A Filtrable Agent Producing Lymphoid Tumors and Osteopetrosis in Chickens


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Lymphoid tumors of the chicken have been shown to be readily transplantable by the use of viable cells. Pentimalli (29) and Olson (25, 27) have described the isolation of such strains from what was thought (28) were rare cases of neoplasia, but which had all the characteristics of the condition designated by Feldman and Olson (9) as lymphocytoma. Burmester and Prickett (7) reported the development of similar tumor strains from cases of naturally occurring visceral lymphomatosis. Earlier, Furth (11-13) described the properties of a filtrable agent (Furth strain 2) that produced lymphomatosis, and occasionally myelomas and endotheliomas. Contrary to the results obtained with agents of other chicken tumors such as the endothelioma of Begg and Murray (1), all sarcomas of Rous and Murphy (31), and similar neoplasms (10), Furth’s strain 2 agent did not produce a tumor at the site of injection. However, viable neoplastic cells implanted in the pectoral muscle produced lymphomatous tumors similar to those described by Pentimalli (29), Olson (25), and Burmester and Prickett (7).

This report describes briefly the manifestations of a filtrable agent associated with a transplantable lymphoid tumor of the chicken (25).

MATERIALS AND METHODS

The lymphoid tumor strain reported in this study, some of whose manifestations and characteristics have been described previously (5, 6, 25, 27, 30), is identified at this laboratory as RPL1-12. The immediate sources of tumor tissue used in these experiments were affected birds in the serial passage of this tumor strain. The transfers were made every 7 days, by injecting a suspension of tumor cells into the pectoral muscle of 6 to 8 week old white leghorn chickens.

Lymphoid tumors in the pectoral muscle and liver used in the preparation of inocula for Experiment 1 were obtained from birds of the 50th RPL passage (inoculum for this passage had been stored at −70° C. for 95 days). The cell-free inocula were prepared from tumor material ground in a mortar with the aid of sterile sea sand to a smooth paste, which was then suspended in 3 parts of 0.85 per cent NaCl solution and centrifuged for 20 minutes at 3,000 r.p.m. The upper three-fourths of the supernatant fluid was carefully transferred to another centrifuge tube with the aid of a slow flowing siphon, and spun for 20 minutes at 3,000 r.p.m. The resulting supernatant was transferred to a serum bottle for inoculation.

Tumors in the pectoral muscle from birds of the 66th RPL passage were used in the preparation of inocula for Experiment 2. The tumor material was first suspended in 3 parts 0.85 per cent NaCl solution with the aid of a mincer (26) and then processed in a bacterial grinder for 2 hours. The resulting suspension was centrifuged for 20 minutes at 19,000 r.p.m. and the supernatant carefully siphoned into a serum bottle preparatory to its inoculation into young chicks. The sediment was resuspended in saline and transferred to another serum bottle for inoculation.

In Experiment 3 blood from birds of the 85th passage with 7 day tumors of the pectoral muscle was used as the source of inoculum. The blood was obtained by cardiac puncture and drawn into a syringe containing 0.1 volume of heparin solution with a concentration of 0.4 gm. in 100 ml. of 0.85 per cent NaCl solution. After the blood cells had been separated in a centrifuge the plasma was filtered through a Seitz sterilizing filter pad. The filter was tested with a broth culture of Serratia marcescens and the filtrate found to be sterile. Positive control broth cultures produced excellent growth of bacteria.

Birds that served as positive controls were injected with cellular inoculum, prepared by passing the tumor through a mincer (26), suspending the mince in 3 parts 0.85 per cent NaCl solution, and filtering through a layer of cheesecloth. In Experiment 3 whole blood was used as the inoculum for the positive control birds.

Inoculations were made at 2 and 3 days of age except when otherwise indicated. The amounts used were 0.25 ml. when injections were made intramuscularly and 0.5 ml. for injections into the peritoneal cavity. One per cent fuller’s earth (by weight) was added to the centrifuged supernatant injected intra-
muscularly in Experiment 2, and to all inocula injected into the left pectoral muscle in the birds of Experiment 1. The right pectoral muscle of the same birds was injected with the inocula before the addition of the diatomaceous earth.

The pedigreed white leghorn chickens used were obtained from laboratory stock bred (32) to supply chicks that were relatively susceptible to lymphomatosis yet did not develop it when maintained under quarantine. However, progeny of this stock, which furnished most of the chicks used in these experiments, have subsequently showed a significant amount of lymphomatosis even though maintained under the same environment as the parental stock. Thus the chicks may not have been entirely free from infection at the time of hatching.

The chicks were raised in wire batteries for the first 3 months and then transferred to a pen with litter on the floor, where they were kept for the remainder of the experimental period of 6 months. The pens in which the birds were kept were under quarantine throughout the experimental period. All apparatus entering the pens was sprayed with a disinfectant, the feed and litter were handled with precautions against contamination, and persons upon entering or leaving the pens changed shoes and outer garments and used a disinfectant on their hands.

RESULTS

The results of Experiments 1, 2, and 3 (Table 1) are similar in that inocula containing viable cells, whether prepared from intramuscular or intrahepatic tumors or from blood of birds bearing tumors, induced the death of all birds inoculated in a short time (average of 10.2 days, including birds injected with centrifuged sediment, Experiment 2). These inoculations produced local tumors in the muscle when the intramuscular route was used, and in the abdominal wall and mesentery when injections were made into the peritoneal cavity. In addition, all birds had extensive involvement of many of the viscera, including the liver, kidney, spleen, pancreas, proventriculus, and gonad.

Chicks inoculated with cell-free preparations, whether these were obtained by the use of a centrifuge (13) or by filtration, did not show evidence of tumor formation (even though a cell irritant, fuller's earth, was used) or other disease until they were at least 10 weeks of age. At this time clinical symptoms resembling osteopetrosis began to make their appearance in all 3 experiments, and by 6 months of age an average incidence of 41 per cent was obtained. During the same period, 56 per cent of the birds inoculated with cell-free material developed macroscopic tumors of the viscera. Most of them had massive tumorous involvement of the liver. Out of a total of 80 birds inoculated with cell-free material, 84 per cent developed tumors of the viscera, bone, or nerve in 6 months' time, to the extent that diagnosis could be made by gross examination. Of the 67 birds that were positive, 20 had osteopetrosis without gross evidence of other pathological lesions, 29 had visceral tumors without osteopetrosis, and 16 had a combination of osteopetrosis and visceral tumors. The remaining 2 had neurolymphomatosis. Only 1 of the 29 birds with tumors of the viscera had gross nerve changes typical of neurolymphomatosis.

The average age at death of birds that died with liver or bone involvement in the experimental period of 6 months was 144.2 days for those inoculated with cell-free material, in contrast to only 10.2 days for those given cellular inoculum. A few of the surviving birds, some with and some without osteopetrosis, were inoculated with a suspension of RPL-12 tumor cells and found to be still susceptible to local tumor development and metastasis to the viscera.

The 15 chicks of Experiment 2 that were not inoculated were raised in the same brooder space, and allowed to intermingle with inoculated birds during the entire course of the experiment. One bird at 67 days of age and another at 89 days showed clinical symptoms of neurolymphomatosis. They were killed and the diagnosis was confirmed at necropsy. At the termination of the experiment one bird, which seemed normal clinically, showed lymphomatous tumors of the liver, heart, and spleen at autopsy. Thus a total of 3 cases of lymphomatosis appeared among 15 birds raised in contact with the inoculated birds.

In the third experiment the noninoculated controls were kept in the same pen but in a separate battery for the first 3 months, thus eliminating the chance for direct contact; however, there may have been indirect contact through the air, dust, flies or other vectors. No evidence of any of the manifestations of the avian leukosis complex (17) was found in any of these controls. Only one death occurred, and that was due to trauma of the poll region caused by cannibalism by the pen mates.

PATHOLOGICAL MANIFESTATIONS

Osteopetrosis.—This condition was easily recognized by clinical examination because the shanks were almost always affected. In the early stages the metatarsus showed irregular surfaces, a convexity of the anterior outline, or a thickening of the diaphysis. Irregular enlargement of other long bones of the leg or wing were detected by digital palpation. The increased diameter of the metatarsal bones resulted in immobilizing the dermal structures, which made them harder to the touch, and they seemed to have an increased surface temperature.
The metatarsus and tibia were found to be the most frequently enlarged; however, almost all the other bones showed similar derangement. The change was usually, but not always, bilateral, and the extent and number of bones that showed gross enlargement seemed to depend upon the duration of the disease.

The bones had a rough, irregular, porous surface, which was covered by a hypertrophied periosteum (Fig. 1). The porous appearance of the bones was due in part to irregularity of the periosteum and to peripheral marrow spaces. On gross examination the osteopetrotic changes seemed to be largely confined to the diaphysis. The marrow cavity was reduced in diameter by abnormal deposition of spongy bone. The bones of some birds showed no enlargement but had a slight surface irregularity and yellow opaque areas, which were believed to be less advanced stages of the disease.

Histological examination of a few cases with the characteristic osteopathy indicated that important changes occurred in the periosteum. Hyperplasia of the periosteal tissues resulted in thickening of this layer with apparent formation of new and abnormal cancellous bone (Figs. 5 and 6). Numerous irregularly placed cavities containing hyperplastic tissue, which appeared to be bone marrow, were found throughout the cancellous structure. The normal diaphyseal architecture was apparently completely altered.

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full or protruding abdomen and often grew pale. Affected viscera usually had nodular or focal tumefaction. The livers of such birds often had comparatively large tumor nodules (1 to 2 cm. in diameter) separated by apparently normal parenchyma or by small focal tumors (Fig. 3). Some livers were a mass of innumerable small focal areas, leaving very little visible liver parenchyma (Fig. 2). Such a tumorous liver viscera in the 45 cases with visceral tumors identifiable at necropsy is given in Table II.

The histological changes observed (Fig. 7) in the viscera were similar to those described by Olson (25), Burmester and Prickett (7), Jungherr (18), and to certain cases of naturally occurring lymphomatosis (30). However, they differed in showing more local leukemoid reactions in the viscera, and by the occurrence of focal areas of necrosis in the liver, probably associated with infarctive processes related to the tumefaction.

**DISCUSSION**

Young chicks inoculated with a cell suspension of the RPL-12 lymphoid tumor developed local tumors with visceral metastases, and died in an average of...
Table II: Gross Involvement of Viscera in 45 Positive Cases That Received Cell-Free Inoculum

<table>
<thead>
<tr>
<th>Lymphomatous organ</th>
<th>Per cent of cases</th>
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<tbody>
<tr>
<td>Liver</td>
<td>93</td>
</tr>
<tr>
<td>Spleen</td>
<td>82</td>
</tr>
<tr>
<td>Kidney</td>
<td>58</td>
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<tr>
<td>Gonad</td>
<td>31</td>
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<td>Heart</td>
<td>22</td>
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<td>Serosa</td>
<td>13</td>
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<td>Pancreas</td>
<td>9</td>
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<td>Proventriculus</td>
<td>9</td>
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<tr>
<td>Adrenal</td>
<td>4</td>
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<tr>
<td>Intestine</td>
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Fig. 7.—Peripheral zone of hepatic lymphomatous nodule showing character of lesion and its influence on adjacent liver parenchyma. Hematoxylin-triosin. Mag. ×165.

10.2 days. Extracts or blood plasma from the same donors made cell-free by either centrifugation or filtration did not induce local tumors or visceral involvement in a short time, but produced visceral tumors and osteopetrosis after an incubation period of 2 to 6 months. The tumefaction of the viscera obtained with the cell-free inocula varied from the diffuse, friable, acute type to the focal, nodular, chronic type. With the differences already noted, the lymphoid tumors were similar in their gross and microscopic appearance to those observed in other malignant tumor strains (7, 25, 29), and to some cases of lymphomatosis (19, 30). The osteopathy described in these experiments was similar in its gross manifestations to the osteopetrosis occurring naturally (2, 16, 20) and following inoculation (4, 8, 16), and the histological changes resembled the osteopetrosis described by Jungherr and Landauer (16). Brandly, Nelson, and Cottral (4) state that augmented cellular activity of the periosteal region, together with the presence of irregular spaces in the bony structure, is characteristic of tarsometatarsal involvement. These changes were typical of the histological alterations found in the experiments described. This is particularly true of the extensive hyperplasia of the periosteal tissues and the lack of medullary fibrosis. Similarity in its transmission characteristics to the osteopetrotic strains previously described (4, 16) was further demonstrated when the blood of an osteopetrotic case of Experiment 1 was injected intravenously, intramuscularly, and intraperitoneally into 45 chicks 10 days of age. Osteopetrosis developed in all 3 groups, giving a total of 24 cases at 96 days of age, when all birds were brought to necropsy. Eleven of the 24 had lymphomatous tumors of the viscera in addition to the osteopetrosis, and 4 others had visceral tumors without gross evidence of osteopetrosis. This is a much higher incidence of osteopetrosis, and at an earlier age, than was obtained by Jungherr and Landauer (16); Brandly, Nelson, and Cottral (4); or Duran-Reynals (8).

That the osteopetrotic propensity is not found in all avian lymphoid tumor strains is indicated by the finding that no osteopetrosis was obtained under similar experimental conditions when strain RPL-16 was used as the source of inoculum. When Seitz-filtered plasma or muscle-tumor extract of an RPL-16 donor was injected into young chicks, 39 per cent developed visceral tumors in the age period of 60 to 180 days, but contrary to the results obtained with RPL-12 no osteopetrosis appeared. Although strain RPL-16 is similar to RPL-12 in many of its manifestations, its source and origin were quite different (7).

Osteopetrosis was not observed by Furth (11-13) in his strain 2, which in other respects was similar to RPL-12 in that inocula containing viable cells induced tumor growth at the site of inoculation whereas cell-free or filtered extracts produced no local reaction but resulted in a systemic disease with lymphomatous tumefaction of the viscera. Furth occasionally observed myelomas and endotheliomas, which were not identified in birds inoculated with strain RPL-12. The latter difference, however, may be due to a lack of unity in nomenclature, or interpretation and identification of the significant cell type. With strain RPL-12 there was a distinct difference in the survival period of birds inoculated with cellular as compared with cell-free inocula. Birds that received cellular inocula died, on the average, in 10.2 days, whereas
Lesions resembling human osteodystrophia fibrosa cystica were obtained by Oberling and his associates (21-24) in birds maintained in outdoor cages on a mineral-poor diet. Gohs (14, 15) produced similar lesions, but without parathyroid hyperplasia, by repeated injections of normal embryonic or adult avian bone marrow treated with x-rays and glycerin. Brandly (3) injected embryonated eggs with whole blood and washed red cells; in 7 to 10 days the spleen, liver, and the diaphysis of the long bones of the legs and wings became enlarged. The microscopic alterations found in the periosteal region were similar in many respects to those obtained in the inoculations described in this report. Gross lesions were the same, irrespective of whether the blood was from apparently normal birds or from birds having lymphomatosis or erythrogranuloblastosis; however, they were absent when cell-free plasma or blood cells killed by x-rays or distilled water were used. Thus similar hypertrophic osteopathies may result from a wide variety of treatments or circumstances, which emphasizes the need for caution in assigning their etiology.

Olson (25, 27) apparently did not obtain bone lesions in the serial passage of this tumor strain by transplantation, nor did they appear while the tumor was maintained in serial passage at this laboratory. Why osteopetrotic alterations developed with cell-free extract but not with cell suspensions is not apparent. The age of birds at inoculation and the dosage used may have been influencing factors, since all serial passage inoculations were made with birds 4 or more weeks of age; whereas the birds of these experiments were inoculated at 2 or 3 days of age. In an immunity experiment to be reported elsewhere, chicks 2 days of age were inoculated intramuscularly with a suspension of cells. The dosage, however, was only 1/40,000 of that used in serial passage and in cell free transmission inoculations. Second and third inoculations were made with a much larger dose, at 23 and 64 days of age respectively. Tumors developed at the site of the first inoculation but no bone lesions were found, even though many of the birds lived over 300 days. However, a high percentage of them developed lymphomatous tumors of the viscera indistinguishable from those elicited by cell-free extracts.

Three birds of 15 uninoculated controls developed lymphomatosis when they were mixed with the inoculated birds at the time of inoculation (Experiment 2); however, no birds of the same families showed evidence of disease when they were brooded separately for the first 3 months. Thus it may be suggested that the agent was transmitted by direct bird-to-bird contact at some time prior to 3 months of age. However, the suggestion is of less significance when we consider the fact that of the 3 birds giving positive results in the contact control group 2 had neural involvement, while only 2 of 22 giving positive results in the inoculated groups had the neural form. That the 3 positive cases were the result of transmission of the agents through the egg remains a possibility, as indicated previously.

The data presented suggest that whereas the transplanted tumor cell is responsible for tumors that develop in a short time (7 to 10 days) at the site of inoculation, with metastasis to other tissues, a filtrable agent or agents will produce lymphomatous tumors of the viscera and osteopetrosis after a much longer incubation period (2 to 6 months) without inducing a tumor at the site of inoculation.

Whether osteopetrosis and the visceral tumors are due to a single entity or result from the action of more than one agent cannot be ascertained from these data. The majority of cases had gross osteopetrosis or visceral tumors, and a combination of the two occurred in only 24 per cent of the total positives. Microscopic examination of 6 osteopetrotic cases without gross tumefaction of the viscera showed that none had lesions typical of lymphomatosis.

The high incidence of visceral tumors obtained without evidence of osteopetrotic manifestations, in birds inoculated with the filtrate of another lymphoid tumor strain (RPL-16), would suggest that the two manifestations are caused by separate entities or that agents of the two lymphoid tumor strains are different. A relation in the occurrence of osteopetrosis and lymphomatosis (including lymphomatous visceral tumors) was noted by Jungherr and Landauer (16) and by Brandly, Nelson, and Cottral (4). It was suggested (16) "that certain transmissible strains of lymphomatosis may have an overt or latent power of bringing about osteopetrotic alterations in various degrees." Both reports state that a complete identity of etiology seems unlikely.

Since the filtrable agent was obtained from a strain after it had been propagated through 200 serial passages by the transplantation of viable tumor cells, it may be suggested that the agent was also thus propagated within or in close relation to the neoplastic cells. Furthermore, relatively large amounts of it must soon have appeared in the blood stream, since filtered plasma from a bird 8 days after intramuscular implantation produced as many positive cases as did centrifuged extracts of the primary tumor.
SUMMARY

1. Manifestations of the filtrable agent or agents of a transplantable lymphoid tumor of the chicken were demonstrated in 3 experiments involving 150 birds.

2. Inocula containing viable tumor cells induced tumor growth at the site of inoculation, metastasis to the viscera, and death of all birds in a relatively short time (average 10.2 days); whereas centrifuged extracts of the same tumors, or filtered plasma of birds bearing tumors, when injected intramuscularly, intraperitoneally, or intravenously into 2 to 3 day old chicks, induced in 6 months a high incidence of osteopetrosis and lymphomatous tumors of the viscera (average of 81 per cent on gross examination) but no tumors at the site of inoculation.

3. These results suggest that the avian lymphoid tumor strain under study, which has been transferred serially in over 200 passages by transplantation of its cells, carries with it a filtrable agent or agents capable of inducing osteopetrosis and lymphomatous tumors of the viscera after an incubation period of at least 2 months.

REFERENCES


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