

## Histopathological Characteristics of Spindle-cell Proliferative Disease in Broiler Chickens and Its Experimental Reproduction in Specific Pathogen-Free Chickens

Shigeaki TAKAMI<sup>1,2)</sup>, Masanobu GORYO<sup>1,2)\*</sup>, Toshiaki MASEGI<sup>1,3)</sup> and Kosuke OKADA<sup>1,2)</sup>

<sup>1)</sup>Department of Pathogenic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193 and <sup>2)</sup>Department of Veterinary Pathology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550 and <sup>3)</sup>Department of Veterinary Pathology, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan

(Received 12 June 2003/Accepted 15 October 2003)

**ABSTRACT.** The livers and spleens of 45 broiler chickens (33 to 79 days old) suspected of Marek's disease (MD) at meat inspection were collected and examined histopathologically. Macroscopically, they were enlarged from two to three times, and multiple, small, white areas of plaque or infrequent, large, white nodules were observed in most cases. Only 9 birds (20%) were diagnosed with MD based on the histological examination, while the other 35 birds (78%) had tumor-like proliferative lesions in the Glisson's sheath of the liver and in the white pulp and around the sheathed arteries of the spleen, which differs from the pattern seen in MD. The proliferating cells were mainly spindle-shaped or pleomorphic, and were variable in size with abundant eosinophilic cytoplasm. The disease giving rise to the present lesions was diagnosed tentatively as spindle-cell proliferative disease. Total 50 1-day-old specific pathogen-free chicks by serial passage were inoculated intramuscularly with 0.1 ml of a 10% homogenate of the affected livers or spleens. Microscopically, one inoculated bird, necropsied at 6 weeks of age, had spindle-cell proliferative lesions in the spleen similar to the lesions of naturally occurring spindle-cell proliferative disease. Some birds had tumorous lesions, including renal adenoma, leiomyosarcoma and myxosarcoma. Reverse transcriptase-polymerase chain reaction performed using primers specific for subgroup J avian leukosis virus (ALV) produced specific amplifications of subgroup J ALV genes in 4 of 5 field cases examined.

**KEY WORDS:** broiler chicken, Marek's disease, spindle-cell, subgroup J avian leukosis virus, tumor-like proliferative lesion.

*J. Vet. Med. Sci.* 66(3): 231-235, 2004

Among the tumors found in broiler chickens at meat inspection in Japan, losses associated with Marek's disease (MD) are taken the most seriously. MD is very important as a lymphoproliferative disease in chickens. MD is caused by the MD virus, which belongs to the family *Herpesviridae* [3]. A disease highly similar to MD at necropsy is lymphoid leukosis (LL). LL is caused by avian retroviruses of the leukosis/sarcoma group belonging to the family *Retroviridae* [7]. Macroscopically, both diseases are characterized by severe enlargement of the liver and spleen with many white plaques and nodules [3, 7]. Microscopically, MD is characterized by proliferation of various-sized lymphoid cells, whereas LL is characterized by follicular proliferation of uniform lymphoblastic cells [3, 7]. Recently, multicentric histiocytosis (MH) [5, 6] and histiocytic sarcomatosis (HS) [1] have been reported as similar diseases associated with enlargement of the liver and spleen. MH and HS resemble each other histopathologically, but clearly differ from MD and LL with respect to the origin of the proliferating cells [1, 3, 5-7]. Experimental induction of MH in broiler and specific pathogen-free (SPF) leghorn chickens has been reported [4].

The aims of this study were to determine the histopathological features of a disease resembling but distinct from MD detected at meat inspection, and to reproduce this disease in SPF chickens by inoculation with a homogenate of the affected liver and spleen, and to clarify the etiology.

### MATERIALS AND METHODS

**Field cases:** Forty-five broiler carcasses (33 to 79 days old) from 17 different flocks were condemned as showing MD because of tumorous lesions in the liver and spleen at meat inspection. The livers and spleens were collected for histopathological and immunohistochemical examinations.

**Experimental cases:** All chicks derived from a SPF White Leghorn line P2 flock, free from antibody to adenovirus, avian infectious bronchitis virus, chicken anemia virus, infectious bursal virus, MD virus, Newcastle disease virus, reovirus and subgroup J avian leukosis virus (ALV), were hatched at our laboratory in Iwate University. The chicks were housed in small isolated boxes with food and water *ad libitum* in sterilized isolated room. One-day-old SPF chicks were inoculated intramuscularly in the thigh muscle with 0.1 ml of a 10% homogenate of the following frozen organs in cell culture medium. The number of prepared chicks for groups 1, 2 and 3 was 16, 20 and 14 birds, respectively. The homogenates were prepared from the spleen of 1 field case (group 1), livers of three 22-day-old chickens of group 1 (group 2) and the liver of one 41-day-old chicken of group 2 (group 3). The experimental periods of groups 1, 2 and 3 were 13, 10 and 10 weeks, respectively. Seven chicks of the same age as the inoculated chicks were prepared as uninfected controls. All dead or killed chickens were necropsied and the tissue samples were collected from them.

**Histopathology:** Tissue samples were fixed in 10% formalin, routinely processed, and embedded in paraffin-wax. Sections were cut approximately 4 µm thick and stained

\*CORRESPONDENCE TO: GORYO, M., Department of Veterinary Pathology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan.

with hematoxylin and eosin (HE). All field-case sections were also subjected to Masson's trichrome staining, the reticulin silver impregnation method and elastica van Gieson staining. All stained sections were examined using an optical microscope.

**Immunohistochemistry:** Indirect immunoperoxidase staining was performed by the avidin-biotin-peroxidase complex (ABC) method using a commercial ABC kit (Vectastain *Elite* ABC kit, Vector Laboratories, Inc., U.S.A.). Paraffin-embedded sections of the liver and spleen from 4 field cases of spindle-cell proliferative disease were deparaffinized, rehydrated in xylene followed by a graded series of ethanol and distilled water, and rinsed in phosphate-buffered saline pH 7.2. After blocking the non-specific reaction, the sections were incubated overnight at 4°C with the primary antibodies. The primary antibodies were monoclonal mouse anti- $\alpha$ -smooth muscle actin, polyclonal rabbit anti-cytokeratin, polyclonal rabbit anti-factor VIII related antigen, polyclonal rabbit anti-S-100 protein (Zymed Laboratories, Inc., U.S.A.), monoclonal mouse anti-desmin, polyclonal rabbit anti-lysozyme and monoclonal mouse anti-vimentin (DAKO A/S, Denmark). The staining was carried out with a biotinylated secondary antibody and ABC reagent. The antigen localization was visualized by incubation of the sections for 3 min at room temperature with 3, 3'-diaminobenzidine- $H_2O_2$  solution. The sections were counterstained with hematoxylin and examined using an optical microscope.

**Reverse transcriptase-polymerase chain reaction (RT-PCR):** Five cases chosen randomly from field cases of spindle-cell proliferative disease were examined. The extraction of RNA from frozen livers was performed using TRIzol Reagent (Gibco-BRL Life Technologies, U.S.A.) according to the manufacturer's instructions. RT-PCR based on specific primers H5 (5'-GGA TGA GGT GAC TAA GAA AG-3') and H7 (5'-CGA ACC AAA GGT AAC ACA CG-3') [8] of subgroup J ALV was performed using a commercial kit (Ready-To-Go RT-PCR Beads, Amersham Pharmacia Biotech, U.S.A.). A mixture (50  $\mu$ l) containing 46 or 47  $\mu$ l of diethylpyrocarbonate-treated water (Bio 101, Inc., U.S.A.), 1  $\mu$ l of each primer and 1 or 2  $\mu$ l of template RNA was added to the reagents for reverse transcription in the kit. Reverse transcription was performed by incubating this mixture at 42°C for 30 min. The PCR conditions were inactivation of the reverse transcriptase and complete denaturation of the template by incubation at 95°C for 5 min, followed by 35 cycles of incubation at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. RNA from the liver of a chicken that had myelocytomatosis demonstrated that it was positive for subgroup J ALV was used as a positive control. RNA from the liver of a 10-day-old SPF chick was used as a negative control.

## RESULTS

**Field cases:** Macroscopically, the livers and spleens of

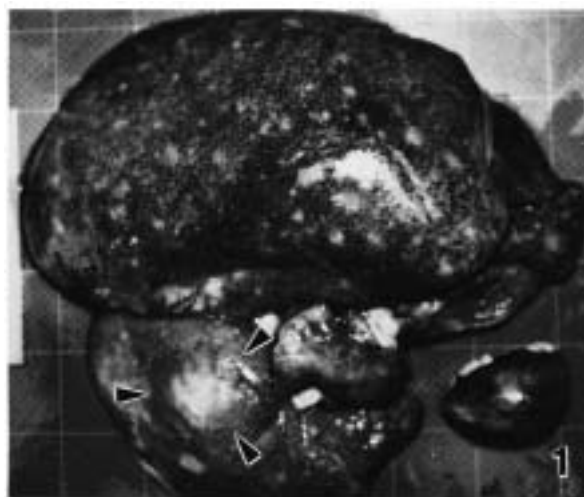


Fig. 1. Liver and spleen of Marek's disease in a field case. Severe enlargement of liver with multiple white plaques and a large nodule (arrowheads).

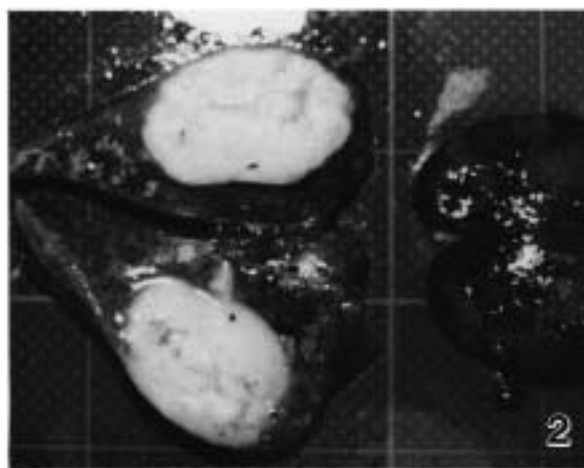


Fig. 2. Liver and spleen of Marek's disease in a field case. Cross section of the large white nodule seen in Fig. 1.

affected chickens were enlarged from two to three times, and multiple, small, white areas of plaque or infrequent, large, white nodules were observed in most cases (Figs. 1–3). The degree of splenomegaly was severer than that of hepatomegaly. The histological diagnosis is summarized in Table 1. Microscopically, only 9 birds (20%) had focal or diffuse proliferation of various-sized lymphoid cells (Fig. 4). Many mitotic figures were observed. Thirty-five birds (78%, 33 to 79 days old) had tumor-like proliferative lesions differing from the lesions of MD. The lesions were multifocal and were composed of proliferating spindle-shaped cells in the Glisson's sheath of the liver and in the white pulp and around the sheathed arteries of the spleen (Fig. 5). The proliferating cells were mainly spindle-shaped or pleomorphic, and variable in size with abundant eosinophilic cytoplasm



Fig. 3. Liver and spleen of spindle-cell proliferative disease in field cases. Severe enlargement of spleen with many small white plaques, but not nodules.

Table 1. Histological diagnosis of 45 field cases

Disease	Number of cases	Percentage
Marek's disease	6	13%
Marek's disease and Spindle-cell proliferative disease	3	7%
Spindle-cell proliferative disease	32	71%
Others	4	9%
Total	45	100%

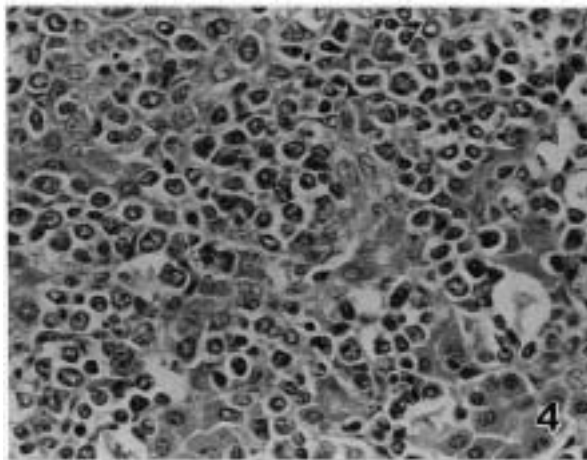


Fig. 4. Marek's disease in the liver of a field case. Proliferation of lymphoid cells is observed. The cells have round nuclei that are variable in size and minimal eosinophilic cytoplasm. HE stain,  $\times 450$ .

(Fig. 6). Mitotic figures were not observed. Three birds (7%) had both types of lesions. Regarding other lesions (4 cases; 9%), severe extramedullary hematopoiesis in the liver of 1 bird and hyperplasia of ectopic lymphoid tissue with germinal centers in the livers and spleens of 3 birds was observed.

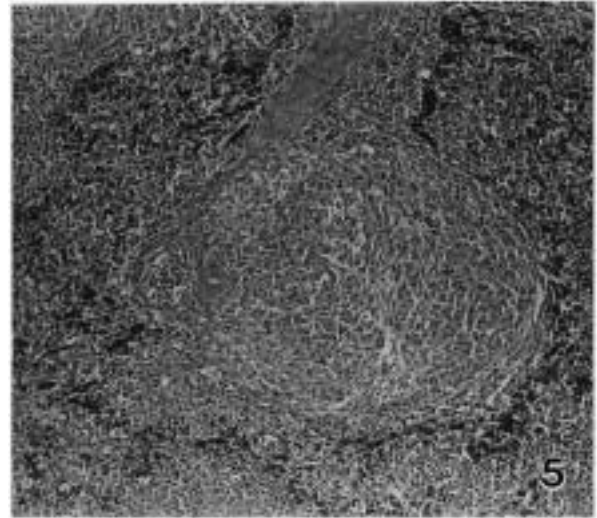


Fig. 5. Spindle-cell proliferative disease in the spleen of a field case. Proliferation of spindle cells differing from lymphoid cells in the white pulp and around the sheathed arteries of the spleen. HE stain,  $\times 110$ .

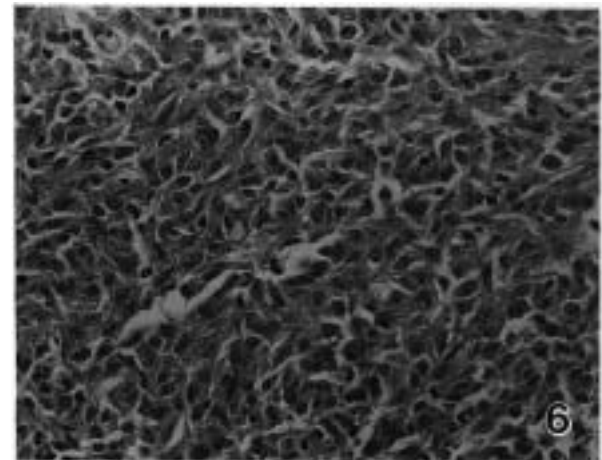


Fig. 6. Spleen of spindle-cell proliferative disease in a field case. Higher magnification of proliferative lesion in Fig. 5. The proliferating cells are mainly spindle-shaped or pleomorphic, and variable in size with abundant eosinophilic cytoplasm. HE stain,  $\times 410$ .

Table 2. Histological lesions of chickens in experimental cases

Group	Spindle-cell proliferative disease	Tumors
1	0/16*	1/16
2	1/20	1/20
3	0/14	2/14
Total	1/50	4/50

\* Number of chickens with lesions/total examined.

In the proliferative lesions that differed from MD, a few fine, blue-colored fibers were observed among the proliferating cells in specimens stained with Masson's trichrome stain. In specimens stained with the reticulin silver impregnation method, the fine fibers were black and seen to be located in the midst of several proliferating cells. No significant finding regarding these proliferating cells was obtained by elastica van Gieson staining. Immunohistochemically, no significant reaction with antibody against any of the antigens examined was detected in the proliferating cells.

**Experimental cases:** At necropsy, the weight of all chickens of the experimental group (the mean weight: group 2: 402 g, group 3: 500 g) was less than that of chickens of the control group (the mean weight: 611 g). Macroscopically, masses in the skin were observed in 2 birds of group 3. The histological lesions are summarized in Table 2. Microscopically, 1 bird of group 2, necropsied at 6 weeks of age, had proliferative lesions differing from MD in the white pulp and around the sheathed arteries of the spleen, similar to the lesions in the naturally occurring field cases (Fig. 7). The proliferating cells were mainly spindle-shaped or pleomorphic, and variable in size with abundant eosinophilic cytoplasm (Fig. 8). Mitotic figures were not observed.

Renal adenoma in 1 bird of group 1, leiomyosarcoma in the kidney of 1 bird of group 2 and myxosarcoma in the skin of 2 birds of group 3 were observed.

**RT-PCR:** Amplification of a 545 bp RT-PCR product comigrating with that of a positive control for subgroup J ALV was seen in 4 of 5 field cases (Fig. 9).

## DISCUSSION

MD Lymphoma is taken the most seriously of all tumors leading to condemnation of broiler chickens at meat inspection in Japan. The diagnosis of MD requires histological differentiation from LL, reticuloendotheliosis (RE) and big liver and spleen disease (BLS) because the signs of these diseases are similar to those of MD at necropsy [2, 3, 7, 9]. In field cases examined here, only 9 of 45 birds (20%) were diagnosed with MD by histopathological examination.

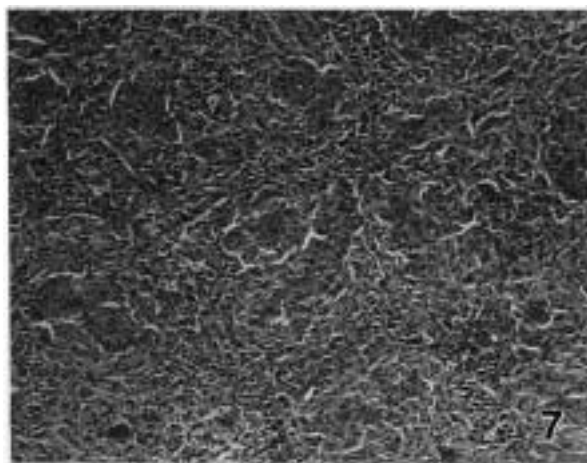


Fig. 7. Spindle-cell proliferative disease in the spleen of a chicken from experimental group 2. Proliferation of spindle cells in the white pulp and around the sheathed arteries. HE stain,  $\times 120$ .

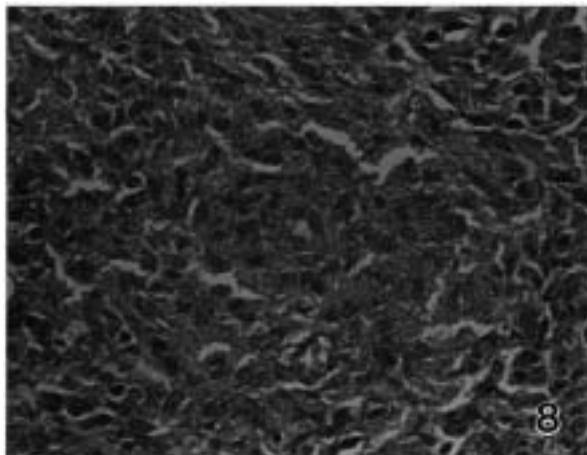


Fig. 8. Spindle-cell proliferative disease in the spleen of a chicken from experimental group 2. Higher magnification of a lesion in Fig. 7. The proliferating cells are mainly spindle-shaped or pleomorphic, and variable in size with abundant eosinophilic cytoplasm. HE stain,  $\times 420$ .

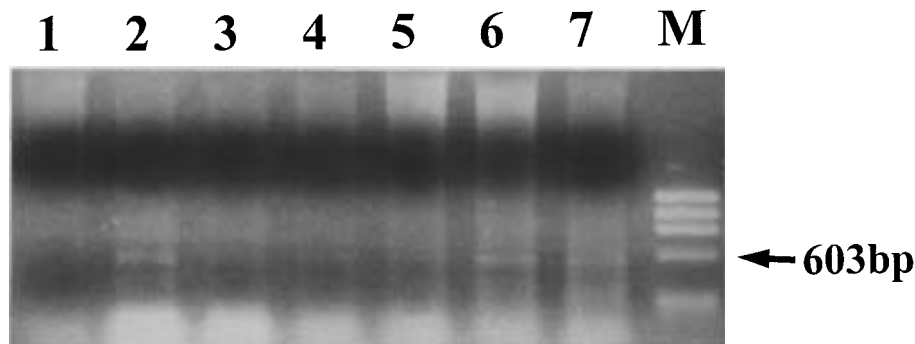


Fig. 9. RT-PCR amplification of RNA isolated from the livers of 5 field cases using primers specific for subgroup J ALV. Lanes: 1: negative control; 2–6: samples; 7: positive control; M:  $\phi$ 174/Hae III digest size marker.

These 9 birds had macroscopically visible large nodules and histopathologically detectable focal or diffuse proliferation of various-sized lymphoid cells. These findings corresponded to those of previous investigations of MD [3].

Tumor-like proliferative lesions differing from MD lesions, the most interesting finding in the field cases, were observed in 35 birds (78%). These lesions were located in the Glisson's sheath of the liver and in the white pulp and around the sheathed arteries of the spleen. The proliferating cells in the lesion were mainly spindle-shaped or pleomorphic, and variable in size with abundant eosinophilic cytoplasm. The disease giving rise to these lesions was diagnosed tentatively as spindle-cell proliferative disease based on the morphological characteristics of the proliferating cells and the areas in which the lesions were forming. Histopathologically, spindle-cell proliferative disease was similar to MH and HS, but clearly differed from MD, LL, RE and BLS [1–3, 5, 7, 9]. In lesions of MH and HS, the origin of the proliferating cells is the histiocyte [1, 5, 6]. Although we performed special staining and immunohistochemical examinations aimed at determining the origin of the proliferating cells of spindle-cell proliferative disease, we could not determine their origin. To determine whether these lesions were related to MH or HS, it will be necessary to determine the origin of the proliferating cells by further detailed examinations.

In our experimental cases, spindle-cell proliferative disease was reproduced in the spleen of 1 bird of group 2. It was suggested that an infectious pathogen might cause the present lesions and that the virulence of the pathogen might be retained during passage through chickens *in vivo*.

Specific amplification of subgroup J ALV genes was obtained in 4 of 5 field cases of spindle-cell proliferative disease by RT-PCR. Some tumors, including leiomyosarcoma, myxosarcoma and renal adenoma, were formed in the experimental cases. Although it may therefore be suspected that spindle-cell proliferative disease is associated with infection by tumorigenic subgroup J ALV [7], we have not yet been able to prove such an association. Therefore the etiology of spindle-cell proliferative disease remains yet unknown. Further studies will be needed to determine the origin of the proliferating cells and the etiology of spindle-

cell proliferative disease.

**ACKNOWLEDGEMENT.** The authors would like to thank Mr. Jouji Honda, The Meat Inspection Center of Fukushima Prefecture, Fukushima, Japan, for the gift of the field cases.

#### REFERENCES

1. Arshad, S. S., Bland, A. P., Hacker, S. M. and Payne, L. N. 1997. A low incidence of histiocytic sarcomatosis associated with infection of chickens with the HPRS-103 strain of subgroup J avian leukosis virus. *Avian Dis.* **41**: 947–956.
2. Barnes, H. J. 1997. Big liver and spleen disease. pp. 1038–1040. *In: Diseases of Poultry*, 10th ed. (Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. eds.), Iowa State University Press, Ames.
3. Calnek, B. W. and Witter, R. L. 1997. Marek's disease. pp. 369–413. *In: Diseases of Poultry*, 10th ed. (Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. eds.), Iowa State University Press, Ames.
4. Goodwin, M. A., Hafner, S., Bounous, D. I., Brown, J., Smith, E. and Fadly, A. 1999. Multi-centric histiocytosis: Experimental induction in broiler and specific pathogen-free leghorn chickens. *Avian Pathol.* **28**: 273–278.
5. Hafner, S. and Goodwin, M. A. 1997. Multicentric histiocytosis. pp. 1047–1048. *In: Diseases of Poultry*, 10th ed. (Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. eds.), Iowa State University Press, Ames.
6. Hafner, S., Goodwin, M. A., Smith, E. J., Bounous, D. I., Puette, M., Kelley, L. C., Langheinrich, K. A. and Fadly, A. M. 1996. Multicentric histiocytosis in young chickens. Gross and light microscopic pathology. *Avian Dis.* **40**: 202–209.
7. Payne, L. N. and Fadly, A. M. 1997. Leukosis/sarcoma group. pp. 414–466. *In: Diseases of Poultry*, 10th ed. (Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. eds.), Iowa State University Press, Ames.
8. Smith, L. M., Brown, S. R., Howes, K., McLeod, S., Arshad, S. S., Barron, G. S., Venugopal, K., McKay, J. C. and Payne, L. N. 1998. Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. *Virus Res.* **54**: 87–98.
9. Witter, R. L. 1997. Reticuloendotheliosis. pp. 467–484. *In: Diseases of Poultry*, 10th ed. (Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. eds.), Iowa State University Press, Ames.