EFFECT OF BETA-PROPIOLACTONE ON AVIAN LYMPHOID TUMOR CELLS

BY

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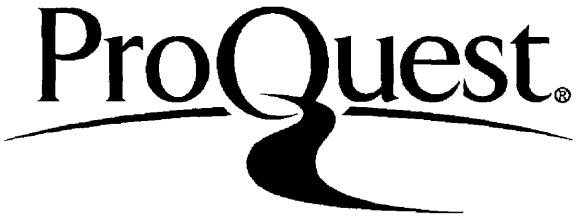

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INTRODUCTION

Lymphomatosis is a fatal infectious neoplastic disease of chickens caused by one or more virus-like agents. It is thought to have two phases, an infectious phase causing non-neoplastic lymphoid proliferations or ectopic lymphoid areas, and a neoplastic stage characterized by the infiltration and multiplication of lymphoid cells in the various tissues of the bird's body resulting ultimately in tumors of the viscera and other tissues. DeOme (1940) classified avian lymphomatosis as a typical neoplastic disease possessing the following properties of neoplastic growth:

1. Uncontrolled and unorganized growth.
2. Strong evidence of metastasis.
3. The ability to invade, displace, and replace normal tissue.
4. Predilection for certain tissues and locations.

There are four types of lymphomatosis dependent upon the tissue involved and all are included under the classification of avian leukosis complex. These four types are visceral, neural, ocular, and osteopetrotic, with visceral lymphomatosis probably the most prevalent. This form of the disease causes single or multiple tumors affecting the liver, lung, pancreas, ovary, muscles, skin, etc. Whatever organ or tissue is affected, it is not unusual also to find diffuse infiltration of a leukemic nature, or sometimes an involvement of the blood which clearly indicates the leukemic nature of the disease.

The ocular form shows itself as a grayish coloration of the iris caused by infiltrations of lymphoid cells. Protrusion of the eyeball may result and impairment of vision, even to total blindness, follows.

The neural form is characterized by the infiltration of the nerves with large lymphocytes causing tumor-like growths. The nerve fibers appear to die for lack of nourishment and are apparently dissolved and carried away by the blood stream, causing an impairment in normal function of structures supplied by these nerves. Paralysis of the wings or legs is the most common and characteristic involvement observed clinically.

The skeletal or osteopetrotic form of lymphomatosis affects primarily the diaphysis of the long bones which show proliferation of the spongy bone both at the periphery and in the interior. The occurrence of this form of the disease is rare as compared to that of the other forms. Present belief indicates that all four types of the disease represent four distinct disease entities caused by four distinct and different agents (Waters, 1954).

An estimated mortality of more than 53,000,000 adult chickens with a value of at least $73,000,000 occurred in the United States in 1953 (13th Report of the Regional Poultry Research Laboratory, 1955). This tremendous loss does not include the impairment of growth of infected young chickens, or the reduction in egg production of laying stock caused by an onset of the disease. There appears to be no period in the life of a chicken when lymphomatosis is not a problem, with birds dying from a few weeks to several years of age with gross evidence of the disease. Losses from lymphomatosis probably exceed those of any other poultry disease and few flocks in the United States have escaped its ravages.

The form of the disease used in these studies was a transplantable lymphoid tumor obtained from the United States Regional Poultry Research Laboratory at East Lansing, Michigan, and designated there as RPL 12. It

was originally described by Olson (1941). He found the tumor to be a spontaneous neoplasm in a 7 month old cross bred chicken. Blood obtained from the heart chamber of this bird was injected into the right wing vein of each of 4 birds. One of these developed a tumor in the region of the right humerus and it was from this bird that serial passage of the lymphoid tumor was initiated. Since 1937 Olson has maintained it by inoculating suspensions of the tumor into the pectoral muscle of Rhode Island red chickens. He found that growth of an implant was generally apparent approximately 7 days after inoculation and that maximum growth usually occurred about 13 days after inoculation. In 1942 the Olson tumor was transferred to the Regional Poultry Laboratory and was designated as RPL 12. It was found to be characterized in serial passage by very energetic growth as evidenced by the rapid rate at which serial transfers could be made (6 to 10 days), and the high incidence (100 per cent).

Lumsden (1931) stated that there are three ways in which a cure for neoplastic disease may be sought:

1. The cause can be investigated and, if found, the disease may prove to be preventable.
2. Some serum, drug, or radiation may be discovered which will be so specifically injurious to cancer cells that these can be destroyed without damage to the normal tissue cells.
3. Some means may be found by which the body can be rendered immune to invasion by the cancer cell.

The present work has been concerned with utilizing the third method, and is an attempt to produce a vaccine against lymphomatosis by attenuating

cells of the lymphoid tumor described above with beta-propiolactone (BPL). This compound was found by Hartman, Piepes, and Wallbank (1951), Stokes and Smolens (1954), and LoGrippo and Hartman (1955) to have some value as a virucide. It is one of the simplest lactones consisting of a four membered ring containing one oxygen and three carbon atoms, and is characterized by great chemical activity because of the tendency for the ring to open. It is a liquid and is soluble in water.

HISTORICAL

Only a brief review of general tumor immunity will be given, with the more detailed review reserved for immunological studies dealing with lymphomatosis. For a historical presentation and description of tissue culture methods the reader is referred to the monographs of Parker (1950) and Cameron (1950). For methods of testing chemicals in tissue culture the reader is referred to the papers by Pomerat (1951), Biesele (1952), Funan (1953), and Rubin (1954).

Richet and Hericourt (1895) inoculated a donkey and two dogs with human osteosarcoma and tested the effects of the antisera thus obtained upon human neoplasms. There was some improvement in the cases treated, but on the whole the results were disappointing and no instance of cure was observed. Vidal (1906), Bosc (1906), and Purvis (1912) by analogous methods obtained similar results. Sometimes emulsions of viable cancer cells were introduced into human beings in attempts to cure neoplasms; however, these cells occasionally survived and gave rise to new tumors, thus proving detrimental instead of beneficial. The discovery by Hanau (1889), Morau (1890), and Jensen (1902) that certain mammalian tumors could be transmitted from one animal to another by implantation gave fresh impetus to investigations of cancer immunity. It was soon noticed that some of the animals, into which tumor fragments were implanted, failed to develop tumors, and that sometimes, after an implanted tumor had grown for a time, spontaneous regression took place. This suggested that possibly the animals exhibited some resistance to the continued growth of tumor cells implanted within them.

Clowes (1905) noticed that a marked effect was exerted on small tumors by the sera of spontaneously recovered mice and some retardation in growth of large tumors. Sera from x-ray treated mice did not produce appreciable effect on other tumors. His experiments indicated the existence of some immunity against cancer.

Tyzzer (1916) produced antibodies in non-susceptible mice by implanting Japanese waltzing mouse tumor grafts in them. Sera from these mice were collected 15 days later and injected into susceptible mice. Immediately after this, tumor grafts were implanted. In another experiment the tumor was first placed in the serum for one hour and then implanted in mice. In both cases it was found that the serum modified the action of the tumor tissues, but not enough to prevent the development of tumors.

Lumsden (1931) injected rabbits and sheep intraperitoneally with fragments of neoplastic growths (mouse cancer M_{63}, Jensen rat-sarcoma, and human mammary cancer) for several weeks. He found the sera of these animals to be highly toxic to tissue cultures of the original malignant cells. In some cases toxicity to normal cells was also noted, but to a lesser degree. In vivo confirmation was obtained by injecting anti-human-breast-cancer serum directly into many rat sarcomata and a rat mammary carcinoma. In almost every case the tumors regressed and the animals were found to be immune to a subsequent test inoculation of tumor. This immunity lasted for over six months. Normal serum had no effect on the tumors. His experiments demonstrated that animals are capable of forming antibodies which have a specific lethal effect upon malignant tumor cells.

Lumsden (1931) also treated Jensen rat-sarcoma tumors with ether and chloroform for varying periods of time but was unable to produce a thoroughly satisfactory vaccine. He found this to be true also when tumor tissue was dried, heated, or frozen. Tumor cells attenuated with formalin and injected into the animals did not induce appreciable immunity; however, by injecting formalin directly into growing tumors Lumsden found that an effective vaccine could be produced in the animal. He called this process auto-vaccination -- an immunity induced by curing in certain ways an implanted tumor already growing in the host. He suggested that immunity may be invoked only when tumor cells actually grow in the treated animal for a time and then regress.

Gardner and Hyde (1932) also attempted to develop an immunity in rats by means of intratumoral injections of formalin, but, although they got a low percentage of regressions after prolonged treatment, they found no immunity to reimplantation. They concluded that success might be attained with some tumors, but that this method was not very promising even for purely experimental investigations.

Suguira and Benedict (1931) demonstrated with certain tumor tissue (Suguira rat sarcoma and Rous chicken sarcoma) the presence of an immunizing agent which was resistant to temperatures which destroyed the proliferating power of the tumor. However, the great majority of investigators studying tumor immunity have found the living cell to be essential in the production of immunity. Tyzzer (1916) found that blood and other normal tissue, as well as tumor tissue, produced some immunity to the latter, and Rhoads and Miller (1935) found that mice, normally susceptible

to transplantable leukemia, could be rendered resistant by intravenous injections of normal spleen and lymph node suspensions as well as by means of sublethal doses of living leukemic cells.

Gross (1943) showed the importance of dosage in the study of tumor immunity. Sarcomata were induced in mice with methyl cholanthrene and then successfully transplanted in serial passage every 7 to 10 days. Those mice showing spontaneous regression of tumors were found to have some immunity to further transplants, but this immunity could be overwhelmed by using massive doses of the tumor suspensions. Gross (1945) also showed that mice rendered immune to one kind of tumor were not necessarily immune to other tumors that arose in the same strain of mice. He stated that the results suggested that acquired immunity is directed specifically against particular tumor cells.

Aptekman, Lewis, and King (1946) extracted rat tumor pulp with 95 per cent alcohol and injected sarcomata of albino rats daily or every other day with this extract. Tumors in 56 of 58 rats treated were destroyed and 78 per cent of these rats, when later challenged with fresh tumor, were immune. Extracts of normal rat tissue in saline solution and alcohol did not destroy the tumors, nor did other control solutions of saline, and various strengths of ethyl alcohol (up to 50 per cent). The offspring of healed immune parents were susceptible to growth of grafts of the tumor. These same authors (1949) found that 50 per cent of 94 rats treated with 10 successive subcutaneous injections (0.5 to 1.0 ml.) of an alcoholic extract of rat sarcomata at 2 to 3 day intervals developed resistance to the growth of transplanted tumor grafts, and remained

resistant during the time they were kept under observation (6 to 12) months).

Lewis and Aptekman (1951) found that rat sarcoma tissue minced with a few drops of acetone; butyl, ethyl, methyl, and propyl alcohol; chloroform, ether, formalin, glycerine, and traces of acids or alkalies failed to protect rats of inbred strains from growth of the sarcoma. Tumor tissue subjected to freezing, heating, and desiccation, and homogenates of tumor tissue in distilled water and physiological salt solution also failed to give protection. However, atrophying tumor tissue (due to occlusion of the blood supply) when transplanted into susceptible rats, brought about the development of some immunity against tumors.

Nagasawa (1951) produced a tumor vaccine against the Yoshida sarcoma but found no difference in protective ability between vaccinated and control animals.

One of the first attempts to develop immunity against lymphomatosis was described by Furth (1934). He injected blood from paralyzed chickens into healthy birds and noted that chickens that failed to develop the disease after one injection resisted repeated intravenous inoculations. Lee (1942) immunized ducks and turkeys by intramuscular injections (15 injections of 4 ml. each during a 30 day period) of a saline tissue extract of ischiatic nerve from a pronounced case of neurolymphomatosis. The sera from these birds neutralized or inhibited the causative agent of neurolymphomatosis. This was demonstrated by injecting chickens with a nerve tissue extract from diseased chickens mixed with the immune serum and also by using a cell-free (Berkefeld) filtrate of nerve tissue with the immune

serum. Control birds received saline solution in place of immune serum. Out of 40 control birds 25 came down with the disease, while only 8 out of 40 of the immunized birds were affected.

Burmester and Prickett (1944) observed that all birds in which an implanted tumor (Olson) had regressed were found to be immune to a second or third implantation of the same tumor. The period of immunity was quite long, lasting at least 202 days. Birds implanted with tumor cells by any of several routes and showing regression of tumors were found to be immune to a second implantation by other routes. Small doses of tumor cells produced a high proportion of takes, but mortality was reduced, the tumors regressed, and the birds developed immunity. This could not be overcome by subsequent inoculation with as many as 10,000 times the number of cells in the first inoculation. Finally, they found that inoculations with inactivated tumor mince (by repeated freezing and thawing) produced no immunity indicating that active growth and regression of the primary tumor was a necessary criterion for immunity. Olson (1945) confirmed some of the work of Burmester and Prickett by noting that chickens which had received viable implants of the tumor exhibited a marked resistance to subsequent inoculation with the same tumor and he also noticed that the ability of the tumor to immunize against itself was enhanced by serial passage.

Johnson (1945) used crystal violet as early as 1938 in attempts to attenuate extracts of spleen tissue taken from birds affected with leukosis, but no significant results were noted. In 1945 he used various tissues from birds having different forms of the avian leukosis complex, but

primarily from birds having lymphomatosis of one type or another, and attempted to make a vaccine by attenuation with formalin. He used the formalin treated tissues for the first 2 injections and followed this by a final injection of unattenuated material. Exposure to the disease in both controls and vaccinated birds was by natural contact primarily, although a few birds were inoculated with fresh tissue from the diseased birds. Some significant results were obtained in several experiments in which no vaccinated birds developed the disease whereas some of the controls did. Out of a total of 256 vaccinated birds 18 came down with some form of leukosis, whereas 26 out of 163 controls were infected.

Olson (1945, 1946, 1947) used several procedures in attempting to make a vaccine from a lymphoid tumor (Olson) which would be effective against the spontaneous disease. He found that the supernatant fluid from centrifuged ground tumor pulp effected some immunity in chickens if it produced growth when first injected. No resistance developed if the supernatant fluid did not produce growth. In another experiment he tried attenuating the tumor tissue by freezing. Repeated freezing and thawing was found to be deleterious to both the ability to grow and the immunizing property. Tumors from different donors required different degrees of attenuation to render the growth innocuous and still retain the ability to immunize. He accomplished this by freezing the different materials for varying periods of time. He found that frozen tumor did bring about some immunity when injected into chickens but that it depended on the conditions of the experiment. He concluded that from a practical point of view, freezing might offer a possibility for making

a tissue vaccine, but that the optimum conditions for doing this would have to be developed.

Olson (1946) also attempted to produce immunity by injecting tumor material, dried over sulphuric acid in vacuum, intramuscularly into chickens. He challenged these birds 19 days later and found no evidence of immunity. Necrotic tumor material also did not induce any resistance. He added phenol to minced and ground tumor pulp in Ringers solution to give a final concentration of 1 per cent and allowed this to react for 1 hour at room temperature. He repeated this procedure using formalin and injected 0.5 ml. of each into chickens. No tumors developed from either the formalin or phenol treated tumor tissue and the birds were challenged 26 days later. All the birds developed tumors except 1 (out of 4) which had received phenolized pulp indicating little if any immunizing action. Heat attenuated tumor tissue elicited some immunity depending upon the time and temperature used in the experiments but it was necessary that the growth capacity of the attenuated tissue not be destroyed and no standardized conditions were obtained.

In another experiment Olson (1947) found that certain miscellaneous tissues of tumor-bearing birds induced resistance when inoculated into healthy chickens. Liver, muscle, kidney, spleen, marrow, and thymus from diseased birds were minced separately with Ringers solution and injected intramuscularly. Their immunizing ability was tested by a subsequent injection of fresh tumor pulp, the activity of which was previously determined in control chickens. These tissues were found to induce resistance against the lymphoid tumor even when they did not induce tumor

growth. Tissues from normal chickens or chicken embryos did not induce resistance. Neoplastic tissue from spontaneous lymphocytoma induced only slight resistance against the lymphoid tumor.

Burmester, Prickett, and Belding (1946) reported that chickens immunized with lymphoid tumor strains, obtained from cases of naturally occurring visceral lymphomatosis, were immune to subsequent implants of tumor tissue. These birds were no more resistant to natural lymphomatosis, however, than non-immunized control birds maintained under the same environment.

Burmester and Belding (1947) found that chickens immunized with frozen tumor cells or cell free tumor extract were resistant to further implants of highly active material. This occurred in many chickens even though palpable tumors were not present, and it was suggested that possibly small tumors occurred within the inoculated tissue and escaped detection. Active growth of the tumor was thought to be a necessary requisite for immunity against a second tumor growth. They found that the injection of normal tissue suspensions of pectoral muscle, spleen, and thymus did not increase resistance to subsequent implants.

Burmester (1947) produced an antiserum against a lymphoid tumor by injecting birds which survived an original and second implant of tumor cells with 10-1 ml. portions of fresh tumor mince intramuscularly at weekly intervals. This was repeated using killed or disintegrated tumor cells. Both living and killed tumor cells caused the formation of an antibody-like factor whereas normal lymphoid tissue did not. This factor partially or completely inhibited the growth of tumor cells <u>in vitro</u>,

and when the anti-serum was injected into birds prior to the implanting of tumor cells there was a marked reduction in mortality and in tumor incidence, indicating in vivo as well as in vitro activity.

Burmester (1955), continuing his studies on antibodies, reported that the virus of visceral lymphomatosis was also capable of inducing antibodies, and that these were transmitted from the adult chicken to the chick, giving the chick a significant immunity to challenge inoculations. It was also reported in 1955 that a vaccine against visceral lymphomatosis had been developed although it was not recommended for use nor in production on a practical basis. No further information on this vaccine has been reported and its status at this writing is not known to the author.

MATERIALS AND METHODS

Avian Lymphoid Tumor. This tumor, designated as strain RPL 12 by the U. S. Regional Poultry Research Laboratory, East Lansing, Michigan, and originally obtained from them, was maintained throughout these studies by serial passage in the pectoral muscle of chickens of varying ages. Chicks from 2 to 5 weeks old were preferred when available, and transfers were made every 6 to 12 days. The tumor was removed aseptically and minced in a pre-cooled Waring blendor for two minutes with about 10 ml. of Hanks' solution. This solution was then filtered through one layer of sterile gauze and 1/2 to 1 ml. injected into the chickens.

Chickens. Chickens were obtained from the poultry department in most cases, although some were purchased from private sources. No attention was paid to sex, kind, or other characteristics, except that healthy chicks were always used.

Tissue Culture Methods. The following techniques were used during this investigation to grow the tissues: Petri dish method as described by Grossfeld (1954), and 25 ml. Erlenmeyer flasks.

Physiological Solution. Hanks' solution was used in all the experiments. It was sterilized by Seitz filtration and stored in 300-ml. Erlenmeyer flasks at refrigeration temperatures. It was prepared as needed according to the following formula:

Phenol Red	0.02g.

NaCl	8.00g.
KCl	0.40g.
$CaCl_2$	0.20g.
$MgSO_4.7H_2O$	0.20g.
$Na_2HPO_4.12H_2O$	0.06g.
KH_2PO_4	0.06g.
Glucose	1.00g.
$NaHCO_3$	0.35g.
H_2O	1000ml.

Plasma and Serum. Adult chickens were bled by cardiac puncture as the plasma or serum was needed. Heparin was used as the anticoagulant in obtaining the plasma. For serum collection, large quantities of blood were placed in 2-liter Erlenmeyer flasks in order to obtain a larger surface area. When the blood had clotted it was kept at 37°C. for 3 to 6 hours and then removed and placed in the refrigerator for several hours or overnight. This was found to be the best method for obtaining large amounts of serum. The serum was removed from the clot by decanting, and was then centrifuged and stored in flasks in the frozen state until needed. The same group of chickens was used for serum and plasma collection throughout the experiments.

Embryo Extract. The embryo extract was prepared from 9-to-11 day old chicken embryos. They were removed from the shells and collected in Petri dishes. After all were collected, they were placed in a 50-ml. syringe, the plunger inserted, and the embryos forced through the small end of the

syringe. The pulp was collected in 300-ml. Erlenmeyer flasks and an equal amount of Hanks' solution added to give a 1:2 dilution of the extract as recommended by Cameron (1950). This was then refrigerated for 24 to 48 hours, centrifuged, and the supernatant fluid removed and stored in Erlenmeyer flasks. Some of the embryo extract from each newly prepared batch was passed through a Seitz filter and then dispensed into ampules. These were flame sealed and stored in the frozen state until needed at a later date for effecting the clotting of the plasma in the tissue culture experiments. It was found that the fresh extract always elicited better clotting than that which was stored for some time.

Nutrient Fluid. This was composed of a mixture of Hanks' solution, serum, and embryo extract in the ration of 40:40:20. The nutrient fluid was sterilized by Seitz filtration and dispensed into 250-ml. Erlenmeyer flasks which were stored in the refrigerator until needed. Sterility examinations were made on all nutrient fluids at the time they were dispensed.

Sterility Control. Bacto Brewer thioglycollate medium (Difco) was used for all sterility controls. It was prepared as needed and was used to test nutrient fluids, embryo extract, and Hanks' solution.

Beta-propiolactone. This was manufactured by the B. F. Goodrich Company. The stock solution was diluted with distilled water to the desired percentage and stored at refrigeration temperature. This was made up fresh for each experiment and not used after one or two weeks.

EXPERIMENTAL

Experiment I

The purpose of the first experiment was to determine the effect of varying concentrations of beta-propiolactone (BPL) on tumor tissue in tissue culture. Preliminary experiments indicated that there was some *in vitro* activity of the BPL against the tumor cells. Tumor material was treated with Hanks' solution in a Waring blendor and BPL added to give various percentages of concentrated BPL. This mixture was thoroughly shaken and a sample injected into the pectoral muscle of chickens. The procedure was repeated with tumor material without added BPL in order to assure tumor activity. The results are given below:

Concentration of BPL in Tumor inocula	No. of birds injected	No. of birds showing growth
0.02 per cent BPL	5	2
None (controls)	5	4
0.03 per cent BPL	5	4
None (controls)	5	5
0.04 per cent BPL	8	2
None (controls)	9	8

The results show that about 44 per cent (8/18 x 100) of the birds injected with the BPL-treated material showed growth while approximately 90 per cent (17/19 x 100) of the controls were affected. It was also

noted that tumors which developed in BPL-treated birds were delayed from 3 to 7 days after growth was first observed in the controls. It is evident from these results that the BPL showed some in vitro activity, and the preliminary experiments thus indicated that it might be advantageous to continue with the investigation.

In order to test the effect of BPL in tissue culture the concentrated BPL was first diluted with distilled water to some particular concentration determined by the percentage to be used in each trial. Dilute BPL was then added to the nutrient fluid as required to give the final concentration as per cent of concentrated BPL in nutrient fluid.

In the first two trials of this experiment (series 1 and 2, table I) the dilute BPL was added to a volumetric flask and this was then made up to volume with either sterile or unsterile nutrient, whichever was available. This solution was then sterilized by filtering through a Seitz filter. This procedure allowed the BPL to be in contact with substances which break down the BPL; therefore, in the last 5 series (3, 4, 5, 6, and 7, table I) the BPL was placed in a volumetric flask and sterile nutrient added to this aseptically. This procedure placed the BPL in contact with the nutrient fluid only a short period of time prior to actual use.

The tissue culture procedure itself consisted of removing the tumor, placing it in a Petri dish, and then cutting it into smaller pieces being careful to avoid necrotic areas. These smaller pieces were placed in another Petri dish containing Hanks' solution, and were then cut into pieces suitable for culturing (approximately 1 cubic mm.). These pieces were transferred to other Petri dishes (about 7 to 12 pieces per plate) and

plasma and embryo extract added. After coagulation of the clot the nutrient fluid containing the BPL was added. Control plates containing nutrient fluid without BPL were also prepared during each trial. The nutrient fluid was changed every 2 to 4 days by removing the old solution by suction and adding fresh nutrient. The plates were observed for 3 to 14 days for visible growth. At the end of the observation period the tissue explants used in 4 trials (series 3, 4, 6, & 7) were removed and injected into baby chicks in an attempt to confirm whether growth had or had not occurred. Tissue pieces from the control plates were pooled, sliced into smaller pieces, taken up in a small volume of Hanks' solution, and injected into several chicks (0.5 to 2.0 ml.). The same procedure was followed with the BPL-treated tissue. The chicks were then observed for several weeks for tumor growth. In series 3 and 4 the control tissue did not elicit tumor formation when inoculated into baby chicks even though growth was noted by gross observation of the tissue in tissue culture. The results of the tissue culture observations were therefore taken as the best single criterion for determining growth, and the effects of the tissue explant injections were used as a second or confirming criterion.

Results. BPL when present in nutrient fluid in a concentration of 0.005 per cent did not inhibit the growth of tumor cells. This was indicated in 3 trials (series 5, 6, & 7, table I) and confirmed in 2 (series 6 & 7) by inoculating tumor pieces from tissue culture into baby chicks. In series 5 there appeared to be some growth in both BPL and control plates but it was very poor in both. The plates were observed for 10 days and the fluid changed once during this time. The experiment was discontinued because of the poor growth.

TABLE I

Results of Experiment I

Series No.	Percent BPL	Effect on Tissue Culture	Control Tissue Culture	No. Birds Inoculated From BPL Plates	No. Birds Inoculated From Control Plates	Reaction of Birds from BPL Tissue[1] Injections	Reaction of Birds from Control Tissue[1] Injections
1.	0.03	No growth	Growth observed	None	None	———	———
2.	0.03	No growth	Growth observed	None	None	———	———
3.	0.02	No growth	Growth observed	3	3	0/3	0/3
4.	0.01	No growth	Growth observed	2	2	0/2	0/2
5.	0.005	Slight growth observed	Slight growth observed	None	None	———	———
6.	0.005	Growth observed	Growth observed	3	3	2/3	2/3
7.	0.005	Growth observed	Growth observed	4	4	3/4	2/4

[1]No. Birds Showing Lymphomatosis / No. Inoculated

All plates in series 6 showed growth and 1 BPL plate was stained and examined microscopically. Fibroblasts and round cells were observed in this plate. Baby chicks were inoculated with tissue pieces obtained from BPL and control plates. Three chicks were inoculated with material from each group of plates. One chick injected with BPL-treated tissue showed evidence of moderate tumor growth and another died 15 days after injection without showing tumor growth. It was autopsied and the lesions indicated that death was caused by visceral lymphomatosis. The third chick showed no symptoms of the disease. Two of the control birds showed symptoms of lymphomatosis and 1 of these died 18 days after inoculation. The third control chick showed no symptoms of the disease.

In the last trial (series 7), in which a concentration of 0.005 per cent BPL was used, growth was observed in all the plates, and after 3 days 4 baby chicks were inoculated with pieces of explants from control or BPL plates. Three of the 4 birds injected with material from the BPL plates showed tumor growth and 2 of these died within 3 weeks. Two of the control birds developed well defined tumors and died within 3 weeks.

All concentrations of BPL above 0.005 per cent were found to inhibit the growth of tumor tissue. Three concentrations were tried: 0.01, 0.02, and 0.03 per cent (series 1 through 4). Growth was observed in the control plates of each series. In 2 trials (series 3, & 4) tissue explants were removed from control and treated plates and injected into baby chicks as previously described. No tumor growth was observed in any of these chicks.

Experiment II

This experiment was designed to prepare a vaccine for lymphomatosis and test its efficiency against a challenge of lymphoid tumor cells. The results of preliminary experiments and experiment I indicated some *in vitro* activity of BPL against the tumor cells. It was hoped that some correlation might exist between the effect of BPL on tumor cells in tissue culture and on tumor cells in a vaccine. Cobb (1955) and Wright (1955) using triethylene melamine against human neoplasms found some correlation between *in vitro* and *in vivo* results. Cobb found that human neoplastic cells in tissue culture were permanently damaged by the triethylene melamine, while Wright found that the same chemical caused some clinical improvement and a few complete regressions in human patients suffering with incurable cancer. It was therefore decided to use a concentration of 0.01 per cent BPL in the preparation of the first vaccine because this was found to be the lowest concentration to inhibit tumor cell growth in tissue culture in the previous experiment.

To prepare the vaccine, 2 tumors were removed from birds which had received injections of tumor tissue 7 to 10 days previously. The tumors were progressive but not necrotic at this time. They were cut into small pieces and were ground in a Waring blendor with 20 ml. of Hanks' solution for 2 minutes. This mixture was then filtered through 1 layer of sterile gauze and a portion of it made up to concentration with the BPL. The diluted BPL was added slowly from a pipette, and the mixture shaken vigorously during the addition and for 5 more minutes. The mixture was allowed to stand for approximately 30 minutes at room temperature before

injection. The control material consisted of tumor cells to which sterile distilled water was added instead of the BPL solution. The vaccine was inoculated into the pectoral muscle of as many baby chicks as the quantity of vaccine allowed, using 0.5 ml. per chicken. Ten to 16 birds were inoculated in each trial. Control birds were inoculated with the same quantity of control tumor suspension immediately after the injection of the vaccinated birds. They were injected in the pectoral muscle and 6 to 8 birds were used in each trial. Control and vaccinated birds were then placed in a small cage with 6 to 10 birds of the same age which received no injections. The non-inoculated birds were later used as controls to ascertain the potency of the challenge dose and are referred to as challenge control birds throughout this report.

Immediately after the inoculations were completed, tissue cultures of both vaccine and control tumor suspensions were made. Small quantities of each suspension were placed in separate Petri dishes with capillary pipettes. Plasma was added and a clot produced by the addition of embryo extract. Nutrient fluid was then added and the plates incubated at 37° C. The plates were observed from 1 to 14 days for growth both grossly and microscopically. If growth was not definitely established by either of these 2 methods of observation, the pieces were cut up and inoculated into baby chicks as in the previous tissue culture experiments.

All vaccinated and control birds were observed daily, following the sixth day of injection, for tumor growth or other symptoms of lymphomatosis. The birds which survived the vaccination were challenged 30 days later with 1 ml. of tumor suspension obtained by mincing tumors from passage birds as

previously described. Challenge control birds of the same age were treated in the same manner. Challenged birds were observed for 30 to 40 days before terminating the experiment. They were examined once each day during most of this period.

Five trials were carried out using the following concentrations of BPL: 0.01, 0.01, 0.03, 0.01, and 0.025 per cent.

Results. In the first trial (series 1, table II) 16 birds, 1 to 2 weeks old, were inoculated with the vaccine. Eight of these developed symptoms of lymphomatosis from the vaccine and all but 1 of the 8 died from the disease. Seven of the 16 birds did not develop lymphomatosis and 1 died as a result of the injection. One control bird died as a result of the injection while the remaining 7 developed lymphomatosis and died. The 8 remaining birds from the BPL group were challenged 30 days later along with 10 challenge control birds. All the control birds showed extensive tumor formation, but only 1 of the 8 vaccinated birds developed tumor growth, and this only to a slight extent. This bird was accidentally given 2 ml. of challenge dose instead of the normal 1 ml.

Tissue cultures of the vaccine in this trial were all found to be contaminated after 2 or 3 days and it was discovered that the embryo extract used in these cultures was contaminated. For this reason the trial was repeated since it was desirable to know whether or not the tumor cells in the vaccine were living. As will be shown later, many investigators have found that living cells are required to induce tumor immunity.

Therefore, series 2 was essentially a repetition of the first trial. Fourteen birds (1 to 2 weeks old) were vaccinated in the breast muscle.

Eleven of these showed moderate to extensive tumor growth and died. Two died without symptoms of tumor growth but upon autopsy lymphomatosis was indicated. The last bird showed only slight symptoms of tumor growth and it was the only bird from this group which was challenged. All the control birds developed the disease and died. After 30 days the 1 surviving vaccinated bird was challenged along with 7 challenge controls. All 7 control birds showed extensive tumor formation while the vaccinated bird remained negative. Growth was observed microscopically in tissue cultures of the vaccine.

In the third trial (series 3, table II) a higher percentage (0.03) of BPL was used in the hope that the tumor cells could be attenuated enough not to cause symptoms of the disease and yet to induce immunity. Fourteen birds, 1 to 3 weeks old, were vaccinated and 8 controls inoculated. Two vaccinated and 1 control bird died from the initial injection. None of the remaining vaccinated birds showed any symptoms of tumor growth, while all control birds developed tumors and died. Tissue cultures of both vaccine and control mixtures appeared to show growth microscopically and the tissue pieces were cut up and injected into baby chicks. None of these chicks developed tumors. Of the 12 birds challenged 30 days after vaccination, 5 showed no symptoms of tumor growth, 5 developed slight growth, 2 developed extensive tumor formation and 1 of these died. Out of 6 challenge control birds receiving the same challenge inocula, 5 showed extensive tumor formation and 1 exhibited only slight growth.

In trial 4 (series 4, table II) the concentration of the BPL used in series 1 and 2 was repeated (0.01 per cent). In this case, however, the

solution of BPL in water was made more dilute than those utilized in series 1 and 2. This enabled a larger volume of the diluted BPL to be added to the tumor mince to obtain the desired final concentration. It was hoped that this procedure would allow more tumor cells to be affected by the chemical and thus give fewer symptoms from the vaccine itself. Ten birds were injected with the vaccine made in this manner. Six birds were inoculated with the control material and all developed extensive tumors and died. Of the 10 vaccinated birds, 1 showed no tumor formation, 6 showed slight growth, and 3 had well developed tumors which regressed in all except 1 bird which died. The bird that died received 1 ml. of vaccine while all others got 0.5 ml. Tissue cultures of both vaccine and control mixtures showed good growth as evidenced macroscopically, and the pieces were cut up and injected into baby chicks. Tumor growth resulted in only 1 out of 6 birds and this resulted from the control tumor tissue. After 30 days the 8 surviving vaccinated birds (1 bird could not be accounted for) were challenged with fresh tumor material which was shown to be active by causing extensive tumor formation in 6 challenge control birds. No signs of tumor growth appeared in any of the vaccinated chickens.

In the last trial (series 5), a concentration somewhat greater than that employed in series 1, 2, and 4 but slightly less than series 3 was used (0.025 per cent). Fourteen birds, approximately 2 weeks old, were injected in the right pectoral muscle with the vaccine. None of these developed symptoms from the vaccine. Eight birds were injected with control tumor material and all developed extensive tumors. Tissue cultures of the vaccine and control suspensions appeared to be growing but caused no tumor

TABLE II

Results of Experiment II

(1)	(2)	(3)	(4)	(5)	(6)
		Vaccination Results[a]	Control Results[a]	Challenge Results[a]	
Series No.	Per cent BPL	(BPL Added)	(No BPL)	Vaccinated Birds	Challenge Control Birds
1.	0.01	8/16[b]	7/8[b]	1/8[c]	10/10
2.	0.01	14/14	8/8	0/1	7/7
3.	0.03	0/14[d]	7/8[e]	7/12	6/6
4.	0.01	9/10	6/6	0/8	6/6
5.	0.025	0/14	8/8	14/14	6/6
TOTAL	-	31/68	36/38	22/43	35/35

[a]No. birds showing lymphomatosis / No. birds inoculated
[b]1 died from initial injection
[c]Bird showing symptoms received 2 ml. of challenge dose instead of 1 ml.
[d]2 died from initial injection
[e]1 died from initial injection

formation when inoculated into baby chicks. These birds were later challenged along with the vaccinated birds but showed no immunity. None of the vaccinated birds showed immunity when challenged, although 3 developed only slight tumor growth. Six challenge control birds inoculated with the same material developed well defined tumors.

DISCUSSION

A total of 43 birds survived vaccination in experiment II. These were all challenged, and 22 birds (approximately 51 per cent) showed symptoms of lymphomatosis. Out of 35 control birds inoculated with the same challenge material, 100 per cent developed symptoms of lymphomatosis. Thus a 49 per cent reduction in tumor incidence was induced by the vaccination procedures. In the 3 trials in which the vaccine contained 0.01 per cent BPL, 17 birds were challenged and only 1 (approximately 6 per cent) of these showed symptoms of tumor growth. This bird received twice the normal challenge dose and its symptoms were very slight. In these 3 trials, therefore, a 94 per cent reduction in tumor incidence was obtained, indicating that an effective vaccine might be produced by attenuating tumor cells with this chemical. From the standpoint of clinical use, however, the vaccines produced in these experiments would not be practical in their present form because of the high percentage of birds developing lymphomatosis from the vaccines (31 out of 68 birds; approximately 46 per cent).

The most effective vaccines prepared (0.01 per cent BPL) were also those which produced the highest incidence of lymphomatosis. This is in line with the widely held view that living cells are necessary for tumor immunity to be induced. Olson (1945) found that supernatant fluid from ground lymphoid tumor pulp would not induce resistance when its growth capacity was lost. He also found (1946) that the immunizing ability of

heat attenuated tumor pulp was lost when the growth capacity of the tissue was destroyed. Burmester and Prickett (1944) observed that inactive tumor mince produced no immunity and their results indicated that active growth and regression of the tumor was a necessary prerequisite to immunity. Burmester and Belding (1947) found that chickens inoculated with frozen tumor cells or cell-free extracts did not develop palpable tumors and yet were immune to further implants, indicating that live tumor cells might not be necessary for immunity. They suggested, however, that possibly small tumors might grow and regress deep within the inoculated tissue and hence escape detection. Olson (1945) also suggested that resistance in birds negative to the original inoculation might be due to a slight unapparent growth of the implant or to natural immunity. Lumsden, Spencer, Tyzzer, and others also are of the belief that living cells are necessary in the development of tumor immunity.

Burmester (1947) found that killed or disintegrated tumor cells caused the formation of antibodies when injected into chickens. Suigura and Benedict (1931) also demonstrated an immunizing agent to be present in dead tissue of certain tumors. As a result of these experiments some evidence is available which indicates that live cells may not be necessary to induce tumor immunity. This may have occurred in some of the vaccines in the present experiment. In the third series, where a concentration of 0.03 per cent BPL was used, none of the 14 vaccinated birds developed symptoms from the vaccine itself, yet 5 of these when challenged remained negative. It is possible that the tumor cells in this vaccine were not living but still caused the development of some resistance to the disease,

or the vaccine may have contained some viable cells which when injected into the chickens produced an immunity but no clearly recognizable symptoms of tumor growth. The resistance may have been due to slight imperceptible growth as Burmester and Belding, and Olson have suggested. Tissue cultures of the vaccine appeared to be growing, although no tumors resulted when pieces were inoculated into baby chickens.

In the fifth series a percentage of BPL (0.025) just below that of the third series was used, and again it was observed that no symptoms resulted from the vaccine itself. The challenge dose in this case, however, caused tumor development in all of the vaccinated as well as control birds. Tissue cultures of the vaccine appeared to be growing and an attempt was made to confirm this growth by injecting tissue explants into baby chicks. Three chicks were injected with vaccine explants and 3 with control explants. They were then observed for 27 days for symptoms of tumor growth, but all remained negative. On the 27th day they were challenged along with the vaccinated birds because it was thought that the injections of the explants might have induced immunity in these birds, particularly if viable cells had been present in the explants. All 6 birds, however, developed tumors. From the results of this trial it appears that viable cells were not present in the vaccine, or that they did not induce immunity. It is surprising that the vaccine with the lower concentration of BPL used here (0.025 per cent) did not induce immunity, since vaccines with both higher (0.03 per cent) and lower (0.01 per cent) concentrations did appear to induce some immunity. One possible explanation for this failure might be the variability of transplanted tumors in their ability

to induce immunity (Spencer, 1942). Thus some tumors when used in a vaccine might induce immunity and others not. In addition, it was noted that the vaccine of this experiment appeared to be slightly more on the acid side (as noted by the change in color of phenol red indicator) prior to injection than previous vaccines, and it is possible that the increased acidity destroyed the ability to grow and immunizing properties of the tumor cells.

From the results of experiment II, it is evident that some immunity was induced in the vaccinated birds by the BPL-treated tumor tissue. It is also clear that more work is required to obtain that percentage which will give the most effective vaccine, both from the standpoint of inducing immunity and of giving minor or no symptoms of the disease. A concentration just slightly above 0.01 per cent may be satisfactory, or possibly a more efficient way of getting the 0.01 per cent BPL to all the tumor cells. This might be accomplished by spreading the tumor mince over a wider area such as a large beaker or a Petri dish and spraying the BPL into this. Or the BPL might be added directly to the tissue in the Waring blendor as the tumor is being minced. A third possibility might be the use of some compound which would more readily penetrate tumor cells, such as glucose or a glucoside (Boyland and Mawson, 1938), to act as a vehicle for the BPL.

If a workable concentration could be attained and an effective vaccine prepared against this lymphoid tumor, there still would remain the difficult task of determining whether or not this vaccine would give protection against the natural disease. Tyzzer (1916) and Spencer (1942)

both stated that immunity to implanted tumors gives no assurance of protection against the subsequent development of spontaneous tumors. Burmester, Prickett, and Belding (1946) found that birds recovering from tumor implants obtained from naturally occurring visceral lymphomatosis, were immune to further such implants but were no more resistant to the occurrence of natural lymphomatosis than non-immunized control birds. Olson (1947) noted that spontaneous lymphocytoma and fowl paralysis appeared in birds which had recovered from previous grafts of a lymphoid tumor; however, he suggested that these may have been present prior to the induction of resistance. He further stated that the question of whether a lymphoid tumor can be utilized in developing a vaccine against the spontaneous disease remains unsettled. It thus remains for further experimentation to determine the best concentration of BPL to use in making a vaccine and to ascertain whether such a vaccine will protect against the natural disease.

SUMMARY

1. Tumor cells treated in vitro with BPL and injected into baby chicks resulted in only 44 per cent tumor growth. Tumor cells not treated with BPL caused tumor growth in 90 per cent of the controls. Tumor growth in the control birds was noted from 3 to 7 days sooner than growth in the birds receiving BPL-treated tissue.

2. BPL when present in nutrient fluid at concentrations of 0.01, 0.02, and 0.03 per cent inhibited the growth of tumor tissue in tissue culture. A concentration of 0.005 per cent did not inhibit tumor tissue in tissue culture.

3. Chickens injected with BPL attenuated tumor tissue vaccines showed an overall reduction of 49 per cent in tumor incidence when challenged with fresh material which caused tumor formation in 100 per cent of the non-vaccinated control birds. In 3 trials, in which a concentration of 0.01 per cent BPL was used to attenuate the tumor tissue, only 42.5 per cent of the birds survived vaccination. Ninety-four per cent of these did not develop lymphomatosis when challenged.

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