Case Report—
Comb Lesions and Mortality Patterns in White Leghorn Layers Affected by Marek’s Disease

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Received 3 August 1982

SUMMARY
Marek’s disease was diagnosed as the cause of prolonged high mortality in two commercial flocks of white leghorn layers by gross and microscopic examination of affected tissues and by immunofluorescent study of live tumor cells using anti-Marek’s disease tumor-associated surface antigen, anti-immunoglobulin M, and monoclonal antibodies. The disease was characterized by swollen, necrotic, lymphomatous combs and mortality that rose above normal at 28 weeks of age, peaked at 35–36 weeks, and returned to normal by 45–46 weeks.

INTRODUCTION
Marek’s disease and lymphoid leukemia are neoplastic diseases of chickens. Although they are etiologically distinct, both are widespread and characterized in part by similar gross lesions (2). This limits the use of serologic and other common diagnostic techniques and makes it fairly difficult to arrive at an early definitive diagnosis of the disease in the field (3,4).

It is important to make a differential diagnosis between the two diseases, because their epizootiology and the methods used for their prevention are entirely different (6). Neumann and Witter (3,4) have described cell immunofluorescence techniques that allow a definitive diagnosis of either disease to be made by detecting cell-surface features

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503
Table 1. Distribution of lymphoid neoplasms\textsuperscript{A} in organs of white leghorn layers affected by Marek's disease.

<table>
<thead>
<tr>
<th>Layer no.</th>
<th>Comb</th>
<th>Liver</th>
<th>Spleen</th>
<th>Gonad</th>
<th>Muscle</th>
<th>Provent.</th>
<th>Nerve</th>
<th>Bursa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{A} Determined by gross and microscopic examinations of affected tissues.

\textsuperscript{B} Lymphoid neoplasm present (+) or absent (-).

associated with the tumors caused by the agents of each. The present report describes an unusual case of a neoplastic disease in two flocks of white leghorn layers that was diagnosed as Marek's disease by the use of such fluorescence techniques.

**CASE REPORT**

**Case history.** *Flock A.* On 6 November 1980, the avian service of the University of California's Veterinary Medical Teaching Hospital in Davis was asked to investigate a case of prolonged high mortality in a flock of white leghorn layers. The client was a commercial egg-type chicken firm with a total population of approximately one million white leghorn layers located in various ranches in California and Oregon. The firm raised its own replacement pullets, which were routinely vaccinated against the major diseases, including Marek's disease. The type of housing used for caged layers varied from ranch to ranch and ranged from open-sided to environmentally controlled houses.

There were about 35,000 layers in the affected flock. Four layers on the average were kept in standard-sized wire cages in a house with a forced ventilation system. Lighting was provided by a combination of sunlight and electric bulbs.

The hospital became aware of the problem when the layers were 32 weeks old. Weekly egg production, expressed as a percentage of layers alive, was 86.6%. Mortality was 0.61%. The highest level of weekly mortality accepted as normal by the firm during the laying season in any of its ranches was 0.25%.

*Flock B.* On 23 March 1981, another case of rising mortality was reported in a different flock of white leghorn layers. These layers, which numbered about 46,000, were 30 weeks old and kept in an open-sided, wild-bird-proof house. Egg production was 88.7%. The weekly mortality was 0.33%, had been higher than 0.25% for each of the previous 2 weeks, and did not seem to be decreasing.

**Clinical signs.** Symptoms of the diseases in the two flocks were identical and included lethargy, anorexia, emaciation, greenish diarrhea, some unilateral paresis, and swollen combs with or without necrotic or vesicular foci (Figs. 1a, 2). Most of the dead layers and virtually all sick layers had comb lesions. Several layers with comb lesions were selected and necropsied after blood had been collected.
Comb lesions and mortality in MD-affected layers

from each by cardiac puncture in bottles containing ethylenediamine tetraacetic acid.

**Gross pathology.** Gross lesions consisted mainly of enlarged visceral organs with or without whitish nodular or miliary foci (Fig. 1b, 1c). Organs affected included the liver, spleen, kidneys, ovary, and different parts of the gastrointestinal tract, especially the proventriculus. Lesions in the enlarged proventriculus consisted

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**Fig. 1.** Marek's disease in white leghorn layers. (a) Comb lesions (I) and unilateral paresis (II). Note pasted vent due to greenish diarrhea. (b) Viscera in situ. Nodular lesions in enlarged liver; enlarged proventriculus. (c) Nodular lesions and atresia of ova in ovary; enlarged spleen and kidney. H = heart; K = kidney; L = liver; O = ovary; P = proventriculus; S = spleen.
of thickened walls and hemorrhagic mucosa. Nodular lesions on atrophied pectoral muscles were also observed. The number and type of organs showing gross neoplastic lesions varied from layer to layer. Table 1 shows the distribution of lesions in the organs of four layers from flock B.

**Histopathology, microbiology, and hematology.** Microscopic examination of all affected organs showed massive infiltration and compression of normal structure by pleomorphic lymphocytes (Fig. 3). Culture of various affected organs and blood for bacteria yielded no pathogenic organisms, except staphylococci in one severely distended and necrotic comb. Suspensions of samples of macerated diseased combs, containing antibiotics to inhibit bacterial growth, were inoculated into chicken embryos for fowl pox isolation; no fowl pox virus was isolated in three embryo passages. There was marked leukocytosis in most of the layers examined. The mean number of white blood cells/μl of blood was 25,250 at 32 weeks of age and 41,250 at 38 weeks.

**Mortality and egg production.** To determine whether the disease had any effect on egg production, graphs of data on weekly egg production and mortality for each flock during the first laying season were plotted.

**Antibody preparation.** Three antibodies were produced for use in the immunofluorescent procedure described below. Antibody against Marek’s disease tumor-associated surface antigen (anti-MATSA serum) was produced by inoculating rabbits with MSB-1 cells (8), a well-characterized Marek’s disease cell-line (1). Anti-chicken immunoglobulin M (IgM) serum, which is specific against IgM on the surface of lymphoid leukemia lymphoma cells, was obtained from rabbits injected with an immunoelectrophoretically pure chicken IgM preparation (7). Monoclonal antibody (RPH-6) for use against Marek’s disease tumor cells was produced by immunizing BALB/c female mice with MSB-1 cells. Spleens from immunized mice were then pooled and fused with SP2/0-Ag 14 myeloma cells in 35% polyethylene glycol 1000. Those hybrid cells that produced antibody in the culture medium were tested against MSB-1 cells by the indirect immunofluorescence test. The use of this monoclonal antibody in the immunofluorescence test for Marek’s disease is a recent modification, and detailed information about its development and characterization is being compiled (L. F. Lee, manuscript in preparation).

**Membrane immunofluorescent study of tumor cells.** Fresh tumor tissues were teased with scissors to make a cell suspension. The suspension was kept on ice for 1 min to allow sedimentation of tissue debris. Tumor cells were removed from the supernatant by centrifugation at 200 × g for 5 min and kept on ice. Groups of these cells (3 × 10⁶ cells per group), suspended in 0.05 ml phosphate-buffered saline (PBS), were pelleted. Membrane immunofluorescent staining of these unfixed living cells (1) was achieved by resuspending the pellets in about 50 μl of monoclonal antibody, rabbit anti-MATSA serum, or rabbit anti-chicken IgM serum for 30 min on ice. The cells were pelleted again after a large volume of PBS had been added. These pellets were resuspended in 50 μl of an appropriate fluorescein-conjugated species of antiglobulin and allowed to react for 30 min on ice. After a final wash in PBS, a drop of cell suspension was placed on a coverslip and immediately examined under a Leitz fluorescence microscope with a vertical illumination system.
Fig. 2. Various manifestations of comb lesions in white leghorn layers affected by Marek's disease. (a) Firm swelling (rear, see arrows). No necrosis. (b) Firm swellings (rear); necrotic spots (see arrow). (c) Generalized swelling and necrosis. Vesicles also present. Wattles affected too. (d) Vesicles and necrotic areas. Necrosis may be a sequela to vesicle rupture.
RESULTS AND DISCUSSION

The disease was diagnosed as Marek's disease on the basis of gross and microscopic examination of affected tissues and by immunofluorescent study of live tumor cells using MSB-monoclonal antibody and the anti-MATSA and anti-IgM sera. As seen in Table 2, tumor cells from all the three hens used for immunofluorescent study reacted positively with MSB-monoclonal antibody and the anti-MATSA serum. None of the tumor cells reacted positively with anti-IgM serum. These results are compatible with those reported earlier by Neumann and Witter (3,4). The percentage of tumor cells from each organ showing fluorescence was consistently greater with MSB-monoclonal antibody than with rabbit anti-MSB serum. This indicates that the monoclonal antibody was probably more sensitive to the presence of Marek's disease tumor cells than rabbit serum was. Provided a fluorescence microscope and the antibodies are available, the immunofluorescence technique readily lends itself to routine diagnostic laboratory use and would be of great value in helping veterinarians arrive at an early and definitive diagnosis of lymphoid leukemia or Marek's disease.

Table 2. Results of immunofluorescent study of tumor cells from white leghorn layers affected by Marek's disease. Data are expressed as percentage of stained cells showing fluorescence.

<table>
<thead>
<tr>
<th>Hen no.</th>
<th>Tumor origin</th>
<th>MSB&lt;sup&gt;A&lt;/sup&gt; monoclonal antibody</th>
<th>Rabbit anti-MSB</th>
<th>Rabbit anti-chicken Ig&lt;sup&gt;B&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
<td>comb</td>
<td>15</td>
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<td>gonad</td>
<td>15</td>
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<td>2</td>
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<td>MSB&lt;sup&gt;C&lt;/sup&gt;</td>
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<td></td>
<td>RP-9&lt;sup&gt;D&lt;/sup&gt;</td>
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<td>90</td>
</tr>
<tr>
<td></td>
<td>normal spleen</td>
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<sup>A</sup> MSB is a well-characterized Marek's disease cell line.
<sup>B</sup> RP-9 is a well-characterized B lymphoblastoid cell line induced by lymphoid leukemia virus.
Fig. 3. Histopathology of organs of white leghorn layers affected by Marek's disease—marked infiltration by pleomorphic lymphocytes, mostly in a perivascular fashion. BV = blood vessel; L = pleomorphic lymphocytes. (a) Liver also showing necrosis of parenchyma. 28×. (b) Comb also showing epidermal, dermal, and muscular necrosis. 70×. (c) Pectoral muscle. 28×. (d,e) Pleomorphic lymphocytes in comb (d) and pectoral muscles (e). 438×.
An examination of the egg production and mortality curves (Fig. 4) revealed no links in either flock between the disease and egg production (expressed as a percentage of the birds alive). However, the mortality patterns were quite similar and were characterized by an increase above 0.25% at 28 weeks of age, a peak at 35-36 weeks, and a return to normal by 45-46 weeks (Fig. 5). This mortality pattern may be useful in the diagnosis of the disease.

In 1967, Purchase and Biggs (5) reported the presence of tumors at the base of the combs of a few 4-to-7-week-old experimental chicks

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**Fig. 4.** Egg production and mortality rates (percent/week) in white leghorn layers affected by Marek's disease.
inoculated with isolates from field outbreaks of Marek's disease. The present report describes outbreaks of Marek's disease in two commercial flocks of white leghorn layers in which swelling of the comb, caused by a massive infiltration of lymphocytes, appears to be a pathognomonic sign. The swellings were firm to the touch, and many of the affected combs also had necrotic lesions. The wattles too were affected in severe cases. These lesions were initially thought to be sequelae to trauma or fowl pox. However, avian pox virus was not isolated from diseased combs in three embryo passages, and the presence of vesicles adjacent to the necrotic areas on some combs suggested that the necrosis might be the result of the disease process itself. To the best of our knowledge, this represents a hitherto unreported manifestation of Marek's disease in layers. Attempts are being made to isolate and characterize the causative virus.

Some of the comb and wattle lesions observed in this disease could also be attributed to other diseases like vesicular dermatitis, chronic fowl cholera, and xanthomatosis. However, the presence of pleomorphic lymphocytes in sections of the swollen combs or wattles examined microscopically would differentiate this from the other diseases.

**REFERENCES**


ACKNOWLEDGMENTS

Photographs were taken and processed by Sam Woo of Medical Illustration Services, University of California, Davis. The manuscript was typed by Yolanda G. Ferguson of the School of Veterinary Medicine, University of California, Davis. Their excellent technical aid is hereby warmly acknowledged.