STUDIES ON LYMPHOMATOSIS IN TURKEYS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

by

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The Ohio State University

1958

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ACKNOWLEDGMENT

I wish first to express my gratitude to Dr. Lloyd C. Ferguson, of the Michigan State University, and to Dr. William D. Pounden, of the Ohio Agricultural Experiment Station, who were jointly responsible for the initiation of this work. Without their interest, encouragement, and help, I would have been unable to start the graduate program which produced this study.

My appreciation to the administrators of Michigan State University and of the Ohio Agricultural Experiment Station for my leave of absence and for financial assistance is heart-felt and sincere.

The assistance of the staff members and technical personnel of the Department of Veterinary Science at the Ohio Agricultural Experiment Station is gratefully acknowledged. Dr. Vance L. Sanger was especially helpful in studying histological preparations in photography.

I particularly appreciate the friendly cooperation of Dr. McCartney and personnel of the Poultry Department in furnishing more than 3,000 of the poults which were used in this work.

I am sincerely grateful to Drs. Bohl, Venzke and Dodd for the attention which I received in formal classes at the Ohio State University and to Drs. Pounden and Helwig for the stimulating and fruitful discussions of problems incident to my dissertation.
Drs. Burmester, Lucas, Waters, Walters and Gross of the United States Federal Regional Poultry Research Laboratory at East Lansing, Michigan, directed by Berley Winton, rendered invaluable technical and material assistance. Two tumor strains and numerous eggs were supplied by that institution.

Photographic work was done by Mr. Robey and Mr. Berkey of the Ohio Agricultural Experiment Station. Their assistance was very much appreciated.

Ralph Cedric Belding
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STUDIES ON LYMPHOMATOsis IN TURKESs

INTRODUCTION

The several pathological conditions included in the avian leukosis complex are collectively conceded to be the most important and costly diseases of adult chickens in North America.

Until quite recently, the condition was uncommon in birds other than chickens. Since about 1950, there has been an apparent increase in incidence of the visceral form of lymphomatosis in turkeys in the United States. The work presented here was undertaken in anticipation that the condition would assume increasing importance as a disease of turkeys, following the pattern of the disease in chickens during the last 30 years.

The objectives of this work were to--

1. Attempt to adapt the present chicken tumor, Strain 12, developed at the Federal Regional Poultry Research Laboratory of East Lansing, Michigan, (RPL 12), to serial transplantation in the muscle of turkeys.

2. Determine the activity of both cells and virus of RPL 12 and RPL 16 in the turkey when inoculated by various routes, including intramuscular, intracocular, intravenous and intraperitoneal.

3. Attempt to adapt a naturally occurring turkey tumor to serial transplantation in turkeys.

4. Determine if a filtrable agent is present in the naturally occurring turkey neoplasm.
5. Determine the antigenic and pathogenic activity of the naturally occurring turkey tumor in the chicken, including both cells and filtrate, by the intraocular, intramuscular, intravenous and intraperitoneal routes.

The writer worked at the Regional Poultry Research Laboratory in 1949 as a Veterinarian P-2. At that time, interest in the problem of producing a vaccine against visceral lymphomatosis, (VL), was very great. The antigenicity of cellular preparations of tumors had been established, but attempts to establish the antigenicity for the "virus-like" agent in an attenuated form were unsuccessful. It was accepted that any method of treatment of the agent which would decrease its virulence would similarly decrease its antigenicity.

The writer participated in unsuccessful and unpublished attempts to grow RPL 12 cells in such foreign hosts as rabbits and guinea pigs, and similar attempts to produce antisera against the agent by inoculating cell-free preparations into animals of the same species. No attempt was made to inoculate birds other than chickens, since the laboratory regulations prohibited the housing of other birds on the premises.

From July, 1949, to December, 1956, the writer was employed by Michigan State University as a Poultry Diagnostician, Instructor in poultry diseases for veterinary students, and research worker in diseases of poultry. In 1950, he saw his first case of VL in a turkey; the diagnosis was confirmed
by touch-preparations and histological sections of the liver. The condition was seen occasionally, but with increasing frequency, during the following six years. The striking similarities in the gross and microscopic appearances of the lesions in the turkey to those in the chicken were impressive; the question of the possible relationship of the causative agents was intriguing. No reference to work dealing with either cellular or cell-free material derived from turkeys with VL could be found. Indeed, excepting for Dr. Logan Julian at Davis, California, no one in this country or abroad was known to be working with any form of lymphomatosis in turkeys when the present work was started in the Department of Veterinary Science at the Ohio Agricultural Experiment Station (OAES) in March, 1957. Since that time, one other worker has succeeded in passing a naturally occurring turkey lymphoid tumor in both poult's and chicks.

At the inception of this work, a great deal of thought was given to the possibility of developing an effective vaccine against the agent of VL. It is widely accepted that a vaccine against VL would be of inestimable value to the poultry industry, which experiences a great annual monetary loss from the effects of the agent. Late developments in this area are very stimulating and encouraging, although it is likely that several additional years of intensive effort will be required before such a product can be made available to the poultry industry.
According to Olson (1910), Gaparini first described fowl leukemia in 1896. Since that time, a great deal of work has been done involving the various conditions classified as the avian leukemia complex. Anyone attempting to study the voluminous accumulation of literature on the subject will soon become aware of the complexity of the problem in the chicken. Gottral (1952) discussed the incidence of lymphomatosis in chickens and presented data on the incidence of lymphoblastomas in other species of animals. According to his data, one of every three chickens observed until the survivors were 1,200 days old developed some form of lymphomatosis in the Federal Regional Poultry Research Laboratory (RPL) flock. Among the domestic animals, sheep and lambs have the lowest incidence; only one in 357,600 suffers from lymphoblastomas. When all types of leukemia are included, one in 5,400 humans suffers from a similar condition. Gottral states specifically that the similarities are limited to the pathology produced; there is no evidence of etiological similarity.

According to Jungherr (1952), comprehensive reviews of the literature have been prepared by Biely and Palmer (1932), Jarmai (1934), Olson (1940), Englebreth-Holm (1942), and Furth (1946). The material included in these publications deals primarily with the disease as it pertains to the chicken; few references are made to other species of birds. Many
references do not specify the type of inoculum used in attempts to reproduce the condition. Since strains of visceral lymphomatosis can be transmitted by both cellular and cell-free material (Burmester, 1947) and because each has a more or less variable incubation period, it is very often impossible to determine which was used from the data presented.

There is still a lack of uniformity in terminology and nomenclature. This work will follow the tentative nomenclature of the avian leukosis complex as given by Jungherr (1952).

It is now well established that turkeys are susceptible to lymphomatosis. Simpson, Anthony and Young (1957) report an outbreak of the disease in which the diagnosis was confirmed by microscopic study. These same authors (1956) reported a case of leucocytozoon infection in turkeys in which visceral lymphomatosis was a complicating factor. Durant (1930) reported two cases of visceral lymphomatosis (VL) in adult turkeys in Missouri, and Prier and Alberts (1950) mention that it occurs occasionally in turkey flocks in Illinois. Jarmai is cited by Olson (1940) as reporting that he isolated a strain of erythroblastosis from a chicken and successfully transferred it to turkeys and that serial passage in turkeys decreased its virulence for chickens. Andrewes and Glover (1939) reported a case of neurolymphomatosis in a turkey.
These two references are the only ones found which report on any form of lymphomatosis other than VL in turkeys.

Julian (1958) has been working with transplants of cells of strain 16 tumor which originated at the Federal Regional Poultry Research Laboratory. This tumor (RPL 16) has consistently killed pouls in his work although he estimates that in the birds which he has used, the pouls are 10,000 times more resistant than are the chicks. This estimate is based on the number of viable tumor cells required to produce deaths in 50 per cent of the inoculated birds.

Twiehaus (1958) states that he has successfully completed transfer of a field strain of turkey VL to both pouls and chicks. This agent, when inoculated into birds up to 19 days of age, produces nearly 100 per cent mortality in both chicks and pouls. After the twenty-first day, both species become somewhat more resistant to the effects of the agent.

Under natural conditions of poultry husbandry, VL is capable of spreading in both the hatcher and during the first few weeks of the growing period (Waters and Bywaters, 1949). During the early growing period, the virus is spread by both oral secretions and feces (Burmester and Gentry, 1954) (Burmester, 1956).

Under laboratory conditions, VL can be reproduced by the inoculation of either a suspension of tumor cells or of a cell-free extract of tumorous tissue (Burmester et al., 1946) (Burmester and Cottral, 1947). Burmester and Cottral (1947) and
Dardiry et al. (1952) made the observation that there was no relation between the malignancy of the tumor cells and the virulence of the filtrable agent.

The methods of preparing cellular and cell-free inocula from lymphoid tumors are described by Burmester and Dennington (1947) and by Burmester (1947). In cellular suspensions of tumors, both viable cells and virus are present. Cell-free preparations produce no tumor at the site of inoculation; the effect of the agent is to cause uninhibited autonomous proliferation of immature lymphocytes. This proliferation tends to occur at pre-existing lymphoid areas; the neoplasms thus usually develop in such organs as the liver, spleen, bursa of Fabricius, pancreas, and kidney, although any living tissue may become involved.

As shown in Figure 1, the virus proliferates within the lymphoid cell. A suspension of neoplastic lymphoid cells thus contains cells which are undergoing mitosis as well as the agent which was freed by mechanical disruption of affected cells. For this reason, a cellular inoculum may produce a tumor at the site of injection within a period of five days (Burmester, 1947). If the bird survives the effects of the primary tumor, and metastasis from the primary site does not occur, the inoculated bird may still die at any time beyond 60 to 90 days after the injection from the effects of the agent on the bird's lymphoid tissues.
Fig. 1 - Electron Micrograph of a Limited Field of Cytoplasm of Cells in Spleen of Chicken with VL. Mitochondria (M); Inclusion-like bodies (IB); Virus-like particles (VP). x 25,000. (Photo reproduced through courtesy of B. R. Burmester.)
Transplantation of neoplastic cells into individuals of a species different from that in which the tumor arose, is, according to Green (1957), dependent on the ability of the tumor to metastasize. He mentions only two exceptions to this, including the virus-induced Shope papilloma and Rous sarcoma. Transplantation of these virus-induced neoplasms is dependent on the concomitant transfer of the virus. Greene (1957) also states, "It should be noted that, although the lymphomas are usually classified as sarcomas, they do not belong to the heterotransplantable group of tumors." Greene stresses the importance of avoiding any normal tissue cells in transplants, since they excite an inflammatory reaction which leads to the destruction of the transplant. At the time when metastasizability is attained by neoplasms, changes occur in the cell wall so that its permeability to proteins of the host is increased. Proteins of the alien species are thus incorporated into the cytoplasmic components of the neoplastic cell without inducing the inflammatory reaction which leads to the rejection of normal heterologous tissue.

"Grafts within an inbred strain are called isografts; grafts between inbred strains, or more broadly, between any two genetically different individuals of the same species, are called homografts. Whereas isografts of tumor or normal tissue regularly succeed, homografts almost always fail" (Snell, 1957). By definition, heterografts are grafts from one species to another.
The avian eye has been used by Burmester and Belding (1949) and Burmester (1952) as a site for isotransplantation of RPL 12. As shown in Table 1, taken from Best and Taylor (1955), the amount of globulin in the aqueous humor is very low as compared with the amount in serum. Since antibodies are found in the gamma globulin, it is obvious that the level of antibody is considerably lower in aqueous humor than it is in serum. Green and Loring (1957) showed that a natural antibody produced by chick embryos at 17 days of incubation was responsible for the arrest in development of a mouse tumor when relatively small numbers of tumor cells were injected intravenously into the embryos.

Algire, Weaver and Prehn (1954) describe the construction of diffusion chambers. Applications of the chambers to studies of cellular immunity by parenteral implantation are given by Algire (1957).

The method used for freezing tumor cells for storage was that given by Burmester et al. (1944), in which the temperature of the sealed tubes of tumor was decreased at the rate of one degree C. per minute from 0 to -20 C. Burmester (1950) reported that RPL 12 cells were still viable and produced intramuscular neoplasms as long as 2,028 days after freezing.

The effect of cortisone on the growth of RPL 12 in chicks was reported by Winton (1951). A daily dose of five mg. did not retard the development of tumors. Treated birds in this
TABLE 1
Composition of Aqueous Humor Compared to that of Serum (Best and Taylor, 1955)

<table>
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<th>Components</th>
<th>Grams per 100 cc.</th>
<th>Aqueous humor</th>
<th>Serum</th>
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<tr>
<td>Water</td>
<td>99.6921</td>
<td>93.3238</td>
<td></td>
</tr>
<tr>
<td>Solids</td>
<td>1.0869</td>
<td>9.5362</td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>0.0201</td>
<td>7.3692</td>
<td></td>
</tr>
<tr>
<td>Albumen</td>
<td>0.0123</td>
<td>4.4135</td>
<td></td>
</tr>
<tr>
<td>Globulin</td>
<td>0.0078</td>
<td>2.955</td>
<td></td>
</tr>
<tr>
<td>Fats</td>
<td>0.0044</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.082</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>0.058</td>
<td>0.070</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>0.365</td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>0.458</td>
<td>0.436</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>0.023</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>0.007</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.017</td>
<td>0.018</td>
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experiment died earlier than the controls, apparently as a result of bacterial infection. Lannek (1952) reported that adrenocorticotropic hormone (ACTH) and hydrocortisone exerted a marked but temporary inhibition on growth of lymphoid tumors, while cortisone had a much less profound action. Lannek used RPL 19 and young crossbred chicks in his work.

Taliaferro (1957) discussed both cortisone and irradiation with X-rays and their effects both on the immune response and on leukocytes. No reference to the application of trypsinization of tumor cells to transplantation has been found.

Burmester et al. (1956b) discussed the antigenicity of three strains of tumor, RPL 12, RPL 15, and RPL 16. Each strain inhibited the development of tumors for period up to about 310 days when the test bird was given a second inoculation of cells of the same strain. Birds which recovered from the first implantation, however, had an incidence of visceral tumors four times as great as did the uninoculated control birds. Burmester (1955) also showed that progeny of hens which had received injections of the virus of VL were much more resistant to inoculation with the same agent than were progeny of hens not so treated. Burmester et al. (1956) showed that virus treated with beta-propriolactone, formalin, or heat was also antigenic, although somewhat less so than untreated virus.
MATERIALS AND METHODS

BIRDS

Turkeys: The turkeys used in this work were from two sources. A total of 600 Beltsville small white poults were purchased from a commercial hatchery in western Ohio. The remaining 3,100 birds were furnished by the Poultry Department at the Ohio Agricultural Experiment Station (OAES), Wooster, Ohio. Both small whites and large whites were used. The OAES flocks were started by purchasing poults from the same commercial hatchery which supplied poults for this work. Visceral lymphomatosis has been found in both OAES stock and in flocks purchased as poults from the hatchery. In no case has the incidence been known to exceed 5 per cent; the incidence in the OAES flock has approximated 1 to 2 per cent per year for the last few years.

Chicks: Some of the chicks used were hatched from eggs supplied by the Federal Regional Poultry Research Laboratory (RPL) in East Lansing, Michigan. These eggs were from Line 15 birds which are highly susceptible to lymphomatosis, yet which are relatively free from the condition unless artificially exposed (Waters and Prickett, 1944). Other chicks were leghorn cockerels purchased from a local hatchery. These were H & N chicks from pullorum-free flocks. They were not vaccinated with any biologic and received no medication of any kind. There were no reported cases of any form of leukosis in pullets of the current year.
Feed and Housing: Poults were started in batteries and usually held in batteries during the observation period. When the period of observation exceeded thirty days, birds were kept in isolation rooms in groups of up to one hundred birds. The 26 per cent protein turkey starter ration used was the one described by Yacowitz and Marsh (1954). In order to insure that adequate levels of vitamins A, D, and E were present, alpha tocopherol and feeding oil containing vitamins A and D were added to the ration. No antibiotic was used in this feed.

TUMORS

A total of six tumor strains were used; two, RPL 12 and RPL 16, were furnished by the Federal Regional Poultry Research Laboratory while three were harvested from turkeys with field cases of visceral lymphomatosis. The sixth was a naturally occurring chicken tumor, N1 C.

RPL 12: RPL 12 represents a strain of tumor originally isolated and passaged by Olson, in Massachusetts, in 1941. It has been well characterized by Burmester et al. (1946). It is known to be capable of causing lymphomatosis by inoculating susceptible chicks with either a cellular or a cell-free preparation.

RPL 16: RPL 16 is a tumor strain originating from an uninoculated bird in the RPL flock. It, too, in susceptible chicks, is capable of reproducing itself when injected as a cellular or cell-free inoculum. The tumor was brought by auto from East Lansing in the frozen state. The specimen was frozen in 1948.
OAES N1 T: Tumor Strain OAES N1 T, designated N1 in this work, was first isolated from a turkey flock near Sugar Creek, Ohio. One of three birds examined in the Laboratory had a very large liver with discrete tumors distributed throughout the parenchyma. The liver also was marked by a few small depressed lesions characteristic of infectious enteritis. Both ceca had thickened walls with ulcerative areas. Other internal organs were grossly normal. Histopathological study confirmed the diagnosis of lymphomatosis and infectious enteritis.

OAES N2 T: A second naturally occurring turkey tumor, strain OAES N2 T, originated in a flock of 25 small white turkeys purchased by a local farmer as pouls from the OAES flock. This tumor has not as yet been successfully transferred.

OAES N3 T: Recently, a third attempt was made to establish a transplantable tumor from a field case. The liver of a freshly killed turkey was brought to the laboratory. The liver, which weighed two pounds, showed no gross pathology other than tumors.

OAES N1 C: This is a naturally occurring chicken tumor adapted to intramuscular transplantation in laboratory chickens by Sanger and Belding (1957). It has not as yet been characterized completely, although it kills from 80 to 100 per cent of intramuscularly inoculated young chicks in from 7 to 14 days.
Since this neoplasm appeared in the livers of a large proportion of birds five to six months after the original intramuscular inoculation with a cellular suspension, it almost certainly can be transmitted by the inoculation of cell-free material. Although this neoplasm will produce local tumors in 90 to 100 per cent of poult's inoculated with cellular suspensions, it has been used in this work primarily to test the antigenicity of N1 T in chicks.

PREPARATION OF INOCULA

Cellular Inocula: Cellular inocula were prepared from frozen RPL 12 in the manner outlined by Burmester et al. (1944). The sealed tubes were thawed rapidly in running tap water and emptied into sterile beakers. The tumor was cut into small fragments with sterile scissors and further reduced in a TenBroeck tissue grinder using 0.85 per cent solution of sodium chloride in sterile distilled water. All inocula contained crystalline penicillin at levels of from 10 to 1,000 units per ml. and dihydrostreptomycin at from 10 to 20 milligrams per ml. The higher levels were used when evidence of bacterial contamination was present in the tumor.

The resulting suspension was strained through fine gauze and transferred to small rubber-stoppered bottles. During preparation, and until inoculations were made, the suspension was kept cool by using ice and water around the glassware.

Preparation of the inoculum was completed as rapidly as possible, and injections into birds were made without delay.
Cellular inocula for serial transfer were made from freshly harvested neoplastic tissue.

As soon as a large palpable mass was formed, the birds were destroyed by cervical luxation. Using aseptic technic, the breast muscle was exposed and affected tissue removed. An attempt was made to trim as much normal muscle as possible from the periphery of the tumor without removing the outermost areas of tumorous involvement. The affected tissue was minced with a small amount of diluent using scissors. Further reduction was carried out either with a TenBroeck tissue grinder or the trypsinizing process and re-inoculated into birds for the next consecutive passage.

Cell-free Inocula: The preparation of cell-free inocula was accomplished by harvesting tumorous tissues and processing them in a Waring blender for 10 minutes, followed by high speed centrifugation for 15 minutes.

Tissues were divided into two equal portions and placed into two containers. Diluent was added at the rate of two parts to each one of tissue. The containers were processed on the blender alternately for periods of two minutes. While one was being run, the second was placed in an ice-water mixture in order to prevent over-heating of the suspension. After each container had been processed for a total of 10 minutes, the suspension was aspirated into lucite tubes and spun for 15 minutes in a refrigerated Spinco centrifuge.
at 29,000 x gravity. The supernatant was saved for inocula-
tion or freezing and storage; the sediment was discarded.

Trypsinization: During the second year of work, most
cellular inocula were prepared by digesting the tumor with
trypsin 1:250 (Difco).

Tumor tissue was harvested and minced in a small beaker
using scissors. A 0.5 per cent solution of trypsin in Hanks' 
balanced salt solution containing antibiotics was prepared
and adjusted to a pH of 7.4 using a sterile 4 per cent solu-
tion of sodium bicarbonate. The finely minced tumor was placed
in a 250 ml. Erlenmeyer flask containing a plastic-coated bar
magnet. The trypsin solution was added at the rate of four
parts to each one of tumor. Heavy aluminum foil was placed
over the mouth of the flask and folded down onto the neck.
A Magnestir unit was used to agitate the tumor suspension.
The flask was either set on several thicknesses of aluminum
foil placed on top of the unit or suspended three to four
inches above the unit on a ring stand in order to prevent
heating of the tumorous material. Agitation was usually
carried out until the supernatant was cloudy and most of the
tumor had been digested. The time required for this was usu-
ally 45 to 90 minutes.

The suspension of cells was strained through fine gauze
and poured into 50 ml. centrifuge tubes. The tubes were spun
for five minutes at 750 RPM in an International portable re fri-
gerated centrifuge, model PR-2, at a temperature of 5° C.
The supernatant was poured from the sedimented cells and replaced by fresh Hanks' solution with antibiotics but without trypsin. The cells were resuspended and again centrifuged for five minutes at 750 RPM.

The supernatant was again replaced using 10 volumes of Hanks' solution to each volume of cells. After resuspending the cells with agitation, the material was examined microscopically by mixing with 1 per cent trypan blue stain and with 2 per cent gentian violet stain. The trypan blue stain was particularly valuable since it gave a relative indication as to the number of viable cells in the inoculum. Usually from 5 to 10 per cent of the cells took the stain and so were judged to be injured or dead.

Early attempts at trypsinization were made by using 0.85 per cent saline solution. During trypsinization and the first centrifugation, results appeared to be very satisfactory. When the cells were resuspended in saline solution however, the presence of large clumps of mucoid material was noted. The few cells remaining in suspension and those trapped in the viscid masses did not remain viable for longer than 60 minutes as judged from their reaction to trypan blue stain. Unless inoculations were made within one-half hour of preparation, tumor formation was either delayed or did not occur.
In the hope that additional electrolytes might improve the inoculum, it was decided to use Locke-Lewis solution (Lillie, 1954) as the fluid for trypsinization and resuspension of cells. Again the process was carried out at an adjusted pH of 7.2 to 7.4, using phenol red as an indicator. When the trypsinized, centrifuged tumor cells were resuspended in Locke-Lewis solution, destruction of cells with the formation of mucoid precipitate again occurred.

In an attempt to improve the quality of the inoculum, brain-heart infusion broth (Difco) was adjusted to a pH of 7.2 to 7.4, using phenol red as an indicator. When this liquid was used in trypsinization and resuspension of cells, the results were far superior to those achieved when 0.85 per cent saline and Locke-Lewis solution were used, although there were still indications of destruction of cells. The cellular suspension still contained many viable cells three hours after preparation as proven by both the trypan blue stain and intramuscular injections into turkeys.

When Hanks' balanced salt solution (Weller et al., 1952) was used as the vehicle for the tumor cells, results were excellent. Hanks' solution was used in the trypsinizing and washing processes as well as in resuspending the cells for inoculation. Cellular suspensions held for as long as seven hours at 4.0°C. apparently retained their pathogenicity and were as effective in producing intramuscular tumors as were freshly prepared suspensions.
ROUTES AND METHODS OF INOCULATIONS

Intra-ocular inoculation: Birds less than two weeks old were preferred for this procedure since the development of the cartilagenous plate of the eye increases the force necessary to insert the needle. Instillation of one drop of a 2 per cent solution of holoacaine three minutes prior to making the injection appeared to reduce the discomfort of the birds.

Cellular suspensions of RPL 12 tumor were prepared using the TenBroeck tissue grinder. The inoculum injected consisted of 0.02 ml. of a 1 to 20 dilution of tumor in saline containing antibiotics and buffered at a pH of 7.2. A one ml. tuberculin syringe and 27 gauge 1/2 inch needle were used. A tuberculin syringe with a white or clear glass plunger was preferred, since the pigment in the suspension made it difficult to see the end of a plunger made of dark material. The needle was inserted two to three millimeters from the lateral margin of the cornea and directed towards the pupil. When the point of the needle was visible in the pupil, the injection was made.

For serial passage, the eye was removed and cut into fragments with sterile scissors in a small beaker with an equal volume of sterile saline containing antibiotics. The minced tissue was further reduced in a tissue grinder, strained through sterile gauze and injected into the anterior chamber.
Intramuscular inoculations: Intramuscular inoculations of both cellular and cell-free preparations were usually made in amounts of 0.5 ml. A 22 or 2½ gauge one inch needle on a 2 1/2 or 5 ml. glass syringe was satisfactory, although a 3 ml. B-D Yale Lok hypoglycemia syringe was superior because its small diameter tended to increase accuracy of dosage, particularly when 0.2 or 0.3 ml. amounts were inoculated.

The deep pectoral muscles on the right side of the breast were used routinely as the site of inoculation. The needle was inserted lateral to the keel and directed anterio-dorsally for about 1/2 inch. Contact with the ribs was avoided, since intrathoracic injections usually resulted in death of the bird. A cotton pledget saturated with 70 per cent ethanol was effective in controlling feathers and down and benefited asepsis.

Injection was made into the posterior muscles of the thigh in unusually small birds. This region produced good tumor development, but harvesting was somewhat more difficult.

Intravenous inoculation: Intravenous inoculations were made into the ulnar vein. A one ml. tuberculin syringe equipped with a 27 gauge 1/2 inch needle was used for amounts up to 0.25 ml. For larger volumes, a three or five ml. syringe was satisfactory, although amounts in excess of 0.5 ml. were used only in birds more than 30 days of age. Down and feathers were removed and the area was swabbed with a pledget moistened with 70 per cent ethanol.
Intraperitoneal inoculation: Injections of both cellular and cell-free materials were made by using a three or five ml. glass syringe and a 22 or 24 gauge 3/4 inch needle. The needle was inserted into the posterior third of the ventrolateral aspect of the abdomen at about a 45 degree angle from a sagittal plane. This site avoided both parenchymatous organs and the abdominal air sacs. Amounts of inoculum were usually 0.5 ml., although as little as 0.2 ml. and as much as 1.0 ml. were used in day-old poult's with no unfavorable effects.

Diffusion chamber implantation: Since lucite rings as described by Algire et al. (1954) were not readily available, the diffusion chambers used in this work were fashioned from a plexiglass tube, 1/2 inch in diameter, which was cut into 1/4 inch long cylinders. The lumen of the tube was 1/4 inch in diameter; the walls were 1/8 inch thick. A small high-speed drill (No. 47) was used to make a hole in the wall through which the chamber could be filled. A standard round applicator stick was cut into 1 inch lengths and one end of each piece was rounded slightly. The rounded end was dipped in a syrupy solution of plexiglass in acetone and allowed to dry in order to form a film of plastic and thus prevent the contents of the chamber from contacting the wood. The coated stick fitted the hole in the chamber and formed an effective plug after the chamber was filled.
The open ends of each cylinder were covered with type TA millipore filter material which was 25 µ thick. Pore size of this cellulose ester filter averages 0.4 µ with but little variation. The material with its protective paper backing was placed on a smooth, flat surface with the filter material up. One end of an open chamber was coated lightly and evenly with MF cement and pressed firmly on the filter sheet. The sheet and backing were trimmed from the cylinder with iris scissors and the opposite end covered in a similar manner. The paper backing was easily removed from the filter material after trimming away the excess, but attempts to remove the backing before the filter had been cemented to the chamber were unsuccessful; the very fragile membrane tore before the adhesive force was overcome.

In a later model, the ends of the filter chamber were protected by a semi-circle of tubing 1/16 inch thick cemented to the rim perpendicular to the filter surface. This device was put on each end immediately after the filter membrane. In addition to protecting the delicate membranes, it served as a handle and thus facilitated the manipulation of the chambers. The completed chamber is shown in Figure 2.

A 1 to 20 suspension of trypsinized RPL 16 tumor cells in Hanks' solution was prepared and 0.25 ml. was injected into each chamber through the small hole in the side wall. This amount nearly filled the device, yet left an air space sufficient to prevent rupture of the membrane when the plug was
Fig. 2 - Improved model of diffusion chamber with handles (1) mounted to protect filter membranes (2) from viscera. The wooden plug (3) is used to seal hole (4) in chamber wall.
inserted. The round plastic-coated end of the plug was inserted into the hole with a minimum of force. The protruding wood was trimmed off close to the chamber and a drop of cement placed on the plug to insure a complete seal. As each chamber was filled, it was placed in a sterile six inch Petri dish containing Hanks' solution.

The birds selected for implantation of diffusion chambers were well-developed 14-day-old cockerels from a local hatchery. Anesthesia was induced by placing cotton, saturated with ethyl chloride and chloroform, in a large glass jar with a closely fitting lid. The bird to be anesthetized was put into the jar and the lid replaced. As soon as the bird relaxed completely, it was removed and placed in lateral recumbent position which was maintained by means of a caponizing bow. Adequate anesthesia was obtained within one to two minutes, depending largely upon room temperature.

The anesthetized bird was placed on its side and the abdominal wall was moistened with 70 per cent ethanol. A 1/2 inch incision was made in the skin and the abdominal muscles were parted by blunt dissection. The aperture into the abdominal cavity was kept open by a self-retaining tissue retractor such as is used in caponization. The filled chamber was introduced into the abdominal cavity and the wound was closed with a single through and through stitch of non-absorbent suture material on a curved needle. Care was taken to avoid puncturing the membrane of the diffusion chamber.
RESULTS

Intraocular inoculations: The first intraocular inoculum consisted of a suspension of cells of RFL 12, the chicken tumor, which it was hoped could be adapted to the turkey. The tumor, as obtained from the Regional Poultry Laboratory, consisted of frozen neoplastic tissue harvested from the breast muscle of birds which had been inoculated intramuscularly with a cellular preparation. The inoculum was prepared in the tissue grinder and injected intraocularly in the manner previously described. The second and subsequent inocula were prepared from tumorous eyes of the next preceding passage.

The first intraocular injection produced neoplasms in 27.16 per cent of the 81 birds inoculated as 2½-hour-old pouls. An inflammatory response was evident within 2½ hours after the injection. Prominent signs of the inflammatory process were photophobia, excessive lacrimation and edematous swelling of the lids. The pouls appeared rough-feathered and depressed. Some anorexia was evident, but water intake was nearly normal. This early reaction was receding by the end of 96 hours and, in most birds, had subsided by the end of seven days.

By the fifth day, a few eyes showed some evidence of tumor development. The margin of the pupil became indistinct
and the ciliary reflex was quite sluggish when compared to that in the eyes which showed only inflammatory changes. The corneas of some eyes appeared thickened, and a dark, mottled discoloration became evident (Figure 3).

Enlargement of the eyeball in affected birds appeared on the seventh day. By the eleventh day, growth and development had apparently ceased; eyes were enucleated and prepared for the second inoculation into young poults.

The second injection was made into 195 poults which again were 24 hours old. The inflammatory response, in this and subsequent passages, remained fairly constant. Tumor incidence in this passage reached 58.34 per cent by the tenth post-inoculation day. The third passage increased tumor incidence to 78.43 per cent of 102 birds injected, while the fourth passage produced 91.0 per cent positive results in nine days.

The fifth and sixth passages produced 94 and 93 per cent positives, respectively, in eight days. Histological examinations showed the presence of lymphoid cells in the ciliary body and surrounding structures, but not to the extent reported by Burmester and Belding (1949) or Burmester (1952) for the same tumor in RPL chicks. There was neither gross nor microscopic involvement of the viscera in any of the poults, although visceral involvement was common in Burmester's intracocularly inoculated chicks.
Fig. 3 - Intraocular involvement in poult inoculated in right eye with cells of RPL 12.
Survivors of this experiment were held for over 300 days but in no instance did any of the 100 adult birds show any evidence of spread of the tumor, although enlargement of the eyeball persisted throughout this period in many individuals.

Control birds for this work were 50, 24-hour-poults each of which received 0.02 ml. of normal breast muscle tissue intraocularly and 50 which were injected with the same amount of inoculum prepared from normal turkey eyes. An inflammatory response resulted in each case which was similar to that produced by the tumorous material. The reaction subsided by the end of the first week. When the birds were destroyed on the fourteenth day, some of them had apparently regained the ability to see out of the affected eye. No neoplasms resulted from the intraocular inoculation either of normal eye or muscle tissue.

Intramuscular inoculations with RPL 12: The first intramuscular injection was made at the same time that the first intraocular injection was made. The inoculum was prepared from the same frozen RPL 12 tumor and in the same manner as was the intraocular material.

The suspension of tumor cells was injected into the deep pectoral muscles in amounts of 0.5 ml. for each bird. Although 87 inoculated birds were observed for 34 days, no palpable neoplasms were found. Starting on the seventh day, when ocular tumors were developing in the birds which received the intraocular injection, two birds were sacrificed every
second or third day. The pectoral muscles of these birds were exposed and examined carefully for gross or microscopic signs of tumor development, but none were found.

When the second intraocular serial passage produced eye tumors in 58 per cent of the inoculated birds, it was decided to prepare an amount sufficient to inoculate additional birds intramuscularly. Although this inoculum produced tumors in over 78 per cent of the eyes into which it was injected, it again failed to produce any intramuscular tumors in any of 143 poults which received the material intramuscularly. This procedure was repeated at the time of the fourth, fifth and sixth intraocular passage with similar results.

The next attempt to reproduce intramuscular tumors was made by procuring frozen RPL 12 and chicks of susceptible line 15 from the Regional Poultry Laboratory. The inoculum was prepared in a TenBroeck grinder and injected intramuscularly into 50 young chicks and 75 young poults. No tumors grew in any of the poult's but eight chicks, killed on the eleventh day after the injection, each had fairly well-developed neoplasms at the site of inoculation. These tumors were again reduced in a TenBroeck tissue grinder and re-inoculated into 120 three-day-old poults and 25 chicks which were thirteen days old.

The second passage in chicks resulted in an increase from 16 per cent to 84.6 per cent in the number of tumor takes. This same inoculum produced intramuscular tumors in 8.33 per cent of the 120 poult's inoculated. Tumors harvested from
poults were used to complete eight serial inoculations. As shown in Table 2, the number of successful takes increased with each transfer through the fourth but was somewhat variable in the fifth, sixth, and seventh passages. The eighth passage was entirely unsuccessful, although the seventh had given very good results.

Fortunately, tumorous tissue had been harvested from poults of the fourth passage and stored in an insulated chest with "dry ice" as a refrigerant. This tissue was processed in a TenBroeck grinder and inoculated into 75 poults which were one week old. Tumors developed in 35 poults, or 58.3 per cent, in ten days. During the next three serial passages of this material, there was again a progressive increase in the number and per cent of successful takes. The fifth passage from the frozen state failed to produce any tumors although some poults were held for twenty-eight days after inoculation.

At the time the inoculum was prepared for serial passage, each bird in the group was examined by digital palpation for the presence of intramuscular tumor. The largest tumors were harvested and used for preparation of inoculum for the next passage. All birds which failed to develop neoplasms and those with small tumors were sacrificed and examined for indications of visceral involvement. In no case did any poults inoculated with a cellular suspension of RPL 12 show any gross or microscopic signs of such metastasis.
Table 2

Effect of Intramuscular Serial Passage of Cells of RPL 12 on Tumor Incidence and Rate of Development in Inoculated Poults

<table>
<thead>
<tr>
<th>Passage</th>
<th>Inoculum Source</th>
<th>Recipients</th>
<th>No. Birds Treated</th>
<th>Nos. Lived</th>
<th>Tumors Percent</th>
<th>Transfer Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Frozen Poults</td>
<td></td>
<td>75</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Frozen Chicks</td>
<td></td>
<td>50</td>
<td>50</td>
<td>16.0</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>Chicks Poults</td>
<td></td>
<td>120</td>
<td>120</td>
<td>8.33</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Poults 1 Poults</td>
<td></td>
<td>103</td>
<td>99</td>
<td>15.15</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>&quot; 2 &quot; Poults</td>
<td></td>
<td>101</td>
<td>98</td>
<td>66.32</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>&quot; 3 &quot; Poults</td>
<td></td>
<td>111</td>
<td>107</td>
<td>89.72</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>&quot; 4 &quot; Poults</td>
<td></td>
<td>87</td>
<td>84</td>
<td>85.71</td>
<td>7</td>
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<tr>
<td>6</td>
<td>&quot; 5 &quot; Poults</td>
<td></td>
<td>64</td>
<td>60</td>
<td>75.0</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>&quot; 6 &quot; Poults</td>
<td></td>
<td>101</td>
<td>96</td>
<td>96.87</td>
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<tr>
<td>8</td>
<td>&quot; 7 &quot; Poults</td>
<td></td>
<td>75</td>
<td>73</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>
One hundred and twenty poults, selected because of the development of large breast tumors, were held for observation. A few were selected from each group of passages number six, seven and eight. One week after the birds were selected, each one was palpated for tumors, and four, selected at random, were destroyed and examined visually for breast tumor and visceral involvement. This procedure was carried out for five weeks at intervals of five to seven days.

During the second week after inoculation, little change in appearance of the tumors was seen. The cut surface was smooth, moist, and glistening. The tumor substance was a pearly-grey in color and was translucent when sliced in thin sheets. The periphery of the mass was indistinct; it merged gradually with the surrounding muscle tissue. Blood vessels in the muscle were slightly enlarged in the area around the neoplasm; other clinical signs of inflammation were usually absent.

At about fourteen to sixteen days after inoculation, the center of the tumor started to change. It became pink in color and appeared dry at the cut surface. In consistency, it approximated a dry cheddar cheese, although it was somewhat more friable.

During the third week, the central core enlarged at the expense of the surrounding neoplasm so that the swelling of the breast subsided. By the fourth week, all visible tumors had disappeared. The tissue in contact with the central necrotic mass was sharply outlined by a hyperemic zone which
resembled granulation tissue. The necrotic material gradually disappeared, being replaced by the apparently normal muscle which had been crowded outward.

By the sixth week after the inoculation, most birds appeared normal. In two which were killed, a remnant of the central core was present and in the other two, a small shred of yellow fibrinous material was found between the deep pectoral muscles.

At the end of 180 days, 40 birds were sacrificed and the breast muscle and viscera examined for signs of neoplasia. No indications of tumor were found. The remaining forty birds were killed at 240 days, but again no tumors were found.

The growth characteristics of RPL 12 tumor in the susceptible RPL line 15 chick have been determined. Turkeys are not a natural host for this neoplasm. It was hoped that serial passage in turkeys would alter the agent for chickens of known susceptibility. In order to determine whether or not changes in the agent had occurred, plans were made to have one-day-old RPL line 15 chicks available at the time when twelfth turkey passage material was harvested.

During the autumn of 1957, eleven successful serial passage were made in young poult's which were purchased from a commercial hatchery. Inoculations were made into 50 poult's once each week on Saturday. Throughout this series of passages, mortality was low but satisfactory growth of tumors was variable from week to week. In some groups, all of the birds produced satisfactory tumors, while in other groups tumor incidence fell to as low as
50 per cent of the inoculated birds. This variation in susceptibility was probably an expression of genetic resistance, since several different flocks supplied eggs to the hatchery from which the poulets were purchased.

During the third and fourth days following the twelfth serial inoculation, all birds which had been injected with tumor cells died, as did also 35 of 50 uninoculated poulets of the same age which were in the same room. Older poulets in this room remained unaffected so it was assumed that chilling of the inoculated birds was responsible for the loss.

For the fourth time, RPL 12 was started in serial passage in poulets. When the fifth passage produced neoplasms in six days, in 38 of 39 survivors (97.4 per cent) of a group of 40 young poulets, cell-free material was prepared for inoculation into chicks.

The only available chicks were from a local hatchery. Sanger (1958) has proven that these birds are susceptible to cells of RPL 12 and is studying their susceptibility to cell-free inocula.

Sufficient tumor of the fifth turkey passage was harvested to prepare both cellular and cell-free inocula according to the procedures previously mentioned. One group of 11 chicks and one group of 11 poulets were each injected intramuscularly with 0.5 ml. of a suspension of trypsinized cells to prove that the cells were capable of reproducing the neoplasm. On the seventh day following the inoculation, all ten surviving
chicks had tumors in the breast and nine of the ten surviving poultus also had breast tumors.

The rest of the fifth passage turkey tumor was used to prepare cell-free inocula. After the tumor was processed for nine minutes in the Waring blender, 11 chicks were inoculated intramuscularly with the disrupted cells and observed for 30 days to see if any tumors would develop. No neoplasms were found on digital palpation, indicating that few if any cells survived in the processed material. When the ten survivors were sacrificed at the end of 132 days, all were negative for any form of leukosis excepting for one bird with neural involvement.

The material from the blender was filtered to remove the coarse particles and the filtrate was spun for three hours in a Spinco ultracentrifuge model L. The material was placed in lucite tubes in the number 30 rotor and run at 17,500 RPM under refrigeration. When centrifugation was completed, the supernatant was removed and the sediment resuspended in Hanks' solution. An amount of Hanks' solution equal to one-half of the original volume was used.

The resuspended sediment was inoculated intraperitoneally into 40 chicks which were three days old. The undiluted supernatant was inoculated intraperitoneally into an additional 40 chicks and 40 poultus, all of which were three days old.

Although survivors of this work have now (November 14, 1958) been observed through 150 days, no gross signs of any form of leukosis have appeared other than one additional case of neural lymphomatosis in a chicken.
RFL 12 cells and Metacortin: On the day before the fourth intramuscular tumor passage was to be made, two groups of fourteen poult concentrations were selected at random and penned in separate wire cages. One group, designated group M, was treated twice daily for the next three days with 2 mg. of Metacortin suspension (Schering) intramuscularly. The second group, group 0, was not given Metacortin.

At the time at which the fourth serial passage was made, birds of both groups were each given an intramuscular inoculation of 0.5 ml. of cellular suspension. Group M received three injections of Metacortin before it was inoculated with tumor cells and three injections after the cellular inoculation.

The proportion of positive birds in the untreated group of fourteen poult concentrations was 92.85 per cent. This figure is comparable to that of the fourth passage birds, which was 89.72 per cent of 107 inoculated survivors, but is in contrast to the figure for the treated group, which was 42.85 per cent positives.

Metacortin inhibited the development of tumors; those present in the treated birds were smaller. The average size of the tumor in untreated birds was 2.92, that in the treated birds was 2.33.

The results of this work are shown in Table 3. The poult concentrations which were given Metacortin were also appreciably smaller at the end of the experiment than were those which were not treated.
Table 3

The Effect of Metacortin on the Development in Poults of Tumors Due to RFL 12 Cells in Fourteen Days when Injected Intraocularly and in Eight Days when given Intramuscularly

<table>
<thead>
<tr>
<th>Route and Treatment</th>
<th>No. of Birds</th>
<th>No. with Tumor</th>
<th>Tumor Sizes</th>
<th>Average Tumor Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Birds</td>
</tr>
<tr>
<td>Intraocular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>10</td>
<td>10</td>
<td>0 1 2 4 3</td>
<td>2.9</td>
</tr>
<tr>
<td>Tumor + Metacortin</td>
<td>10</td>
<td>5</td>
<td>5 2 3 0 0</td>
<td>0.8</td>
</tr>
<tr>
<td>Intramuscular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>14</td>
<td>13</td>
<td>1 2 2 4 5</td>
<td>2.7</td>
</tr>
<tr>
<td>Tumor + Metacortin</td>
<td>14</td>
<td>6</td>
<td>8 2 1 2 1</td>
<td>7.9</td>
</tr>
</tbody>
</table>
An additional group of ten birds was similarly treated with Metacortin to determine the effect on the development of intraocular tumor. Ten untreated birds were penned separately as controls. From the results shown in Table 3, it is apparent that Metacortin reduced tumor incidence by approximately fifty per cent. Tumors were classified according to size. Small tumors were graded as one, two and three were progressively larger, while four indicated a very large, fully developed neoplasm. The average size of the tumor in untreated birds was 2.55 while that in the treated birds was 1.5.

Intramuscular RPL 16: On July 18, 1958, one tube of tumor strain RPL 16 was brought from East Lansing in the frozen state. The specimen was frozen on March 5, 1948 and stored under dry-ice refrigeration at the Regional Poultry Laboratory for ten years, four months and thirteen days.

The specimen was thawed rapidly, reduced in a TenBroeck tissue grinder and injected intramuscularly into twelve chicks and six poults, all of which were one day old.

One drop of the cellular suspension was stained with trypan blue and examined microscopically before injections were made. Careful inspection of the stained material failed to reveal any unstained cells. Many deeply stained and a few lightly stained cells were seen, indicating that deterioration had occurred.
On the eleventh day after the inoculation was made, one poult and three chicks had a fairly large tumor in the breast muscle. The poult was sacrificed and the muscle tumor was prepared into a cellular suspension which was inoculated into the breast muscle of twelve poults. This injection produced large tumorous masses, in a period of six days, in eleven of the twelve inoculated birds.

The third serial passage was made into fifteen birds. This passage produced eight deaths on the sixth day; the last survivor in the group died on the eighth day after the injection was made. The breast tumor was very large and the liver was large and friable and had multiple pin-point grey foci on the surface. The histological picture of the local tumor is shown in Figure 4; that of the pancreas in Figure 5.

In order to prolong the life of inoculated birds, the inoculum was reduced in volume from 0.5 ml. to 0.2 ml. for the fourth serial passage. The decrease in dosage of cells was effective in that survival rate of inoculated poults went up to 50 per cent, although nine chicks died out of ten which were given the same amount of inoculum.

Naturally occurring turkey tumor N1 T: A cellular suspension was prepared from the liver of a turkey suffering from naturally occurring lymphomatosis. This bird also had lesions of infectious enterohepatitis on the liver. Preparations of this tumor and preparations of serial passages in turkeys are designated N1 T in this work. A numeral following
Fig. 4 - Intramuscular involvement in poult inoculated intramuscularly with cells of RFL 16. Notice extreme invasion with only remnants of muscle (M) remaining. H and E. X345
Fig. 5 - Pancreas of poult inoculated intramuscularly with cells of RPL 16. Metastatic focus (F) of lymphoid tumor. H and E. X120
the T indicates the number of serial passages; thus N1 T10 indicates the tenth consecutive passage in turkeys.

The original inoculum was injected into a total of 104 poults which were two weeks of age. Of this number, 64 were given the material intramuscularly, while 40 were injected intraperitoneally. The same inoculum was also injected intramuscularly into 25 one-week-old chicks. Of the 64 intramuscularly inoculated poults, 28 received material prepared in a TenBroeck tissue grinder, while 36 were given a trypsinized preparation.

On the twenty-first day following the inoculations, only the poults which had received trypsinized tumor cells intramuscularly showed any indication of the development of tumors; all other birds were negative.

The tumors in the breast muscles produced in the first passage were very firm and contained much necrotic material. The second passage tumors, which developed in seven days, and third, which were harvested on the eleventh day, were also very necrotic. In the third passage, only five survivors remained on the eleventh day; eleven deaths occurred. Survivors were sacrificed and a cellular suspension made from their tumors by trypsinization. This material was inoculated into eighteen poults and twelve chicks. In harvesting tumor for this and other passages, an attempt was made to exclude necrotic tissue.

A small portion of the cellular suspension was streaked on blood agar culture medium made with blood agar base (Difco)
with 5 per cent bovine blood added. At the end of 24 hours, the resulting bacterial growth was removed with saline solution and injected in 0.5 ml. amounts into the breast muscle of ten young poults. No detectable swelling occurred at the site of inoculation. When the birds were sacrificed and examined on the twelfth day after the inoculation, no signs of necrosis or acute inflammation remained in the area where the injection was made.

Of the twelve chicks inoculated with material harvested from the third passage in poults, one died on the tenth day with a large neoplasm and no necrosis in the breast muscle. When a suspension of this tumor was again passed in chicks, it produced tumors in 20 of 25 inoculated birds with no detectable necrosis. The same suspension inoculated into poults again produced necrotic tumors. All remaining chicks were sacrificed and the neoplastic tissue harvested for freezing and storage identified as N1 T3 G2.

Of the eighteen one-day-old poults inoculated with N1 T3 material, twelve died within eleven days. The six survivors were sacrificed for passage. Three of the six showed evidence of liver involvement; the organs were enlarged, friable and had small, raised grey foci on the surface. Microscopic studies of the liver revealed metastasis as shown in Figure 6. A photomicrograph of the tumor in the breast is shown in Figure 7.
Fig. 6 - Metastasis in liver of poult inoculated intramuscularly with cells of Ni T. H and E. X503
Fig. 7 - Intramuscular tumor resulting from local inoculation with cells of NL T. Notice tumor cells invading muscle (M). H and E. X231
This naturally occurring turkey tumor is now (November 14, 1958) in the nineteenth serial passage in pouls. Since the seventh passage, intramuscular doses of 0.5 ml. have regularly produced mortality of from 90 to 100 per cent in young pouls. In order to prolong the lives of inoculated birds, dosages of from 0.1 to 0.25 ml. have been used. The lesser amounts of inoculum produce a mortality of about 50 per cent; most of the deaths occur from the eighth to the twelfth days.

Since the seventh passage in pouls, repeated attempts to reproduce the turkey tumor in the chicks which are available have not been successful. No local tumors have resulted, even when dosages of one ml. of suspension have been used.

Diffusion chamber implantation: A total of 20 chicks were used in this study; ten 1½-day-old chicks were each given 0.2 to 0.3 ml. of a suspension of trypsinized cells of RPL 16 intraperitoneally. These birds served as controls, proving both that the cells were viable and that the birds were susceptible. The ten chicks which received the diffusion chamber, filled with the same amount of the same cellular suspension, were from the same hatch as the controls. These birds were wing-banded for identification.

The filter membrane was examined critically under a binocular dissecting microscope immediately before implantation into the abdominal cavity of the bird. One membrane on each of two chambers was found to be ruptured at the time it was implanted; the remaining eight chambers were intact when placed into the bird.
On the sixth day after this experiment was started, one of the birds implanted with the chamber having a ruptured membrane died; the second bird, which died on the seventh day, had also been implanted with a known defective filter membrane. Both of these birds had developed masses of tumor tissue which surrounded the diffusion chambers and which involved the mesentery, liver, pancreas and kidney. Figure 8 shows the presence of tumors developing in the liver.

On the seventh day, five "no chamber controls" died, followed by four deaths on the eighth day. Considering that nine of the ten "no chamber controls" had died and that both birds known to have been given the ruptured chambers had also died, it seemed advisable to destroy one bird from the group implanted with intact chambers for histological study. This was done on the eighth day after implantation.

It was necessary to sacrifice three birds before one was found with the filter membranes on the diffusion chamber still intact. Chambers in the other two sacrificed birds were surrounded by masses of tumors. The pathology in the viscera was similar to that shown in Figure 8.

Microscopic study of slides prepared from the viscera of the chick with the intact chamber was negative for foci of tumors. No indication that any cells had escaped from the chamber could be found.

On the ninth day the last of the "no chamber controls" died as did also two more which had received intact chambers.
Fig. 8 - Tumor in abdomen of chick with metastases in liver as a result of ruptured diffusion chamber containing cells of RPL 16. Metastatic lesions in liver (A); tumor mass resulting from rupture of diffusion chamber (B). H and E. X77.
In both birds, the filter membrane had been disrupted at some time after implantation. The last three birds to die were from the group implanted with chambers which were intact when put in place. One bird died on each of the twelfth, thirteenth and seventeenth post operative days. In each case, the chamber had at least one of the two filters disrupted.

An improved chamber (Figure 2) has been designed since the above birds were implanted. It is expected that the semi-circular guards will protect the filter membrane while the chamber is in place, and will thus give improved results. This work will be repeated in the immediate future, using the improved chambers. Before any valid conclusions can be drawn, it will be necessary to study several birds in which the chamber remained intact until the bird was destroyed. The very limited apparent success in retaining the neoplastic cells within the chamber in one of the eight birds is, however, rather encouraging.

Serology and immunology: The results reported here are preliminary to a concerted effort to study the antigenicity of turkey passaged tumors in the chick.

Intramuscular inoculation of 0.25 ml. of a suspension of RPL 12 T6 cells was made into 20 one-week-old small white turkeys. On the eighth post-inoculation day, the birds were examined and three which failed to produce palpable tumors were destroyed. During the third week after the inoculation, 12 of the largest and healthiest birds were selected and re inoculated with 0.5 ml. of RPL 12 cells which had been passaged
in poults. No local tumor developed as a result of this or subsequent cellular inoculations into these poults. Two additional injections of RPL 12 turkey cells were made at weekly intervals. All preparations were made with a Ten-Broeck tissue grinder.

When these birds were four months old, a second series of three inoculations of 1.0 ml. of cellular suspension derived from turkey tumors was given. Two weeks later, the immunized birds were bled and turkey immune serum (TIS) prepared and stored in the frozen state.

A trypsinized preparation of cells of a field strain of chicken tumor N1 C was made. After the cells were washed with Hanks' solution, they were centrifuged and resuspended in turkey serum at a one to ten dilution. One portion was resuspended in TIS; a second in normal turkey serum (NTS). Both suspensions were incubated for 30 minutes at room temperature.

Twenty chicks of the same hatch were divided into two groups. The birds in one group were inoculated intramuscularly with 0.5 ml. of the one to ten dilution of N1 C cells in NTS, while each bird in the second group was given 0.5 ml. of the one to ten dilution of N1 C cells in TIS.

On the eighth day following the inoculation, the first bird died in the group given cells incubated with NTS. On the eleventh day, five survivors remained. All 10 birds in the group were dead by the fourteenth day. Tumor development in the breast muscle was good in each bird.
No deaths occurred in the birds inoculated with N1 C cells incubated in TIS until the thirteenth day. On the eighteenth day, five survivors remained in the group. The last surviving bird died on the twenty-fifth day after the inoculation was made. The tumors in these birds were not significantly different from those in the birds given chicken tumor cells and NTS, excepting that their development was delayed to a considerable extent.

Since the TIS was produced in turkeys by immunization with RPL 12 cells which had been passed in poults for a minimum of six serial passages, it is apparent that the inhibitory factor was specific for the lymphoid cells in the chicken neoplasm.

As previously stated, the naturally occurring turkey tumor, N1 T, has repeatedly been inoculated into chicks. Since the seventh serial passage in turkeys was completed, this tumor has consistently failed to produce local neoplasms in chicks, even when as much as 1.0 ml. of a one to ten dilution of cells was inoculated.

For some time it has been known that lymphoid tumor cells are antigenic; they stimulate the production of specific antibodies by the inoculated bird. In chicks which survive the primary tumor, these antibodies prevent the development of local tumors when the bird is subsequently re-inoculated with cells of the same tumor strain. Unfortunately, these anti-cellular antibodies do not afford protection against the virus. Birds which survive the primary tumor may die at a later time from the effect of the viral agent.
To determine the antigenic activity of turkey tumor strain NL T in the chicken, ten chicks were inoculated with 0.5 ml. of a one to twenty dilution of trypsinized cells of NL T. One week later, each chick was given 0.5 ml. of a suspension of cells of the naturally occurring chicken tumor, NL C, prepared in a TenBroeck grinder. At the same time, the same amount of the suspension of NL C cells was injected into 40 chicks of the same breeding, but one week younger. On the seventh day after these 40 birds were inoculated, four died with 3+ and 4+ tumors. Six others were sacrificed for serial passage. On this same day, ten survivors were palpated for breast tumors. The ten birds inoculated with NL T cells, followed by cells of NL C, were also palpated. The results of palpation of both groups at one week are shown in Table 4. It is apparent that inoculation with cells of NL T prior to injecting cells of NL C did not prevent the development of NL C tumors. The failure of local tumor development in two of the vaccinates was probably due to experimental factors rather than to the vaccine, since there was no great difference in size of the tumors which did develop.

Two weeks after both groups were inoculated with cells of NL C tumor, all 30 birds in the unimmunized group were dead, the local tumor was very large in each bird. No deaths had occurred in the 10 birds which were inoculated with NL T cells prior to receiving the inoculum of cells of NL C. By the twenty-first day after challenge, five of these birds had no local tumors. Two of the ten survivors had 1+ tumors, one
Table 4
The Resistance of Chicks to Cells of a Field Strain of Chicken Tumor when Previously Inoculated Intramuscularly with Cells of a Field Strain of Turkey Tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Birds</th>
<th>No. with Tumor</th>
<th>Tumor Sizes</th>
<th>Average Tumor Size*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Turkey tumor cells</td>
<td></td>
<td></td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>followed by Chicken tumor cells</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Chicken tumor cells only</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* One week after inoculation with Chicken tumor cells.
had a 2+ and two had 4+ neoplasms at the site of inoculation with N1 C cells.

On the twenty-third post inoculation day, the first death occurred in the vaccinated birds; the second occurred on the following day. Both of these birds had 4+ tumors in the breast muscle but no visible involvement of the viscera.

Digital palpation of the breast muscles of the eight survivors on the thirty-third post inoculation day revealed the presence of a small tumor in one bird; the other seven were negative. This bird and one of those with no tumor were sacrificed and histological study for metastasis to the liver and pancreas was made. No increase in lymphoid tissue in the liver or pancreas was apparent in either bird, although tumor cells were found at the site of inoculation in the bird with the palpable neoplasm. The six surviving birds in the vaccinated group are being held for an extended period of observation. Although 45 days have passed since the challenge inoculum was given, these survivors all appear to be normal, healthy birds.

This limited experiment is being repeated at the present time. A group of eleven chicks were inoculated at seven and at fourteen days of age with 0.5 ml. of a one to ten dilution of cells of N1 T. A second group of ten chicks of the same hatch was not injected. On the twenty-first day after hatching, each bird in both groups was given 0.5 ml. of a suspension of cells of tumor strain N1 C. On the twelfth day after inoculating N1 C cells into the breast muscle, all eleven
previously vaccinated birds have well developed local neoplasms. Two of the ten unvaccinated controls failed to produce tumors. No deaths have as yet occurred in either group.
DISCUSSION

One of the objectives of this work was to attempt to adapt chicken tumor strain RPL 12 to growth in the turkey. Since the anterior chamber of the eye has been proven to be one of the best sites for transplantation of heterologous tissues, it was decided that probably the most logical approach was to implant the chicken tumor cells in the turkey eye. It was hoped that adaptation to the eye would increase the potential of the neoplasms for growth in the muscle of the heterologous host.

Intraocular neoplasms persisted for over 300 days, but no metastasis developed. Coraan (1953) observed that metastasis is dependent more on the duration of growth of the primary tumor than on the size of the growth. According to this observation, RPL 12 should certainly have spread from the eye to the viscera if the strains of turkeys used were susceptible to the cells of RPL 12.

It is important to keep in mind that both OAES poults and those purchased from a commercial hatchery were apparently completely resistant to the virus of RPL 12, and also moderately resistant to cells of the same neoplasm. Although several hundred poults have been inoculated with preparations of this tumor, no deaths attributable to the action of these preparations have resulted.
RPL 12 is known to produce three different manifestations in susceptible RPL line 15 chicks. These three manifestations are lymphomatosis, osteopetrosis, and erythroblastosis (Burmester, 1947a). The turkey has never been reported as being susceptible to osteopetrosis. The only reference to erythroblastosis in the turkey is the report by Jarmai (1934). None of the turkeys used in this work have shown any indication of either osteopetrosis or erythroblastosis. It is obvious that the work with cell-free material reported in this dissertation should be repeated, but using RPL line 15 birds as recipients for the inocula prepared from RPL 12 tumor after serial passage in turkeys.

Both cortisone preparations and irradiation with X-rays are known to increase the susceptibility of animals to many neoplasms and bacterial infections. Since both of these agents inhibit the production of lymphocytes and may also destroy them, their inhibitory effect on a lymphoid neoplasm is easily understood.

Metacortin (Schering), as used in this work, appeared to be superior to cortisone (Winton, 1951) and to both cortisone and hydrocortisone as used by Lannek (1952) in inhibition of development of lymphoid tumors. Two factors must be remembered in reference to this apparent superiority; both the preparation used and the birds which received it in this work were different from those reported by the two authorities cited. The chicks, probably, and the turkeys, certainly, had a natural resistance to the neoplasm different from the birds reported by Winton and by Lannek.
A comparison of the results obtained by the inoculation of RPL 16 cells intramuscularly into poult's with those obtained by the similar use of RPL 12 emphasizes the difference in susceptibility of the same strain of birds to two similar, but different, neoplastic cell strains.

Since there is no correlation between susceptibility of a bird to cells of any given tumor and susceptibility to the virus of the same tumor (Dardiry et al. 1952), one cannot assume that the virus of RPL 12 and that of RPL 16 are dissimilar in infectivity for OAES poult's. It is entirely possible that there are strains of turkeys which are more resistant to RPL 16 than they are to RPL 12. Much of the confusion in the literature on avian lymphomatosis can be attributed to the failure of research workers to grasp the basic concept of variation in resistance between strains of birds to any given agent. Workers have been criticized because others, working with different strains of birds, have been unable to duplicate their results.

Successful adaptation of a field strain of turkey lymphomatisis, NL T, was accomplished June 20, 1958. Mortality following intramuscular inoculations of 0.5 ml. of a 1 to 20 dilution of cells into poult's has been high. Metastasis occurs commonly in poult's which survive longer than about eight days after inoculation.

Although early attempts to reproduce this neoplasm in chicks were successful, repeated attempts made since the seventh turkey passage, to grow the tumor in chicks have been completely
unsuccessful. As shown in Table 4, a single intramuscular inoculation into chicks affords appreciable protection against the cells of a highly virulent naturally occurring chicken tumor. The "vaccinating dose" of Nl T cells was prepared from chicken tumor in a TenBroeck tissue grinder, without the use of trypsin. This was done to avoid the possibility of sensitizing the chick against antigens in the challenge material other than those present in the lymphoid cells.

Although chicks inoculated with Nl T cells have shown no local reaction, the possibility exists that they may prove susceptible to a filtrable agent assumed to be present. This possibility must be ruled out by inoculating chicks with cell-free tumor filtrates and observing the inoculated birds for long period of time. The limited work reported here indicates only that Nl T cells, inoculated into chicks, stimulate antibody against Nl C cells. That the inoculated chicks do not show an increase in lymphoid foci in the liver and pancreas is presumptive evidence that anti-viral antibody is also produced by the inoculated chick. Plans to investigate this point more fully are being formulated.

"Vaccines" which produce antibodies against lymphomatosis have already been reported (Burmester et al., 1957). These vaccines do not affect the incidence of naturally occurring lymphomatosis; their effect is apparent only when "protected" birds are challenged. Although progeny of the vaccinated birds may themselves have some protection against the agent, they
are theoretically capable of transmitting the agent to susceptible birds.
SUMMARY

Attempts were made to adapt cells of avian lymphomatosis from chickens to passage in turkeys. Two tumor strains, RPL 12 and RPL 16, were obtained from the Federal Regional Poultry Research Laboratory (RPL) at East Lansing, Michigan. These tumor strains have been used for more than 15 years at the Regional Poultry Laboratory, in studies conducted on the chicken. To the writer's knowledge, only RPL 16 has been transmitted to turkeys (Julian, 1958).

Both RPL 12 and RPL 16 were reproduced in turkeys during the two years necessary to carry out the present work. Although RPL 12 cells and cell-free inocula were injected into more than 1,000 young poults by each of several routes, this neoplasm failed to metastasize to any visible extent and was not proven to be the cause of death of any poults. Several hundred young chicks were inoculated with similar preparations by Sanger (1958) during this same period. Visceral metastasis and death occurred in a large proportion of inoculated chicks, proving that the agent inoculated into the poults had not lost its pathogenicity.

Poults, inoculated several times with cellular suspensions of RPL 12, produced serum which had an attenuating effect on the cells of a highly virulent field strain of chicken lymphoid tumor (N1 C). A suspension of N1 C cells of chicken origin was incubated for 30 minutes in turkey immune serum (TIS) and a second suspension of N1 C cells
was similarly incubated in normal turkey serum (NTS). Ten young chicks inoculated with NL C cells + NTS died within a period of from eight to 14 days. Ten chicks of the same hatch died in from 13 to 25 days when inoculated at the same time with the suspension of NL C cells + TIS.

Cells of RFL 16 proved to be lethal for the poults inoculated with them. This tumor killed up to 100 per cent of intramuscularly inoculated poults; visceral metastasis was common during the second week after the inoculation was made.

Most suspensions of turkey tumor cells were prepared by trypsinization, rather than by mincing or grinding, during the second year of this work. This process yielded a greater number of living tumor cells in proportion to the amount of tissue debris than did the TenBroeck tissue grinder. Cells were more evenly dispersed and so were easier to count accurately. It was felt that the antibiotics used, which were penicillin and dihydrostreptomycin, were more effective in the relatively pure suspension of tumor cells produced.

Diffusion chambers were constructed from lucite rings and Millipore filter membranes with a pore size of 0.4 μ. One limited study of metastasis was made. This study, which is to be repeated, indicated that the spread of the neoplasms from the initial intramuscular tumor to the viscera is due to metastasis of tumor cells and not to the effects of the virus.

A naturally occurring turkey tumor (NL T) was isolated and successfully carried through at least 19 consecutive serial passages in young poults by the use of cellular
suspensions injected intramuscularly. Early attempts to implant cells of this tumor in chicks were successful but, since the seventh passage in poults was completed, the cells have apparently completely lost their virulence for chicks. The single injection of the cellular suspension of this neoplasm into chicks increased their resistance to challenge with cells of the highly virulent tumor, Nl C. Nl C cells alone killed 100 per cent of a group of 30 chicks within 14 days after the intramuscular inoculation of 0.5 ml. of suspension. Ten chicks of the same breeding, inoculated with Nl T cells one week prior to being given the same inoculum of Nl C, survived for at least three weeks. Histological study of the liver and pancreas of two birds in this group was done on tissues harvested on the thirty-third day after the inoculation of cells of Nl C. There was no apparent increase in lymphoid tissue in the liver or pancreas of either bird.

On the basis of the results obtained in these preliminary experiments, the possibility is discussed that a naturally occurring turkey lymphoid tumor may prove to be a source of vaccines against lymphomatosi in chicks.
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In 1946, I graduated from Michigan State College with the degree of Doctor of Veterinary Medicine. After some experience in small animal practice, I was employed for one year at the Federal Regional Poultry Research Laboratory at East Lansing, Michigan, as a Veterinarian P-2.

In 1949, I was employed at Michigan State College as an Instructor in the Department of Bacteriology and Public Health. I received the Master of Science Degree in Bacteriology from Michigan State College in 1954. Since January 1, 1957, I have been an Assistant in the Department of Veterinary Science at the Ohio Agricultural Experiment Station in Wooster, Ohio, and have completed the requirements for the degree of Doctor of Philosophy in the Department of Veterinary Preventive Medicine at the Ohio State University. I expect to return to Michigan State University as an Assistant Professor on January 1, 1959.