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

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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 tumors such as lymphomas, and in 3 cases, meningioma and hemangiosarcoma. 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.

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 disease virus, MD virus, Newcastle disease virus, reovirus and subgroup J avian leukosis virus (ALV), were hatched at the 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 MD virus, infectious bursal disease virus, Newcastle disease virus, avian infectious bronchitis virus, and subgroup J avian leukosis virus (ALV) were 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 chicks 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...
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 (Vectorstain 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-α-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-H₂O₂ 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 µl) containing 46 or 47 µl of diethylpyrocarbonate-treated water (Bio 101, Inc., U.S.A.), 1 µl of each primer and 1 or 2 µl 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 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). 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...
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.

Table 1. Histological diagnosis of 45 field cases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marek’s disease</td>
<td>6</td>
<td>13%</td>
</tr>
<tr>
<td>Marek’s disease and Spindle-cell proliferative disease</td>
<td>3</td>
<td>7%</td>
</tr>
<tr>
<td>Spindle-cell proliferative disease</td>
<td>32</td>
<td>71%</td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>9%</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 2. Histological lesions of chickens in experimental cases

<table>
<thead>
<tr>
<th>Group</th>
<th>Spindle-cell proliferative disease</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/16*</td>
<td>1/16</td>
</tr>
<tr>
<td>2</td>
<td>1/20</td>
<td>1/20</td>
</tr>
<tr>
<td>3</td>
<td>0/14</td>
<td>2/14</td>
</tr>
<tr>
<td>Total</td>
<td>1/50</td>
<td>4/50</td>
</tr>
</tbody>
</table>

* 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 fragment 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.
These 9 birds had macroscopically visible large nodules and histopathologically detectable focal or diffuse proliferation of various-sized lymphoid cells. These findings correspond 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]. 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.

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REFERENCES


