

## Tumors Associated with Avian Leukosis Virus Subgroup J in Layer Hens during 2007 to 2009 in China

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**ABSTRACT.** In the 3 years leading up to November 2009, 6 different types of naturally occurring neoplasms associated with avian leukosis virus subgroup J (ALV-J) were diagnosed by histopathology, polymerase chain reaction (PCR) and immunohistochemistry (IHC) in 140 layer hens out of approximately 100,000. The most prevalent tumor type was hemangioma (50%) in commercial layer flocks; the second most prevalent neoplasm type was myelocytoma (38.6%); a small number of ALV-J positive lymphomas (4.3%) that were not associated with Marek's disease (MD) or lymphoid leukosis (LL) was observed. Histiocytic sarcomas (2.1%) were found mainly in the spleen, liver and kidney. Fibrosarcomas (2.8%) presented as metastatic thigh, liver, lung and kidney neoplasms. Three cases of intestinal adenocarcinoma (2.1%) were found associated with ALV-J. Chickens with multiple tumors were a common phenomenon. Usually, hemangiomas plus myelocytomas (8.6%), myelocytomas plus histiocytic sarcomas (2.1%), hemangioma plus myelocytoma and lymphoma (3.6%) were found in various viscera organs. The present report describes the occurrence of multiple neoplasms associated with ALV-J in field layer hens.

**KEY WORDS:** avian leukosis virus subgroup J (ALV-J), layer chickens, multiple neoplasms.

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Avian leukosis virus subgroup J (ALV-J), first described in the United Kingdom in 1991 and later in other countries, has been associated primarily with myeloid leukosis in meat-type chickens [8, 11–15]. ALV-J has caused severe economic losses in broiler breeders throughout the world. Vertical transmission from broiler breeders to progeny is more frequent with ALV-J than with other ALV subgroups [25].

ALV-J in the field has occurred almost entirely in meat-type chickens several years ago. Layers have rarely become infected, but experimental studies with Rous sarcoma virus having the inserted envelope gene of ALV-J by Payne [16] have shown that both layers and turkeys are susceptible to ALV-J infection, while other poultry and game birds appear to be resistant.

The frequencies of different tumor types seen by Payne [11] during 1996–1998 from suspect ALV-J infected flocks were 58% for myeloid leukosis (ML), 12% for histiocytic sarcoma (HS), 9% for erythroblastosis and 5% for blast cell tumors. Numerous birds had more than one type of tumor, and some had up to four different types.

In chickens, layer strains are considered to be at risk in China and their exposure to ALV-J contamination has already been demonstrated by Xu [23, 24, 26] and Cheng [6]. ALV-J in egg-type chickens with ML has already resulted in significant economic losses in China. Gingerich [9] also reported that a recombinant ALV containing the long terminal repeat (LTR) of the subgroup J and the envelope of subgroup B was isolated from commercial White

Leghorn flocks with ML. Cheng [5] reported cases of ML diagnosed in a local Chinese breed that is commonly recognized as a dual purpose type of chicken, both for eggs and meat, in Shandong Province, P.R. China.

The naturally occurring neoplasms encountered during necropsy in many layer chickens from a population of about 100,000 populations in China during 2007–2009 and the relative incidence of the more common hemangiomas neoplasms are documented in this article. Some of the more unusual cases are described in detail.

### MATERIALS AND METHODS

**Cases history:** Between November 2007 and November 2009, 140 commercial brown (97.9%) and white (2.1%) egg-type hens, from different breeder sources that were 26 to 29 weeks of age were submitted to the College of Veterinary Medicine for investigation of mortality and production problems. After diagnosis, we visited some of the farms that submitted hens. During the visits, some common symptoms were seen in different flocks. Approximately 5–15% of the birds in the flocks were obviously out of production and had shriveled combs. Bluish colored blister were seen in several flocks. These blood blisters varied in size from a few millimeters to 5 cm. Bleeding frequently occurred from larger blood blisters. Most of the chickens submitted here exhibited stunting, paleness, paralysis, depression clinical signs of disease.

**Histopathology:** Live birds were euthanized by cervical dislocation. All birds were given a thorough necropsy examination, and tissues were fixed in 10% buffered formalin, embedded in paraffin wax; 4 to 6  $\mu$ m sections were routinely stained with hematoxylin and eosin (HE) and

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examined for microscopic lesions. Tumors were diagnosed on the basis of characteristic gross and microscopic lesions.

**Immunohistochemistry:** The tissues selected for IHC were from the liver, spleen and kidney. IHC examination was carried out to detect ALV-J and reticuloendotheliosis virus (REV) *in situ* by probing respective tissue samples with mouse monoclonal anti-ALV-J (G2-3) [18] or mouse monoclonal anti-REV (11B118+11B154) [4] (provided by Professor Zhizhong Cui).

All incubations were carried out at room temperature. Neutral buffered formalin-fixed tissue sections were cut to 4  $\mu$ m and mounted on poly-L-lysine-coated slides. The sections were deparaffinized and rehydrated, and the endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. After washing with distilled water 3 times for 3 min per wash, sections were treated with 0.1% trypsin in 0.1% calcium (pH 7.8) for 10 min to expose epitopes. The sections were then washed 3 times for 5 min per wash with phosphate buffered saline (PBS pH 7.6) and blocked with 5% bovine serum albumin and 10% fetal calf serum in PBS for 10 min. The slides were then incubated with primary antibody (1:400) for 1 hr, washed with PBS and incubated with biotinylated secondary anti-mouse antibody for 30 min. Reactivity was assessed by observing the formation of a brown precipitate following an incubation with 3,3'-diaminobenzidine-hydrogen peroxide substrate solution. The reaction was stopped with water, and the slides were counterstained with hematoxylin. The slides were then examined under light microscopy using an Olympus DP20 (Olympus, Center Valley, PA, U.S.A.).

**Polymerase chain reaction (PCR):** PCR was used to identify the proviral gp85 for ALV-J, ALV-A, ALV-B and LTR for REV and MDV, respectively. The PCRs were performed as described previously [5, 19, 20]. Samples PCR-positive for ALV-A, ALV-B, REV and MDV were excluded in this report. The primers used for PCR are shown in Table 1.

## RESULTS

The types of neoplasms were identified by pathology, and then confirmed by PCR and IHC (double positive). The tumor locations and numbers are provided in Table 2. Details of the neoplasms are provided below. All tumors of PCR and IHC negative for ALV-J were not included in this study.

**Hemangiomas:** Hemangiomas were found on the skin of the trunk, digitus (Fig. 1A), joint (Fig. 1B), face and wing, and in the liver (Fig. 1C), spleen, heart (Fig. 1D), small intestine (Fig. 1E) and pancreas of 70 hens. Most of the affected birds were derived from the same brown Hy-line strains, and these cases occurred as several distinct outbreaks.

The early hemangiomas lesions were bluish in color and relatively soft in consistency; however, as they developed, the bluish color was slowly replaced by a pink color identical to that of the adjacent skin. Microscopically, the tumors were both reticular (Fig. 1F) and capillary (Fig. 1G) in type, with infiltration of myeloid cells and lymphocytes. Endothelial-like cells were frequently found within the lumen of

Table 1. Primer sequences of ALV-A, B, J, MDV and REV

Virus	Primer sequence		Fragment sizes/bp
ALV-J (env)	Forward	5'-ATGGGAGTTCATCTATTGCAACAACCAG-3'	924
	Reverse	5-TTAGCGCCTGCTACGGTGGTGACC-3'	
ALV-B (env)	Forward	5'-CGAGAGTGGCTCGCGAGATGG-3'	1,300
	Reverse	5'-AGCCGGACTATCGTATGGGGTAA-3'	
ALV-A (env)	Forward	5'-CGAGAGTGGCTCGCGAGATGG-3'	1,100
	Reverse	5'-CCCATTTCCTCCTCTCCTTGTA-3'	
MDV (132 bp)	Forward	5'-TACTTCCTATATAGATTGAGACGT-3'	132
	Reverse	5'-AGCTAGGCTCGTATGAA-3'	
REV (env)	Forward	5'-AGCTAGGCTCGTATGAA-3'	438
	Reverse	5'-TATTGACCAGGTGGGTTG-3'	

Table 2. Type, location and number of naturally occurring neoplasms identified in layer chickens submitted for necropsy in 2007–2009

Type of neoplasm (No. cases)	Locations and other details (No. cases)
Hemangiomas (HG; 70)	Skin of the trunk (20), digitus (51), face (5), and wing (10); liver (7), spleen (6), heart (3), small intestine (3), ovary (4), pancreas (3)
Myelocytomas (ML; 54)	Inner sternum (5), rib (3), sacrum (3), cervical vertebra (1); liver (54), spleen (47), kidney (44), ovary (8), proventriculus (22), intestine (6), pancreas (6), lung (8), heart (18), bone marrow (54), thymus (1)
Lymphomas (LM; 6)	Liver (2), spleen (2), heart (1), kidney (2), proventriculus (2), ovary (1)
Fibrosarcomas (FS; 4)	Thigh (2), lung (1), kidney (3), liver (1)
Histiocytic sarcomas (HS; 3)	Liver (2), spleen (3), kidney (3), bone marrow (1), lung (1)
Intestinal adenocarcinoma (IA; 3)	Small intestine (3)
HG+ML (12)	Liver (12), spleen (12), heart (1), small intestine (1), pancreas (2)
HG+HS (3)	Liver (2), spleen (3), kidney (3)
HG+ML+LM (5)	Liver (2), spleen (2), ovary (1)

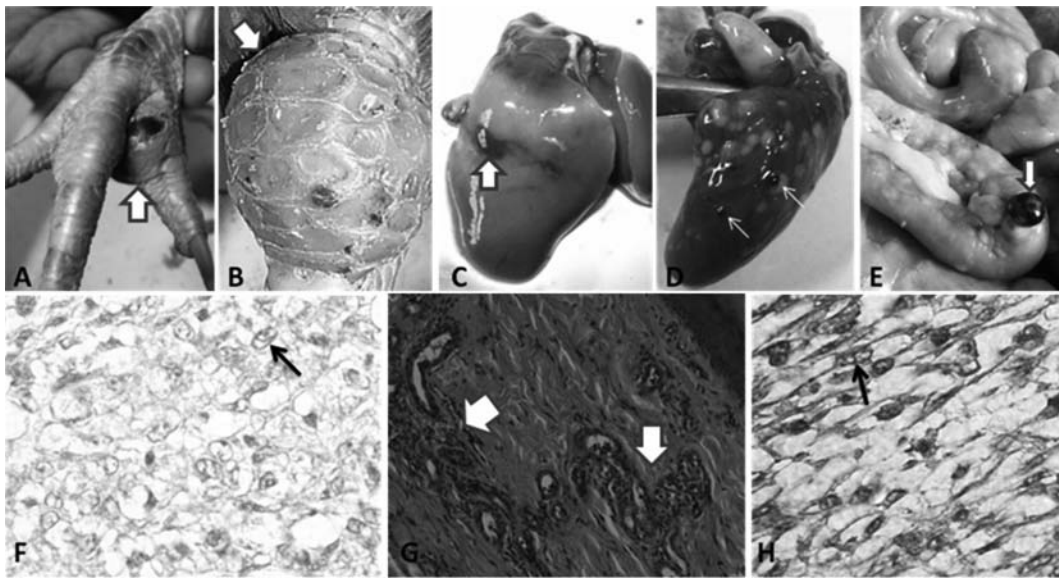


Fig. 1. Hemangioma. Superficial hemangioma on the digitus (A), joint (B), liver (C), heart (D) and duodenum (E). Histopathology of reticular type (F) and capillary type (G) hemangioma were observed. HE stain. Immunohistochemistry showed that the endothelial cells of the hemangioma were positive for ALV-J antibody (G2-3)(H).

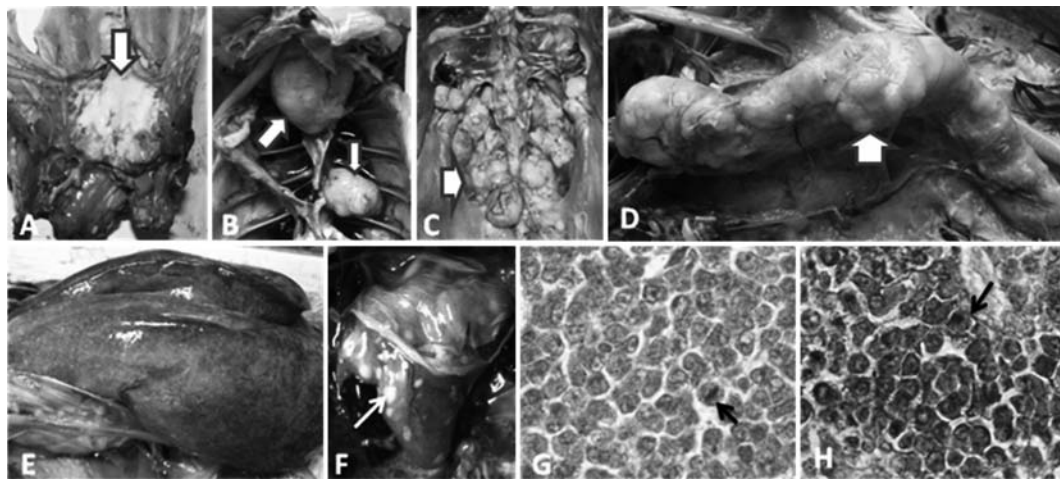


Fig. 2. Myelocytomas. Myelocytomas were found on the inner sternums (A), ribs (B), sacrum (C), and cervical vertebra (D) and in the livers (E) and hearts (F) of the chickens. Histologically, the cells were relatively uniform large myeloid cells with a large number of acidophilic granules in the cytoplasm and were pleiomorphic with a vesicular or lobulated nucleus with a few chromatin clumps (G). Mitotic figures were prominent (arrow) (G). Diffuse expression of G2-3 (ALV-J gp85) antigen was found within the myelocytes in the myelocytoma (H).

the blood channels, apparently proliferating from the intima. Mitotic figures were not observed. Free erythrocytes were present in the lumina, and local hemorrhage was common. Endothelial-like cells expressing ALV-J antigen were detected by IHC (Fig. 1H).

**Myelocytomas:** Myelocytomas were found on the inner sternum (Fig. 2A), rib (Fig. 2B), sacrum (Fig. 2C), cervical vertebra (Fig. 2D), and in the liver (Fig. 2E), spleen, kidney, ovary, proventriculus, intestine, pancreas, lung, heart (Fig.

2F) and bone marrow of the chickens. No tumors were observed in the bursa of Fabricius and brain. Histologically, the cells were relatively uniform large myeloid cells with a large number of acidophilic granules in the cytoplasm and were pleiomorphic with a vesicular or lobulated nucleus with a few chromatin clumps. Mitotic figures were prominent (Fig. 2G). The cells were present as sheets or columns. Most of the affected birds were between 180 to 200 days of age. Some tumor cells expressing ALV-J antigen were

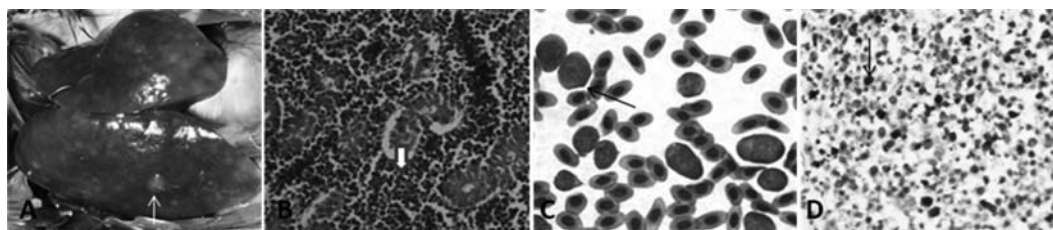


Fig. 3. Lymphomas. The liver (A) and kidney (B; HE stain) contained numerous small discrete foci of uniform lymphoblastoid cells with intense nuclei and a high mitotic rate. Tumor cells were detectable in blood (C). Immunohistochemistry showed diffuse expression of G2-3 (ALV-J gp85) antigen within lymphocytes in lymphomas (D).

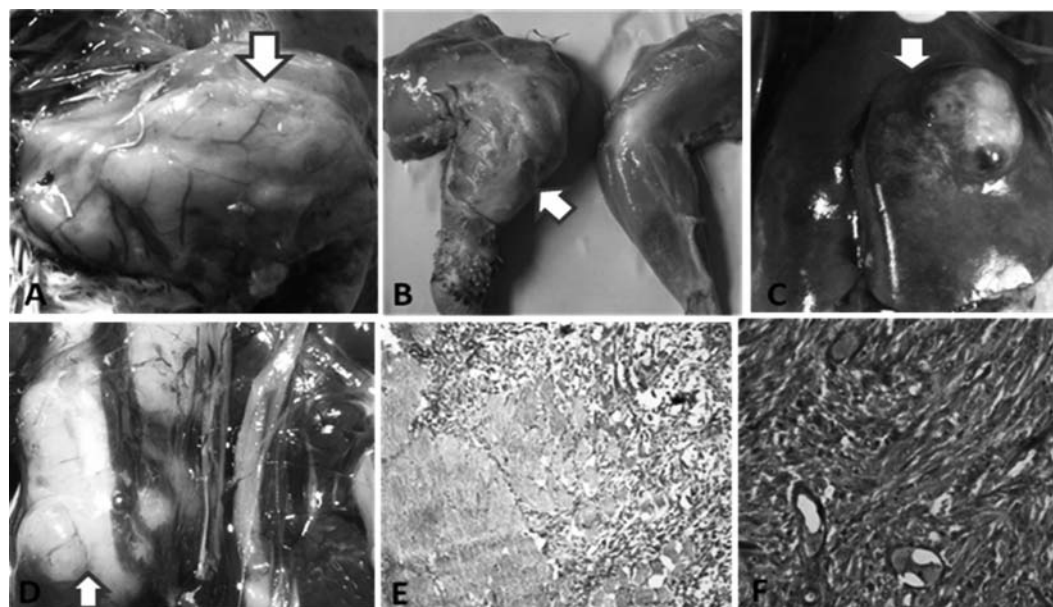


Fig. 4. Fibrosarcomas. Fibrosarcomas were on the thigh (A and B) and in the liver (C) and kidney (D). Neoplasms were firm, pale, encapsulated and composed of plump mature fibrocyte-like cells arranged as whirling bundles in a dense collagenous matrix (E and F). HE stain.

detected by IHC (Fig. 2H).

**Lymphomas:** The spleen, liver (Fig. 3A), kidney (Fig. 3B) and other unspecified viscera contained numerous small discrete foci of uniform lymphoblastoid cells with large vesicular nuclei and a high mitotic rate. In a 26 week-old hen, the ovary, liver, spleen and duodenal Peyer's patch were enlarged, with numerous small grey nodules. We also observed a great many small lymphocytes in the blood (Fig. 3C). The proliferating cells were small lymphocytes that were ALV-J antibody positive (Fig. 3D).

**Fibrosarcomas:** Four cases of fibrosarcomas were found on the thigh (Fig. 4A and 4B), and in the lung, liver (Fig. 4C) and kidney (Fig. 4D). Neoplasms that were firm, pale and encapsulated and composed of plump mature fibrocyte-like cells arranged as whirling bundles in a dense collagenous matrix were considered to be fibromas (Fig. 4E and 4F). Mitotic figures were not common.

**Histiocytic sarcomas:** The histiocytic proliferative

lesions described in the present study were found in Hy-line white layer chickens. There was uniform splenomegaly (Fig. 5A), hepatomegaly (Fig. 5B) and renomegaly characterized by diffuse miliary off-white 1 to 3 mm foci throughout the capsular surface as well as on the cut surface. In addition, the bone marrow (14%) and lungs (7%) occasionally exhibited HS lesions similar to those noted in the spleen.

Histologically, histiocytic cells were found lying particularly in the red pulp and sometimes surrounding sheathed capillaries and in the white pulp (Fig. 5C). The histiocytic cells showed some variability between and within birds. They had a conspicuous eosinophilic cytoplasm, and in some instances, there was slight vacuolation and coalescence of cell margins. The characteristics of the nuclei varied; some nuclei were large and polygonal with an open vesicular structure and obvious nucleolus, and others were smaller, elongated and without a clear nucleolus. Mitoses

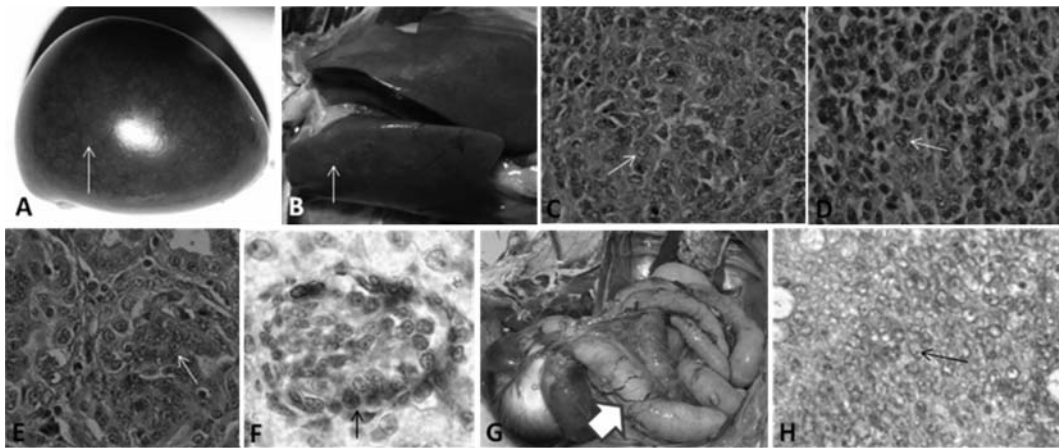


Fig. 5. Histiocytic sarcomas and intestine adenocarcinoma. Putative early histiocytic sarcomas in a chicken (spleen, A). The neoplastic histiocytic population with prominent spindle cell morphology diffusely effaces the splenic architecture (liver, B). Multifocal circumscribed, unencapsulated histiocytic nodules expand the hepatic sinusoids in the periportal areas (HE stain, C). In addition, there is mild to moderate infiltration of lymphocytes and plasma cells (HE stain, E). The neoplastic histiocytic population with prominent glomerulus cells in the kidney (HE stain, E). Diffuse expression of G2-3 (ALV-J gp85) antigen within glomerulus cells (immunohistochemistry, F). Intestinal adenocarcinoma (G). Many cuboidal epithelial cells crowded together and lost their tubule-like structures. No mitotic figures were observed (HE stain, H).

were present, and in some cases, the proliferating histiocytes formed a whorled pattern. Lymphocytes were often present among the histiocytes. Reticulin fibers were found sparsely in the proliferative areas. In the livers, similar diffuse and often extensive proliferations of histiocytes were found around portal areas and in the parenchyma (Fig. 5D). Similar neoplastic cells were also observed within other involved organs (Fig. 5E). Infiltration of variable numbers of lymphocytes, plasma cells and heterophils was also observed in some histiocytic proliferative lesions. Diffuse expression of G2-3 (ALV-J gp85) antigen within glomerulus was observed (Fig. 5F).

**Intestinal adenocarcinoma:** Three cases of intestinal adenocarcinoma were found (Fig. 5G). Many cuboidal epithelial cells crowded together, and lost tubule-like structures. No mitotic figures were observed (Fig. 5H). The nodule was located predominately on the mucosal aspect, but it traversed the muscularis, and that portion penetrating through to the serosal surface was covered by a thick fibrous capsule. Similar nodules were not found elsewhere; the ovary was quiescent, and the mucosa of the oviduct was normal.

**Multiple tumors:** Chickens with multiple tumors were a common phenomenon detected with histopathology. Usually, hemangiomas plus myelocytomas (8.6%), myelocytomas plus histiocytic sarcomas (2.1%), hemangioma plus myelocytoma and lymphoma (3.6%) were found in various viscera organs (Table 2). One kind of tumor cells usually formed one focus.

## DISCUSSION

ALV-J infection in broiler breeder flocks is associated

with the occurrence of ML (myelocytomatosis). ML is a tumor condition that is readily characterized as being comprised of transformed white blood cells from the bone marrow. However in this report, hemangiomas were the most prevalent tumors associated with ALV-J breakout in several commercial layer flocks. Zavala [27] noted that the timing, however, may vary according to "factors such as genetics, environment, management, nutritional status, concomitant infections, immunocompetence and the actual form of transmission".

Hemangiomas were ever the most prevalent neoplasm in the survey of broiler chickens in the U.K. reported by Campbell [3]. He noted that pullet broilers were more commonly affected than cockerels, but the basis for that was not known. Burstein [2] reported an outbreak of hemangiosarcomas in young White Leghorn layers in Israel, with up to 20% mortality.

A strain of ALV was isolated from those affected birds and was shown to induce hemangiosarcomas following experimental inoculation [21]. It is probable that the similar neoplasms reported in this survey also had a viral etiology, as they occurred in related White Leghorn cross birds.

Myelocytomas can be induced by ALV-J and other certain strains of ALV [17]. However in some cases, we found that the myelocytomas caused by ALV-J in layer chickens were more extensive and various than in meat-type chickens. We had checked the env gene of ALV-J from these cases and found an 85 bp fragment depletion (data not shown). We did not know if the depletion caused the severe lesion in the layer chickens. This requires further study.

Arshad *et al.* [1] reported a very low incidence of HS in meat-type chickens infected with ALV-J at hatching (1.1%).

They suggested that the HS lesions may be specific to meat-type chickens and that the absence of HS lesions in meat-type chickens inoculated in ovo may be a result of the low incidence of this tumor type. In our report, HS accounted for 2.1% of the tumors associated with ALV-J.

Fibromas were less prevalent in the domestic fowl than fibrosarcomas, and most of the fibrous neoplasms reported in this article were considered to be fibrosarcomas (4/140=2.8%) because of local invasion, cell pleomorphism and/or metastases.

Histiocytic sarcomas were differentiated from fibrosarcomas because of their greater diversity of cell types and the collagenous matrix in which the histiocytes were embedded [14].

Lymphomas in the domestic fowl were usually considered to be the consequence of virus transformation (MDV, ALV or REV). However, Crittenden [7] reported 10 cases of lymphoid leukosis like lymphomas in SPF birds free from ALV: four of these were also free from Marek's disease, whereas the others had been vaccinated with herpes virus from turkeys, and their status for MDV was not determined. Those cases, together with these two cases in SPF birds, indicated that some lymphomas in domestic fowls may be due to other causes.

Adenocarcinomas originating from the intestines are rare in the domestic fowl and critical evaluation is necessary to differentiate intestinal adenocarcinomas from intestinal serosal implants of metastatic abdominal adenocarcinomas of ovarian or oviductal origin [3]. A few spontaneous cases have been reported in domestic fowls [22], and duodenal adenomas have been induced in guinea fowl following intravenous inoculation with a particular strain of ALV [10].

The neoplasm designated as an intestinal adenocarcinoma in this survey had penetrated the muscularis, so it was locally invasive. The lack of involvement of other organs indicated that it was probably of intestinal origin.

The present article is the first to describe tumors associated with ALV-J in layer chickens. We performed PCR and immunohistochemistry to confirm the results of pathology. The most prevalent neoplasms encountered were hemangiomas, and the second most prevalent neoplasms were myelocytomas. Some other neoplasms, such as fibrosarcomas, histiocytic sarcomas and lymphomas were also demonstrated to be associated with ALV-J.

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