coulter Counter® Model
D2N automated cell counter, Coulter Corp., Hialeah, FL) (Rup et al., 1982). For each chicken, 2 X 10^6 cells, Con A (Sigma, St. Louis, MO) at 10 µg/ml, and tritiated thymidine (NEN Life Science Products, Boston, MA) at 5 µCi/ml were added to a 96 well round bottom plate and incubated for 72 hours at 41°C as described previously (Bounous et al., 1995). For cell control wells, the cell media (RPMI 1640, Life technologies, Grand island, NY) was added instead of Con A. Test and control wells were run in triplicate for each chicken. Cells were harvested using a Skatron 11019 cell harvester (Skatron AS, Tranby, Norway) and radioactivity measured using a Beckman LS3801 liquid scintillation counter (Beckman Instruments, Irvine, CA) (Rup et. al., 1982). The radioactivity of the cells harvested onto filtermats was assayed on a scintillation counter (Beckman, USA) and recorded as counts per minutes (cpm). Stimulation index (SI) of each samples were calculated as follows: SI = [(cpm of stimulated)-(cpm of unstimulated)] / (cpm of unstimulated)]

**Flowcytometry.** Splenocytes prepared as described above were suspended to a concentration of 1 X 10^7 cells/ml. Cells (1 X 10^6) were incubated with a mouse monoclonal antibody, chicken CD3-FITC (Southern Biotech, Birmingham, AL), for 1 hour at 4°C. Isotype controls (nonspecific mouse IgG labeled with FITC, Southern Biotech, Birmingham, AL) were used in each labeling series to identify the region of the histogram containing cells positive for surface antigen. After washing twice with 2 ml HBSS 1% FBS, relative immunofluorescence of cells was analyzed using a flow cytometer (EPICS Coulter Flowcytometer, Florida, USA). Analytical gates were chosen based on forward and side scatter to include lymphocytes and to exclude debris, dead cells, and red cells.

**RNA extraction.** Total RNAs were extracted from 250 µl of each of plasma samples collected at 0.3, 1, 3, 6 and 9 weeks post-infection using a commercial reagent and
according to manufacturer’s recommendations (Tri Reagent BD, Molecular Research Center Inc. Cincinnati, OH). Each RNA sample was resuspended in 20 µl of diethyl pyrocarbonate (DEPC) treated water and stored at –80 C until used.

**Real time RT-PCR.** RT-PCR was performed using reagents from the Light Cycler-RNA Amplification SYBR Green® I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used have been described (Smith *et al.*, 1998) and produced an amplicon of approximately 545 bp. Amplification and detection of specific products was undertaken by a Light Cycler system (ROCHE Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s recommendations (ROCHE Light Cycler version 3.0, ROCHE Molecular Biochemicals, Indianapolis, IN). Briefly, reverse transcription was done at 55 C for 10 minutes and denaturation was done at 95 C for 30 seconds. Forty PCR cycles were done with denaturation at 95C, hybridization at 55 C for 10 seconds, and extension at 72 C for 13 seconds. The melting curve analysis was done with an initial denaturation at 95 C. DNA melting was accomplished with an initial temperature of 65 C for 10 seconds and a gradual temperature increase with a transition rate of 0.1 per seconds until reaching 95 C. The melting temperature of the expected 545 bp amplicon was estimated to be 83 C to 85 C, as determined using cell lysates infected with an ALV-J isolate and control RNA. This estimated melting temperature was used to confirm the identity of the products obtained using real time RT-PCR (ROCHE Molecular Biochemicals, Indianapolis, IN).

**Quantitation of viral RNA.** To quantitate the viral RNA in plasma, we used ten-fold serial dilution of control RNA produced by *in vitro* transcription as standard (Kim *et al.*, 2002). We performed real time RT-PCR with RNAs from cell lysates with different
TCID_{50}s to determine correlation between control RNA and TCID_{50}s. We divided the results from real time RT-PCR into three categories: low (V<0.1 pg), medium (0.1 < V< 10 pg) and high (V > 10 pg).

**Serology.** At the end of the experiment, serum samples collected during the experimental period were tested for antibody against poultry pathogens including Marek’s disease virus (MDV), *Mycoplasma spp.*, avian influenza virus, chicken anemia virus, infectious bursal disease virus, infectious bronchitis virus, New castle disease virus and reovirus by routine diagnostic tests such as HI, HA, ELISA. Neutralizing antibody against ALV-J was determined using a microneutralization test (Fadly & Witter, 1998).

**Histopathology.** At necropsy, heart, proventriculus, kidney, liver, lung, spleen, bursa, thymus, bone marrow, peripheral nerve, brain, pancreas, duodenum, large intestine and skeletal muscle from each chicken were collected and fixed by immersion in 10% neutral buffered formalin for less than 36 hours and embedded in paraffin for sectioning. Tissue sections were stained with H&E and examined microscopically.

**Immunohistochemistry (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. 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). After IHC staining, sections were counter-stained with hematoxylin, air dried, cover slipped, and examined using light microscopy. Staining intensity and extent were converted to scores as previously described (Arshad et al., 1997): 0 = negative; 1 = few positive cells; 2 = many positive cells.

Statistical analysis. The body weight gain, relative bursal weight and data from mitogenesis assay and flow cytometry were analyzed using two-tailed Student t-test with assumption of different variance. Significance of differences in percentage of viremia, antibody and the results of histopathology was determined by Chi-square analysis, and mean tissue scores from immunohistochemistry were analyzed using Kruskal-Wallis analysis of variance. Significance was assumed at the 0.05 level of probability.

RESULTS

Body weight, relative bursal weight and lymphocyte mitogenesis assay. The results of body weight, relative bursal weight and lymphocyte mitogenesis assay are summarized in Table 5.1. Body weights of the chickens treated with CY were significantly lower than those that were PBS treated. However, no significant difference was induced by the virus infection within the same treatment group.

Relative bursal weights of CY treated chickens were significantly lower than those that were PBS treated throughout the experiment. No significant difference was observed between infected and uninfected within the same treatment group.
Con A stimulated lymphocyte proliferation throughout the experiment in all of the groups. As shown in Table 5.1, no difference of the stimulation index was noticed between any of the groups.

**Flowcytometry.** Relative subpopulation of CD3-positive cells in CY-treated and PBS-treated groups were shown in Fig 5.1. Relative population CD3-positive cells out of gated lymphocyte population was significantly higher in CY treated group than that of the PBS treated group. However, no significant difference was noticed between infected and non-infected chickens (Data not shown).

**Serology.** Two out of 7 sera submitted were positive for antibody against Marek’s disease virus (MDV) by agar gel immunodiffusion test (California Animal Health Food Safety Laboratory System, University of California, Davis). No evidence of other poultry pathogens was detected.

**Viremia.** Presence of virus within the plasma samples was successfully detected by real time RT-PCR using SYBR green dye. Positive samples were determined by melting curve analysis, and presence of a peak between 83C and 85C. Based on the results of real time RT-PCR using cell culture lysates with a known TCID$_{50}$ (Fig 5.2), we categorized the virus titer as high (10 pg $> V$, corresponding to $> 10^5$ TCID$_{50}$), medium ($0.1 < V < 10$ pg, corresponding to $10^3$ to $10^5$ TCID$_{50}$) and low ($V < 0.1$ pg, corresponding to $< 10^3$ TCID$_{50}$). The results of the real time RT-PCR are summarized in Table 5.2. Positive samples for viremia were detected only in infected groups. The overall percentage of positive samples was significantly higher in the CY group than in the PBS group ($p<0.001$). Early in the experiment, virus titer was similar in both PBS treated and CY
treated groups. However, more individual chickens had a medium to high titer of virus in CY treated group compared to the PBS treated group.

**Virus neutralizing antibody.** The results of virus neutralization test were summarized in Table 5.3. Presence of neutralizing antibody was first observed at 6 weeks post-infection in the PBS treated group. More than half of the samples tested had neutralizing antibody at the end of the experiment. As expected, neutralizing antibody was not present in any of the serum from CY treated and uninfected groups.

**Histopathology.** All of the tissue samples collected from necropsy were examined microscopically. The bursas from the chickens treated with CY had markedly decreased numbers of lymphoid follicles separated by increased interfollicular connective tissue (Fig. 5.3). The results of the histopathology are summarized in Table 5.4. Nodular to diffuse infiltrations of lymphoid cells were present in variable organs including liver, heart, lung, kidney, bone marrow (Fig 5.4), spleen, proventriculus, ventriculus (Fig. 5.5), small and large intestines, and pancreas. In most of chickens, lymphocytic infiltrations were present in multiple organs.

Minimal to mild foci of myeloid cell infiltrates were present in the lung (Fig. 5.6), heart (Fig. 5.7), liver (Fig. 5.8), and kidney from some chickens. Most of these were present in one organ per bird. No morphologically distinctive neoplastic cells or changes pathognomonic for ALV infection were present in any tissues examined.

**Immunohistochemistry.** The tissue distribution of viral antigen is summarized in Table 5.5. Tissue staining for ALV-J was significantly higher in the CY infected group than in PBS infected group at 3 weeks. The CY infected group had an overall mean tissue score greater than that of the PBS infected group at 3 weeks and 9 weeks. In the PBS
treated group, tissue expression was higher at 9 weeks than at 3 weeks post-infection. Greatest antigen staining (mean scores per tissue > 1.0) was present in the kidney (Fig. 5.9), ventriculus and proventriculus (Fig. 5.10). Many other tissues were variably positive. In addition to staining in these specific tissues, viral antigen was also widely stained in smooth muscle cells and connective tissues of many tissues (Fig. 5.11).

**DISCUSSION**

CY treatment has been used as a specific suppressor of B-cell dependent humoral immunity. However, T-cells may also be killed or slowed in proliferation for less than 2 weeks by single or multiple, high dose CY treatments (Glick, 1971; Linna, Frommel & Good, 1972; Sharma & Lee, 1977). In our current study, the immune status of chickens was confirmed by relative bursal weight, flowcytometry, and lymphocyte blastogenesis assay. Bursas from chickens treated with CY were significantly smaller than those sham treated with PBS. Histologically, bursal follicles were also smaller and depletion of lymphocytes was prominent after CY treatment. By flow cytometric analysis, the relative population of CD3-positive lymphocytes was higher in CY treated birds, indicating a decrease in CD3-negative lymphocytes, presumably the B-cell population. There was no significant difference in blastogenetic activity of the splenocytes stimulated by Con A, indicating intact T cell activity in CY treated birds.

In our experiment, most of the chickens had Marek’s disease virus (MDV) infection before two weeks of age, as indicated by the presence of lymphocytic infiltrations in multiple organs and the presence of antibody. In addition to the results of mitogenesis assay and flow cytometry, the minimal histologic changes within the bursa of Fabricius
and thymus in PBS-treated chickens suggested that the immunosuppression caused by MDV infection was not significant. However, the degree of immunosuppression caused by MDV infection is variable with different isolates (Lee et al., 1978; Liu & Lee, 1983; Calnek et al., 1998). Calnek et al. (1998) investigated immunosuppressive effect of vMDV, VVMDV and vv+MDV and the results indicated that the degree of immunosuppression is linked to the virulence and that a simple measure of atrophic changes in the bursa of Fabricius and thymus might be useful in determining the pathotype classification of new MDV isolates.

Enhancement of ALV pathogenesis by serotype 2 Marek’s disease virus (MDV) has been reported (Campbell & Frankel, 1979; Fadly & Ewert, 1994; Witter, 1995). Coinfection with ALV-J and vvMDV was conducive to an increased expression of lymphomas, myelocytomas, and lymphocytic infiltrative peripheral neuritis (Zavala et al., 2000). In chickens with dual infection of MDV and ALV-J, ALV-J viremia progressed more rapidly and was more persistent than when chickens were vaccinated against MDV (Zavala, 2001). In our experiment, we could not determine whether the MDV infection enhanced pathogenicity of ALV-J or not. However, the effects of the MDV infection might be similar in both treatment group because most of the chickens had MDV infection in our experiment.

Congenital or neonatal infection of ALV-J can significantly decrease body weight gain (Stedman & Brown, 1999). In our experiment, no significant difference in body weight gain was induced by ALV-J infection. This suggests ALV-J induced body weight suppression may be present with congenital infection but not with infection at 2 weeks of age. Birds exposed to the virus at a very young age more frequently develop tolerant
viremia. This may be due to the constitutive embryonic expression of EAV-HP env sequences and induction of tolerance to those sequences (Benson et al., 1998; Smith et al., 1999; Sacco & Venugopal, 2001).

We measured viremia in chickens using real time RT-PCR with SYBR green I dye. SYBR green I dye binds to any double-stranded DNA which is generated during a PCR reaction. Therefore this system will not differentiate primer dimers from an expected PCR product. To correct this problem, we used a melting curve analysis. PCR products from standard RNA and cell lysates infected with ALV-J, ADOL-7501 isolates, exhibited a melting peak between 83 C and 85 C. We determined a positive and negative based on the presence of a melting peak within this range. As expected, the amplification plot was also affected by presence of primer dimers in the PCR reaction. However, in our experiment this effect was minimal even in negative samples (Data not shown). In addition, we successfully quantified viral RNA in plasma using control RNA as a standard. The result of real time RTPCR strongly correlated with the TCID$_{50}$s of cell lysates.

Chemically or virus-induced immunosuppression lead to an increase in rates of viremia and shedding of subgroup A ALV in chickens infected with virus after hatching (Fadly et al., 1985). Cloacal shedding, viremia, and tumor development were significantly lower in chickens with maternal antibody following exposure to subgroup A ALV at hatching (Fadly, 1988; Fadly et al., 1989). However, induced moulting or raised circulating corticosterone in adult hens did not influence of ALV infection or shedding. Similarly, actively acquired antibody induced by inoculation of infectious ALV at 8 weeks of age prevented shedding and congenital transmission to the subsequent
In our study, CY-treated chickens exhibited a significantly higher rate of viremia compared to that of PBS treated birds. The CY treated group had more chickens with high titered viremia late in the experiment compared to that of the PBS treated group. However, there was little correlation between viremia and antibody status in our study.

As expected, neutralizing antibody was not observed in any of the chickens in the CY treated group, while more than 60% of the chickens had neutralizing antibody at the end of the experiment in PBS treated group. The result also indicates that CY treatment induced complete ablation of humoral immunity in our experiment.

Microscopically, there were no evident neoplastic foci consistent with ALV-J infection in our experiment. Tissue myeloid cell infiltrates were present in both infected and uninfected chickens early in the experiment. This suggests these infiltrates were extramedullary hematopoietic foci rather than an effect of ALV-J. Distribution of viral antigen was investigated by immunohistochemical staining using monoclonal antibody against envelope glycoprotein. The distribution of the viral antigen was consistent with previous reports (Arshad et al., 1997; Gharaibeh et al., 2001). At 3 weeks post-infection, the frequency and intensity of the staining was significantly higher in the CY treated group than in the PBS treated group.

In our experiment CY treatment increased the rate of viremia, titer of the virus, and viral antigen expression and induced no significant effect on body weight gain and tumor formation. Those results indicate that B-cell suppression caused by CY treatment only affected virus replication but did not change the clinical effects of ALV-J on chickens infected at 2 weeks of age.
REFERENCES


Table 5.1. Summary of body weight gain, relative bursal weight and lymphocyte mitogenesis assay (mean ± standard deviation).

<table>
<thead>
<tr>
<th>WPI</th>
<th>Group</th>
<th>Body weight</th>
<th>Bursal weight*</th>
<th>Stimulation index**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>PBS</td>
<td>189 ± 16.6a</td>
<td>0.34 ± 0.02a</td>
<td>69.8 ± 22.2a</td>
</tr>
<tr>
<td></td>
<td>PBS/J</td>
<td>186 ± 22.3a</td>
<td>0.29 ± 0.05a</td>
<td>78.6 ± 29.4a</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>106 ± 24.4b</td>
<td>0.11 ± 0.04b</td>
<td>65.9 ± 24.0a</td>
</tr>
<tr>
<td></td>
<td>CY/J</td>
<td>101 ± 16.9b</td>
<td>0.10 ± 0.04b</td>
<td>76.0 ± 21.5a</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>283 ± 34.7a</td>
<td>0.43 ± 0.03a</td>
<td>16.1 ± 4.9a</td>
</tr>
<tr>
<td></td>
<td>PBS/J</td>
<td>280 ± 26.5a</td>
<td>0.45 ± 0.04a</td>
<td>18.2 ± 7.6a</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>174 ± 33.6b</td>
<td>0.12 ± 0.03b</td>
<td>16.4 ± 5.9a</td>
</tr>
<tr>
<td></td>
<td>CY/J</td>
<td>166 ± 26.9b</td>
<td>0.18 ± 0.14b</td>
<td>11.8 ± 7.1a</td>
</tr>
<tr>
<td>3</td>
<td>PBS</td>
<td>612 ± 76.9a</td>
<td>0.36 ± 0.02a</td>
<td>28.3 ± 9.5ab</td>
</tr>
<tr>
<td></td>
<td>PBS/J</td>
<td>540 ± 50.6a</td>
<td>0.37 ± 0.08a</td>
<td>18.3 ± 6.4c</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>442 ± 52.0b</td>
<td>0.06 ± 0.01b</td>
<td>37.8 ± 13.8b</td>
</tr>
<tr>
<td></td>
<td>CY/J</td>
<td>376 ± 96.6b</td>
<td>0.06 ± 0.04b</td>
<td>22.9 ± 5.6ac</td>
</tr>
<tr>
<td>6</td>
<td>PBS</td>
<td>1102 ± 131.2a</td>
<td>0.28 ± 0.04a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS/J</td>
<td>982 ± 122.4ab</td>
<td>0.34 ± 0.14a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>848 ± 109.2bc</td>
<td>0.04 ± 0.01b</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CY/J</td>
<td>697 ± 187.6c</td>
<td>0.05 ± 0.02b</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>PBS</td>
<td>1669 ± 203.7a</td>
<td>0.06 ± 0.02a</td>
<td>22.5 ± 18.3a</td>
</tr>
<tr>
<td></td>
<td>PBS/J</td>
<td>1480 ± 230.3ab</td>
<td>0.08 ± 0.04a</td>
<td>49.3 ± 32.8a</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>1201 ± 197.5bc</td>
<td>0.03 ± 0.02a</td>
<td>33.3 ± 12.7a</td>
</tr>
<tr>
<td></td>
<td>CY/J</td>
<td>1186 ± 165.9c</td>
<td>0.04 ± 0.02a</td>
<td>27.8 ± 16.1a</td>
</tr>
</tbody>
</table>

1: Weeks post-infection

a, b, c: Values within a block followed by different letters are significantly different (p <0.05).

* Bursal weight: relative bursal weight (bursal weight / body weight) X 1000

** Stimulation index = [{(cpm of stimulated)-(cpm of unstimulated)} / (cpm of unstimulated) ]

ND: not done
Table 5.2. ALV-J viremic status measured by Real time RT-PCR

<table>
<thead>
<tr>
<th>Group</th>
<th>WPI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Low&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Medium&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>High&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>7/14 (50)</td>
<td>3/13 (23)</td>
<td>4/10 (40)</td>
<td>4/8 (50)</td>
<td>4/6 (67)</td>
</tr>
<tr>
<td>PBS/J</td>
<td></td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Low&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medium&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>High&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>10/15 (67)</td>
<td>6/10 (60)</td>
<td>10/11 (91)</td>
<td>5/5 (100)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>CY&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Low&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medium&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
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<tr>
<td>High&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>10/15 (67)</td>
<td>6/10 (60)</td>
<td>10/11 (91)</td>
<td>5/5 (100)</td>
<td>2/2 (100)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Weeks post-infection

<sup>2</sup> Number of positive / Number of tested ( percentage), determined by real time RT-PCR using H5/H7 primers

<sup>3</sup> Number of samples. ALV-J Virus titers in plasma measured by real time RT-PCR using H5/H7 primers was divided into low, medium and high.
Table 5.3. ALV-J virus neutralizing antibody tested by microneutralization test

<table>
<thead>
<tr>
<th>WPI&lt;sup&gt;1&lt;/sup&gt; Group</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>ND</td>
<td>ND</td>
<td>0/11</td>
<td>0/7</td>
<td>0/3</td>
</tr>
<tr>
<td>PBS/J</td>
<td>ND</td>
<td>ND</td>
<td>0/11</td>
<td>3/8 (4-16)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4/6 (4-1024)</td>
</tr>
<tr>
<td>CY</td>
<td>ND</td>
<td>ND</td>
<td>0/9</td>
<td>0/6</td>
<td>0/3</td>
</tr>
<tr>
<td>CY/J</td>
<td>ND</td>
<td>ND</td>
<td>0/7</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

<sup>1</sup> Weeks post-infection

<sup>2</sup> Number of positive / Number of tested (Range of virus neutralizing titer)

ND: not done
Table 5.4. Summary of histopathologic findings

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphocytic infiltration</th>
<th>Myeloid cell infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>PBS</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>PBS/J</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>CY</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>CY/J</td>
<td>1/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>

1 Weeks post-infection

2 Number of chickens with infiltration / Number of chickens examined.
Table 5.5. Viral antigen expression* at 1, 3 and 9 weeks post-infection in tissues infected with ALV-J (ADOL-7501) as 2 weeks of age.

<table>
<thead>
<tr>
<th>Tissue**</th>
<th>Weeks post-infection</th>
<th>1 weeks</th>
<th>3 weeks</th>
<th>9 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/2 (0)</td>
<td></td>
</tr>
<tr>
<td>Bursa</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 1/2 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/2 (0)</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0/3 (0) 0/3 (0)</td>
<td>1/3 (0.7) 3/3 (2)</td>
<td>2/3 (1) 2/2 (2)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/2 (0)</td>
<td></td>
</tr>
<tr>
<td>Marrow</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/2 (0)</td>
<td></td>
</tr>
<tr>
<td>Nerve</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/2 (0)</td>
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</tr>
<tr>
<td>Pancreas</td>
<td>0/3 (0) 0/3 (0)</td>
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<td>1/3 (0.3) 1/2 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Proventriculus</td>
<td>0/3 (0) 0/3 (0)</td>
<td>1/3 (0.3) 3/3 (2)</td>
<td>1/3 (0.7) 1/2 (1.5)</td>
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</tr>
<tr>
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<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 1/2 (1)</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 0/3 (0)</td>
<td>0/3 (0) 1/2 (1)</td>
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</tr>
<tr>
<td>Ventriculus</td>
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<td>1/3 (0.7) 1/2 (1)</td>
<td></td>
</tr>
<tr>
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<td>0/3 (0) 0/3 (0)</td>
<td>1/3 (0.3) 1/2 (0.5)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of birds positive / total number of birds examined (mean score for each tissue: 0 = negative; 1 = few positive cells; 2 = many positive cells).

** Tissue-specific cells evaluated
Fig. 5.1. Flowcytometric analysis of splenic lymphocytes. Relative population of CD3-positive lymphocytes from spleen of cyclophosphamide treated chickens was significantly higher than that of PBS treated. No significant differences between infected and uninfected chickens within same treatment were observed (Data not shown).
Fig 5.2. Quantitative real time RT-PCR by H5/H7 primers and Light Cycler system using SYBR green I dye. Strong correlation ($R^2=0.993$) was observed between TCID$_{50}$s and amount of ALV-J RNA measured by real time RT-PCR.
Legends for microscopic pictures (Fig. 5.3 – 5.12).

Chickens were daily treated with PBS or 4mg of cyclophosphamide for 4 days from hatching. Some of the chickens from each treatment were infected with an avian leukosis virus subgroup J (ALV-J) isolate, ADOL-7501, at 2 weeks of age.

Fig 5.3. Bursa. H&E. A 3 week-old chicken from CY treated/ uninfected group. Markedly decreased number of follicles separated by increased interfollicular connective tissue. 100X

Fig. 5.4. Sternum. H&E. A 5 week-old chicken from PBS treated/ uninfected group. A discrete nodular infiltration of lymphocytes displacing bone marrow space. 100X

Fig. 5.5. Ventriculus. H&E. A 5 week-old chicken from PBS treated/ infected group. Multiple nodular infiltrations of lymphocytes within the serosa. 100X

Fig. 5.6. Lung. H&E. An 8 week-old chicken from PBS treated/ uninfected group. Small aggregates of myeloid cells within the interstitium. 400X

Fig. 5.7. Heart. H&E. An 11 week-old chicken from CY treated/ infected group. Minimal aggregates of myeloid cells within the interstitium of the myocardium. 400X

Fig. 5.8. Liver. H&E. An 8 week-old chicken from CY treated/ infected group. Minimal aggregates of myeloid cells within the periportal area. 400X

Fig. 5.9. Kidney. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from PBS treated/ infected group. Expression of the viral antigen was detected within the lumenal surfaces of the renal tubular epithelial cells. 200X
Fig. 5.10. Proventriculus. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 5 week-old chicken from CY treated/infected group. Expression of the viral antigen was observed within the basaloid aspects of the mucosal lining epithelial cells. Scattered positive cells are present in the connective tissue. 400X

Fig. 5.11. Heart. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from CY treated/infected group. Expression of the viral antigen was observed within a few myocardial cells. 400X

Fig. 5.12. Bursa. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from CY treated/infected group. Expression of the viral antigen was observed within the surface lining epithelial cells, cells in the medullae of bursal follicles and connective tissue cells. 400X

Fig. 5.13. Small intestine. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 5 week-old chicken from PBS-treated/infected group. Viral expression was observed in the smooth muscle cells and connective tissue cells of the intestinal wall. 400X

Fig. 5.14. Liver. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from CY treated/infected group. Viral expression was observed in the lining cells of the sinusoids and Kupffer cells. 400X
CHAPTER 6
EFFECTS OF CYCLOSPORIN TREATMENT ON THE PATHOGENESIS OF SUBGROUP J AVIAN LEUKOSIS VIRUS (SUBGROUP J) INFECTION IN BROILER CHICKENS WITH MAREK’S DISEASE VIRUS EXPOSURE

1 Kim, Y., M. J. Pantin and T. P. Brown. To be submitted to Avian Pathology
ABSTRACT

In this study, we investigated the effects of T-cell suppression on the pathogenesis of subgroup J avian leukosis virus (ALV-J). Chickens were treated with cyclosporin A (CSP) 50 mg/Kg body weight or a corresponding volume of olive oil per every three days after hatching until the end of experiment. Some of the chickens from each treatment group were infected with an isolate of ALV-J, ADOL-7501, at 2 weeks of age. The effects of viral infection were compared to uninfected birds in same treatment group. Intramuscular injection of CSP induced significant T-cell specific immunosuppression determined by decreased cutaneous basophilic hypersensitivity response and decreased lymphocyte mitogenetic activity using concanavalin A.

Most of the chickens examined had Marek’s disease virus (MDV) infection prior to 3 weeks of age determined by the presence of multifocal lymphocytic infiltrations and antibody to MDV. Virus neutralizing antibody against ALV-J was first observed at 4 weeks post-infection. The percentage of antibody-positive birds and antibody titers were similar in infected chickens between both treatment groups. The percentage of chickens with viremia measured by real time RT-PCR using SYBR green I dye was higher in CSP treated group than that of the Oil treated group (p=0.05). Microscopically, one CSP treated chicken had a nephroblastoma at 10 weeks post infection. At 7 and 10 weeks post-infection, more chickens had infiltrations of myeloid cells in multiple organs including heart, liver and occasionally lung (p<0.01). Expression of ALV-J viral antigen determined by immunohistochemical staining using monoclonal antibody specific for viral envelope glycoprotein was higher at 10 weeks post-infection than that of the 4
weeks post-infection. And overall mean score of viral antigen staining was significantly higher in CSP treated chickens than Oil treated chickens at 10 weeks post-infection.

INTRODUCTION

Cyclosporin, a selective T-cell immunosuppressant drug, depresses cell-mediated immunity in chickens, causing prolonged skin graft survival, depressed proliferative responses in mitogen-stimulated lymphocytes and decreased wattle responses to injected antigen (Hill et al., 1989). Cyclosporin have been used as a means of inhibiting the cell-mediated immune response in order to determine the role of T-cells in protective responses to infectious pathogens of chickens (Lillehoj, 1987; Hill, Rowland, Latimer & Brown, 1989; Fitzgerald et al., 1996; Isobe et al., 2000).

The role of immune system in the pathogenesis of avian leukosis virus (ALV) infection has been studied. Chickens infected with ALV after hatching transmit virus at a much lower rate than congenitally-infected, immune tolerant chickens (Rubin et al., 1962; Weyl & Dougherty, 1977; Okazaki et al., 1980; Fadly & Okazaki, 1982; de Boer et al., 1981). Viremia, antibody development, cloacal and albumen shedding, and tumor incidences were significantly lower in chicks with maternal antibody following massive exposure by a strain of ALV subgroup A at hatching (Fadly, 1988). However, with certain strains of ALV, immunosuppression can increase the frequency of ALV shedding with a consequent increase in congenital transmission in chickens infected with the virus after hatching (Crittenden et al., 1982; Crittenden et al., 1984; Fadly et al., 1985; Crittenden et al., 1987; Fadly, 1987). The incidence of regression of wing-web tumors induced by Rous sarcoma virus was shown to be dependent on the quantity of thymus
tissue remaining after neonatal thymectomy in chickens of inbred line 6 (Cotter et al., 1975).

Subgroup J ALV (ALV-J) has caused significant economic loss in the broiler industry because of increased mortality, decreased weight gain, and an increased incidence of tumors in broilers (Payne et al., 1997; Stedman & Brown, 1999). ALV-J induces late-onset myeloid leukosis (Payne et al., 1992). Renal tumors and other sarcomas such as histiocytic sarcoma, hemangiosarcoma, mesothelioma, granulosa cell tumors, pancreatic adenocarcinoma, fibroma, and an unclassified leukemia are also observed (Payne et al., 1992; Arshad et al., 1997a; Hafner et al., 1998; Payne, 1998).

Eradication programs applied for ALVs are essentially based on the experience with lymphoid leukosis, where the virus is primarily transmitted vertically. In vertical transmission, ALV-J behaves like other exogenous ALVs and an established ALV eradication programs (Spencer, 1984) should be effective in eradicating an ALV-J infection (Witter et al., 2000). However, horizontal transmission of the ALV-J is more significant than for other subgroups of ALV, therefore a different eradication strategy is needed.

This study was performed to determine the effects of suppression of the cell-mediated immune system on ALV-J infection, as a part of the study determine the role of the immune system in the control of ALV-J infection in broiler chickens.

**MATERIALS AND METHODS**

**Chickens.** White Plymouth Rock eggs (SEPRL, USDA, Athens, GA, USA) were obtained from a flock that was free of avian leukosis viruses and other common poultry
diseases. Chickens were hatched and reared on wire-floored isolation units until 2 weeks of age, then transferred to plastic isolation units. Feed and water provided ad libitum.

**Virus.** ADOL-7501 isolate of ALV-J (ADOL, East Lansing, MI) was cloned by three limiting dilutions in secondary line 0 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 with cell culture medium and 0.1 ml containing 10\(^{4.5}\) TCID\(_{50}\) was inoculated into chickens intraperitoneally. A virus neutralization (VN) test was carried out on secondary line 0 chicken embryo fibroblast (CEF) cultures as a microneutralization assay using 100 TCID\(_{50}\) / well (Fadly & Witter, 1998).

**Experimental design.** Chicks (n=123) were hatched from fertilized eggs (n=150). The hatched chicks were divided into a Oil treated group (n=43 chicks) and a cyclosporin A (CSP) treated group (n=80 chicks). Chicks of CSP group were injected in alternating pectoral muscles with a 26-gauage needle every third day until the end of the experiment with 50mg Cyclosporin A (CSP) oral suspension (Sandimmune\textsuperscript{®} oral solution, Novartis Pharma AG, Basle, Switzerland) per kg body weight. The stock solution containing 100 mg of CSP was diluted with olive oil and the dilutions of the drug were adjusted as body weights increased. Birds in the Oil group were similarly injected with same volume of olive oil. At 2 weeks of age, 40 chickens from each of the PBS and CY treated group were randomly selected. Groups were then subdivided into the following treatments: Oil without ALV-J (n= 20), Oil + ALV-J (n= 20), CSP without ALV-J (n= 20), CSP + ALV-J (n= 20).

At 1, 2, 4, 7, and 10 weeks post-infection, all chickens were bled to test their viremia and antibody status of ALV-J. At 1, 2, 4, 7, and 10 weeks post-infection, three to seven
chickens from each of the four groups were killed by cervical dislocation and sampled for lymphocyte blastogenesis assay, flow cytometry, and histopathology as described below, and necropsied. Body weights and relative thymic weights were also measured at this time using the formula [Relative thymic weight = (thymic weight / body weight) X 1000].

**Isolation of splenocytes.** Approximately half of the spleen was harvested from chickens from each group at necropsy. Spleens were collected individually in Hank’s balanced salt solution (HBSS, Sigma, St Louis, MO) and prepared as described previously with minor modifications (Bounous et al., 1995). Briefly, spleens were homogenized using a Tissue Tearor (Biospec Products Inc., Racine, WI) and splenocytes were resuspended in HBSS-CMF (Sigma, St Louis, MO) with 1% fetal bovine serum (FBS). Splenocytes were centrifuged over 3 ml Histopaque 1077 (Sigma, St Louis, MO) for 30 minutes at 400 g. The recovered mononuclear cell fraction was washed and resuspended as described previously at 2.67 X 10^7 cells per ml using a Coulter Counter® Model D2N automated cell counter (Coulter Corp., Hialeah, FL) (Rup et al., 1982).

**Lymphocyte mitogenesis assay.** For each chicken, 2 X 10^6 cells, Con A (Sigma, St Louis, MO) at 10 µg/ml, and tritiated thymidine (NEN Life Science Products, Boston, MA) at 5 µCi/ml were added to a 96 well round bottom plate and incubated for 72 hours at 41°C as described previously (Bounous et al., 1995). For cell control wells, cell culture media (RPMI 1640, Life technologies, Grand island, New York) was added instead of Con A. Test and control wells were run in triplicate for each chicken. Cells were harvested using a Skatron 11019 cell harvester (Skatron AS, Tranby, Norway) and radioactivity measured using a Beckman LS3801 liquid scintillation counter (Beckman Instruments, Irvine, CA) (Rup et al., 1982). The radioactivity of the cells harvested onto filtermats was measured by a scintillation counter (Beckman, USA) and recorded as counts per minutes (cpm). Stimulation index (SI) of each samples were calculated as follows: SI = [{(cpm of stimulated)-(cpm of unstimulated)} / (cpm of unstimulated)]
Flow cytometry. Splenocytes prepared as described earlier were suspended to a concentration of $1 \times 10^7$ cells/ml. Cells ($1 \times 10^6$) were incubated with monoclonal antibodies, CD3-FITC (Southern Biotech, Birmingham, AL) or Ia-PE (Southern Biotech, Birmingham, AL), for 1 hour at 4°C. Isotype controls (nonspecific mouse IgG labeled with FITC or PE, Southern Biotech, Birmingham, AL) were used in each labeling series to identify the region of the histogram containing cells positive for surface antigen. After washing twice with 2 ml HBSS 1% FBS, relative immunofluorescence of cells was analyzed by flow cytometer (EPICS Coulter Flowcytometer, Florida, USA). Analytical gates were chosen based on forward and side scatter to include lymphocytes and to exclude debris, dead cells, and red cells.

Cutaneous basophil hypersensitivity (CBH) response. The test was performed to evaluate T-cell function in the CSP treated chickens at 2 weeks of age as described by Corrier and DeLoach (1990). Ten chickens were injected intradermally in the skin between 3rd and 4th digits of the left foot 200 ug of Phytoagglutinin-P (PHA-P, Sigma, St. Louis, MO) in 100 µl of sterile physiological saline solution (PSS). The right foot of each chicken was similarly injected with 100 µl of PSS to serve as a control. The CBH response to PHA-P was evaluated by determining the thickness of the interdigital skin before injection and at 12 and 24 hours after infection with a constant-tension, digital micrometer (Mitutoyo Co., Kanagawa, Japan). The CBH response was calculated by two methods: 1) CBH-1 or increased skin thickness = (post-injection skin thickness, left foot) – (pre-injection skin thickness, left foot); and 2) CBH-2 response = (PHA-P response, left foot) – (PSS response, right foot).
RNA extraction. Total RNAs were extracted from 250 µl of each of plasma samples collected at 1, 2, 4, 7 and 10 weeks post-infection using a commercial reagent and according to manufacturer’s recommendations (Tri Reagent BD, Molecular Research Center Inc. Cincinnati, OH). Each RNA sample was resuspended in 20 µl of diethyl pyrocarbonate (DEPC) treated water and stored at –80 C until used.

Real time RT-PCR. RT-PCR was performed using reagents from the Light Cycler-RNA Amplification SYBR Green® I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used have been described (Smith et al., 1998) and produced an amplicon of approximately 545 bp. Amplification and detection of specific products was undertaken by a Light Cycler (ROCHE Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s recommendations (ROCHE Light Cycler version 3.0, ROCHE Molecular Biochemicals, Indianapolis, IN). Briefly, reverse transcription was done at 55 C for 10 minutes and denaturation was done at 95 C for 30 seconds. Forty PCR cycles were done with denaturation at 95C, hybridization at 55 C for 10 seconds, and extension at 72 C for 13 seconds. The melting curve analysis was done with an initial denaturation at 95 C. DNA melting was accomplished with an initial temperature of 65 C for 10 seconds and a gradual temperature increase with a transition rate of 0.1 per seconds until reaching 95 C. The melting temperature of the expected 545 bp amplicon was estimated to be 83 C – 85 C, as proved using cell lysates infected with an ALV-J isolate and control RNA (data not shown). This estimated melting temperature was used to confirm the identity of the products obtained using real time RT-PCR (ROCHE Molecular Biochemicals, Indianapolis, IN).
Quantitation of viral RNA. To quantitate the viral RNA in plasma samples, we used ten-fold serially diluted control RNA produced by in vitro transcription as standard RNA (Kim et al., 2002). We performed Real time RT-PCR with RNA from cell lysates with different TCID$_{50}$s to determine correlation between real time RT-PCR and TCID$_{50}$s. We divided the results of real time RT-PCR into three categories: low ($V<0.1$ pg), medium ($0.1 < V< 10$ pg) and high ($V > 10$ pg).

Serology. At the end of the experiment, serum samples collected during the experimental period were tested for antibody against poultry pathogens including Marek’s disease virus (MDV), Mycoplasma spp., avian influenza virus, chicken anemia virus, infectious bursal disease virus, infectious bronchitis virus, New castle disease virus and reovirus by routine diagnostic tests such as HI, HA, ELISA. Neutralizing antibody against ALV-J was determined using a microneutralization test (Fadly & Witter, 1998).

Histopathology. At necropsy, samples of heart, proventriculus, kidney, liver, lung, spleen, bursa, thymus, bone marrow, peripheral nerve, brain, pancreas, duodenum, large intestine and skeletal muscle from each chicken were fixed by immersion in 10% neutral buffered formalin for less than 36 hours and embedded in paraffin for sectioning.

Immunohistochemistry (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. 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). After IHC staining, sections were counter-stained with hematoxylin, air dried, cover slipped, and examined using light microscopy. Staining was converted to scores as previously described (Arshad et al., 1997b): 0 = negative; 1 = few positive cells; 2 = many positive cells.

**Statistical analysis.** The body weight gain, relative thymic weight and data from mitogenesis assay and flow cytometry were analyzed using two-tailed Student t-test with assumption of different variance. Significance of differences in percentage of viremia, antibody and the results of histopathology was determined by Chi-square analysis, and mean tissue scores from immunohistochemistry were analyzed using Kruskal-Wallis analysis of variance. Significance was assumed at the 0.05 level of probability.

**RESULTS**

**Body weight gain, relative thymic weight and lymphocyte mitogenesis assay.**

The results of body weight gain, relative thymic weights and lymphocyte mitogenesis assays were summarized in Table 6.1. No significant differences in body weight gain and relative thymic weights were observed in any of the groups.

Stimulation index determined by Con A treatment on splenocytes was significantly higher in Oil group than that of CSP group throughout the experiment. However, no significant difference in stimulation index was induced by the ALV-J infection in either treatment group.
Flow cytometry. The results of the flow cytometric analysis are summarized in Table 6.2. There were no significant differences in relative subpopulation of CD3-, CD4-, CD8- and Ia- positive cells out of gated lymphocytes in any of the groups throughout the experiment.

CBH response. The effect of CSP treatment on the CBH response was evaluated in chickens at 2 weeks of age (Table 6.3). The CBH-1 response was significantly decreased (p < 0.001), from .69 ± .14 mm (mean ± SD), in the oil group to .29 ± .6 mm in the CSP group. Similarly, the CBH-2 response was significantly decreased (p < 0.001), from .65 ± .15 mm (mean ± SD) in the oil group to .21 ± .9 mm in the CSP group.

Serology. Three out of eight sera submitted were positive for antibody against Marek’s disease virus (MDV) by agar gel immunodiffusion test (California Animal Health Food Safety Laboratory System, University of California, Davis). No evidence of infection with other pathogens was detected in the chickens used in the experiment.

Viremia. Presence of virus was successfully detected in plasma from infected chickens by real time RT-PCR using SYBR Green I dye. As shown in Table 6.4, viremia was detected only in infected groups throughout the experiment. Early in the experiment, the ratio of positive samples to negative samples was similar but at the end of experiment the ratio was significantly higher in CSP group compared to that of Oil group (p < 0.01). Based on the results of real time RT-PCR using cell culture lysates with known TCID_{50} (unpublished data), we divided virus titer into high (10 pg > V, corresponding to > 10^{5} TCID_{50}), medium (0.1 < V < 10 pg, corresponding to 10^{3} to 10^{5} TCID_{50}) and low (V < 0.1 pg, corresponding to 10^{3} TCID_{50}). As shown in Table 6.4, the composition of the virus titer in the Oil group was similar to that of the CSP group early in the experiment.
However, more chickens had medium to high titered viremia in the CSP group compared to the PBS group.

**Virus neutralizing antibody.** The results of virus neutralization tests are summarized in Table 6.5. Neutralizing antibody was first detected at 4 weeks post-infection in the Oil group. More than half of the samples tested had neutralizing antibody at the end of the experiment. The percentage and titers of the neutralizing antibody positive samples in the Oil group was similar to those given CSP.

**Histopathology.** All of the tissue samples collected from necropsy were examined microscopically and the results are summarized in Table 6.6. Most of the chickens had lymphocytic infiltrates. Nodular infiltrates of lymphocytes were present in multiple organs including brain (Fig. 6.1), heart, lung, kidney, liver (Fig. 6.3), proventriculus (Fig. 6.2), ventriculus, spleen, small and large intestines, bone marrow and pancreas. Frequency of these lymphocytic infiltrates did not correlate with treatment.

One chicken from the CSP treated group examined at 10 weeks post-infection had a nephroblastoma in the kidney (Fig 6.4). Minimal to mild focal myeloid cell infiltrates were present in heart (Fig. 6.5), liver, lung (Fig. 6.6), and kidney in some chickens. At 7 and 10 weeks post-infection, myeloid infiltrates were more severe and were more common compared to chickens examined at earlier periods. In addition to that, significantly more chickens had myeloid infiltrates in the CSP group compared to the Oil group.

**Immunohistochemistry.** Monoclonal antibody against ALV-J successfully detected expression of viral antigen within the formalin fixed tissue sections. The distribution of viral antigen among the tissue-specific components of the standard tissues was
summarized in Table 6.7. The greatest antigen expression (mean scores per tissue > 1.0) was observed in the heart (Fig. 6.7) and kidney (Fig. 6.8). Many other tissues including lung (Fig 6.9), ventriculus (Fig. 6.10), bursa of Fabricius (Fig. 6.11) and liver (Fig. 6.12) were variably positive. In addition to staining of tissue specific components, viral antigen also stained in smooth muscle cells and connective tissues of multiple tissues.

There was no significant difference in the frequency of viral antigen staining in chickens between the PBS infected group and the CSP infected group in this experiment. However overall mean tissue score of the CSP infected group was significantly higher than that present in the Oil treated infected group at 10 weeks post-infection (p < 0.05). In each treatment group, staining of viral antigen was higher at 10 weeks than at 4 weeks post-infection.

**DISCUSSION**

In this study, intramuscular injection of chickens every 3 days with 50 mg/kg body weight CSP caused a significant reduction in response to the T-cell mitogen, Con A. In addition to that, the CSP group exhibited significantly decreased cutaneous basophilic hypersensitivity response in our experiment similar to that described in a previous study (Corrier & Deloach, 1990). Nowak *et al.* (Nowak *et al.*, 1982) showed that CSP acts as a selective T-cell suppressor in chickens. Suresh & Sharma (1995) found a similar injection of CSP did not decrease the humoral response to sheep red blood cells and brucella antigens in turkeys.

In our experiment, CSP injection did not cause significant alteration of thymic morphology and size, in contrast to results in a previous study (Hill et. al., 1989). The
lymphocytic composition of splenocytes estimated by flow cytometric analyses using monoclonal antibody against chicken CD3, CD4, CD8, and Ia was not significantly altered by CSP treatment or ALV-J infection. Thus the apparent disruption of T-cell function in this study was most likely due to toxic principle of cyclosporin on T-cell function. Cyclosporin prevents synthesis of cytokines by T cells by blocking a late stage of the signaling pathway initiated by the T-cell receptor. This especially affects the production of interleukin-2 (IL-2), hence T cell proliferation is affected (Ho et al., 1996; Resch & Szamel, 1997). As a consequence IL-2 dependent functions which include T-helper activities, cytotoxicity, natural killer cell activity and antibody dependent cell cytotoxicity would be decreased after cyclosporin treatment (Hill et al., 1989), even though antibody-based flow cytometric analyses appeared unaffected.

The degree of immunosuppression caused by MDV infection is variable with different isolates (Lee et al., 1978; Liu & Lee, 1983; Calnek et al., 1998). In our experiment, most of the chickens acquired Marek’s disease virus (MDV) infection before three weeks of age, indicated by the presence of lymphocyte infiltrations in multiple organs and presence of antibody determined by AGID. Histologic changes within the bursa of Fabricius and thymus in Oil treated chickens were minimal in our experiment, indicating that primary organs may not be significantly affected by this MDV infection.

Enhancement of lesions due to serotype 2 Marek’s disease virus (MDV) by ALV has been reported (Campbell & Frankel, 1979; Fadly & Ewert, 1994; Witter, 1995). Coinfection with ALV-J and vvMDV is associated with an increased expression of lymphomas, myelocytomas, and lymphocytic infiltrative peripheral neuritis (Zavala et al., 2000). In chickens with dual infections of MDV and ALV-J, ALV-J viremia
progressed more rapidly and is more persistent compared to chickens that were well vaccinated against MDV (Zavala, 2001). The potential effect of MDV infection on ALV-J pathogenesis in our experiment requires further studies. However, overall objectives of our study did not appear to be affected by this MDV infection, since all treatment group had MDV to a similar extent.

Congenital infection and neonatal infection with ALV-J causes significant decrease in body weight in broilers (Stedman & Brown, 1999). Viral infection of thyroid and the pituitary gland may be the cause for this effect (Stedman et al., 2001). In our experiment, there was no significant body weight suppression in any of the groups. This could be due to timing of the ALV-J exposure at 2 weeks of age. Birds exposed to ALV-J at much younger age developed tolerant viremia, increased incidence of tumors, and more body weight suppression. This difference may be due to constitutive embryonic expression of EAV-HP env sequences and the induction of tolerance in these birds (Benson et al., 1998; Smith et al., 1999; Sacco & Venugopal, 2001).

Real time RT-PCR using the Light Cycler system with SYBR Green I dye, was very efficient in detecting and quantifying the viral RNA in plasma in our experiment. However, it did not yield an absolute copy number of viral RNA. Because SYBR Green I dye binds to the double stranded DNA produced during PCR amplification, primer dimers as well as the specific amplicon can be added to the amplification plot. In our experiment, primer dimmers only minimally affected the results of quantitative real time RT-PCR even in negative samples (data not shown). The percentage of birds with viremia was higher in the CSP treated group than in the Oil treated group. In addition, more chickens had higher titer viremia in the CSP treated group than in the Oil treated
group. The percentage and titer of bird with neutralizing antibody were similar in both groups. Those results may indicate that other immune functions related to cell-mediated immunity is involved in controlling the viremic status in chickens.

Minimal to mild foci of myeloid cell infiltrations were present early in the experiment even in the uninfected groups, and there was no significant difference in frequency between groups. The nature of these myeloid infiltrates could not be determined, and they may be extramedullary hematopoietic foci. Later in the experiment (7 and 10 weeks post-infection), myeloid infiltrates were present only within the ALV-J infected groups and the extent of these infiltrates was more severe than those present earlier. At same time, significantly increased numbers of birds in the CSP treated group had myeloid infiltrates in multiple organs, compared to a smaller numbers of organs with the infiltrates in the Oil treated group. Also one nephroblastoma was observed in a CSP treated chicken at 10 weeks post-infection.

Distribution of the viral antigen was similar to that previously reported (Arshad et al., 1997b; Gharibeh et al., 2001). Not all congenitally infected birds have the same level of viremia, indicating embryos infected at different stages of development and may resulted in different levels of expression of viral antigen in tissues (Rubin et al., 1962). In our experiment, CSP treated chickens had higher intensity of viral antigen staining compared to that present in the control group at 10 weeks post-infection. This may indicate T-cell specific immunosuppression results in an increased viral load in tissues of ALV-J infected broiler chickens.
REFERENCES


Table 6.1. Summary of body weight gain, relative bursal weight and lymphocyte mitogenesis assay (mean ± standard deviation).

<table>
<thead>
<tr>
<th>WPI</th>
<th>Group</th>
<th>Body weight</th>
<th>Thymic weight*</th>
<th>SI**</th>
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<tr>
<td>0.3</td>
<td>Oil</td>
<td>191 ± 17.5</td>
<td>ND</td>
<td>ND***</td>
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<tr>
<td></td>
<td>Oil/J</td>
<td>182 ± 19.9</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>CSP</td>
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<td>ND</td>
<td>ND</td>
</tr>
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<td>ND</td>
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<td>ND</td>
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<tr>
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<tr>
<td></td>
<td>CSP/J</td>
<td>707 ± 82.3</td>
<td>4.04 ± 1.05</td>
<td>3.8 ± 1.9\textsuperscript{b}</td>
</tr>
<tr>
<td>7</td>
<td>Oil</td>
<td>1251 ± 193.8</td>
<td>2.92 ± 0.48</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>1235 ± 239.2</td>
<td>3.50 ± 0.47</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>1114 ± 157.3</td>
<td>3.16 ± 0.36</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>1154 ± 149.9</td>
<td>4.32 ± 1.89</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>Oil</td>
<td>1930 ± 366.9</td>
<td>2.29 ± 0.38</td>
<td>15.6 ± 5.4\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>1803 ± 414.4</td>
<td>3.17 ± 0.99</td>
<td>23.9 ± 8.7\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>1612 ± 348.9</td>
<td>2.94 ± 1.25</td>
<td>2.7 ± 1.4\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>1677 ± 338.9</td>
<td>2.72 ± 0.31</td>
<td>4.4 ± 1.9\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}: Weeks post-infection

* Thymic weight: relative bursal weight (bursal weight / body weight) X 1000

** SI (Stimulation index) obtained from mitogenesis assay using con A. SI = \[ \frac{(cpm \text{ of stimulated})-(cpm \text{ of unstimulated})}{(cpm \text{ of unstimulated})} \]

*** ND: not done

Values within a block followed by different letters are significantly different (p <0.05).
Table 6.2. Flowcytometric analysis of splenocytes using monoclonal antibodies

<table>
<thead>
<tr>
<th>WPI</th>
<th>Group</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>Ia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oil</td>
<td>46.65 ± 4.65(^2)</td>
<td>24.72 ± 0.33</td>
<td>32.92 ± 2.18</td>
<td>30.03 ± 6.05</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>54.76 ± 9.66</td>
<td>20.45 ± 2.94</td>
<td>36.16 ± 11.3</td>
<td>39.19 ± 5.13</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>48.50 ± 4.39</td>
<td>20.5 ± 8.51</td>
<td>38.21 ± 12.41</td>
<td>36.73 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>51.37 ± 10.56</td>
<td>23.29 ± 2.30</td>
<td>36.16 ± 1.10</td>
<td>34.36 ± 5.24</td>
</tr>
<tr>
<td>2</td>
<td>Oil</td>
<td>41.88 ± 11.40</td>
<td>24.29 ± 5.91</td>
<td>35.97 ± 4.98</td>
<td>40.91 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>50.67 ± 15.45</td>
<td>31.06 ± 1.56</td>
<td>36.10 ± 15.20</td>
<td>39.72 ± 6.88</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>47.20 ± 6.22</td>
<td>18.53 ± 0.10</td>
<td>32.00 ± 2.96</td>
<td>37.58 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>48.85 ± 14.12</td>
<td>26.80 ± 12.68</td>
<td>35.64 ± 3.07</td>
<td>41.44 ± 4.86</td>
</tr>
<tr>
<td>4</td>
<td>Oil</td>
<td>47.58 ± 3.34</td>
<td>ND(^3)</td>
<td>35.97 ± 4.98</td>
<td>42.93 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>49.05 ± 13.15</td>
<td>ND</td>
<td>32.11 ± 8.21</td>
<td>40.49 ± 4.82</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>48.70 ± 4.10</td>
<td>ND</td>
<td>33.47 ± 3.02</td>
<td>43.73 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>50.49 ± 11.81</td>
<td>ND</td>
<td>31.92 ± 5.26</td>
<td>39.75 ± 6.23</td>
</tr>
<tr>
<td>10</td>
<td>Oil</td>
<td>41.35 ± 3.04</td>
<td>21.28 ± 2.76</td>
<td>28.03 ± 5.30</td>
<td>34.35 ± 5.72</td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>42.51 ± 1.79</td>
<td>15.5 ± 2.63</td>
<td>28.38 ± 4.99</td>
<td>38.48 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>CSP/J</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\) Weeks post-infection

\(^2\) Relative lymphocytes subpopulation (%) ± standard deviation

\(^3\) ND: Not done
Table 6.3 Effect of cyclosporin A (CSP) treatment\(^1\) on the cutaneous basophil hypersensitivity (CBH) response\(^2\) induced by injection of PHA-P\(^3\) 200 ug and a physiological saline solution (PSS) in 2 week-old chickens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Olive oil</th>
<th>Cyclosporin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH-1 response (mm)(^4)</td>
<td>(0.69 \pm 0.14)</td>
<td>(0.29 \pm 0.6^*)</td>
</tr>
<tr>
<td>CBH-2 response (mm)(^5)</td>
<td>(0.65 \pm 0.15)</td>
<td>(0.21 \pm 0.9^*)</td>
</tr>
</tbody>
</table>

\(^1\) Intramuscular injection of 50 mg/ kg body weight every third days after hatching.

\(^2\) Data are expressed as mean ± standard deviation; n = 10.

\(^3\) PHA-P = phytohemagglutinin P.

\(^4\) CBH-1 = (skin thickness at 12 h postinjection, left foot) – (preinjection skin thickness, left foot)

\(^5\) CBH-2 = (skin thickness, PHA-P-injected foot) – (skin thickness, PSS-injected foot)

* Significantly different from oil treated birds (p < 0.001)
Table 6.4. ALV-J viremic status measured by Real time RT-PCR

<table>
<thead>
<tr>
<th>WPI Group</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low³</td>
<td>7</td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Medium⁴</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>High⁴</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total²</td>
<td>7/19 (37)</td>
<td>12/12 (100)</td>
<td>9/14 (64)</td>
<td>7/10 (70)</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>CSP²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low³</td>
<td>9</td>
<td>12</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medium⁴</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>High⁴</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total²</td>
<td>9/15 (60)</td>
<td>12/12 (100)</td>
<td>6/11 (55)</td>
<td>7/9 (77)</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>

¹ Weeks post-infection

² Real time RT-PCR for ALV-J using H5/H7 primers was done on RNA extracted from plasma. Number of positive / Number of tested ( %)

³ ALV-J Virus titer in plasma measured by real time RT-PCR using H5/H7 primers was divided into low, medium and high. Number of samples.
Table 6.5. Virus neutralizing antibody against ALV-J tested by microneutralization test

<table>
<thead>
<tr>
<th>WPI(^1)</th>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>ND(^3)</td>
<td>ND</td>
<td>0/5 (0)(^2)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td></td>
</tr>
<tr>
<td>Oil/J</td>
<td>ND</td>
<td>ND</td>
<td>3/11 (4-16)</td>
<td>4/9 (64-1024)</td>
<td>4/5 (64-1024)</td>
<td></td>
</tr>
<tr>
<td>CSP</td>
<td>ND</td>
<td>ND</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td></td>
</tr>
<tr>
<td>CSP/J</td>
<td>ND</td>
<td>ND</td>
<td>0/8</td>
<td>5/9 (64-1024)</td>
<td>3/5 (64-1024)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Weeks post-infection

\(^2\) Number of positive / Number of tested (Range of virus neutralizing titer)

\(^3\) ND: not done
Table 6.6. Summary of histopathologic findings

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphocytic infiltration</th>
<th>Myeloid cell infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Oil</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Oil/J</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>CSP</td>
<td>3/3</td>
<td>2/2</td>
</tr>
<tr>
<td>CSP/J*</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

1 Weeks post-infection

2 Number of chickens with infiltration / Number of chickens examined.

* At 7 weeks post-infection, one nephroblastoma was observed in the kidney.
Table 6.7. Viral antigen expression* at 1, 4 and 10 weeks post-infection in tissues infected with ALV-J (ADOL-7501) as 2 weeks of age.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weeks post-infection</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 weeks</td>
<td>4 weeks</td>
<td>10 weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oil/J</td>
<td>CSP/J</td>
<td>Oil/J</td>
<td>CSP/J</td>
<td>Oil/J</td>
</tr>
<tr>
<td>Brain</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Bursa</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>1/3 (0.3)</td>
</tr>
<tr>
<td>Heart</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>1/3 (0.7)</td>
</tr>
<tr>
<td>Intestine</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>1/3 (0.3)</td>
<td>1/3 (0.3)</td>
<td>2/3 (0.7)</td>
</tr>
<tr>
<td>Liver</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>1/3 (0.7)</td>
</tr>
<tr>
<td>Lung</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Marrow</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Nerve</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Proventriculus</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Thymus</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Ventriculus</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
</tbody>
</table>

* No. birds positive/total no. birds examined (mean score for each tissue: 0 = negative; 1 = few positive cells; 2 = many positive cells).

** Tissue-specific cells evaluated
Fig. 1. Brain

Fig. 2. Proventriculus

Fig. 3. Liver

Fig. 4. Kidney

Fig. 5. Heart

Fig. 6. Lung
Legends for microscopic pictures (Fig. 6.1 – 6.12).

Chickens were daily treated with Oil or 50 mg of cyclosporin A (CSP) every three days till the end of the experiment. Some of the chickens from each treatment were infected with an avian leukosis virus subgroup J (ALV-J) isolate, ADOL-7501, at 2 weeks of age.

Fig 6.1. Brain. H&E. A 12 week-old chicken from CSP treated/ uninfected group. Mild perivascular cuffing of lymphocytes. 100X

Fig. 6.2. Proventriculus. H&E. A 6 week-old chicken from CSP treated/ uninfected group. Multifocal infiltrations of lymphocytes within muscle layer and serosa. 100X

Fig. 6.3. Liver. H&E. A 6 week-old chicken from CSP treated/ infected group. Multifocal infiltrations of lymphocytes within the hepatic parenchyma.

Fig. 6.4. Kidney (nephroblastoma). H&E. A 12 week-old chicken from CSP treated/ infected group. Infiltrating foci of neoplastic cells forming occasional tubule and primordial glomeruli with abundant fibroblastic connective tissue. 200X

Fig. 6.5. Heart. H&E. A 12 week-old chicken from CSP treated/ infected group. Infiltrating multiple aggregates of myeloid cells within the myocardium. 400X

Fig. 6.6. Lung. H&E. A 12 week-old chicken from Oil treated/ infected group. Focal aggregate of myeloid cells within the interlobular connective tissue. 400X

Fig. 6.7. Heart. Kidney. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from Oil treated/ infected group. Expression of the viral antigen was detected within the myocardial fibers. 400X

Fig. 6.8. Kidney. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from CSP treated/ infected group.
Expression of the viral antigen was detected in the lumenal surface of the renal tubular epithelial cells. 200X

Fig. 6.9. Lung. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from CSP treated/infected group. Viral antigen expression was detected within the lining epithelium of the parabronchioes and air capillaries.

Fig. 6.10. Ventriculus. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from CY treated/infected group. Expression of the viral antigen was observed within basaloid aspects of the lining epithelial cells of the mucosa. Occasional positive staining cells are present within the lamina propria. 400X

Fig. 6.11. Bursa. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from PBS-treated/infected group. Viral antigen expression was observed cells within the medullae of the bursal follicles. Occasional positive staining also observed in the surrounding connective tissue. 400X

Fig. 6.12. Liver. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 12 week-old chicken from Oil treated/infected group. Viral expression was observed in the lining cells of the sinusoids and Kupffer cells. 400X
CHAPTER 7
CONCLUSION AND DISCUSSION

This project was designed to determine the effect of immunosuppression on the pathogenesis of the subgroup J avian leukosis virus (ALV-J) using chemically immunosuppressed broilers. In addition, we developed and validated multiple techniques for reverse transcription polymerase chain reaction (RT-PCR) based detection and quantitation of ALV-J genomic RNA. We investigated the pathologic changes caused directly by cyclophosphamide treatment.

RT-PCR based detection and quantitation methodologies for ALV-J viral RNA:

We developed and validated new methodologies for quantitation of plasma viral RNA, rather than proviral DNA. Both Quantitative competitive RT-PCR (QC-RT-PCR) and real time RT-PCRs (TaqMan® probe system and SYBR Green I dye system) were developed and found to be strongly correlated with tissue culture infective dose 50 (TCID$_{50}$s) as determined by conventional system using a cell culture system and Ag-ELISA. RT-PCR-based viral quantification has many advantages over conventional cell culture methods, however, there are several precautions that must be incorporated into these methodologies. Attention to the issues unique to testing for viral RNA using RT-PCR techniques were found to be critical for maintaining high assay sensitivity.
First, extraction of viral RNA from samples is an important source of variability that may impact results obtained. Plasma (rather than serum) was used in our studies to limit release of endogenous ALV into samples. This choice resulted in the lack of endogenous ALV being present in samples. Future studies might consider use of an exogenous control for the extraction process if plasma is to be used in that work. Such an exogenous control could be added prior to extraction as a measure of nucleic acid extraction efficiency.

Secondly, RNA is more rapidly degraded than DNA. This occurs by contamination with RNase which is ubiquitous in laboratory environments. RNA is also slowly degraded even at –20 C in clean storage conditions with no contamination. The mechanism for this degradation at freezing temperatures is unknown.

Thirdly, the reverse transcriptase enzyme itself is very inefficient and error prone. This will alter the sensitivity of the quantitative methods. Finally, insensitivity of the fluorescent dye and the detection of bands in the agarose gel were other complicating factors.

In spite of these analytical issues, absolute and precise quantification of ALV-J RNA using QC-RT-PCR was possible, but the limit of quantification was higher than expected. Real time RT-PCR method using the TaqMan® probe system could specifically quantitate PCR products from the target RNA. This assay was difficult to set up and the sensitivity was poor. Low sensitivity in our assays was most likely due to the limitation of having to choose compatible primer sets and probe. In contrast to that, real time RT-PCR using SYBR Green I dye was a very sensitive and was easy to set up. This system
quantitates RT-PCR product from target RNA as well as primer dimers generated during the PCR reaction.

Based on these findings, we chose real time RT-PCR using SYBR Green I dye for all our further studies because it was rapid, easy and simple to run. And furthermore, the sensitivity of this method was much better than that of any other quantification methods developed in our study. The results were strongly correlated with TCID₅₀s. The influence of primer dimers was minimal in our experiments, and melting curve analysis on the PCR product was a very effective tool to determine positive samples.

**Toxicologic changes caused by cyclophosphamide treatment:** As expected, 4mg of CY treatment for 4 days after hatching caused severe damage to bursa and spleen. The changes persisted until the end of our experiment. Also we noticed approximately 50% mortality and significant depression of body weight gain. This relatively high mortality and body weight suppression should be taken into account if studies are planned using this mechanism of immunosuppression. Late feathering, as reported by other researchers previously using this protocol, was present in our experiment although feather follicles were not examined histologically to determine the mechanism for this affect. There were mild to moderate, transient histologic changes in liver, kidney and bone marrow. These direct toxicologic effects have not been reported previously and need to be considered in future experiments as representing toxicologic tissue alternations of cyclophosphamide rather than being due to other treatments. Further investigations on morphologic changes using electron microscopy or functional analysis of affected organs can be done to determine the extent of toxicity.
Effect of immunosuppression on ALV-J pathogenesis: In our experiments, we succeeded in individually suppressing either the B-cell or T-cell system. The immune status of the bird was determined by relative weight of immune organs, mitogenesis assay and flow cytometric analysis of splenocytes. These assays required sacrificing experimental animals to obtain the required samples for analysis. If it were necessary to keep the birds alive for subsequent testing or challenge work, assays using injections of B- or T-cell specific antigens such as Brucella sp. and sheep red blood cells could have been employed. Additionally, T-cell mediated function could have been assayed by measuring cutaneous basophilic hypersensitivity assays on interdigital skin and wattle could be useful in young and old chickens, respectively.

According to published studies, the procedure of CY treatment used in our experiment caused transient but significant T cell suppression which recovered by 2 weeks of age. This was the reason why we set the timing of infection with ALV-J in our experiments. However, as previously described, infection with the virus at 2 weeks of age did not induce major pathogenic effects on body weight suppression and tumor formation. In other studies, these effects were observed in chickens infected congenitally or at one day of age. Therefore, if analysis of these effects associated with perinatal infection are required, other methods for immunosuppression such as in ovo inoculation of CY and testosterone, surgical removal of bursa or thymus, or injection of monoclonal antibody against lymphocyte surface antigen may be useful.

In our study, cyclophosphamide treatment suppressed B cell immunity, enhanced viremia, virus titer, and tissue expression of viral envelope glycoprotein. No affect was found on tumor incidence in the small numbers of birds included in the study. In a
separate experiment, cyclosporin A treatment suppressed T cell immunity, and also enhanced viremia and the tissue expression of the viral antigen compared to those of control group. Though the number of the tumors observed histologically was not enough to draw a conclusion, the results of histological examination indicated there may be enhanced tumor formation by cyclosporin A treatment.

It was difficult to compare the effects of cyclophosphamide treatment to those of the cyclosporin A treatment due to differences in extent of immunosuppression produced, different treatment schedules, and different toxicologic effects of each chemical. Further difficulties in comparing the two drugs stem from our use of viremia as one reaction variable. Cyclosporin A, but not cyclophosphamide, has direct antiviral activity. It suppresses the replication of human immunodeficiency virus type I by binding to cyclophilin present in the virion. In our experiment we did not determine the direct effect of either of the chemicals we used on virus replication. Though there is no cyclophilin-like protein within the ALV-J virion, direct pro- or anti-viral effect of chemicals should be further investigated.

Theoretically, the intensity of viral antigen expression has been a good indicator for the pathogenicity of virus infection. In our experiments, both cyclophosphamide and cyclosporin A treatments enhanced the viral antigen expression. This could be an indication of the role of B- and T-cell function in controlling the pathogenesis of subgroup J ALV.

Like other subgroups of ALV, ALV-J transmits congenitally as well as horizontally. For control programs in field situations, neonatal horizontal transmission of ALV-J is important. Therefore, the effects of immune system on virus shedding should have been
determined. In our experiment, we found intensive staining in renal tubules and it is possibly a good indicator of viral shedding, however, the relationship between shedding rate and viral antigen expression in renal tubules has not been reported.

In the data set obtained from our experiments, we observed minimal to mild correlation between the presence of antibody and viremic status in chickens. However, the number of chickens in our experiment was too small to make statistically certain conclusions. The relationship of antibody status and viremia should be determined in further studies using increased numbers of chickens and longer experimental period. To confirm the result of our experiments, further studies using immune-enriched chickens are required. Those chickens may be produced by injection of specific cytokines such as IL-2, IL-12, IL-4 etc.

**MDV infection in chickens**: In our experiment, most of the chickens were infected with Marek’s disease virus. Due to the long duration of our experiments, maintaining birds free of MDV was very difficult. In future studies vaccination of MDV *in ovo* or at day of age should be incorporated. The enhancement of ALV-J pathogenicity by MDV infection has been described. This could be due to enhanced transcription of viral genome or immunosuppressive effect. The effects on virus replication could not be determined in our experiment.

Altogether the most relevant findings of this research are listed as follows:

1) RT-PCR-based quantitative methodologies had a strong correlation with a conventional titration method using cell culture and ag-ELISA.

2) Quantitative competitive RT-PCR was a good method for absolute quantiation of ALV-J viral RNA
3) Real time RT-PCR using Taqman® probe system was difficult to set up for quantitation of ALV-J viral RNA.

4) Real time RT-PCR using SYBR Green I dye is easy to set up and perform and useful for detection and semi-quantitation of ALV-J viral RNA.

5) Cyclophosphamide treatment caused decreased body weight gain, complete ablation of B cell function and also induced toxicity in other organs including skin, liver, spleen, kidney and bone marrow.

6) Cyclophosphamide treatment exacerbated the severity of ALV-J infection, enhancing viremia and expression of viral envelope glycoprotein in ALV-J infected broilers. No differences in body weight gain and tumor formation were induced by the viral infection.

7) Cyclosporin A treatment caused significant suppression of T-cell mediated immunity but left antibody formation intact.

8) Cyclosporin treatment enhanced ALV-J viremia and tissue expression of viral envelope glycoprotein, but did not affect the body weight gain or virus neutralizing antibody production. The effect on tumor formation was inconclusive, although a suggestion of enhanced tumor frequency was seen.
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