

## Effects of cyclosporin A treatment on the pathogenesis of avian leukosis virus subgroup J infection in broiler chickens with Marek's disease virus exposure

Yongbaek Kim\*, Thomas P. Brown and Mary J. Pantin-Jackwood

*Departments of Veterinary Pathology and Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA*

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 mitogenic activity using concanavalin A. Most of the chickens examined had Marek's disease virus infection prior to 3 weeks of age. The percentage of antibody-positive birds and antibody titers were similar in infected chickens between both treatment groups. The ratio of viremic chickens was significantly higher in CSP treated group than that of the Oil treated group. Microscopically, one CSP treated chicken had a nephroblastoma at 10 weeks post infection. At 7 and 10 weeks post-infection, more chickens had myeloid cell infiltrations in multiple organs including heart, liver and occasionally lung. Expression of ALV-J viral antigen determined by immunohistochemical staining was significantly higher in CSP treated chickens than Oil treated chickens at 10 weeks post-infection. This study indicated that chemically-induced T-cell suppression may enhance pathogenicity of the AVL-J virus in broilers.

**Key words:** Avian leukosis virus subgroup J, cyclosporin A, chickens

### Introduction

Cyclosporin A, 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 [21]. Cyclosporin A 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 [18,21,23,26].

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 [12,13,29,34,43]. 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 [17]. 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 [9,10,11,14,16]. 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 [8].

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 [31,40]. ALV-J induces late-onset myeloid leukosis [30]. Renal tumors and other sarcomas such as histiocytic sarcoma, hemangiosarcoma, mesothelioma, granulosa cell tumors, pancreatic adenocarcinoma, fibroma, and an unclassified leukemia are also observed [1,20,30,32]. Eradication programs applied for ALVs are essentially based on the experience

\*Corresponding author  
Phone: +1-919-316-4559; Fax: +1-919-541-4714  
E-mail: kim16@niehs.nih.gov

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 [39] should be effective in eradicating an ALV-J infection [45]. 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<sub>50</sub>) of 10<sup>6.5</sup>/ml. It was diluted with cell culture medium and 0.1 ml containing 10<sup>4.5</sup> TCID<sub>50</sub> 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<sub>50</sub>/well.

### 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-gauge needle every third day until the end of the experiment with 50mg Cyclosporin A (CSP) oral suspension (Sandimmune® 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 Oil and CSP 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) × 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 [4]. 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 × 10<sup>7</sup> cells per ml using a Coulter Counter® Model D2N automated cell counter (Coulter Corp., Hialeah, FL) [35].

### Lymphocyte mitogenesis assay

For each chicken, 2 × 10<sup>6</sup> 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 41C as described previously [4]. 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) [35]. 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 × 10<sup>7</sup> cells/ml. Cells (1 × 10<sup>6</sup>) were incubated with monoclonal antibodies, CD3-FITC (Southern Biotech, Birmingham, AL), CD4-PE (Southern Biotech, Birmingham, AL), CD8-FITC

(Southern Biotech, Birmingham, AL) or MHC II-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 [7]. Ten chickens were injected intradermally in the skin between 3<sup>rd</sup> and 4<sup>th</sup> digits of the left foot 200 µg 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 manufacturers 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 [37] 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 manufacturers 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 83-85°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 [24]. We performed Real time RT-PCR with RNA from cell lysates with different TCID<sub>50</sub>s to determine correlation between real time RT-PCR and TCID<sub>50</sub>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.

### 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 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 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 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.

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

WPI <sup>1</sup>	Group	Body weight	Thymic weight*	SI**
3 days	Oil	191 $\pm$ 17.5	ND	ND***
	Oil/J	182 $\pm$ 19.9	ND	ND
	CSP	189 $\pm$ 15.5	ND	ND
	CSP/J	181 $\pm$ 20.3	ND	ND
1	Oil	283 $\pm$ 24.8	5.03 $\pm$ 1.28	ND
	Oil/J	267 $\pm$ 31.7	ND	ND
	CSP	275 $\pm$ 22.9	5.63 $\pm$ 0.88	ND
	CSP/J	261 $\pm$ 34.1	ND	ND
2	Oil	427 $\pm$ 47.7	3.76 $\pm$ 1.18 <sup>ab</sup>	65.2 $\pm$ 18.7 <sup>a</sup>
	Oil/J	417 $\pm$ 41.1	4.47 $\pm$ 0.52 <sup>a</sup>	81.3 $\pm$ 28.4 <sup>a</sup>
	CSP	408 $\pm$ 41.2	3.02 $\pm$ 0.78 <sup>b</sup>	5.4 $\pm$ 0.2 <sup>b</sup>
	CSP/J	386 $\pm$ 48.6	3.07 $\pm$ 0.37 <sup>b</sup>	5.28 $\pm$ 2.6 <sup>b</sup>
4	Oil	782 $\pm$ 94.1	3.30 $\pm$ 0.86	60.0 $\pm$ 31.2 <sup>a</sup>
	Oil/J	760 $\pm$ 111.4	4.20 $\pm$ 1.07	67.2 $\pm$ 26.9 <sup>a</sup>
	CSP	718 $\pm$ 92.4	4.00 $\pm$ 0.42	3.1 $\pm$ 2.5 <sup>b</sup>
	CSP/J	707 $\pm$ 82.3	4.04 $\pm$ 1.05	3.8 $\pm$ 1.9 <sup>b</sup>
7	Oil	1251 $\pm$ 193.8	2.92 $\pm$ 0.48	ND
	Oil/J	1235 $\pm$ 239.2	3.50 $\pm$ 0.47	ND
	CSP	1114 $\pm$ 157.3	3.16 $\pm$ 0.36	ND
	CSP/J	1154 $\pm$ 149.9	4.32 $\pm$ 1.89	ND
10	Oil	1930 $\pm$ 366.9	2.29 $\pm$ 0.38	15.6 $\pm$ 5.4 <sup>a</sup>
	Oil/J	1803 $\pm$ 414.4	3.17 $\pm$ 0.99	23.9 $\pm$ 8.7 <sup>a</sup>
	CSP	1612 $\pm$ 348.9	2.94 $\pm$ 1.25	2.7 $\pm$ 1.4 <sup>b</sup>
	CSP/J	1677 $\pm$ 338.9	2.72 $\pm$ 0.31	4.4 $\pm$ 1.9 <sup>b</sup>

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

\* Thymic weight: relative thymic weight (thymic weight / body weight) X 1000

\*\* SI (Stimulation index) obtained from mitogenesis assay using Con A. SI = [((cpm of stimulated)-(cpm of unstimulated)) / (cpm of unstimulated) ]

\*\*\* ND: not done

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

**Table 2.** Flowcytometric analysis of splenocytes using monoclonal antibodies

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

<sup>1</sup> Weeks post-infection<sup>2</sup> Relative lymphocytes subpopulation (%) ± standard deviation<sup>3</sup> ND: Not done**Table 3.** ALV-J viremic status measured by Real time RT-PCR

Group	WPI <sup>1</sup>	1	2	4	7	10
	Oil <sup>2</sup>	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
Oil/J	Low <sup>3</sup>	7	5	8	3	2
	Medium <sup>3</sup>	0	5	1	2	1
	High <sup>3</sup>	0	2	0	2	0
	<b>Total<sup>2</sup></b>	7/19(37)	12/12(100)	9/14 (64)	7/10 (70)	3/4 (75)
	<b>CSP<sup>2</sup></b>	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
CSP/J	Low <sup>3</sup>	9	12	4	1	1
	Medium <sup>3</sup>	0	0	2	2	3
	High <sup>3</sup>	0	0	0	4	3
	<b>Total<sup>2</sup></b>	9/15 (60)	12/12 (100)	6/11 (55)	7/9 (77)	7/7 (100)

<sup>1</sup> Weeks post-infection<sup>2</sup> Real time RT-PCR for ALV-J using H5/H7 primers was done on RNA extracted from plasma. Number of positive / Number of tested (%)<sup>3</sup> 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.

### Flow cytometry

The results of the flow cytometric analysis are summarized in Table 2. There were no significant differences in relative subpopulation of CD3-, CD4-, CD8- and MHC II- 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. 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

Fifteen out of twenty sera submitted were positive for antibody against Mareks disease virus (MDV) by agar gel immunodiffusion test (California Animal Health Food Safety Laboratory System, University of California,

**Table 4.** Virus neutralizing antibody against ALV-J tested by microneutralization test

Group	WPI <sup>1</sup>	1	2	4	7	10
Oil		ND <sup>3</sup>	ND	0/5 (0) <sup>2</sup>	0/5 (0)	0/5 (0)
Oil/J		ND	ND	3/11 (4-16)	4/9 (64-1024)	4/5 (64-1024)
CSP		ND	ND	0/5 (0)	0/5 (0)	0/5 (0)
CSP/J		ND	ND	0/8	5/9 (64-1024)	3/5 (64-1024)

<sup>1</sup>Weeks post-infection<sup>2</sup>Number of positive / Number of tested (Range of virus neutralizing titers)<sup>3</sup>ND: not done**Table 5.** Summary of histopathologic findings

Group	Lymphocytic infiltration					Myeloid cell infiltration				
	1 <sup>1</sup>	2	4	7	10	1	2	4	7	10
Oil	2/2 <sup>2</sup>	3/3	3/3	3/3	3/3	1/2	0/3	1/3	0/3	0/3
Oil/J	1/3	3/3	4/4	3/3	7/7	0/3	1/3	4/4	0/3	4/7
CSP	3/3	2/2	3/3	2/2	3/3	2/3	0/2	1/3	0/2	0/3
CSP/J*	3/3	3/3	4/4	2/2	7/7	3/3	1/3	3/4	2/2	7/7

<sup>1</sup>Weeks post-infection<sup>2</sup>Number of chickens with infiltration / Number of chickens examined.

\* At 7 weeks post-infection, one nephroblastoma was observed in the kidney.

Davis). No evidence of infection with other pathogens was detected in the chickens used in the experiment.

### Viremia

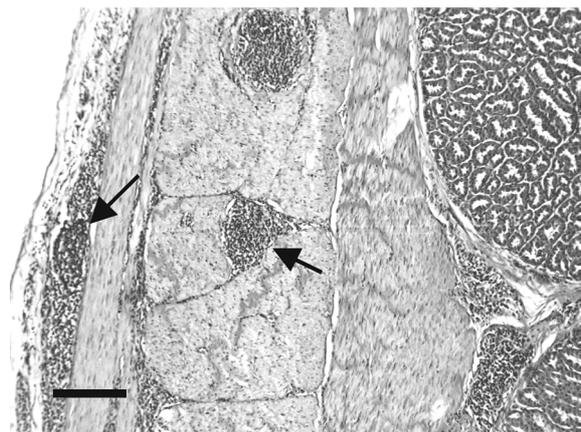
Presence of virus was successfully detected in plasma from infected chickens by real time RT-PCR using SYBR Green I dye. As shown in Table 3, viremia was detected only in infected groups throughout the experiment. Early in the experiment, the ratio of positive samples to negative samples was similar but at 10 weeks postinfection 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<sub>50</sub> (data not shown), we divided virus titers into high ( $10 \text{ pg} > V$ , corresponding to  $> 10^5$  TCID<sub>50</sub>), medium ( $0.1 < V < 10 \text{ pg}$ , corresponding to  $10^3$  to  $10^5$  TCID<sub>50</sub>) and low ( $V < 0.1 \text{ pg}$ , corresponding to  $10^3$  TCID<sub>50</sub>). As shown in Table 4, the composition of the virus titers 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

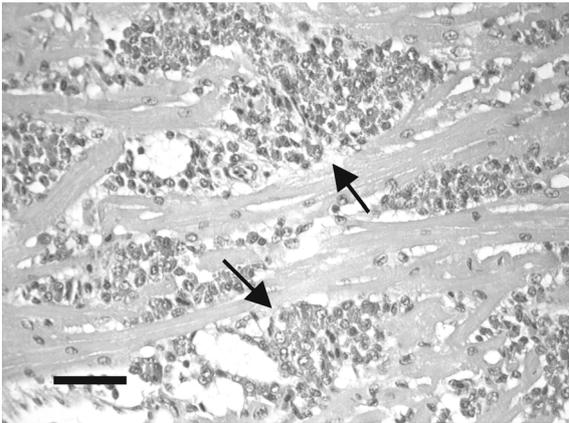
The results of virus neutralization tests are summarized in Table 4. Neutralizing antibody was first detected at 4 weeks post-infection in the Oil group. More than half of the samples tested had neutralizing antibody at the end of the experiment. The percentage and titers of the neutralizing antibody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

### Histopathology

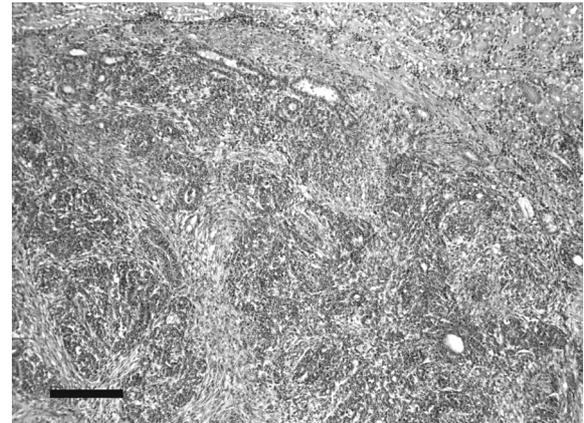
All of the tissue samples collected from necropsy were examined microscopically and the results are summarized in Table 5. Most of the chickens had lymphocytic infiltrates. Nodular infiltrates of lymphocytes were present in multiple organs including brain, heart, lung, kidney, liver, proventriculus (Fig. 1), ventriculus, spleen, small and large intestines, bone marrow and pancreas. Frequency of



**Fig. 1.** Proventriculus. H&E. A 6 week-old chicken from CSP treated/ uninfected group. Multifocal infiltrations of lymphocytes within muscle layer and serosa (arrow). Bar=400  $\mu\text{m}$ . 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. 2.** Heart. H&E. A 12 week-old chicken from CSP treated/infected group. Infiltrating multiple aggregates of myeloid cells (arrow) within the myocardium. Bar=100  $\mu$ m.



**Fig. 3.** 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. Bar=200  $\mu$ m.

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. 3). Minimal to mild focal myeloid cell infiltrates were present in heart (Fig. 2), liver, lung, 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

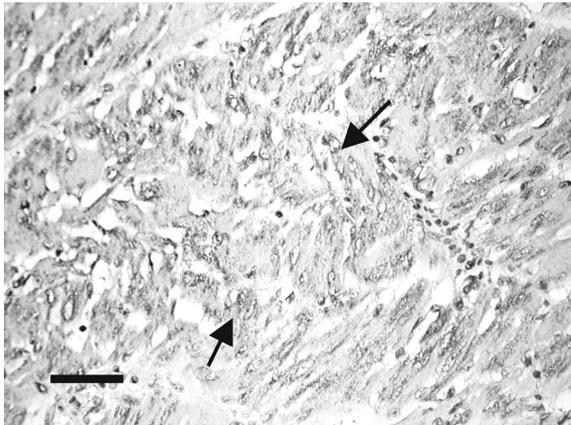
Monoclonal antibody against ALV-J successfully detected expression of viral antigen within the formalin fixed tissue sections. The distribution of viral antigen among the tissue-specific components of the standard tissues was summarized in Table 6. The greatest antigen expression (mean score per tissue >1.0) was observed in the heart (Fig. 4) and kidney (Fig. 5). Many other tissues including lung, ventriculus, bursa of Fabricius and liver (Fig. 6) were variably positive. In addition to staining of tissue specific components, viral antigen also stained in

**Table 6.** Viral antigen expression\* at 1, 4 and 10 weeks post-infection in tissues infected with ALV-J (ADOL-7501) as 2 weeks of age

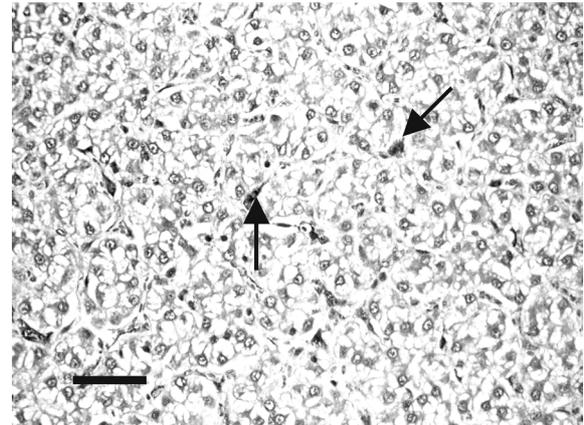
Tissue	Weeks post-infection					
	1 weeks		4 weeks		10 weeks	
	Oil/J	CSP/J	Oil/J	CSP/J	Oil/J	CSP/J
Brain	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Bursa	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)	1/3 (0.7)
Heart	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.7)	2/3 (1.3)
Intestine	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Kidney	0/3 (0)	0/3 (0)	1/3 (0.3)	1/3 (0.3)	2/3 (0.7)	2/3 (1.3)
Liver	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.7)	1/3 (0.7)
Lung	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)
Marrow	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Nerve	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Pancreas	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Proventriculus	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)
Spleen	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	2/3 (1)
Thymus	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.7)
Ventriculus	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (0.3)

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

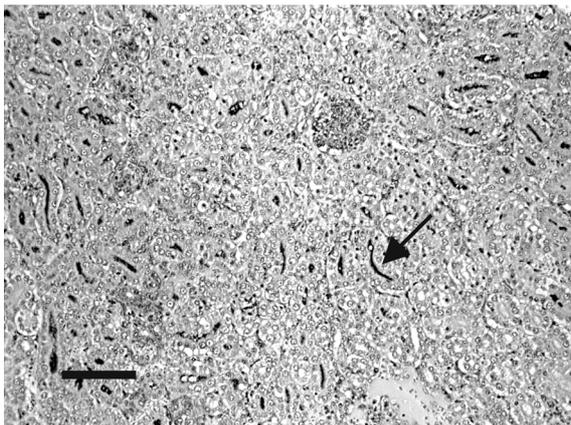
\*\* Tissue-specific cells evaluated



**Fig. 4.** 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 diffusely stained within the myocardial fibers (arrow). Bar=100  $\mu$ m.



**Fig. 6.** 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 (arrow). Bar=100  $\mu$ m.



**Fig. 5.** 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 luminal surface of the renal tubular epithelial cells (arrow). Bar=200  $\mu$ m.

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 [7]. Nowak *et al.* [28] showed that CSP acts as a selective T-cell suppressor in chickens. Suresh and Sharma [42] 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 [21]. 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 A on T-cell function. Cyclosporin A 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 [22,33]. 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 A treatment [21], even though antibody-based flow cytometric analyses appeared unaffected.

The degree of immunosuppression caused by MDV infection is variable with different isolates [5,25,27]. In our experiment, most of the chickens acquired Mareks 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 [6,15,44]. Coinfection with ALV-J and vvMDV is associated with an increased expression of lymphomas, myelocytomas, and lymphocytic infiltrative peripheral neuritis [46]. 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 [47]. 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 groups had MDV to a similar extent.

Congenital infection and neonatal infection with ALV-J causes significant decrease in body weight in broilers [40]. Viral infection of thyroid and the pituitary gland may be the cause for this effect [41]. 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 [3,36,38].

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 dimers 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 [2,19]. 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 [34]. 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.

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