TISSUE CULTURE STUDIES ON AVIAN LYMPHOID TUMOR

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Robert H. Bussell

1952

This is to certify that the

thesis entitled

Tissue Culture Studies on Avian Lymphoid Tumor.

presented by

Robert H. Bussell

has been accepted towards fulfillment
of the requirements for

M.S. degree in Bacteriology

Walter N. Mack
Major professor

Date January 30, 1952

TISSUE CULTURE STUDIES ON AVIAN LYMPHOID TUMOR

By

ROBERT H. BUSSELL

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology and Public Health

1952

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. Walter N. Mack for his supervision, interest and many valuable suggestions which have made this investigation possible.

Grateful acknowledgment is also due to Dr. H. J. Stafseth for his assistance during the writing of this thesis.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
HISTORICAL	8
MATERIALS AND METHODS	14
EXPERIMENTAL	17
Experiment I	17
Experiment II	22
Plates I-XIV	27-40
Experiment III	41
DISCUSSION	43
SUMMARY	49
BIBLIOGRAPHY	50

INTRODUCTION

There have been many experimental approaches to the cancer problem. Three of the main ones are those concerned with (1) genetics (2) cellular physiology and biochemistry and (3) attempts to demonstrate viruses and virus-like agents as the cause of cancer. During the past 50 years an ever-widening number of tumors and tumor-like processes of animals have been discovered. Many of these have been shown to have etiological relationships with viruses and virus-like agents. Possibly the only reason why such agents have not been demonstrated in some of the human tumors is the high degree of specificity shown by these tumors and their agents. In general their specificity is such that only homologous hosts can be used in experimental work with these tumors. Thus, the same situation has risen that has always plagued microbiologists working in the realm of human diseases, namely, that experimental progress is the lack of a satisfactory experimental host. In the absence of such a host, similar diseases in animals must receive the attention of the laboratory investigator. This is the situation that prevails in a great deal of the cancer research

done today. The fowl is among the animals which have contributed greatly to the study of neoplastic diseases.

The first tumor-like process shown to be caused by a filterable virus was fowlpox (Marx and Sticker, 1902). Since that time many viruses have been shown to cause proliferative lesions of many different types, both in animals and in man (Kidd, 1948, 1950).

The first neoplastic disease transmitted by a filterable agent was demonstrated by Ellerman and Bang (1908). They were able to transmit both lymphomas and leukemia of chickens by inoculating blood and cell-free filtrates of organs from the diseased birds into healthy birds. A few years later Rous (1911) succeeded in transmitting a sarcoma of fowl, which had been previously identified as a neoplasm, by means of a filterable agent. A year later Rous (1912) suggested a more widespread use of this and other transplantable tumors of the fowl as material for cancer research.

The description of many transmissible tumors of the fowl have followed the work of Rous. Practically all these tumors have been transmitted by the use of agents other than living tumor cells. This property has been accepted as

characteristic of this group of tumors by Claude and Murphy (1933). However, there are several characteristics of this group of cell-free and filterable agents which cause many to hesitate calling them viruses. First of all, when filtrates are inoculated into animals there is a latent period before tumors and death are produced. This not the case when live tumor cells are transmitted directly, tumors and death following in a relatively short period of time. As Claude and Murphy (1933) pointed out, bacteria and viruses show the same degree of susceptibility to ultraviolet light whereas the filterable tumor agents are far more resistant.

They also pointed to the fact that these agents have a definite selective action with regard to the type of tissues which they will affect. This, of course, is a property they have in common with true viruses. However, some of the tumor agents are bound or inactivated by the tissues to which they show their specific affinity. Claude and Murphy (1933) stated that this was not true of the viruses. However, since the discoveries of Hirst (1941, 1943) and the development of the cell receptor theory, this view becomes more questionable. Hirst (1941) first discovered that influenza virus would agglutinate red blood

cells and later (1943) showed that the cells of the respiratory tract would absorb the influenza virus. These are the cells which influenza virus selectively attack.

The sporadic occurrence of tumors in nature has also been advanced as an argument against any theory proposing viruses as their etiological agents. Andrews (1934) proposed that a latent virus exists in apparently normal tissues, which in turn requires some sort of stimulation before malignancy is produced. This would provide an excellent explanation if the theory could be proved by experimental evidence. As yet, the presence of such an agent in normal tissues has not been adequately demonstrated. If some method, possibly a serological test, for demonstrating infection with such a latent agent could be developed, this problem might be solved. An analogy may be drawn here between a well-known virus and these tumor agents to show the significance of their detection if they exist in supposedly normal tissues. Before adequate means of detecting the presence of poliomyelitis virus were available, investigators were unable to explain the sporadic occurrence and the seemingly noncontagious nature of the disease. The discovery of a suitable experimental animal (Landsteiner and

Popper, 1909) definitely established poliomyelitis as an infectious disease and provided a means for the detection and identification of the etiological agent. A year later Netter and Levaditi (1910) developed the neutralization test. This provided an indirect method for determining the distribution of the virus in nature, which was shown to be widespread in spite of the sporadic occurrence of paralytic cases.

Shrigley (1951) stated that probably the greatest handicap to the study of the agent of Rous' sarcoma is the lack of a means to determine it quantitatively. This is also true of the other agents producing tumors in fowls and other animals. It is also a situation which must be solved if these agents are to be considered viruses in the sense that we think of them today.

It may be said that these tumor-producing agents do have properties in common with the well-known viruses. They are both ultramicroscopic, filterable and their multiplication is intimately associated with living susceptible tissues and cells.

It was not long after the development of techniques for the cultivation of living cells in vitro that Carrel (1925, 1926) applied these techniques to the study of the agent of Rous'

sarcoma. He was able to propagate this agent in the presence of chick embryo tissue, chicken monocytes and chicken spleen. Since that time relatively few investigations have appeared in the literature applying these techniques to the study of tumor-producing agents. However the literature dealing with the cultivation of the tumors in vitro is voluminous.

The lymphoid tumor used in the following studies is a transplantable tumor originally described by Olson (1941). Later, in the hands of Burmester et al. (1946, 1947), this tumor was shown to contain a filterable agent. The filtered plasma from tumor-bearing birds was also shown to contain this agent. It failed to produce tumors at the site of inoculation and required incubation periods of the order of four to six months before tumors developed. This tumor may be considered to be a form of visceral lymphomatosis or a lymphocytoma which may be defined as a malignant neoplastic disease, the undifferentiated lymphocyte being the type cell of the tumor (Olson, 1940).

Chrétien (1951) was able to show that this tumor would maintain its malignancy when cultivated in vitro by reinoculation of tumor tissue into chickens and the resultant production of tumors at the site of inoculation. Working with the filterable

agent described by Burmester et al. (1946, 1947), she was unable to produce tumors by the inoculation of normal spleen material, growing in vitro, to which this agent had been added.

This thesis is essentially a continuation of the work by Chrétien (1951) on attempts to detect the presence of this agent using tissue culture techniques. An effort was also made to determine if a magnetic field would affect the growth of this tumor in vitro.

HISTORICAL

There are many excellent reviews concerning viruses and filterable agents and their relationships to tumors. One of the more recent ones (Shrigley, 1951) discusses a wide variety of tumors of animals. Kidd (1948, 1950) discussed various proliferative lesions caused by viruses and the reasons for placing some of these viruses along with sunlight, tar and many other substances in the category of carcinogenic agents. Claude and Murphy (1933) gave a summary of the work done up to that time on the various transmissible tumors of the fowl and presented several good reasons for not placing many of the filterable agents isolated from these tumors in the category of viruses.

For discussions concerning the tumor with which this thesis deals and other closely-related conditions in the fowl, the reader is referred to the works of Olson (1940), Jungherr (1948) and Chrétien (1951).

For a historical presentation and descriptions of the methods of tissue culture the reader is referred to the monographs of Parker (1950) and Cameron (1950). An excellent

summary of tissue culture techniques, as applied to the study of viruses, may be found in the paper by Robbins and Enders (1950).

The present literature review will deal only with the cultivation of tumor-producing agents in vitro.

Carrel (1925), while working with the agent of Rous' sarcoma, was the first to attempt to change normal cells into malignant ones in vitro by means of a tumor-producing agent. By isolating pure cultures of cells from the Rous sarcoma tumor growing in vitro, he was able to show that it was the macrophages and not the fibroblasts which are responsible for the malignancy of this tumor. He then proceeded to add the Rous sarcoma agent to pure cultures of monocytes. They rapidly acquired the characteristics of malignancy, as shown by inoculation into chickens. Some of these cultures appeared as normal growing tissue although they produced tumors when inoculated; however, more often specific changes took place in the infected cultures. The cultures which showed the specific changes produced tumors more rapidly than the ones which showed no such changes. Carrel and Ebeling (1926) were able to show that, although monocytes under ordinary cultural conditions

never were transformed into fibroblasts, the Rous sarcoma agent produced this change in such cultures. They described other conditions which will produce this change in the absence of the Rous sarcoma agent. They attempted to explain this phenomenon on the basis of an adaptive change, the sensitive cell transforming itself into a type of a cell which is not sensitive to the action of the Rous agent. Carrel (1926) proved conclusively that this agent multiplies in vitro and that this multiplication depends upon the presence and the nature of the cells contained in the cultures. Tumors were produced after inoculation of cultures having incubation periods ranging from 4 to 30 days after addition of the agent. Successful results were obtained with leucocytes, spleen fragments and embryo pulp. As he had shown before, experiments utilizing pure cultures of fibroblasts were all negative.

Ludford (1937) was the next to study the infection of cells in tissue cultures with the Rous sarcoma agent. He also performed similar experiments with the agent of the Fujinami sarcoma, which is a similar tumor of the fowl. His object of reopening these studies was to clarify the confusion which existed at that time as to whether the monocyte or the fibroblast

was the malignant element of these sarcomas. Berkefeld filtrates of both the Rous and the Fujinami sarcomas were added to cultures of fibroblasts and cultures of the buffy coat obtained from fowl blood. Inoculation of the fibroblasts treated with these agents produced tumors, but in only one case did cultures of the buffy coat produce a tumor. After varying periods of time the cultures were treated with immune serum to inactivate any free virus in the cultures. When the cultures were treated in this manner only the inoculation of the fibroblast cultures resulted in tumor formation. This suggested to Ludford that in Carrel's previous experiments the production of the tumors by the monocyte cultures was due to the presence of the agent in the medium. His conclusion was that the fibroblast and not the monocyte was the cell which was sensitive to infection with these agents.

Furth et al. (1934, 1937) described experiments dealing with the cultivation of agents producing various types of leukosis in fowls. The majority of their experiments deal with the cultivation of the tumor-producing cells. Their observations concerning the cultivation of the various agents in the presence of normal cells are as follows: Working with their virus 13

(a complex sarcoma leukosis agent), they failed to demonstrate survival of the agent in tissue cultures of normal fibroblastic cells from chicken embryonal leg muscle and in adult chicken spleen cultures. They concluded that this agent was destroyed in the presence of these normal cells. Their agent was prepared by freezing the tumor tissue at -31° C. After thawing, the material was centrifuged and the supernatant fluid used as a source of the agent. The same results were obtained by cultivation of their virus 1 (which produces a form of erythroleukosis) in the presence of normal cells. In another series of experiments they attempted to cultivate a virus which produces leukosis only (virus 1), in the presence of sarcoma tissue. The results of their inoculations showed that only sarcomas were produced and no leukosis, thus proving that the leukotic agent did not survive in the presence of common sarcoma cells.

Doljanski and Pikovski (1942) were able to show that the agent of hemocytoblastosis (strain T_1 of Engelbreth-Holm) would survive in the presence of normal bone marrow and normal fibroblasts for as long as 178 days. In the absence of living cells the agent lost its activity within 24 hours. Because of the large number of serial transfers made, the authors concluded

that there was a real increase of the leukotic agent in vitro. Both the cell cultures and the cell-free supernatant fluid remained infective throughout these experiments. However, they failed to observe any changes in the appearance of the cultures or the individual cells when compared with similar cultures without the addition of the agent.

Chrétien (1951), working with the agent of the avian lymphoid tumor, with which this thesis deals, was unable to produce tumors by the inoculation of normal spleen fragments which had been cultivated in the presence of this agent in vitro. However, she was able to show that the addition of the agent produced morphological changes in the growth pattern of the normal spleen fragments cultivated in vitro.

MATERIALS AND METHODS

Avian Lymphoid tumor. This tumor, designated as strain RPL 12 by the U. S. Regional Poultry Research Laboratory, East Lansing, Michigan, was maintained throughout these studies by serial passage in the pectoral muscle of chickens. Alternate passages of this tumor material from chickens to tissue cultures were maintained throughout the majority of these experiments. This technique was first used by Chrétien (1951), whereby she proved that this tumor would maintain its malignancy when cultivated *in vitro*.

Tissue culture methods. The following techniques were employed during this investigation: Carrel flask, 25-ml. Erlenmeyer flasks, and the double cover slip method described by Parker (1950).

Physiological solution. Hanks' solution was used throughout these experiments. It was prepared according to a modification of the formula given by Hanks and Wallace (1949). The solution was prepared from stock solutions as needed and stored in 25- or 50-ml. Erlenmeyer flasks. Hanks' solution was

prepared from the stock solutions at least once a week. Penicillin and streptomycin were added to Hanks' solution to make a final concentration of 25 units of penicillin per ml. and 125 micrograms of streptomycin per ml.

Plasma and serum. The same group of chickens was used throughout these experiments. They were bled by cardiac puncture as the plasma or serum was needed. Heparin was used as the anticoagulant in obtaining the plasma. After removing the cells from the blood, the plasma and serum were stored in the refrigerator until used.

Embryo extract. The embryo extract was prepared from 9- to 11-day-old chicken embryos. They were removed from the shells aseptically and then rinsed in Hanks' solution to remove any excess red blood cells. The eyes and feet were removed and the embryos placed in a mortar. After cutting up the embryos with a pair of curved scissors and grinding them lightly with a pestle, two ml. of Hanks' solution was added for each embryo used. This made approximately a 1:2 dilution of the embryo pulp as recommended by Cameron (1950). The mixture was then allowed to stand at room temperature for 30 minutes

before centrifuging at approximately 2,000 r.p.m. for 10 minutes. The supernatant fluid was drawn off, sealed in small test tubes and stored in the frozen state until used. Just before use, the extract was thawed at room temperature and clarified by light centrifugation.

"Agent." All samples of the "agent" used in these experiments were obtained from the U. S. Regional Poultry Research Laboratory, East Lansing, Michigan. Burmester and Cottral (1947) described the methods of preparation of the "agent" in that laboratory. In general, either filtration or centrifugation or both methods have been used to render the tumor suspensions free of intact cells. Plasma from chickens with active growing tumors was also used as a source of the agent in one experiment.

Sterility checks. All nutrient fluids added to the tissue cultures were checked for sterility. Plain nutrient broth (Difco) was the medium employed. Only occasionally was contamination encountered, this usually being caused by a mold. Occasionally contamination of the Hanks' stock solutions occurred, making it necessary to prepare new solutions.

EXPERIMENTAL

Experiment I

The first group of experiments was concerned with attempts to detect the presence of the agent when it was added to normal lymphoid tissue growing in vitro. After the addition of the agent to the cultures, which consisted of normal spleen fragments from 17-day-old chicken embryos, daily observations were made. Controls of normal spleen without added agent were used to make comparisons. The cultures were inoculated into chickens at the end of observation periods which ranged from 7 to 12 days. These inoculations were made to determine if the normal cells had become malignant, or if by some mechanism the incubation period of this agent could be shortened. Burmester and Cottral (1947) found that this agent requires an incubation period of four to six months before tumors may be demonstrated in the inoculated chickens.

The Carrel flask technique was used throughout this group of experiments. Briefly it consisted of embedding small fragments of spleen (approximately one cubic mm.) in a plasma

clot consisting of: 0.3 ml. plasma, 0.6 ml. Hanks' solution and 0.1 ml. of embryo extract. After complete coagulation of the plasma clot a liquid nutrient (Cameron, 1950) consisting of 40 per cent serum, 40 per cent Hanks' solution and 20 per cent embryo extract was added to the cultures. The nutrient fluid was changed every two or three days. This procedure consists of removing the old nutrient, bathing the culture in fresh Hanks' solution for 15 to 30 minutes and then adding fresh nutrient. The agent, in varying quantities, was added to the cultures after they had been allowed to proliferate for different intervals of time. In each case the nutrient fluid was changed just prior to the addition of the agent so that the cultures could be left undisturbed for at least two days before it was necessary to change the nutrient fluid.

In one series of cultures in this experiment the agent was diluted 1:2 with the plasma used in the formation of the clot. A similar method was used by Doljanski and Pikovski (1942) in their studies of the agent of fowl leukosis in tissue cultures.

At the end of the observation periods the tissue fragments were removed from the cultures and separated from the

clot. After the addition of a small amount of Hanks' solution, these fragments were then ground in a mortar to make a cell suspension. Each such cell suspension was inoculated into the pectoral muscle of two chickens (approximately 1-2 months old), each chicken receiving half of the inoculum which was usually between 0.5 and 1.0 ml.

A synopsis of the cultures in this experiment is given below.

Amount of Agent Added (ml.)	Age of Cultures When Added (days)	Age of Cultures When Inoculated (days)
dil. 1:2 with plasma	0	7
0.4	2	9
0.4	5	11
0.25	2	10
0.25	5	12

Results. Active migration of the round type of cells could be seen within a few hours after the planting of the spleen fragments. After 24 hours an extensive proliferation of the fragments had taken place. During the third day of cultivation the

fibroblast-like cells began to make an appearance and they increased rapidly thereafter.

Difficulty was encountered in making observations of the cultures which were made after diluting the agent with the plasma. This was due to the cloudy nature of the solution which contained the agent, leaving the plasma clot slightly opaque. The cultures which received 0.4 ml. of the agent were slightly cloudy but observations were possible. This difficulty required alteration of the methods in later experiments.

As far as could be determined, no specific morphological changes were observed in the cultures to which the agent was added. On the third day of incubation, one of the cultures (0.4 ml. agent added on the second day) seemed to show a greater proportion of the fibroblast-like cells than the controls. The digestion of the plasma clot seemed to be retarded in the cultures which contained the agent. This might have been due to an interference with the multiplication of the cells, although no gross differences were observed in the extent of proliferation shown by these fragments.

Chickens inoculated with these cultures were observed for 3 months and all failed to develop tumors within this period

of time. Several of the birds died after two months but all failed to show signs of visceral lymphomatosis or tumors at the site of inoculation.

Experiment II

A second series of tissue cultures was set up to eliminate some of the difficulties encountered in the first experiment.

The controls in the previous experiment consisted of normal spleen fragments to which no agent had been added. To eliminate personal error in the observation of the normal spleen fragments, and spleen fragments to which the agent had been added, "unknowns" were prepared. Samples of chicken plasma as well as tissue extracts, some containing the agent and some not, were prepared at the U. S. Regional Poultry Research Laboratory. These preparations appeared the same when observed and were designated by numbers only. The identity of these "unknowns," was not revealed to the investigator until the experiments were completed. Attempts were then made to determine which of these "unknowns" contained the agent. The cultures were observed daily to determine if there were any differences in their gross or microscopic characteristics. Tissue cultures of spleen fragments, one containing the agent and a second culture not containing the agent, were prepared at the same time to serve as controls.

In addition to the Carrel flask type of cultures as described in Experiment I, the double cover-slip method, as described by Parker (1950), was also used. This technique consists of attaching a small cover-slip to a larger one by means of a drop of Hanks' solution. A small fragment of the tissue is then placed into the plasma clot which is prepared by mixing one drop of plasma with one drop of embryo extract on the small cover-slip. A drop of the "unknown" specimen was also mixed with the two substances making up the clot. A few drops of the nutrient fluid described in Experiment I were added to the cultures and a large cover-slip was placed over a depression slide and sealed with paraffin.

It was not possible to test all tissue cultures for in vivo production of tumors because of the limited space available for housing the chickens. In the first series of cultures, the individual cultures were carried in duplicate. After 9 days of cultivation (0.4 ml. "unknown" added on second day), the cells from the duplicate cultures were removed from the plasma clot and placed in a mortar. The nutrient fluid from both cultures and enough physiological saline were added to make approximately two ml. of fluid. The cells were ground with a

pestle and the resulting cell suspension inoculated into the pectoral muscle of two chickens (approximately 2-4 months old), each receiving one-half of the cell suspension or approximately a one-ml. inoculum.

Table I gives a summary of the details and results of the chicken inoculations in this experiment.

Results. It was not possible to determine which of the "unknown" samples contained the agent and which did not. As far as could be determined, no specific morphological differences were observed in the cultures which received the unknown samples of the agent.

The results of the animal inoculations were also negative. The chickens inoculated with these cultures were observed for a period of three months. Several of the chickens died from other causes during the observation period; all failed to show signs of visceral lymphomatosis or tumor formation at the site of inoculation.

During the course of later experiments, two of the above inoculated chickens (series No. 1) were inoculated with one ml. of a 20 per cent fresh tumor cell suspension for serial propagation of the tumor. These two chickens failed to develop

TABLE I

Summary of details and results in Experiment II

Type of Culture	Series No.	Source of Spleen Tissue	Amount of "Unknown" Added	"Unknown" Sample No.	Presence of Agent in "Unknown"	Days of Cultivation Before Added	Days of Cultivation Before Terminated	No. of Chickens Inoculated	Results of Inoculation
Carrel Flask	1	17 da. chick embryo	0.4 ml.	3629	+	2	9	8	Neg.
				3630	-				
				3627	+				
				3628	-				
	2	18 da. chick embryo	0.25 ml.	3629	+	1	11	-	-
				3630	-				
				3627	+				
				3628	-				
	3	18 da. chick embryo	0.4 ml.	3647	+	3	11	4	Neg.
				3652	-				
Double Cover Slip	4	3-5 mo. old chicken	1 drop	3629	+	0	5	-	-
				3630	-				
				3627	+				
				3628					
	5	17 da. chick embryo	1 drop	3629	+	0	5	-	-
				3630	-				
				3627	+				
	6	18 da. chick embryo	1 drop	3647	+	0	5	-	-
				3652	-				
				3548	+				

tumors. All other chickens used for serial propagation of this tumor received similar inoculations. They developed tumors at the site of inoculation within a period of 5 to 11 days. When the identity of the "unknown" samples was revealed, it was found that one of the chickens had been originally inoculated with material from cultures containing normal spleen fragments to which no agent was added. The other chicken had been originally inoculated with material from cultures containing normal spleen fragments to which the agent was added.

Plate I - Tissue Culture - 4-hour Growth. Normal Spleen Fragment. Magnification - 120X.

Plate II - Tissue Culture - 24-hour Growth. Normal Spleen Fragment. Magnification - 36X.

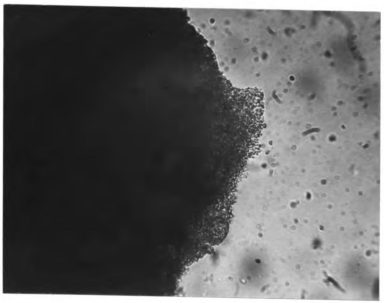

Plate I

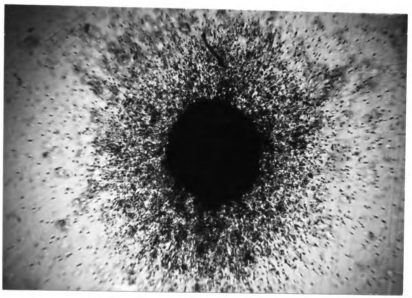

Plate II

Plate III - Tissue Culture - 72-hour Growth. Normal Spleen Fragment. Magnification 36X.

Plate IV - Tissue Culture - 72-hour Growth. Normal Spleen Fragment, Agent Added 24 hours Previously. Magnification 36X.

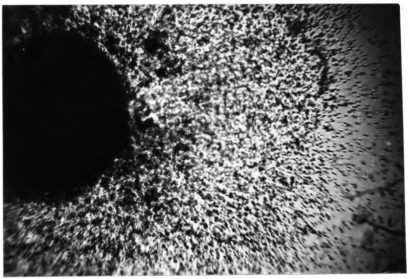

Plate III

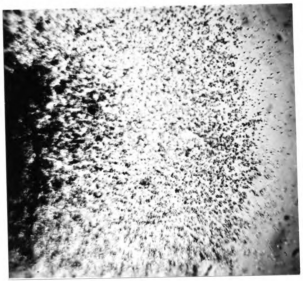

Plate IV

Plate V - Tissue Culture - 72-hour Growth. Normal Spleen Fragment. Magnification Approximately 72X.

Plate VI - Tissue Culture - 72-hour Growth. Normal Spleen Fragment, Agent Added 24 hours Previously. Magnification Approximately 72X.

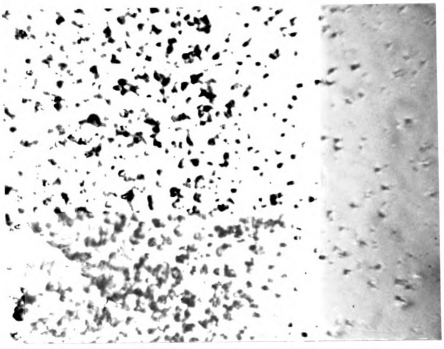

Plate V

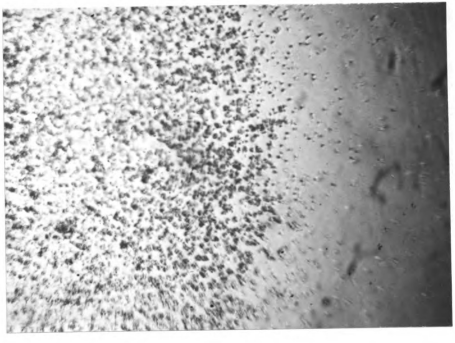

Plate VI

Plate VII - Tissue Culture - 5-day Growth. Periphery of Normal Spleen Fragment. Magnification 36X.

Plate VIII - Tissue Culture - 5-day Growth. Periphery of Normal Spleen Fragment. Magnification Approximately 72X.

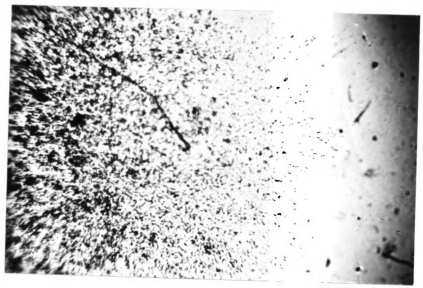

Plate VII

Plate VIII

Plate IX - Tissue Culture - 5-day Growth. Normal Spleen Fragment, Showing Outgrowth of New Cells. Magnification 516X.

Plate X - Tissue Culture - 5-day Growth. Normal Spleen Fragment, Showing Outgrowth of New Cells, Agent Added 3 days Previously. Magnification 516X.

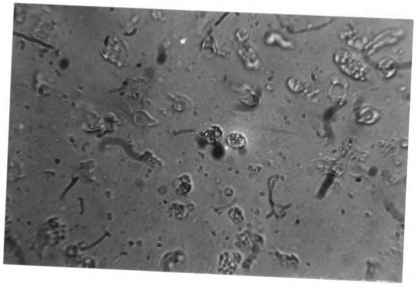

Plate IX

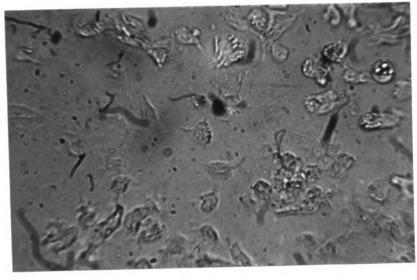

Plate X

Plate XI - Tissue Culture - 7-day Growth. Periphery of Normal Spleen Fragment, Agent Added 5 days Previously. Magnification 36X.

Plate XII - Tissue Culture - 7-day Growth. Periphery of Normal Spleen Fragment, Agent Added 5 days Previously. Magnification Approximately 72X.

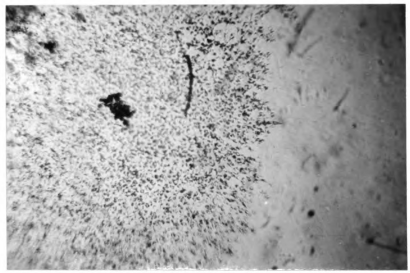

Plate XI

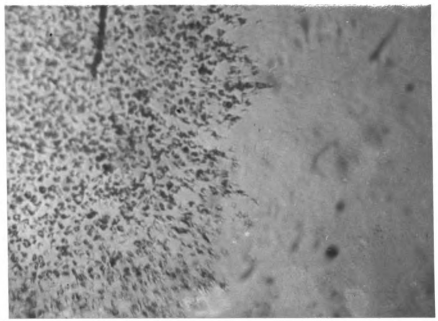

Plate XII

Plate XIII - Tissue Culture - 7-day Growth. Normal Spleen Fragment, Showing Outgrowth of New Cells. Magnification 516X.

Plate XIV - Tissue Culture - 7-day Growth. Normal Spleen Fragment, Showing Outgrowth of New Cells, Agent Added 5 days Previously. Magnification 516X.

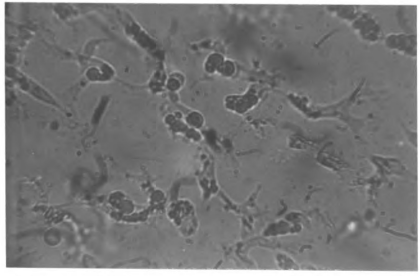

Plate XIII

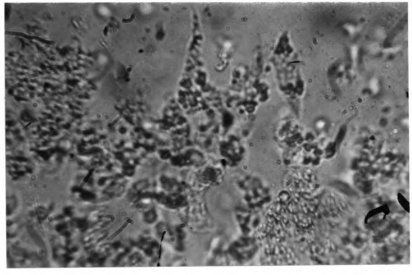

Plate XIV

Experiment III

Fardon (1940) and Katzberg (1951) observed that when two small fragments of tissue are cultivated in vitro there occasionally is observed a field of attraction between the two fragments. The pattern formed resembled the lines of force in a magnetic field.

With this in mind an experiment was set up to determine if a magnetic field would affect the in vitro growth of the avian lymphoid tumor. Normal spleen and heart fibroblasts growing in vitro were also placed in a magnetic field.

The only reference in the literature found, dealing with a similar experiment, was that of Ingvar (1920). He applied weak galvanic currents to tissue cultures consisting of central nervous system tissue of the chick. He was able to show that the current had a directing influence upon the cells and fiber outgrowth. This occurred almost entirely along the lines of force in the galvanic field. The cell processes growing toward the cathode differed morphologically from those growing toward the anode. He concluded that electrical forces play a role in the formative processes in morphogenesis.

Two horseshoe magnets with a field strength of approximately 125-150 gauss were used in this experiment. The Carrel flask type of culture as described in previous experiments, was used to cultivate the tissues.

The first trial consisted of placing one culture containing a single fragment of tumor in the magnetic field. A control culture, which was not placed in the magnetic field, consisting of a single fragment of tumor, was used to make comparisons. The culture placed in the magnetic field grew rapidly, but the control culture failed to grow.

The experiment was repeated, but this time two control cultures were used. This time the results of the first trial were reversed. The fragment in the culture placed in the magnetic field failed to grow and the fragment in each control culture grew. Similar experiments were set up using cultures of normal spleen and heart fibroblasts from a 17-day-old chick embryo.

Results. These experiments failed to indicate that the magnetic field influenced the growth of tissues in vitro. It should be stated that the experiments were of a preliminary nature and should receive more investigation.

DISCUSSION

As Robbins and Enders (1950) pointed out, there are two general methods of demonstrating the presence of a virus in tissue cultures. The first method is to show that the material removed from the culture exhibits the characteristic activity of the virus in question. This method of demonstrating the presence of the agent of the avian lymphoid tumor was impractical because of the unusually long incubation periods (up to 300 days) required. Experiments of this nature should be performed with this agent to determine if the agent will remain active in the presence of living cells.

It was hoped that the incubation period could be shortened by the addition of this agent to normal lymphoid tissue growing in vitro. If this agent was able to convert the normal cells into malignant ones, the incubation period would be expected to be somewhat like that following the inoculation of live tumor cell suspensions (approximately 7-15 days). If this had happened, a more rapid method of detecting the agent would be available.

Inoculation with the cultures containing normal spleen fragments, to which the agent was added, and subsequent failure of tumor formation in the chickens could be explained in several ways.

Direct inoculation of this agent into chickens requires a long incubation period before tumors develop. Thus, continued stimulation over a long period of time seems to be necessary for the conversion of the normal into the malignant cell in vivo. If this continued stimulation is necessary for the conversion to take place in vitro, the failure could be explained on the basis of the characteristics of spleen cultures growing in vitro. Maximow and Bloom (1948) showed that lymphocytes cultured in vitro rapidly develop into macrophages and then turn into fibroblasts. Since the lymphocyte is the type-cell of the tumor, its apparent absence from cultures of spleen tissue within a few days would explain the failure of these cultures to produce tumors. However, this fails to explain why the tumor may be cultivated in vitro (Chrétien, 1951) without loss of malignancy. It may be possible that the malignant lymphocytes do not undergo the changes which have been observed of the normal ones. Intimate physiological mechanisms, lacking

in tissue cultures, may be necessary for the conversion of the normal into the malignant cell in this case. The conditions used for the cultivation of tissues in vitro are a great deal different from those which involve the growth of tissues in vivo. Such factors as the presence or absence of inhibiting factors, the growth-promoting principles involved and the influence of the animal as a biological unit must be considered. Influenza virus, in the presence of chick embryo tissue, multiplies in vitro at 37° C. At a temperature of 41° C. (approximately the normal body temperature of the chicken) multiplication does not take place (Enders and Pearson, 1941). Enders (1948) used these facts to explain the resistance of the chicken to infection with influenza virus. A similar explanation might possibly account for the failure here, since the cultures were incubated at 37° C. instead of 41° C.

The number of cells contained in the inoculum may not have been adequate to elicit tumor formation had they been malignant. An attempt was made in the second experiment to overcome this difficulty. Some of the cultures to be inoculated were carried in duplicate. This resulted in the chickens receiving an inoculum containing approximately twice the number

of cells inoculated in Experiment I. The results of these tests were also negative.

A second method of demonstrating the presence of a virus in tissue cultures is by detecting some abnormal change in the tissue or cells which is caused by the virus in question (Robbins and Enders, 1950).

Chrétien (1951) was able to detect morphological changes in tissue cultures of spleen fragments to which the agent of this tumor was added. Similar experiments were performed during this investigation but all failed to indicate that morphological changes took place in cultures to which the agent was added.

Carrel and Ebeling (1926) were able to convert pure cultures of monocytes into fibroblasts by the addition of the Rous sarcoma agent, but they also described other conditions during which this conversion may take place. When the monocytes were observed to congregate in masses of dead tissue the conversion took place in the absence of the agent.

Doljanski and Pikovski (1942) were unable to demonstrate an abnormal change in cultures to which the agent of fowl leukosis (hemocytoblastosis, strain T_1) was added.

The fact that two of the chickens, used for inoculation of cultures containing normal spleen incubated with the agent, later resisted an inoculation of live tumor cells must be explained. Burmester and Prickett (1944), working with the same lymphoid tumor, were able to show that chickens surviving appropriate dosages of live tumor cells were later resistant to subsequent challenge inoculations with these cells. Active growth and later regression of the tumors had taken place in all cases. At first it was believed that the chickens surviving the challenging inoculations in this experiment may have developed a similar type of resistance. When the condition of the unknown samples was revealed, it was discovered that one of the chickens had received a control preparation which did not contain the agent. It is believed that these chickens survived the challenge inoculations due to their age (approximately 4-1/2 months) or to their state of health. The chickens were kept under very crowded conditions and when they were inoculated with the tumor cell suspension it was noted that they were in poor physical condition. It is known that young chickens and those in good physical condition are more susceptible to the transplantation of tumors than older and unhealthy ones (Claude and

Murphy, 1933). It is possible that the inoculum may have been improperly administered, however, 30 other such inoculations during these experiments, proved to be 100 per cent successful.

The experiments dealing with the effects of a magnetic field on the growth of the tumor in vitro were of a preliminary nature only. During the first trial it was noted that a tumor fragment grew better when the culture was placed in a magnetic field. The control culture failed to show growth. The experiment was repeated with proper controls and no stimulation in growth could be detected.

Similar experiments were performed to see if a magnetic field would affect the growth of fragments of spleen and heart fibroblasts from a chick embryo. These experiments failed to show that the magnetic field had any consistent effect on the growth of these tissues in vitro.

It is suggested that this possibility be investigated further. The magnets employed had a field strength of 125-150 gauss. It may be that a stronger magnet could influence the growth of these tissues. An experiment using growth measurements similar to those described by Parker (1950) would also be valuable.

SUMMARY

1. Tissue cultures of normal spleen tissue to which the cell-free agent of an avian lymphoid tumor was added failed to produce tumor formation upon inoculation into chickens.

2. Attempts to detect morphological changes in cultures receiving the agent were entirely negative.

3. Placing tissue cultures containing avian lymphoid tumor tissue, normal chick embryo spleen and heart fibroblasts in a magnetic field (125-150 gauss) failed to reveal any differences in the rate of growth in these cultures when compared with controls.

BIBLIOGRAPHY

1. Andrewes, C. H. 1934. Viruses in relation to aetiology of tumors. Lancet, 227, 117-123.

2. Aycock, W. L. 1948. The epidemiology of poliomyelitis. Chapter in Virus and Rickettsial Diseases, Harvard University Press, Cambridge, Mass., pp. 555-580.

3. Burmester, B. R., and Cottral, G. E. 1947. The propagation of filtrable agents producing lymphoid tumors and osteopetrosis by serial passage in chickens. Cancer Research, 7, 669-675.

4. Burmester, B. R., and Prickett, C. O. 1944. Immunity reactions obtained with a transmissible fowl tumor (Olson). Cancer Research, 4, 364-366.

5. Burmester, B. R., Prickett, C. O., and Belding, T. C. 1946. A filterable agent producing lymphoid tumors and osteopetrosis in chickens. Cancer Research, 6, 189-196.

6. Cameron, G. 1950. Tissue Culture Technique, Academic Press, New York.

7. Carrel, A. 1925. Essential characteristics of a malignant cell. J. Am. Med. Assn., 84, 157-158.

8. Carrel, A. 1926. Some conditions of the reproduction in vitro of the Rous virus. J. Exptl. Med., 43, 647-668.

9. Carrel, A., and Ebeling, A. H. 1926. The transformation of monocytes into fibroblasts through the action of Rous virus. J. Exptl. Med., 43, 461-468.

10. Chrétien, M. M. A. 1951. Cultivation in vitro of the tumor of lymphomatosis. Unpublished M. S. Thesis. Michigan State College.

11. Claude, A., and Murphy, J. B. 1933. Transmissible tumors of the fowl. Physiol. Rev., 13, 246-275.

12. Doljanski, L., and Pikovski, M. 1942. Agent of fowl leukosis in tissue cultures. Cancer Research, 2, 626-631.

13. Ellerman, V., and Bang, O. 1908. Experimentelle leukamie bei huhnern. Zbl. Bakt. Orig., 46, 595. (Cited by Kidd, 1948.)

14. Enders, J. F. 1948. Propagation of viruses and rickettsiae in tissue cultures. Chapter in Viral and Rickettsial Infections of Man, T. M. Rivers, Ed. J. B. Lippincott Co., Philadelphia, pp. 114-127.

15. Enders, J. F., and Pearson, H. E. 1941. Resistance of chicks to infection with influenza virus. Proc. Soc. Exptl. Biol. Med., 48, 143-146.

16. Fardon, J. C., Brotzge, G., Loeffler, M. K., and Breit, J. 1940. Attraction fields in vitro. Nature, 146, 619-620.

17. Furth, J., and Breedis, C. 1937. Attempts at cultivation of viruses producing leukosis in fowls. Arch. Path., 24, 281-302.

18. Furth, J., and Stubbs, E. L. 1934. Tissue culture studies on relation of sarcoma to leukosis of chickens. Proc. Soc. Exptl. Biol. Med., 32, 381-383.

19. Hanks, J. A., and Wallace, R. E. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med., 71, 196-200.

20. Hirst, G. K. 1941. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science, 94, 22-23.

21. Hirst, G. K. 1943. Adsorbtion of influenza virus on cells of the respiratory tract. J. Exptl. Med., 78, 99-109.

22. Howe, H. A. 1948. Poliomyelitis. Chapter in Viral and Rickettsial Infections of Man, T. M. Rivers, Ed. J. B. Lippincott Co., Philadelphia, pp. 245-268.

23. Ingvar, S. 1920. Reaction of cells to the galvanic current in tissue cultures. Proc. Soc. Exptl. Biol. Med., 17, 198-199.

24. Jungherr, E. 1948. The avian leukosis complex. Chapter in Diseases of Poultry, ed. 2, H. E. Biester and L. H. Schwarte, Eds. Iowa State College Press, Ames, pp. 421-474.

25. Katzberg, A. A. 1951. Distance as a factor in the development of attraction fields between growing tissues in culture. Science, 114, 431-432.

26. Kidd, J. G. 1948. Viruses and virus-like agents as causes of cancer. Bull. Johns Hopkins Hosp., 82, 583-600.

27. Kidd, J. G. 1950. Proliferative lesions caused by viruses and virus-like agents. Chapter in Pathogenesis and Pathology of Viral Diseases, J. G. Kidd, Ed. Columbia University Press, New York, pp. 161-180.

28. Landsteiner, K., and Popper, E. 1909. Uebertragung der poliomyelitis acuta auf affen. Ztschr. f. Immunitatsforsch. u. exper. Therap., 2, 377-390. (Cited by Howe, 1948.)

29. Ludford, R. J. 1937. The production of tumours by cultures of normal cells treated with filtrates of filterable fowl tumours. Am. J. Cancer, 31, 414-429.

30. Marx, E., and Sticker, A. 1902. Untersuchungen uber das edpitelioma contagiosum des geflugels. Deutsch.

31. Netter, A., and Levaditi, C. 1910. Action microbicide exercee par le serum des malades atteints de paralysie infantile sur le virus de la poliomyelite aigue. Compt. rend. Soc biol., 68, 617. (Cited by Aycock, 1948.)

32. Olson, C., Jr. 1940. Transmissible fowl leukosis. A review of the literature. Mass. Agr. Exp. Sta. Bull. 370.

33. Olson, C., Jr. 1941. A transmissible lymphoid tumor of the chicken. Cancer Research, 1, 384-392.

34. Parker, R. C. 1950. Methods of Tissue Culture, ed. 2, Paul B. Hoeber, Inc., New York.

35. Robbins, F. C., and Enders, J. F. 1950. Tissue culture techniques in the study of animal viruses. Am. J. Med. Sci., 220, 316-338.

36. Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exptl. Med., 13, 397-411.

37. Rous, P. 1912. Transplantable tumors of the fowl: a neglected material for cancer research. J. Am. Med. Assn., 58, 1682-1683.

38. Shrigley, E. W. 1951. Virus-induced tumors of animals. Chapter in Annual Review of Microbiology, vol. V, C. E. Clifton, Ed. Annual Reviews, Inc., Stanford, Calif., pp. 241-264.

ROOM USE ONLY