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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

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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 flow cytometry. Chickens in this experiment had Mareks 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.

Key words: Avian leukosis virus subgroup J, cyclophosphamide, B-cell, real time RT-PCR, chickens

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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 [16,17]. Cyclophosphamide treatments have been used to inhibit humoral immunity in order to determine its role in the pathogenesis of infectious pathogens of chickens [1,25].

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 [19]. Subgroup J ALV (ALV-J) induces tumors and decreased weight gain in experimentally or naturally infected chickens [22,23,32]. 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 [20,21]. Since horizontal transmission of the ALV-J is more significant, eradication programs for this subgroup have to be applied more intensely [24].

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 [10].

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) × 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 Hanks balanced salt solution (HBSS, Sigma, St. Louis, MO) and prepared as described previously with minor modifications [4]. 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 2.67×10^7 cells per ml (Coulter Counter® Model D2N automated cell counter, Coulter Corp., Hialeah, FL). For each chicken, 2×10^6 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 [4]. 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) [27]. 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 1×10^7 cells/ml. Cells (1×10^6) 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 use.

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 and produced an amplicon of approximately 545 bp [30]. 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 95°C, 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 [14]. 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 [10].

Hisopathology

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 manufacturers 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 (2): 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 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 1, no difference of the stimulation index was noticed between any of the groups.

Table 1. Summary of body weight gain, relative bursal weight and lymphocyte mitogenesis assay (mean ± standard deviation)

WPI[1]	Group	Body weight	Bursal weight*	Stimulation index**
3days	PBS	189 ± 16.6[a]	0.34 ± 0.02[a]	69.8 ± 22.2[a]
	PBS/J	186 ± 22.3[a]	0.29 ± 0.05[a]	78.6 ± 29.4[a]
	CY	106 ± 24.4[b]	0.11 ± 0.04[b]	65.9 ± 24.0[a]
	CY/J	101 ± 16.9[b]	0.10 ± 0.04[b]	76.0 ± 21.5[a]
1	PBS	283 ± 34.7[a]	0.43 ± 0.03[a]	16.1 ± 4.9[a]
	PBS/J	280 ± 26.5[a]	0.45 ± 0.04[a]	18.2 ± 7.6[a]
	CY	174 ± 33.6[b]	0.12 ± 0.03[b]	16.4 ± 5.9[a]
	CY/J	166 ± 26.9[b]	0.18 ± 0.14[b]	11.8 ± 7.1[a]
3	PBS	612 ± 76.9[a]	0.36 ± 0.02[a]	28.3 ± 9.5[ab]
	PBS/J	540 ± 50.6[a]	0.37 ± 0.08[a]	18.3 ± 6.4[c]
	CY	442 ± 52.0[b]	0.06 ± 0.01[b]	37.8 ± 13.8[b]
	CY/J	376 ± 96.6[b]	0.06 ± 0.04[b]	22.9 ± 5.6[ac]
6	PBS	1102 ± 131.2[a]	0.28 ± 0.04[a]	ND
	PBS/J	982 ± 122.4[ab]	0.34 ± 0.14[a]	ND
	CY	848 ± 109.2[bc]	0.04 ± 0.01[b]	ND
	CY/J	697 ± 187.6[c]	0.05 ± 0.02[b]	ND
9	PBS	1669 ± 203.7[a]	0.16 ± 0.02[a]	22.5 ± 18.3[a]
	PBS/J	1480 ± 230.3[ab]	0.18 ± 0.04[a]	49.3 ± 32.8[a]
	CY	1201 ± 197.5[bc]	0.03 ± 0.02[a]	33.3 ± 12.7[a]
	CY/J	1186 ± 165.9[c]	0.04 ± 0.02[a]	27.8 ± 16.1[a]

[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

Flowcytometry

Relative subpopulation of CD3-positive cells in CY-treated and PBS-treated groups were shown in Fig. 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

Thirteen out of 22 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 83°C and 85°C. Based on the results of real time RT-PCR using cell culture lysates with a known $TCID_{50}$ (Fig. 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 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 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

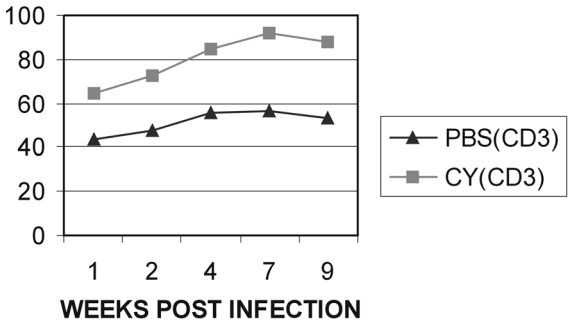

Fig. 1. Flowcytometric analysis of splenic lymphocytes. Relative population of CD3-positive lymphocytes from spleen of cyclophosphamide treated chickens was significantly higher than that of PBS treated. No significant differences between infected and uninfected chickens within same treatment were observed (Data not shown).

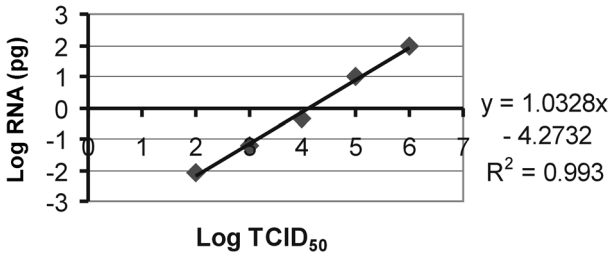

Fig. 2. Quantitative real time RT-PCR by H5/H7 primers and Light Cycler system using SYBR green I dye. Strong correlation (R^2=0.993) was observed between $TCID_{50}$s and amount of ALV-J RNA measured by real time RT-PCR.

treated with CY had markedly decreased numbers of lymphoid follicles separated by increased interfollicular connective tissue (Fig. 3). The results of the histopathology are summarized in Table 4. Nodular to diffuse infiltrations of lymphoid cells were present in variable organs including liver, heart, lung, kidney, bone marrow, spleen, proventriculus, ventriculus (Fig. 4), small and large intestines, and pancreas. In most of chickens, lymphocytic infiltrations were present in multiple organs. There was no difference in organ distribution of the lymphocytic infiltration between the groups.

Minimal to mild foci of myeloid cell infiltrates were present in the lung (Fig. 5), heart, liver, 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.

Table 2. ALV-J viremic status measured by Real time RT-PCR

Group		WPI[1] 3days	1	3	6	9
PBS[2]		0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
PBS/J	Low[3]	4	2	2	4	1
	Medium[3]	3	1	2	0	3
	High[3]	0	0	0	0	0
	Total[2]	7/14 (50)	3/13 (23)	4/10 (40)	4/8 (50)	4/6 (67)
CY[2]		0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
CY/J	Low[3]	6	1	3	0	0
	Medium[3]	4	5	5	1	1
	High[3]	0	0	1	4	1
	Total[2]	10/15 (67)	6/10 (60)	10/11 (91)	5/5 (100)	2/2 (100)

[1] Weeks post-infection
[2] Number of positive / Number of tested (percentage), determined by real time RT-PCR using H5/H7 primers
[3] 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 3. ALV-J virus neutralizing antibody tested by microneutralization test

Group	WPI[1] 3days	1	3	6	9
PBS	ND	ND	0/11	0/7	0/3
PBS/J	ND	ND	0/11	3/8 (4-16)[2]	4/6 (4-1024)
CY	ND	ND	0/9	0/6	0/3
CY/J	ND	ND	0/7	0/3	0/3

[1] Weeks post-infection
[2] Number of positive / Number of tested (Range of virus neutralizing titer)
ND: not done

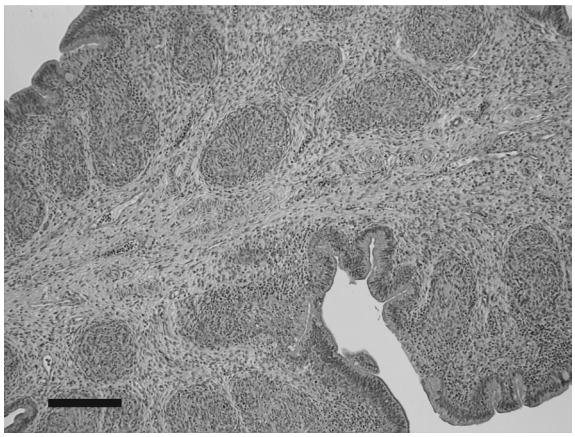

Fig. 3. Bursa. H&E. A 3 week-old chicken from CY treated/ uninfected group. Markedly decreased number of follicles separated by increased interfollicular connective tissue. Bar=400 μm. Chickens were daily treated with PBS or 4mg of cyclophosphamide for 4 days from hatching. Some of the chickens from each treatment were infected with an avian leukosis virus subgroup J (ALV-J) isolate, ADOL-7501, at 2 weeks of age.

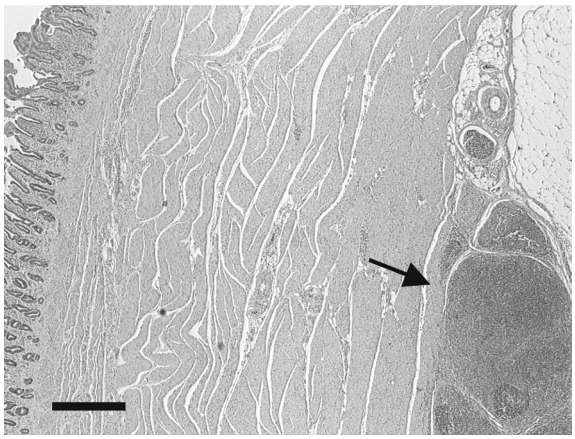

Fig. 4. Ventriculus. H&E. A 5 week-old chicken from PBS treated/uninfected group. Multiple nodular infiltrations of lymphocytes (arrow) within the serosa. Bar=600 μm

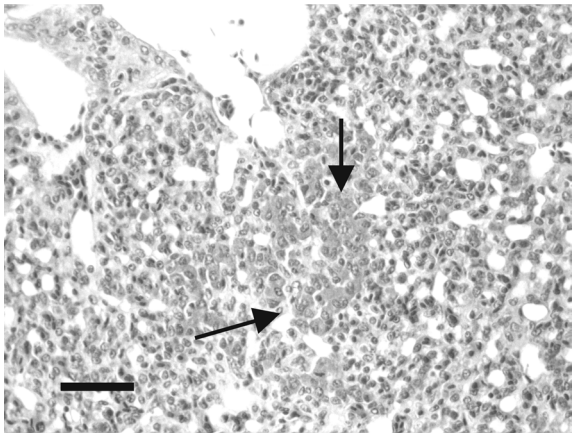

Fig. 5. Lung. H&E. An 8 week-old chicken from PBS treated/ infected group. Small aggregates of myeloid cells within the interstitium. Bar=100 μm

Immunohistochemistry

The tissue distribution of viral antigen is summarized in Table 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. 6), ventriculus and proventriculus (Fig. 7). Many other tissues including liver (Fig. 8) 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.

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 [13, 17,29]. 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 signifincantly smaller than those sham treated with PBS. Histologically, bursal follicles were also smaller and depletion of lymphocytes was prominent after

Table 4. Summary of histopathologic findings

Group	Lymphocytic infiltration					Myeloid cell infiltration				
	3days	1[1]	3	6	9	3days	1	3	6	9
PBS	1/1[2]	1/1	1/1	2/2	2/2	0/1	1/1	1/1	0/2	0/2
PBS/J	1/3	3/3	3/3	3/3	6/6	1/3	1/3	2/3	2/3	4/6
CY	0/1	1/1	1/1	2/2	2/2	0/1	0/1	0/1	0/2	0/2
CY/J	1/3	2/3	4/4	2/2	2/2	1/3	0/3	0/4	1/2	2/2

[1]Weeks post-infection
[2]Number of chickens with infiltration / Number of chickens examined.

Table 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

Tissue**	Weeks post-infection					
	1 weeks		3 weeks		9 weeks	
	PBS/J	CY/J	PBS/J	CY/J	PBS/J	CY/J
Brain	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/2 (0)
Bursa	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/ 2 (0.5)
Heart	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/2 (0)
Intestine	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Kidney	0/3 (0)	0/3 (0)	1/3 (0.7)	3/3 (2)	2/3 (1)	2/2 (2)
Liver	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/2 (0)
Marrow	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/2 (0)
Nerve	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/2 (0)
Pancreas	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)	1/ 2 (0.5)
Proventriculus	0/3 (0)	0/3 (0)	1/3 (0.3)	3/3 (2)	1/3 (0.7)	1/ 2 (1.5)
Spleen	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/ 2 (1)
Thymus	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/ 2 (1)
Ventriculus	0/3 (0)	0/3 (0)	1/3 (0.3)	3/3 (1.7)	1/3 (0.7)	1/ 2 (1)
Lung	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)	1/ 2 (0.5)

*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

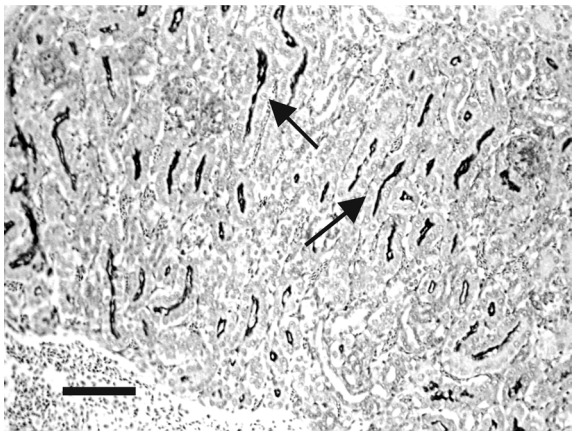

Fig. 6. Kidney. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from PBS treated/infected group. Expression of the viral antigen was detected within the lumenal surfaces of the renal tubular epithelial cells (arrows). Bar=200 μm

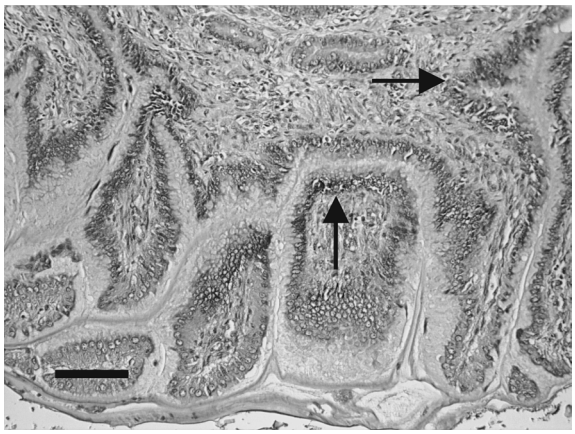

Fig. 7. Proventriculus. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. A 5 week-old chicken from CY treated/infected group. Expression of the viral antigen was observed within the basaloid aspects of the mucosal lining epithelial cells (arrows). Scattered positive cells are present in the connective tissue. Bar=200 μm

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 Mareks 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 [15,18,5)]. 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

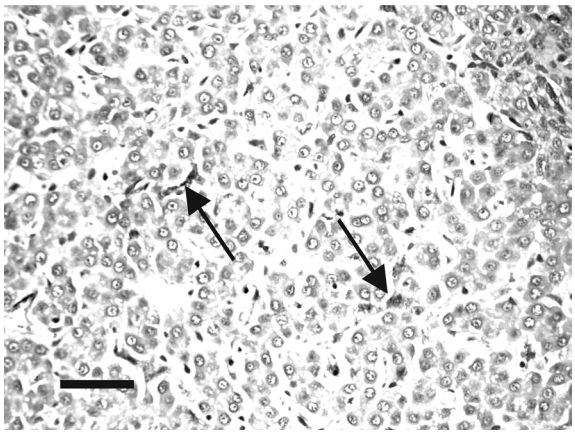

Fig. 8. Liver. Immunohistochemical staining with monoclonal antibody against ALV-J envelope glycoprotein. An 11 week-old chicken from CY treated/infected group. Viral expression was observed in the lining cells of the sinusoids and Kupffer cells (arrows). Bar=100 μm

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 Mareks disease virus (MDV) has been reported [6,8,33]. Coinfection with ALV-J and vvMDV was conducive to an increased expression of lymphomas, myelocytomas, and lymphocytic infiltrative peripheral neuritis [34]. 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 [35]. 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 [32]. 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 [3,28,31].

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 [8]. Cloacal shedding, viremia, and tumor development were significantly lower in chickens with maternal antibody following exposure to subgroup A ALV at hatching [7,11]. 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 generation [26]. 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 [2,12]. 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.

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