CULTIVATION IN VITRO OF THE TUMOR OF LYMPHOMATOSIS

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CULTIVATION IN VITRO OF THE TUMOR OF LYMPHOMATOSIS

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INTRODUCTION

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The cause of cancer has been an inexhaustible source of theories: some scientifically logical, others merely existing on philosophical hypotheses.

Most cancer workers seem to favor the endogenous theories, which are numerous. All of these theories, however, include the realization that after the cause or carcinogen provokes a sudden change in living cells, directly or by the removal of an inhibitor, these cells multiply indefinitely. Thus, to the "mutationist", this change in cell behavior is the disease itself, and the impulse causing the cells to multiply after the initial stimulus is gone becomes identified with the intrinsic forces of the cell itself.

In support of this, Haddow (1947) applies the conclusion which Sonneborn (1945) derived, from his studies of the Paramecium, to the Rous sarcoma agent. Considering the Rous agent as a plasmagene, Haddow states that the penetration of the mutant plasmagene into the cytoplasm of a susceptible normal cell results in: 1) the continued production of the cytoplasmic substance, i.e., the virus, in the development of the character of malignancy; 2) the hereditary maintenance of malignancy in successive cell generations. The above theory, at least, favors the individual specificity of various tumor agents.

Murphy (1935) interprets results of studies with the Rous chicken tumors No. 1 and No. 7, as opposed to the parasite theory, on the basis that an already differentiated cell becoming infected with a

growth-stimulating parasite might be expected to retain its essential characteristics in the malignant state. On the contrary, as he states, there is evidence that the complex differentiation of cells is just as much the result of the agent as is the growth, which would indicate that the agent is probably closely related chemically to those factors normally determining cell differentiation.

Altenburg's (1946) "viroid" theory presupposes the idea that the virus (as in Rous sarcoma) arises as a mutation, produced by a carcinogen, from a viroid normally present in the animal. It is claimed, by the author, that normal fowl tissue can cause the production of antibody against Rous' sarcoma. According to the above-mentioned theory, this could be explained on the ground that the viroid, in normal tissue, and the virus in sarcoma, are very much alike, and so it follows that an antibody against one would be an antibody against the other. He states further that cancer due to nuclear gene mutation in the host is unlikely, as shown by Auerbach (working under Muller) using chemical carcinogens, since there was no increase observed in the mutation rate of genes in the nucleus of Drosophila. Such an increase would be expected if nuclear gene mutation were the cause of cancer.

While on the subject of chemical carcinogens, mention should be made also of Andrewes' (1936) attempts to show the presence of a virus in a nonfilterable tar sarcoma. Antibodies were developed in the birds inoculated with a tar-induced tumor which neutralize the virus of Rous' sarcoma No. 1, antibodies which were not demonstrable upon inoculation of normal fowl tissue. Gottschalk (1943) also reports neutralizing

antibodies for sarcoma No. 13 produced upon injection of rabbits with nonfilterable methylcholanthrene-induced sarcoma No. 16 stating that this serum contained antibodies in much larger amounts than that of normal birds. Even if this is insufficient evidence to show the presence of a not-yet-demonstrated virus in chemically induced tumors, it seems, at least, to support the postulate of antigenic relationship between those factors determining cell differentiation.

To most biologists, the virus theory still seems the most plausible, because the characteristics associated with many neoplasms resemble those of the ultrafilterable agents as we see them in non-cancerous diseases; moreover, agents answering specifically to the description of viruses have actually been isolated from various types of tumors. It is easy to imagine that, since certain viruses, such as the pox group, produce proliferative changes in cells, as shown by Andrewes (1934[a]), they can also give rise to true neoplasms. Rous (1943) points out, also, that in kidney tumors of swamp frogs, Rana pipiens, where virus is the known etiological agent, large inclusion bodies are found in the nuclei, as seen in other virus diseases.

In certain neoplasms, the infective nature, as shown by transmissibility, is demonstrated through the work of Foulds (1937[b]) with a fowl carcinoma, Duran-Reynals and Shrigley (1946) with chicken sarcomas, and Olson (1941), and Burmester and Prickett (1945) with lymphoid avian tumors.

Whether this theory can be made applicable to all cancer growths

is impossible to say at present, particularly since the virus alone is not a satisfactory answer, even when isolated from tumor tissue.

There are, therefore, certain digressions from the strict acceptance of a virus as the exogenous causative agent of cancer, depending upon the associated factors. Andrewes' (1934[b]) explanation exemplifies the picture of latent viruses existing in seemingly normal cells which cause cell malignancy upon stimulation by a carcinogen, be it a hormone factor as suggested by Bittner (1948) and Lacassagne (1932), or a labile "specific factor" which ruptures cell defenses and allows the virus to infect as demonstrated by the studies of Gye (1925), or perhaps an "age factor" as Duran-Reynals (1950) calls it.

Regardless of theory or particular manifestation of neoplasm, any agent to which is attributed the etiology of the disease, according to Duran-Reynals (1950), should show an affinity for cells and induce them to grow while multiplying along with them. It should be specific for cells in that the same characteristic lesions are produced, and yet be able to change (vary) so that various lesions of different characteristics can be produced. It should be capable of remaining latent in tissues until it is altered by environmental conditions, and have the ability to live in a free or in a recondite state.

With this in mind, together with the work of Carrel (1924[a,b,c]) dealing with the artificial cultivation of tissue cells from the sarcoma of Rous, a study was outlined for the attempted cultivation <u>in vitro</u> of the lymphomatosis (Strain RPL 12) tumor tissue in an endeavor to show

whether neoplastic characteristics are preserved in vitro. An effort was also made to "infect", in vitro, normal spleen tissue with the lymphomatosis "agent".

HISTORICAL

HISTORICAL

The first reported case of "lymphosarcomatosis" was published by Roloff in 1868, according to Engelbreth-Holm (1942). The latter author also relates that it was not until 1908 that the transmissibility of "fowl leukemia" was discovered by Ellermann and Bang when they realized that by intravenous or by intraperitoneal inoculation of blood, emulsions of organs or cell filtrates from diseased birds, the condition could be transmitted to healthy birds. These observations were actually made two years prior to Rous' work with sarcoma of fowl, but the authors were not deservedly credited as being the first discoverers of the fact that avian mesenchymal tumors were transmissible by a cell-free agent because, at that time, there was doubt as to the neoplastic nature of leukemia.

Since that period, a great many bewildering and conflicting publications have appeared about allied conditions bearing close morphological relationships, which Feldman (1932) grouped under the heading of lymphoblastoma, such conditions as lymphadenoma, pseudoleukemia, lymphoma, leukemia, lymphocytoma, alveolar sarcoma, lymphosarcomatosis, round-cell sarcoma, lymphatic leukemia, lymphosarcoma, and Hodgkin's disease. Even the terms "fowl leukosis", "leukosis complex", and lymphomatosis used interchangeably present definite complications, in that they include the common group of transmissible leukemic and aleukemic or leukemic-like diseases of fowls.

Besides the complexity of terminology, there seems to be little agreement as to whether some or all of these neoplasms are caused by filterable agents. As to the subject of those accepted virus-caused tumors, there is also dissension as to whether all the various manifestations of the diseases mentioned are caused by the same virus. This was expressed by Furth (1931), Stubbs and Furth (1931), Furth and Seibold (1933), Furth and Breedis (1933) and Furth (1934[a]), the particular condition depending on such factors as dosage, age and resistance of the host, portal of entry, and route of metastases. As opposed to this, Shrigley, et al (1945) and Shrigley (1947), Andrewes and Shope (1936), Stubbs and Furth (1935), and Furth (1936[a], 1936[b]) considered other neoplastic diseases as being caused by entirely unrelated viruses possessing specific tissue affinity, thus always producing similar type tumors and possibly changing their tissue specificity only upon variation or mutation.

Then again, there is the more prominent concept, that of various strains of presumably the same virus being antigenically related, each strain producing its specific typical disease upon transfer. For those who follow this latter train of thought, it is easy to suspect, when certain experimental reports indicate a complexity of symptoms, that perhaps the process involves not one, but many such strains working together.

In order to avoid more confusion than is necessary, some space, is here devoted to the explanation of terminology.

According to Engelbreth-Holm (1942), the original Ellermann classification was changed only in that the group called lymphogenous leukemia (lymphatic leucosis) should not include leukemic conditions, since intravascular infiltration occurred only rarely in this condition.

Feldman (1932) proposed a classification which was adopted by Olson in 1933 (Feldman and Olson, 1933). They divided the group of leukoses into myeloid leukosis, erythroleukosis, and suggested the term lymphocytoma for the aleukemic neoplasm (which was later called lymphomatosis).

The myeloid leukosis is described as a neoplastic-like hyperplasia of the granuloblastic elements of the bone marrow, with subsequent invasion of the bloodstream by an excessive number of immature myeloblastic cells. The formation of heteroptic myeloid foci may occur in the liver and marked leukemia with definite anemia results.

Erythroleukosis is characterized by vigorous proliferation of erythroblastic cells and appearance in the bloodstream of hemoglobin-free precursors of red blood cells in such numbers as to constitute leukemia. Usually severe anemia and an intravascular accumulation of primitive erythroblasts occur in the bone marrow, spleen and liver. This condition was shown to be transmissible by Oberling, Guérin, and Boic (1933), Beard et al (1950).

Lymphocytoma (or lymohomatosis) is defined as a malignant neoplasm, the undifferentiated lymphocyte being the type cell involved.

It is characterized by the presence of diffuse or nodular, neoplastic, extravascular pinkish-gray to grayish-white deposits in various parts of the body. The visceral organs are most frequently affected, there being a predilection for the liver; the spleen and kidney are also commonly involved, with occasional nodular masses occurring in the gizzard, proventriculus, intestines, mesentery, omentum, ovary, pancreas, heart and lungs. Although leukemia does not occur ordinarily, Feldman stated that there is a reduction in hemoglobin from the normal 50-60 per cent to 15 per cent, and in red blood cell count from three million to one million, and that blood coagulates slowly.

The microscopic characteristics of the tumor consist of compact masses of proliferated lymphoid cells supported by irregular strands of fibrous stroma, and a delicate reticulum. The type cell is usually comparable in size to the large lymphocyte of normal fowl blood.

Furth has mentioned various strains of viruses which causes one to wonder at the great ambiguity of the so-called "leukosis complex".

Furth and Breedis (1933) and Furth (1934[a]) described a Strain 2 which produces lymphatic tumors upon intramuscular injection, causes infiltration throughout the blood-forming organs, and is almost always associated with a lymphatic leukemia, an anemia, and often an infiltration of the nerves which seldom causes clinical paralysis. The same strain, on the other hand, is later reported (Furth 1936[a]) as producing a transplantable osteochondrosarcoma.

Furth's (1934[b], 1935[a]) Strains 5 and 6 are said to produce neurolymphomatosis, similar to the spontaneous disease, upon intravenous

inoculation of blood cells or plasma from diseased animals. The same author (1936[b]) later states that as a result of an injection of Strain 5, in one case,a sarcoma was formed characterized by peculiar giant cells. He called the new agent Strain 15 since it no longer caused neurolymphomatosis nor leukosis.

Another strain called Strain 11 (Furth, 1936[b]) originated spontaneously. It generally produced a spindle-cell sarcoma morphologically similar to Rous'. Still another Strain called 13 is said to produce a sarcoma (type endothelioma) with an erythroleukosis.

In 1932 (a,b,c) Furth's work with a leucosis agent showed that when blood cells were inoculated in decreasing amounts, the period of incubation was not so frequently nor so greatly prolonged as when plasma or silicous filtrates were used. Collodion filtration determined the agent as being much smaller than 250 mu. The agent was found to resist drying at least 54 days, and to remain active with glycerine preservation at 104 days. Furth also found that this agent was not inactivated by freezing in liquid air; that at 37.5° C., it lost its activity, whereas at 4° C., it retained its activity at least 14 days.

The condition called lymphomatosis (lymphocytoma or leukosis complex) itself embodies a number of varied representations: 1) The ocular form produces a pathological aggregation of lymphocytes in the vascular tissues of the eye. 2) The visceral form is characterized by large masses of lymphoid cells in any of the organs of the viscera. 3) The neural type shows infiltration of lymphocytes, histocytes, and

plasma cells in the nerve trunks. 4) The osteopetrosis form is manifested by thickening of the long bones, particularly the shanks. 5) The leukosis type produces an excessive number of leucocytes in the bloodstream. (This, however, was never reported with Strain 12 used in these studies).

The agents causing various types of lymphomatosis may or may not be antigenically related, but they, at least, have in common the undifferentiated cells of the lymphatic series as the type cell with which the neoplasm is associated.

Much of the experimental work on lymphomatosis has been concentrated on studies of the natural and artificial trasmissibility and the factors on which it is dependent. The commendable research by Waters (1945) brought out the significance of transmission to progeny through the egg by showing that lymphomatosis occurred in chicks under four months of age, in spite of the fact that there was no direct contact with previously infected birds. His studies with Bywaters (1949) presented evidence to the effect that the disease is also transmitted by contact, since chicks from a free source, bred in isoletion do not contract the condition whereas those from free source bred with chicks from a known contaminated source show a high percentage of lymphomatosis. This was similarly observed by Brewer and Brownstein (1946) who pointed out, in addition, that a significantly higher incidence of infection than in controls is observed when infected tissues are fed to chicks. In addition, Waters and Bywaters (1949) showed that age is an

important factor in the outcome, the hatching period being the time of greatest susceptibility, and the older the bird the more resistant. However, according to Waters (1947) there are certain limitations to definite conclusions as to transmission by contact since it is almost impossible to be sure that the experimental animals are actually free of the tumor agent prior to the time of exposure; besides, the length of time elapsing before the tumor is grossly visible is generally too great to assure that the tumor was produced as a result of contact.

Cole (1949) observed that embryonic mortality was not adversely affected by the development of leucosis in dam or sire during or shortly after the breeding season. Barber (1947) also found that between the progeny of random bred and inbred males (fifth generation brother and sister matings) there was no significant difference in the incidence of leucosis up to one year.

These observations would lead one to believe that if the disease is transmitted through the egg, the agent often remains latent for a period of one year.

Asplin (1947) tried to associate "Chick Disease" with lymphomatosis by assuming that it was an early acute form of the latter. His attempt was unsuccessful, however.

Burmester (1945) pointed out that twice as many females as males develop lymphomatosis spontaneously, but upon inoculation of infected blood into normal chicks, there was no significant difference between sexes.

In regard to artificial transmission, Beach (1938) claimed failure to transmit two strains of the agent associated with neurolymphomatosis, whereas Furth (1934[b]) and Blakemore (1945) wrote of success in transmitting a neural form. Duran-Reynals (1946) reported inability to transplant twelve lymphoid tumors. Nevertheless, Olson (1941) reported a serially transmissible lymphoid tumor which he felt has no etiological connection with fowl paralysis or leukosis. A tumor answering to a similar description was developed by Burmester and Prickett (1945) which upon intraperitoneal inoculation caused visceral lymphomatosis and gross neoplastic lesions of the pectoral muscle when injected intramuscularly. Brewer and Brownstein (1946) also claimed transmission success.

Burmester et al (1946) reported that besides transmission of the agent by means of viable tumor cells, if centrifuged extracts of tumors or filtered plasma of infected fowls were injected intramuscularly, intraperitoneally or intravenously, a high incidence of osteopetrosis and lymphatic metastases in viscera occurred. No tumors were observed at the site of inoculation.

Burmester and Denington (1947) studied four lymphomatosis tumor strains, called RPL 18, 19, 20, and 21, developed from cases of naturally occurring visceral lymphomatosis. The same results were obtained as mentioned above, osteopetrosis being observed only with Strains 18 and 21, however. Strain 19 produced hemangiomatosis rather than the usual extravascular infiltration (the comparatively short incubation period being suggestive of erythroleukosis).

It should be emphasized, however, that all positive results with

filtrates were characterized by an unusually long (latent) period of incubation, 116 to 162 days.

Emmel (1950), working with Strain RPL 16 developed by Burmester et al, substantiated the latter's results as to transmission, except that he did not produce tumor formation with plasma inoculations (intramuscularly nor intravenously), and did not agree to the extensive gross tumor involvement of the visceral organs as described by Burmester (1947[b]). A study of the physical properties of the transmissible agent made by Emmel led to the conclusion that the factor responsible for tumor development was intimately associated with the nucleoprotein of the tumor cells, particularly the nucleoli. Nucleoprotein preparations from muscle tumors and from blood cells of infected birds produced typical tumors in 32 out of 52 birds during eight trials when injected intramuscularly.

A good deal of research has also been conducted on the immunology of the disease. Rous (1913), during his studies with fowl sarcoma, concluded that birds manifest two kinds of resistance to avian tumor independent of one another, one against the implanted tumor cells as such, and the other against the action of the etiological agent causing the neoplastic change.

Furth (1934[b]), dealing with the agent associated with neurolymphomatosis, observed that chicks which failed to develop the disease after the first injection, subsequently resisted repeated intravenous reinoculations.

Foulds (1937[a]) observed that noninfective extracts of a tumor

induced by 1:2:5:6 dibenzanthracene produced sera which neutralized filtrates of Rous sarcoma No. 1. His deduction was that a virus was present, but a virus-protoplasm complex was formed which prevented the demonstration of the virus by filtration.

Duran-Reynals and Estrada (1940) in reference to Rous sarcoma, made the discovery that serum from normal adult chickens contained an antibody-like factor, which serum, upon injection with infected tissue, protects the animal in such a way as to cause a shift from hemorrhagic into neoplastic lesions or suppression of all lesions.

Furth and Kabat (1941), Kabat and Furth (1941) showed that neutralizing antibodies against fowl tumor agents of leukosis and sarcoma could be produced in rabbits by the injection of heavy material sedimented at 27,000 r.p.m. obtained from chicken tumor, (after first clarifying at 2000 and 8000 r.p.m.). Similar sediments from normal chick spleen did not produce such antibodies. Thus the complement fixing antibodies produced by both normal and tumor materials are unrelated to neutralizing antibodies.

Burmester and Prickett (1944) found that the immunity produced after regression of local tumors, caused by inoculation of transmissible tumor, appeared in all birds surviving an active growth of the tumor, and could not be overwhelmed by very large and repeated doses of the same agent. The immunity remained over a prolonged period.

Olson (1945[a], 1945[b], 1946) agreed with these findings and added that the immunizing action is not affected by freezing or heating of tumor pulp although this treatment reduced growth activity. In 1947,

he pointed out that tumor cells, however, are necessary to induce immunity since he found that whole blood did, but plasma did not, stimulate the production of antibodies.

Unfortunately, the immunity acquired by means of cell transplants was found not to protect the animals against the natural occurrence of visceral or neural lymphomatosis, as shown by Olson (1947) and Burmester et al (1947). The latter authors, however, showed that plasma from hyperimmunized birds, when injected into chicks having received implants of the same tumor strain, reduced the mortality resulting from tumor involvement and the incidence of "takes".

Burmester (1947[a]) also demonstrated in vitro cytotoxic activity of lymphoid tumor cell antiserum by allowing the tumor cells to incubate 24 hours in the antiserum before injection. The activity of the antiserum was measured by the partial or complete suppression of the growth and malignancy of cells, not duplicated by incubation with normal serum.

Contrary to the results of Olson, Burmester and Belding (1947) reported that survivors of cell-free tumor extract injections of Strains 14 and 15 (from naturally occurring visceral form) were found immune to highly active tumor cell suspensions. Cross immunity was also demonstrated between most of the avian lymphoid tumor strains investigated.

Only a brief summary of some of the reported tissue culture studies of related neoplasms will be discussed.

Claude Bernard (Parker 1950), as early as 1878, pointed out the importance of the "milieu intérieur" (internal environment) in regulating the activities of living tissue. He realized that to study the

functional property of cells, it would be necessary to eliminate the influence of the organism by isolating the different parts in artificial systems.

Jolly (1903), using hanging drops, conducted extensive studies on leucocytes in vitro.

It was not until 1907, however, that Harrison devised a simple technique which permitted explanted parts to continue to grow and develop outside the organism, again by means of the "hanging drop" method.

Indebtedness for the development of more precise techniques is extended to Carrel (1924[a,b,c]; 1925, 1926). He was able to separate, in vitro, the mononuclear blood cells from the fibroblasts present in the sarcoma of Rous; and having obtained pure cultures of each type cell, he demonstrated that the malignancy factor was not present in fibroblast cultures but in cultures of macrophages. He further tried to infect cultures of normal fibroblasts and also of normal mononuclear blood cells with the filterable agent causing the sarcoma and the same conclusion was derived, that infection was not produced in the fibroblasts because "les macrophages du sang sont sensibles au virus du sarcome de Rous et qu'à son contact, ils se transforment en cellules sarcomateuses d'une grande malignité."

Furth and Stubbs (1934) in their tissue culture studies attempted to correlate sarcoma to leukosis of chickens. Using Carrel's method, they grew a pure culture of chicken sarcoma Strain 13 (which produced

both sarcoma and erythroleukosis) until the leukotic blood cells disappeared, to see if the culture still had the ability to produce erythroleukosis. They found the culture still highly virulent after in vitro cultivation for 67 days, with the ability to produce either sarcoma or sarcoma with erythroleukosis. These authors concluded that either two viruses were present in the cells, or else the one agent stimulated neoplasia in both reticular and endothelial cells.

Furth and Breedis (1937) cultured leukemic myeloblasts (associated with Virus I) in a liquid medium for 30 days and produced leukosis upon inoculation of tissue cells. Tissue cultures (plasma clot method) of spleen and bone marrow, grown from a case of myeloid leukemia showing myeloblasts and fibroblast-like cells, also produced leukosis. A fibroblast-like culture from bone marrow of an erythroleucotic case, not showing leukotic blood cells, failed to produce leukosis. In support of the opinion that oncogenic viruses multiply in vitro only in the presence of cells on which they confer neoplastic properties, results were obtained showing that Virus 13 (sarcoma) perishes in vitro within two weeks in the presence of normal fibroblasts of spleen, although the sarcoma cells, grown in vitro for 158 days (free of primitive blood cells), still produced sarcoma and leukosis. It was also shown that Virus I which produces leukosis, perished in the presence of sarcoma cells produced by a virus that does not stimulate primitive blood cells.

Verne, Oberling, and Guérin (1936), using the "hanging-drop" method, attempted to transmit leukosis (Engelbreth-Holm Strain) by

means of leukemic bone marrow cultivated with the agent. Transmission was observed only in a small percentage of cases after a relatively short in vitro culture period (never after 15 days), so it was concluded by the workers that probably the culture supplied media only for survival of the agent, and not for multiplication.

The work of Doljanski and Pikovski (1941, 1942) with the same strain contradicted this conclusion. Their experiments showed not only that leukotic blood cells could be cultivated in vitro without loss of malignancy but also that leukosis could be produced upon injection of cultures of normal bone marrow or of normal fibroblasts from myocardium grown with leukotic plasma. Since the agent remained active as long as the artificially infected cells were cultivated, even after many transfers (178 days), the authors decided that there was real multiplication of the leukotic agent in vitro, and that the agent did not require primitive blood cells for maintenance since activity continued long after the disappearance of blood cells.

So far as it is known, no attempts have been made to cultivate in vitro the agent causing the aleukemic type of lymphomatosis (Strain RPL 12) prior to the herein described experiments.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

Materials and Methods

The neoplastic tissue of Strain RPL 12, used as beginning material for tissue culture studies, was obtained from the Regional Poultry Research Laboratory,* in the form of an actively growing lymphatic tumor of the pectoral muscle.

Serum expressed from blood taken from a single chicken was used at first; finally the serum obtained was in the form of pooled samples. The latter were always Seitz filtered. An attempt was made to select birds free of lymphomatosis and other diseases.

Plasma, prepared by using sodium citrate as an anticoagulant, was generally pooled and then Seitz filtered.

Physiological Solutions were made up. Tyrode's Solution was used for the first experiment; a modification of Hanks' Solution was used the rest of the time.

Phenol Red was incorporated in the physiological solution (Hanks) as an indicator to a concentration of 0.002 per cent, the pH being adjusted to 7.8 with the addition of 1.4 per cent sodium bicarbonate after autoclaving for 10 minutes at 10 pounds pressure, or at any time the pH fell below 7.8.

When Hanks' Solution became too alkaline due to the loss of carbon dioxide, upon exposure to air, a N/10 solution of sodium dihydrogen phosphate was used to adjust to the proper pH.

Embryo Extract was made up 1:1 with Hanks' Solution after breaking

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* Located in East Lansing, Michigan.

up the nine to 11-day old embryos by various methods. The first method used, as suggested in Cameron (1950) consisted principally of chopping up the embryos with the curved scissors. The second method, which was preferred, made use of the Waring blendor for comminution of the tissues. The third method consisted of pushing embryos through a 20 ml. syringe. In each case, embryos were first thoroughly rinsed with Hanks' Solution, and after the tissues were broken up and the physiological solution was added, the mixture was allowed to stand at least 30 minutes before centrifugation and separation of the supernatant fluid.

The ultrafiltrate was of ox origin and prepared according to Simms and Sanders (1942).

Penicillin and Streptomycin were both added to Hanks' Solution to a concentration of 25 units/ml. of Penicillin and 125 micrograms/ml. of Streptomycin, in order to avoid bacterial contamination.

The "agent" obtained for "infecting" normal tissue was brought from the Regional Poultry Research Laboratory, and prepared by dilution of the plasma from a chicken infected with Strain 12 visceral type lymphomatosis.

Normal spleen for tissue cultures was obtained once from a three-day old White Leghorn, and twice from a 17-day old chick embryo.

Sterility Media employed were dextrose peptone broth and thioglycollate broth. Agar slants were utilized at first, but it was found that because antibiotics were added to the nutrient, the broth as a dilution factor, was necessary to overcome the effect of the antibiotics

in testing for sterility. All nutrients used and all supernatant fluids, removed when nutrient was changed, were tested for sterility.

Equipment and Techniques. These were modifications, depending on the materials available, of those suggested by Cameron (1950) and Parker (1950). Although some usage was made of concave slides for "hanging drop" cultures, and D_3 Carrel flasks for photographic purposes, most of the experimental procedures involved the use of 25 ml. Erlenmeyer flasks. Pasteur and serological pipettes were both employed. An attempt was made to use Roller tubes, but this method was abandoned because of unsatisfactory results obtained due to inaccessibility of a Roller drum.

Perforated cellophane was obtained from the Microbiological Laboratories in Coral Gables, Florida.

Animals. These were mainly chickens obtained from various places. No age standardization was possible, and no attempt was made to choose birds which were naturally susceptible and free from contact with lymphomatosis because of difficulty in obtaining such animals.

Two guinea pigs were also used for ocular inoculations. All other inoculations were given intramuscularly in the left pectoral muscle, except for the first experiment when one half the animals were inoculated intraperitoneally. The cell suspension for injection was made up either with Hanks' Solution or Normal saline (0.85 per cent), and 0.5 ml. was injected in every case. The technique adopted at first involved the use of alumdum for grinding up the tissues with a mortar and pestle,

but finally alumdum was not utilized. There was always a possibility that some of the alumdum might be injected and act as an irritant, causing a foreign body reaction, which might be a contributing factor in tumor development.

Experimental

Experiment I

The first attempt to grow lymphomatosis tissue was by the "Maitland" type culture, as described by Maitland (1928), Rivers, Haagen and Muckenfuss (1929), Rivers and Li (1930), all working with the vaccine virus, and Maitland (1931) working with the foot-and-mouth disease virus. These authors, using the suspended cell method, claimed that tissue cells survived a considerable amount of time and even multiplied, and that the virus increased in the suspension.

Tyrode's solution was used with chicken serum, the proportions varied at first until a 1:10 dilution (serum-Tyrode's Solution) was decided upon. No phenol red nor antibiotics were used at the beginning. For each specimen, four flasks were inoculated with tissue cells: one containing serum and one without serum incubated at 37° C., one with and one without serum incubated at refrigerator temperature, the last three flasks being used as controls. A very finely minced tissue suspension, 0.1 ml. per flask, was inoculated. Every two days, the nutrient was changed by centrifuging the mixture, washing the sediment with physiological solution, discarding the supernatant except for 0.5 ml. of the last sedimentation which was mixed, and 0.1 ml. of it transferred to fresh nutrient.

The first two minced tumors (P 120 and P 121) were discarded because of contamination. Tumor P 122, also very finely minced tissue, was treated with antibiotics overnight before being used for planting. After nine days *in vitro*, only the material in the flask containing serum and kept at 37° C. was used for intramuscular inoculation because of the inavailability of chickens at the time.

Tumor P 123 was divided: one part being used from which to cut fragments of larger size (2-4 mm.) for planting in flask A, one part being used again for fine mincing for flask B, except that this time 0.5 ml. of the latter cell suspension was used instead of the 0.1 ml. used formerly. Antibiotics were included in the medium hereafter. After 23 days, cellular material from flasks A and B was inoculated separately into baby chicks, using four birds for each flask (two receiving intramuscular and two intraperitoneal inoculations).

The next tumor received, called No. 2 was minced with curved scissors to a one to 1.5 mm. size and rinsed thoroughly with Hanks' Solution. Nutrient was changed to 1:3 parts of serum and Hanks' Solution using phenol red as an indicator. A pH of 7.8 was maintained. This nutrient was again changed every two days. Fifteen days later, the tissue fragments were broken up as much as possible in the flask with glass beads and the cells were suspended in fresh Hanks' Solution and inoculated intramuscularly in the pectoral muscle. Three animals were inoculated from each of the two flasks, kept at 37° C.

Results: No gross tumor formation was observed in any of the chickens inoculated with P 122 and P 123, although two chicks injected

with the latter tumor fragments showed enlarged spleens and slight discoloration of the livers seven weeks after inoculation. In the six chicks inoculated with tumor No. 2, neither gross tumors nor visceral alterations were seen.

Experiment II.

Because of the lack of success in keeping the neoplasm active in a "Maitland" type culture, and suggestions of Carrel and Baker (1926), Jacoby et al (1937), and Hoffman et al (1940) that extracts of tissue have a stimulating effect on cell multiplication, and the advice of Plotz (1937) that the addition of plasma provides support for cell growth and permits an increased opportunity for cellular respiration, a different method of cultivation was utilized. The formula used was obtained from Cameron (1950).

The fragments of a freshly harvested tumor were cut to one mm. size, washed with Hanks' Solution and embedded in a plasma clot, consisting of:

0.3 ml. Plasma
0.6 ml. Hanks' Solution containing Phenol Red
0.1 ml. Embryo Extract

After coagulation of the mass, a liquid phase nutrient was added. In one flask

40 per cent Hanks' Solution
40 per cent Serum
20 per cent Embryo Extract

was used, as suggested by Cameron. In the other, the proportions were made up as recommended by Grand (Cameron, page 90)

50 per cent Hanks' Solution
30 per cent Serum
20 per cent Embryo Extract

The Cameron nutrient was finally adopted for all succeeding flasks.

The liquid nutrient was changed every two to three days after first washing the plasma clot with fresh Hanks' Solution for ten to 15 minutes. Growth of the fragments was obvious as a loose network of cells could be observed macroscopically extending from the tissue explant, and the pH of the nutrient became more acid.

Growth was allowed to continue for a week when the cells were released from the fibrin clot and suspended in Hanks' Solution with a minimum of cellular breakdown, and three chicks were inoculated from each flask. Two to three-day old chicks were injected.

Results: Three chicks died soon after inoculation due to traumatic injury or other non-specific causes. The three remaining ones all developed large breast tumors, appearing within about ten days and showing hemorrhagic infiltration; the one allowed to grow for a month contained also a large area of necrosis in the center of the tumor. One showed paralysis of one leg and died 19 days after inoculation. All three showed visceral metastasis and generalized lymphomatosis.

These tumors were harvested on different days and divided: one piece was used for serial transfer into three more chicks, one piece was used for tissue culture in two flasks (one to be employed for re-inoculation, A, and the other to be kept growing in vitro, B), and one small section was placed in 10 per cent formalin for sectioning and histological examination.

This process was continued, beginning with these three tumors produced from tissue cultures, except that hereafter only one tumor from each group of three chicks, inoculated from each specimen, was harvested.

Upon each harvesting, three divisions were made as mentioned above. The tissue cultures to be reinoculated into animals (A), whenever possible, were allowed to grow in vitro only ten to 14 days before reinoculation, changing the nutrient every two to three days and repairing the plasma clot whenever necessary due to proteolytic digestion.

Fragments from the (B) flasks were transferred to fresh plasma clots every ten to 14 days (except for the material in the beginning two flasks which were not transferred until 22 and 16 days).

All tumors examined were typical of those associated with lymphomatosis Strain 12, showing hyperplasia due to lymphatic infiltration, capillary engorgement, sometimes with hyaline degeneration and bloody sinuses, and in older tumors, areas of necrosis. All histological specimens were examined by a worker familiar with the pathology.

Several tumors, allowed to grow longer than the usual period, showed rapid regression within a few days. Those tumors produced by the tumor tissue cultures often appeared larger and more cartilaginous than those produced by direct cell transfer from a chicken, although the period of incubation was somewhat extended as a rule.

Some form of visceral metastasis was observed in all animals showing gross tumor formation, and in some having no gross tumors. Visceral

symptoms also followed the usual pattern, involving one or more organs of the viscera. The liver was usually enlarged and appeared discolored with bright red streaks of infiltration, the spleen was generally greatly enlarged and ruptured easily with little pressure applied. Kidneys often appeared enlarged and pink or grayish-white due to nodular deposits. The same grayish-white spots of lymphocytic aggregation were also observed on the spleen. In one chicken inoculated with tumor fragments cultivated in vitro, there was a growth found attached to the heart which was as large as the organ itself.

Histological sections, stained with hematoxylin and eosin, also confirm the type of malignancy typical of this tumor. The proliferative areas were found infiltered with immature lymphocytes and the percentage of cells in mitosis greatly exceeded that observed in normal cells.

Colonies of the following different ages (according to the time of planting in vitro) were mounted in celloidin, sectioned and stained with hematoxylin and eosin:

No. 3632 - 13 days
No. 3729 - 3 months
No. 3654 - 2 months
No. 3743 - 3 months

All colonies still showed the presence of cells with typical malignant characteristics, with a high percentage of cells in mitosis, similar to that indicated by Fischer and Parker (1929) as evidence of malignancy in vitro.

Five other cultures are being processed in the same manner, as indicated on the chart, and consequently have not yet been examined.

Theiler and Smith (1937) pointed out that there was a change in pathogenicity of the yellow fever virus upon prolonged incubation in vitro. In order to test the malignancy of tumor cells after prolonged cultivation in vitro, half the colonies (fragments) from No. 3729 and No. 3743 and all the growth in flask No. 3654 were inoculated into three chickens each (as recorded in the charts) after the following number of days in vitro:

No. 3729 - 99 days
No. 3654 - 69 days
No. 3745 -105 days

The last examination of the chickens was made 34 days after inoculation and, as yet, no tumors had been produced, in spite of the fact that sections made of fragments from the same flasks (as previously mentioned) on the day of inoculation still indicated malignancy.

During the process of in vitro cultivation, different techniques and nutrient substances were tried, which will be described separately. This explains the loss of some tissues in vitro.

Results of all inoculations made during this experiment can be seen in Charts I and II.

Explanation of the Charts

The words Grand or Cameron referring to the nutrient (See page 25) in flasks used during the first trial are only to indicate that the tumor tissue culture used for inoculation was taken from two different flasks of fragments taken from the same bird No. 1620. All four-figured numbers refer to the chick tags, so tumor cultivated in vitro from this source is also identified as such.

CHART I

LYMPHOMATOSIS - STRAIN 12

TISSUE CULTURE 1620 (CAMERON) 10-13-50

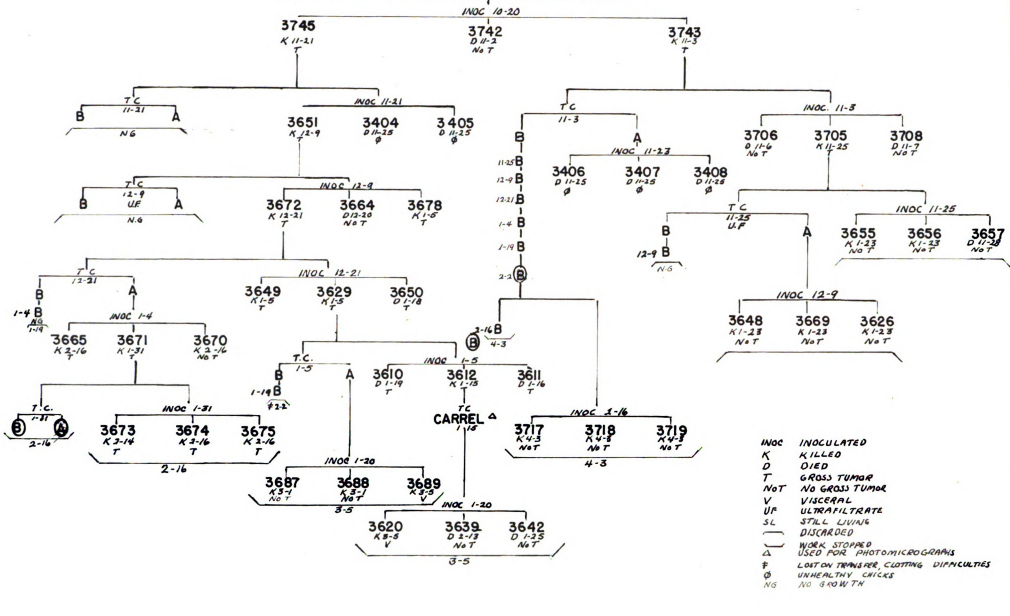
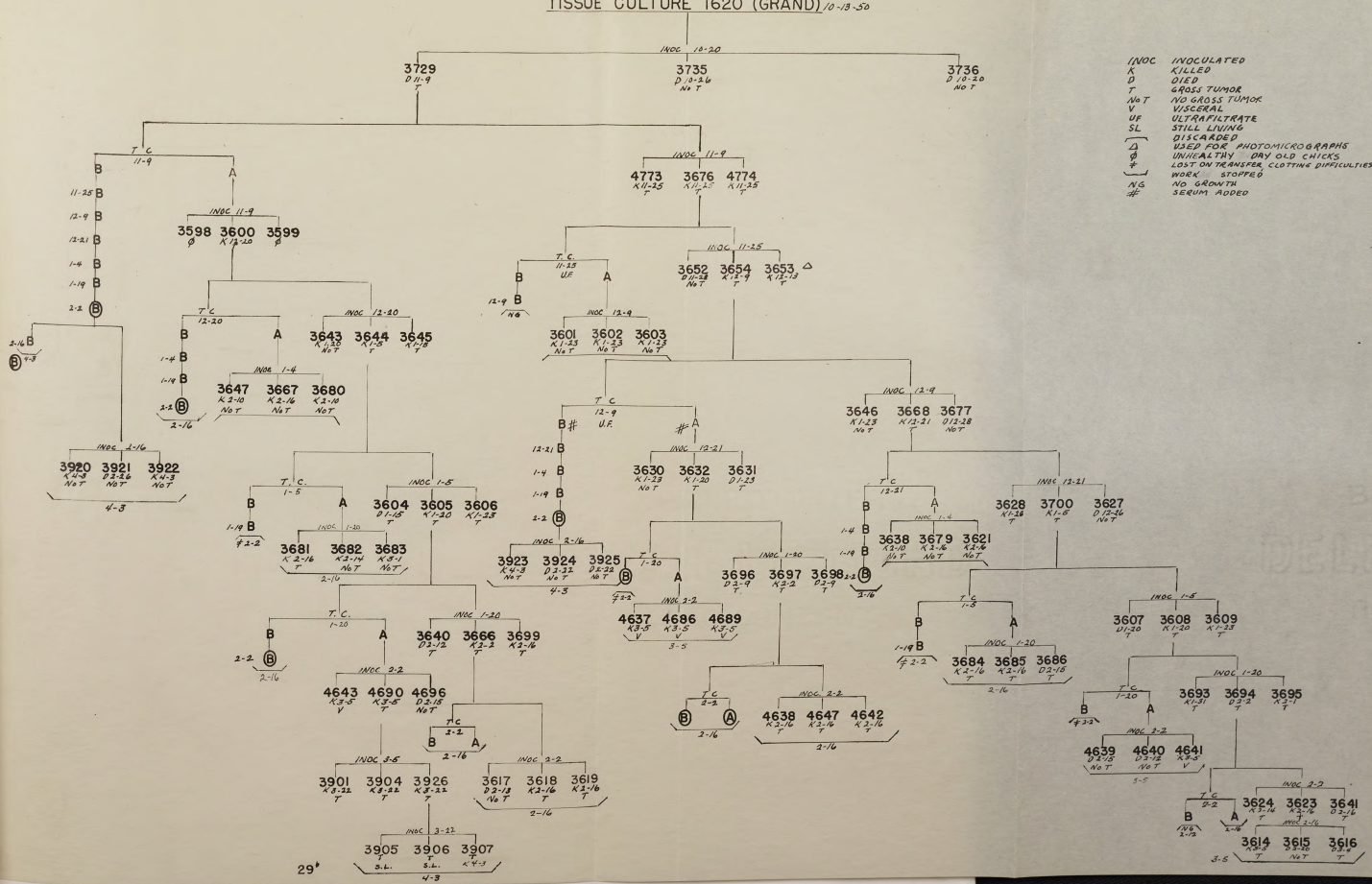


CHART 11

LYMPHOMATOSIS STRAIN 12

TISSUE CULTURE 1620 (GRAND) 10-13-50



Dates of inoculation (Inoc) and of killing (K) or death (D) are recorded to show the time allowed for tumor development in order that the outcome of injections be properly evaluated. For instance, animals occasionally died from causes other than lymphomatosis before adequate time was allowed for tumor formation. Dates aside of flasks indicate the date of transfer.

T = the formation of a tumor on the left pectoral muscle.

no T = no grossly observed tumor was formed during the period of observation.

Not all inoculated chickens free of gross muscle tumor formation were autopsied. Those which were, in spite of having no externally obvious neoplasm, and showed evidence of visceral form of lymphomatosis infiltration were marked (V), for Visceral.

U. F. = ultrafiltrate, indicating the use of such in the nutrient, since this is significant to the results (as described in later experiments).

It will be noted that cultures were occasionally lost for one reason or another.

N. G. = no growth

† = lost on transfer due to clotting difficulties

ϕ = chicks died shortly after inoculation due to ill health; lot was hatched under poor conditions at the laboratory and was used when only one day old.

Ⓐ or Ⓑ (circled) = colonies from particular flask were embedded in celloidin for histological sections.

= Serum was used instead of ultrafiltrate in the last nutrient change before inoculation of the A flask and thereafter for the B flask.

Carrel = flask which had been used for photomicrographs had to be used for animal inoculation since the other two chicks with tumors from same group died unnoticed at a time when tumors could not be harvested.

Δ = used for photomicrographs

Experiment III

Simms and Sanders (1942) suggested the use of serum ultrafiltrate as a substitute for serum for certain studies with tissue cultures. This serum ultrafiltrate was prepared by dialysis through collodion membranes impermeable to proteins. The 'B' factor associated with normal serum and tissues, said to cause deposition of fat, is also removed in this process. Since it is desirable to eliminate lipid deposition because it exhibits some toxicity and also interferes with the clear outline of cells in photomicrographs, ox ultrafiltrate, made according to Simms and Sanders, was tested in the outlined studies.

Tumor cultures Nos. 3676, 3705, 3654 and 3651 received ox serum ultrafiltrate as a substitute for serum in the nutrient. In other words, the fragments were embedded in a plasma clot as in the previous experiment but the liquid nutrient added was comprised of:

40 per cent Hanks' Solution
40 per cent Ox serum Ultrafiltrate
20 per cent Embryo Extract

Cultures No. 3676 and No. 3705 were cultivated in vitro for 14 days before inoculation into animals, which involved removing fluid layer in order to add fresh ultrafiltrate nutrient four times; No. 3651 and No. 3654 were cultivated in vitro 12 days, involving three nutrient changes.

Cultures No. 3676 and No. 3705 were inoculated into chickens in spite of the fact that growth was very poor. Culture No. 3651 was not used for inoculation, since no visible growth remained after 12 days *in vitro* cultivation. These flasks were discarded. Culture No. 3654 which had not exhibited much growth with three applications of ultrafiltrate nutrient, showed considerable more cellular outgrowth after one application of serum nutrient (used when an insufficient amount of ultrafiltrate nutrient was prepared) prior to inoculation. Growth, however, was still not as extensive as when ordinary serum was used instead of serum ultrafiltrate.

Results: All tissues in flasks in which serum ultrafiltrate was used instead of plain serum exhibited very sparse growth, and even the small halo of cells seen macroscopically around the fragment seemed to disintegrate after several days.

As indicated on Charts I and II, none of the chicks inoculated with tumor tissue cultures grown with serum ultrafiltrate nutrient produced tumors, except one inoculated with No. 3654 in which plain serum was finally incorporated in the medium. When it was realized that serum ultrafiltrate did not support growth, the B flask of No. 3654, after transfer, was continued on serum nutrient, and was still growing prolifically when it was finally inoculated into chickens 69 days later.

Cultures No. 3675 (B) and No. 3705 (B), after the first transfer to fresh plasma clots and continuation of serum ultrafiltrate nutrient, did not show any evidence of growth and were finally discarded.

Experiment IV

The value of using perforated cellophane instead of the plasma clot was pointed out by Evans and Earle (1947). This method was attempted. The tissue fragments were placed under the perforated cellophane and the liquid nutrient used, consisted of serum ultrafiltrate nutrient of the same concentration as used in the previous experiment. The method was tried with No. 3676 and No. 3705 in Carrel D_3 flasks and handled in conjunction with the regular plasma clot flasks to test the efficiency of the technique.

Results: The data of this experiment are not included in the Charts. Neither flask showed the expected growth as described by the above authors. The fragments merely appeared to break up. It must be emphasized, however, that instead of using the suggested nutrient containing plain serum, serum ultrafiltrate was used. It was not surprising, therefore, after the observations described in the last experiment, to find this outcome.

The results, then, are not to be considered significant, and the idea of replacing plasma by the perforated cellophane should not be abandoned until the same method has been tried with unfiltered serum. The technique was not pursued further, since the plasma clot technique had been so successful thus far.

Experiment V

An attempt was also made to use the hanging drop technique introduced by Harrison (1907), except that plasma and embryo extract 1:1

were used instead of serum with a physiological solution (as described in Parker, 1950). This was done for the purpose of taking photomicrographs, and met with some degree of success. A normal spleen fragment showed macroscopically visible growth within two hours; the lymphomatosis tissue fragment, however, grew much more slowly and not as extensively.

Due to the extreme thickness of coverglasses provided with the large depression slides, and the moisture which collected in the sealed chamber, difficulty was encountered in taking satisfactory photomicrographs with the low power objective, and it was impossible to focus with the high power objective. Besides this, since it would have been necessary to transfer sections of fragments every two days to insure continued growth, the process of using the hanging drop technique for photomicrographs was abandoned since the intention was to follow a tissue fragment through for several days without disturbing its growth pattern.

The Roller tube technique (as described in Cameron, 1950) was also tried and again the advantage of using this method for the study of cellular structure was readily realized because of the ease with which the thin layer of cells could be stained. However, since it was impossible to obtain a rotor drum with a properly geared motor, and growth was not extensive without such a system, this project was put aside after trying the method with eight tubes, using normal spleen and tumor tissue.

Experiment VI

A naturally occurring case of ocular form of lymphomatosis was brought into the Poultry Clinic (Bacteriology Building). The eye was obtained and sections from the ciliary body and also from the muscle in the back of the eye were cut, and fragments of each section were embedded in plasma clots in separate flasks. The nutrient used was the same as that adopted in Experiment II.

Growth of fragments from both sections was rapid and extensive. Growth in vitro was allowed for ten days after which 0.03 ml. of a cell suspension of tissue culture from each specimen (from both flasks) was inoculated each one into the anterior chamber of a guinea pig eye and the eye of a 15-day old chicken.

Results: Both guinea pigs and chickens were examined periodically for evidence of ocular symptoms similar to those seen in natural cases. No such symptoms appeared, the animals still being negative after weeks of observation. It may be that too dilute a cell suspension was used. Different results may have been obtained if the entire fragment colony had been transplanted.

Experiment VII

The second intended objective of this project was to "infect" normal lymph tissue in vitro with the lymphomatosis "agent", thus producing lymphoid tumors upon the inoculation of chicks with "infected" normal tissue, and showing a change in vitro in the growth of normal tissue after "infection".

No control animals were available, nor was housing of animals for the prolonged experiments (100-300 days) available to make certain that the "agent" used would actually produce the disease.

Spleen tissue was chosen. Carrel D_3 flasks and plasma clot technique were used throughout this study.

The first attempt involved the use of the spleen tissue from a six-day old chicken, and in this case, serum ultrafiltrate nutrient was used. The fragments grew rapidly at first, probably due to the presence of plasma; "infection" with 0.4 ml. of the "agent" was made on the fifth day after planting the tissue. Cultivation in vitro was continued for six additional days and then the normal and the "infected" spleen cultures were each inoculated in three chickens.

Results of Animal Inoculations: After approximately nine weeks' observation of the injected animals, there was still no evidence of gross muscular tumor formation, nor of visceral indications of lymphomatosis. However, these results were not held as being significant, since serum ultrafiltrate nutrient had been used and five days in vitro were allowed before "infection" was attempted.

For the second and third trials, the regular Cameron nutrient was used, and spleen from a 17-day old chick embryo was planted. In both studies, a comparison was made between the growth and cellular orientation of tumor fragments, normal spleen and "infected" spleen in vitro.

For the second trial, the "agent", in the same amount as was previously used, was added to a flask of the normal spleen tissue on

the third day of cultivation. After nine days *in vitro*, both the normal and the "infected" spleen tissue cultures were each inoculated into three chicks.

On the same day that the spleen tissue was planted, tumor No. 3655, which had an 18-day incubation period, was also cultivated *in vitro* for comparative study.

Photomicrographs were taken periodically of fragments and/or the periphery of the outgrowth of cells from all three flasks until the ninth day when inoculations were made.

Results of the Animal Inoculations: None of the chickens inoculated with either normal spleen or "infected" spleen showed signs of lymphomatosis, after approximately two months' observation.

The most satisfactory photomicrographs are herewith submitted on pages 40 to 47. The oil droplet formation on the cells is due, it is believed, to deposition of fat from an excessive amount of lipid in the serum.

Plates I, II, III, V, and VII are photomicrographs of tissue cultures of normal spleen during different stages of growth. Plate I shows mostly mononuclear blood cells and some granulocytes. Plate II shows more of the granulocyte-type cells. In Plate III (92-hour growth), fibroblast-like cells make their appearance. This is in accordance with Maximow and Bloom (1950).

Plates IV, VI, and VIII are photomicrographs which illustrate the unique growth pattern assumed by normal spleen which had been "infected" with the lymphomatosis "agent".

Upon comparison of Plates III and IV, it will be observed that even 24 hours after the addition of the "agent" (Plate IV), there is some difference in the orientation of the cells along the periphery of the new cellular growth.

Plate VI shows still more variation of spleen tissue from the five-day normal spleen growth (Plate V). The cellular outgrowths of the "infected" spleen (Plate VI) show extreme proliferation in "tentacle" fashion.

A more extended cellular proliferation can be observed on Plate VIII after nine days' growth. The non-infected normal spleen, (Plate VII), on the other hand, shows only a slight diversion from the usual continuous periphery containing a loose network of fibroblast-like cells.

Macroscopically, entire fragment colonies of the normal non-'infected' spleen had a smooth halo-like appearance whereas the 'infected" spleen fragments were "starfish" shaped.

Plates IX, X, XI, XII are photomicrographs of the growth, at different intervals, of the same fragment of lymphomatosis tumor tissues. Plate IX (48-hour growth) and Plate X (70-hour growth) show a section of the embedded fragment with small round mononuclear blood cells and larger granulocytes migrating from the piece of tissue. Plates XI (9-days' growth) and Plate XII (15 days' growth) both illustrate the fibroblast-like appearance of the majority of the cells at this age of in vitro cultivation.

Plates XIII, XIV, and XV are also photomicrographs of lymphomatosis tumor tissue. This particular fragment was minute in size and

smaller than those usually planted. The characteristic pattern, already described for the infected spleen, of haphazard proliferations irregularly spaced around the colony, can be observed particularly in Plate XIII. Plates XIV and XV are photomicrographs taken one day later, of the same colony, which had grown so that only the lower left portion of fragment on Plate XIII (not entirely included) could be photographed at one time with the same magnification.

Plates on pages 40 to 47 are all photomicrographs taken with a Spencer microscope with a magnification of 120X.

PLATES I AND II

Plate I - Tissue Culture - 24-hour Growth. Normal Spleen Section of Fragment and Migrating New Cells

Plate II - Tissue Culture - 48-hour Growth. Periphery of Outgrowth of New Cells

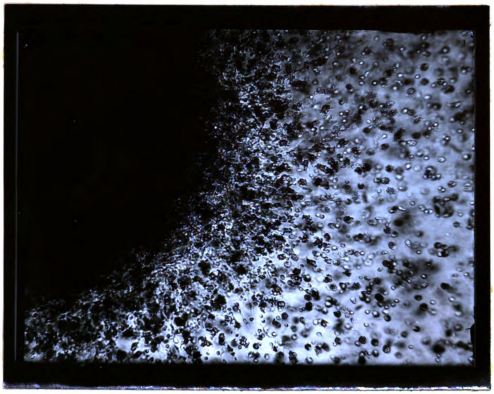


Plate I

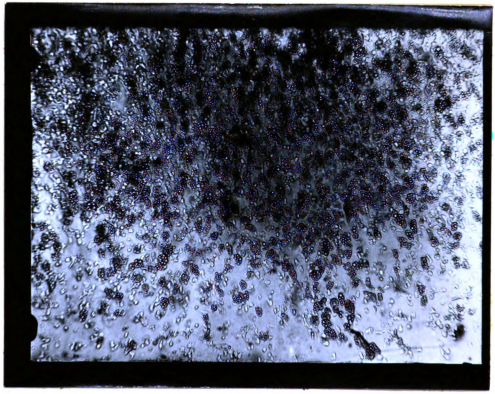


Plate II

PLATES III AND IV

Plate III - Tissue Culture, 92 hour growth. Normal Spleen Colony periphery.

Plate IV - Tissue Culture, 92 hour growth. Normal Spleen "infected" with "agent" 24 hours previously. Colony periphery.

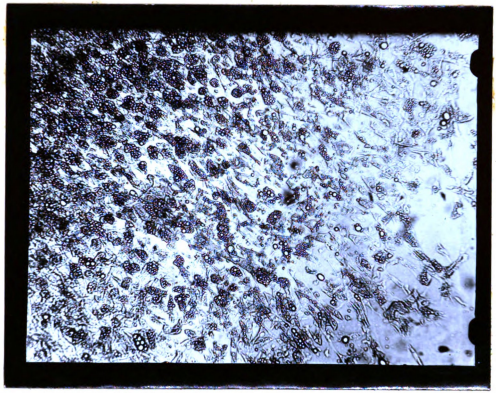


Plate III

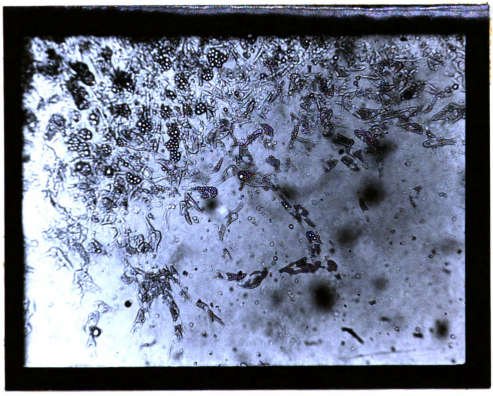


Plate IV

PLATES V AND VI

Plate V - Tissue Culture, five days' growth. Normal Spleen Colony periphery.

Plate VI - Tissue Culture, five days' growth. Normal Spleen "infected" with "agent" two days previously. Colony periphery.

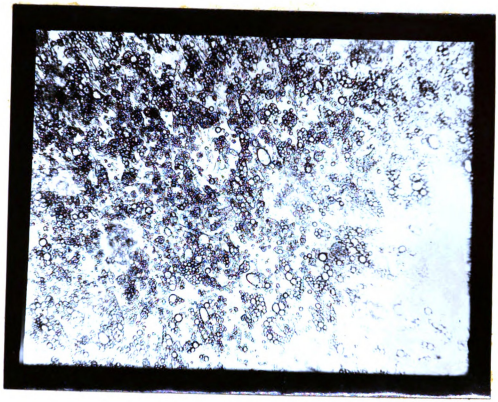


Plate V

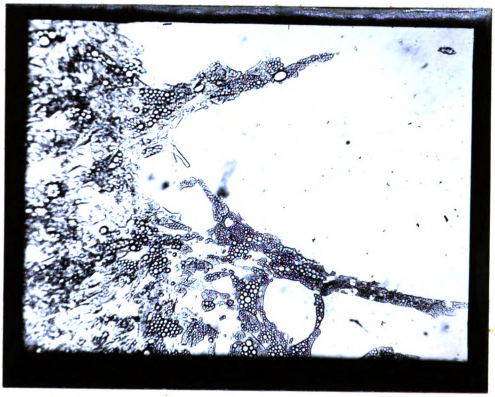


Plate VI

PLATES VII AND VIII

Plate VII - Tissue Culture, nine days' growth. Normal Spleen Colony periphery.

Plate VIII - Tissue Culture, nine days' growth. Normal Spleen "infected" with "agent" five days previously. Colony periphery.

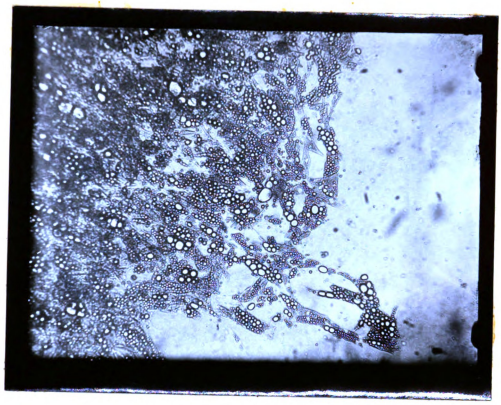


Plate VII

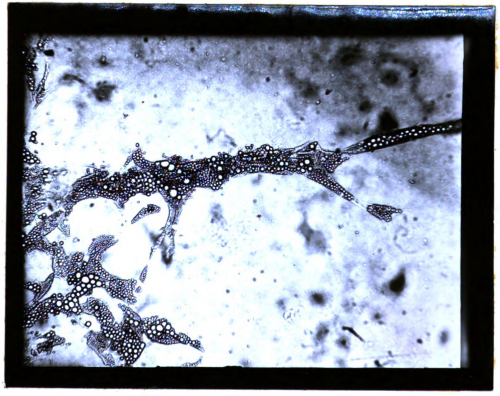


Plate VIII

PLATES IX AND X

Plate IX - Tissue Culture, 48 hour growth. Lymphomatosis tumor fragment 1, with migrating cells.

Plate X - Tissue Culture, 70 hour growth. Lymphomatosis tumor fragment 1, with migrating cells.

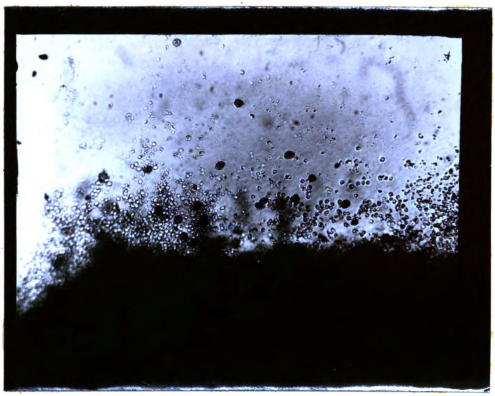


Plate IX

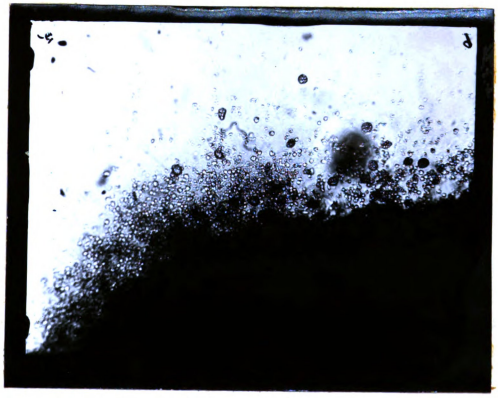


Plate X

PLATES XI AND XII

Plate XI - Tissue Culture, nine days' growth. Lymphomatosis tumor, fragment 1; outgrowth of cells near periphery.

Plate XII - Tissue Culture, 15 days' growth. Lymphomatosis tumor, fragment 1; outgrowth of cells near periphery.

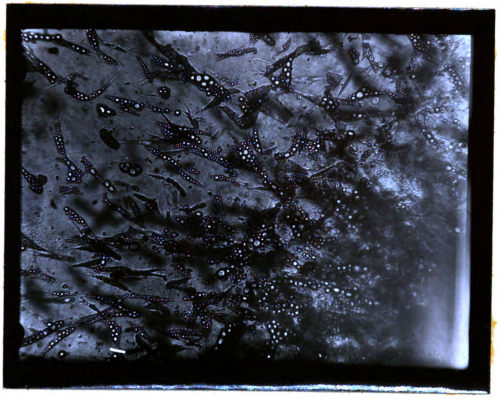


Plate XI

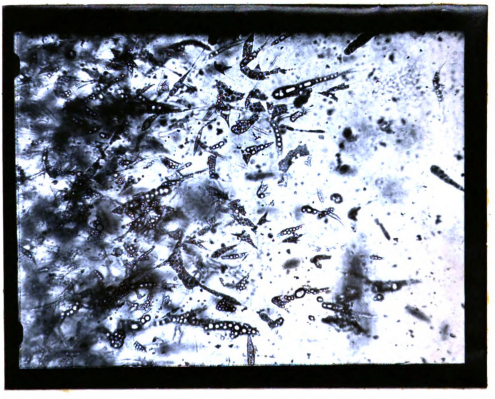


Plate XII

PLATE XIII

Plate XIII - Tissue Culture, 92 hour growth. Lymphomatosis tumor fragment 2.

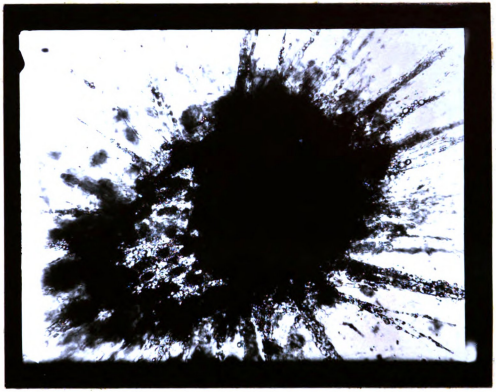


Plate XIII

PLATES XIV AND XV

Plate XIV - Tissue Culture, five days' growth. Lymphomatosis tumor fragment 2.

Plate XV - Tissue Culture, five days' growth. Lymphomatosis tumor fragment 2 (same field as on Plate XIV using a different focus).

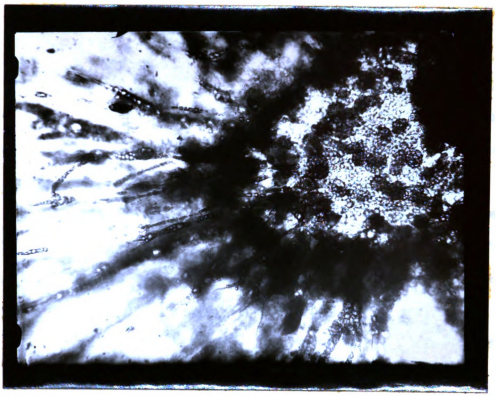


Plate XIV

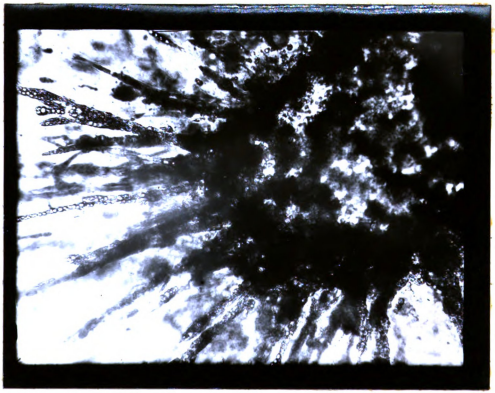


Plate XV

The third project was conducted primarily to repeat photographic observations. This time, however, a dissecting microscope was used for magnification instead of the standard microscope (Spencer) used previously.

Tumor No. 3012 was removed to supply the tumor tissue culture, and the chick embryo spleen in one flask was again "infected" on the third day of cultivation, except that this time 0.25 ml. of the "agent" was used instead of 0.4 ml. added during the previous project.

Photomicrographs were again taken of normal spleen before and after "infection", and of tumor tissue during various periods of growth. No inoculations were made from the flasks containing spleen tissue.

Few of the photomicrographs taken were successful, so that it was impossible to carry out the objective of comparing entire fragments of normal spleen with "infected" spleen.

Nevertheless, the best photomicrographs of normal spleen and of tumor tissue fragments are submitted on pages 49 and 52, for comparison with the photomicrographs of higher magnification already presented.

Plates XVI, XVII, XVIII, and XIX are photomicrographs of normal spleen fragments. The smooth cellular periphery can be easily seen.

Plates XX, XXI, XXII, and XXIII are photomicrographs of tumor tissue fragments. The irregular orientation of cells and proliferation is characteristic.

Magnifications are included in Plate identifications.

PLATES XVI AND XVII

Plate XVI - Tissue Culture, 48 hour growth. Normal Spleen fragment 1. (Magnification 18X)

Plate XVII - Tissue Culture, 48 hour growth. Normal Spleen fragment 1. (Magnification 36X)

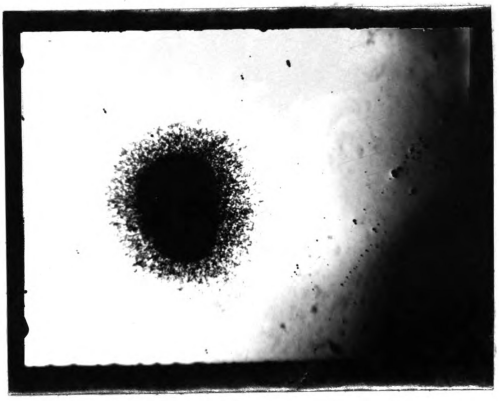


Plate XVI.

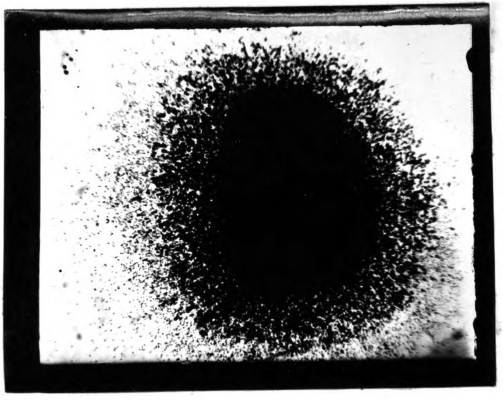


Plate XVII

PLATES XVIII AND XIX

Plate XVIII - Tissue Culture, 48 hour growth. Normal Spleen fragment 2. (Magnification 3cX)

Plate XIX - Tissue Culture, 72 hour growth. Normal Spleen fragment 2. (Magnification 3f.

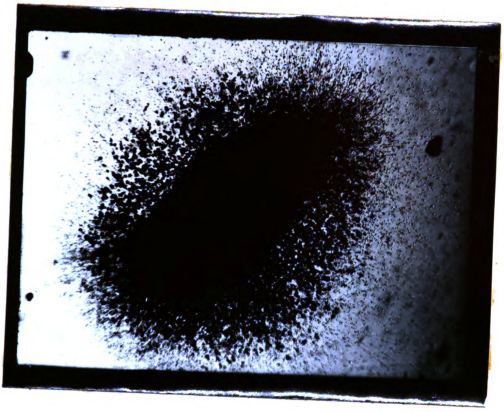


Plate XLII

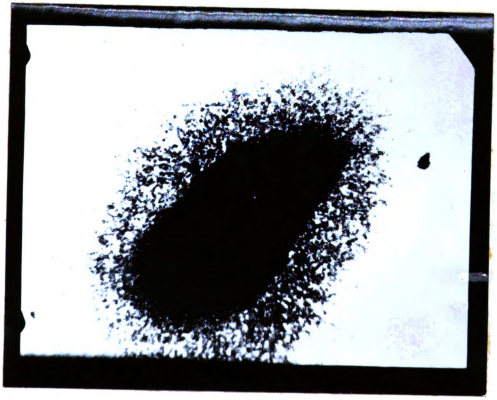
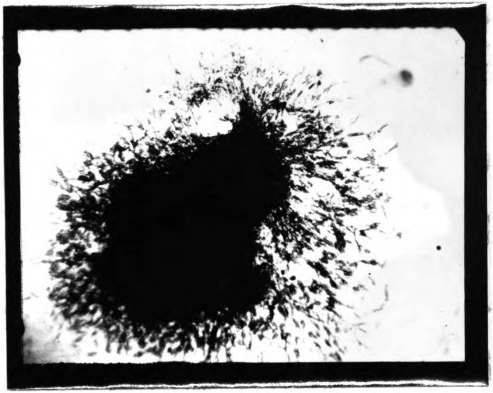


Plate XIX

PLATES XX AND XXI

Plate XX - Tissue Culture, four days' growth. Lymphomatosis tumor fragment 1. (Magnification 36X)

Plate XXI - Tissue Culture, six days' growth. Lymphomatosis tumor fragment 2. (Magnification 36X)



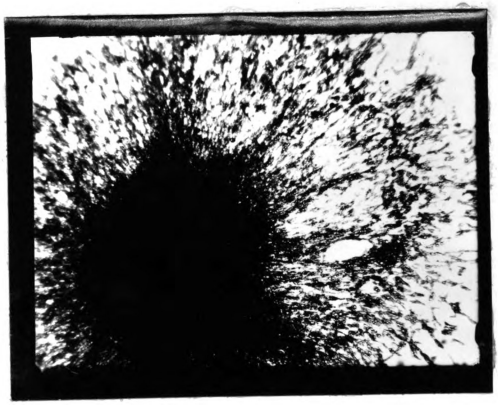


Plate I

PLATES XXII AND XXIII

Plate XXII - Tissue Culture, three days' growth. Lymphomatosis tumor fragment 3. (Magnification 10X)

Plate XXIII - Tissue Culture, three days' growth. Lymphomatosis tumor fragment 3. (Magnification 36X)

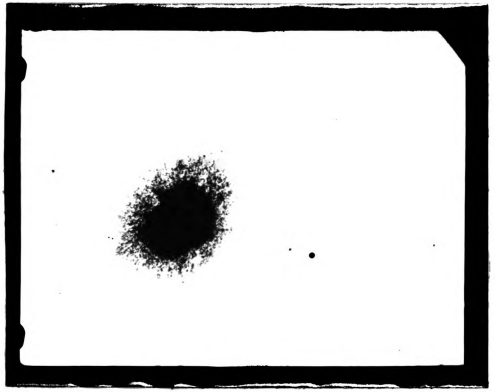


Plate XXII

PLATE XXIII

DISCUSSION

DISCUSSION

It is realized that at the beginning of Experiment I, the techniques used were inadequate. Ignorance of proper nutrient proportions and unfamiliarity with the correct size of cellular fragments to be used undoubtedly influenced the outcome of the inoculations. However, by the time of the last trial, the method used was considered adequate enough so that the results obtained were of some significance.

It is doubtful whether the suspended cell technique could ever be so adapted that lymphomatosis tumor elements would remain active long enough to be used for serial transfer.

There is still disagreement as to how long the cells survive in such a medium, whether the tissue cells actually multiply, and whether the living cells provide a medium for subsistence or for multiplication of the virus.

It would be expected, if the causative agent of the tumor disease were a virus, that there would be multiplication or at least persistence of this virus as long as the cells were alive. Therefore, the fact that the injection of neoplastic cellular material, after cultivation in the "Maitland" type culture, did not produce tumor symptoms, may mean that the tumor is a true neoplasm, and that these neoplastic cells did not survive the period of incubation in vitro.

Assuming that a virus is involved in the lymphomatosis tumor development, it could mean that even if the cells did remain alive for a period, the cell metabolism in this resting state may not have

provided the right conditions for actual propagation of the virus, and that the virus, already present in a surviving state, became so diluted by the nutrient replacement that activity was destroyed. Or again, the virus may have been so integrated within the cells that when cellular multiplication stopped, virus activity was automatically destroyed.

The formation of a fibrin clot with the addition of plasma, as in Experiment II, provided adequate support for the cellular outgrowth so that a network of new cells could be easily demonstrated macroscopically. It was not too surprising to find that upon injection of these tumor fragments, actively growing in vitro, the typical tumor could be produced. This accomplishment has been previously reported in this paper referring to the work of Carrel, Furth, and Dolzanski with other types of tumors.

The ability to produce the tumor after several days of in vitro cultivation of tumor fragments may indicate that a virus, in combination with the cytoplasm as a complex, is kept in a state of multiplication and activity. Again, it may signify that the malignant cells, being permanently altered cells as described by Lewis (1935), merely breed true as a species of cells in vitro just as the neoplasms are seen to do in vivo.

It seems safe to state, however, that either the neoplastic cells must be in a state of active growth to initiate tumor formation or else certain elements present in serum are essential for neoplastic activity. This was concluded since tissues maintained in a resting state through the use of serum ultrafiltrate (as shown by Layton and Tutelman, 1949)

did not produce tumors; yet, upon one addition of chicken serum to tissue culture No. 3654, (which had been kept in a resting state with serum ultrafiltrate), growth was again stimulated and a tumor was developed upon inoculation of cellular fragments.

If serum is the essential element, there is yet to determine what factor in serum is involved. Is it the "B" factor of Simms (1942), missing in the serum ultrafiltrate, or that substance having growth-stimulating activity associated with a protein fraction as described by Waymouth (1947)? It cannot be the mere presence of any protein, since the protein in the embryo extract, used with the serum ultrafiltrate, did not aid tumor formation.

It had been hoped that the addition, in vitro, of a diluted serum from a chicken with Strain 12 visceral type lymphomatosis, which had been shown to contain innumerable infective units of the tumor, to normal spleen tissue would "infect" the cells so that malignancy would become inherent in the cellular elements and the injection of the latter into chickens would stimulate the development of the characteristic tumor. The possibility of doing this seemed quite plausible because Carrel demonstrated infection with the Rous sarcoma agent in this way, and Earle (1943[a,b]) and des Ligneris (1935) even accomplished the production of malignancy in vitro by means of a carcinogen. Nevertheless, results were disappointing.

It is always possible that the frozen "agent" was no longer active, due to the method of storage or to the presence of neutralizing antibodies (cytotoxic elements).

However, even though it was impossible to show an infectivity which produced tumor formation in the animal upon injection, it is significant that the addition of the "agent" to the tissue fragments in vitro stimulated a pattern of cellular outgrowth in the normal spleen tissue which is similar to that observed in the outgrowth of tumor fragments. The uninfected normal spleen cultures, in comparison, grew as a loose meshwork of cells in a rather uniform pattern (as can be viewed in Plates III to VIII).

The pattern of the "infected" spleen and of the tumor tissue outgrowth is shown to be "starfish-like" in appearance (Plates XIII to XV and XX to XXIII), both macroscopically and microscopically, due to the extreme proliferation of cells causing the periphery of the tissue colony to become very irregular. This is in contrast to the smooth regular edge which forms the periphery of the normal spleen tissue (Plate III and XVI to XIX).

The fact that attempts to "infect" normal tissues in vitro with the "agent" were not successful does not necessarily mean that it cannot be done, nor does it dismiss the possibility that a virus may be involved in the process of this particular tumor development.

Assuming that the "agent" used was still active, it may have been impossible for a virus to enter cells under the in vitro conditions existing. The pH (7.8) may have been inappropriate for penetration of the cells by a virus, an excess of lipoids in the serum may have been inhibitory, or the plasma clot may have rendered the cells impervious to the virus. "Infection" may have been more likely if fragments had

been suspended in the "agent" dilution for a short period before tissues were embedded in the plasma clot, or entry of a virus may have been eased by placing the tissue fragments with the "agent" at refrigerator temperature before planting.

Because of the work of Carrel (1924[b]) with the Rous agent, in which he showed that leucocytes alone, and not the fibroblasts were responsible for inherent malignant characteristics, it is suggested that "infection" may have been more likely using only the mononuclear cells obtainable in the buffy coat layer of sedimented defibrinated blood.

The attempt to "infect" normal tissue cells in vitro with the cell-free agent of lymphomatosis, therefore, should not be abandoned.

There were certain technical difficulties arising during the experimental work, which will be mentioned only briefly.

Contamination did occur even in the presence of antibiotics, but none of the cultures were lost because of it. Bacterial colonies in the plasma clot were kept at a minimum by the antibiotics until transfer to a fresh clot could be made. The more frequent fungus spore contamination (although seldom encountered) did not interfere with tissue growth, since colonies were easily cut out from the plasma clot.

An excessive amount of lipid material was present in certain batches of serum used, which may have been the cause of the inhibition of growth of certain tissue cultures, as shown by Baker and Carrel (1925), and certainly caused havoc with the photomicrographs because of fat deposition. This could have been avoided by starving the

chickens before taking blood, but because of the source of blood, it was impossible to request such a procedure.

Several cultures were lost upon transfer (as indicated in the Charts) because of clotting difficulties. This was of greater concern than any other difficulty encountered. Citrated blood was used as a source of plasma, which may account for the difficulties because, as Parker (1950) states, the presence of an anticoagulant makes different samples of plasma from the same batch irregular in their clotting time (perhaps due to an unequal distribution of the anticoagulant) and this is what was found. Some samples from the same batch contained plasma which clotted rapidly, and others contained plasma that simply would not coagulate even when the proportions normally used to form the clot were changed to include less Hanks' Solution in comparison to the plasma and embryo extract. This problem could have been corrected by using plasma prepared by the tedious method of centrifuging the blood withdrawn, without an anticoagulant, immediately in iced cups and storing the plasma removed in paraffin-lined tubes; or by using a standard amount of purified bovine fibrinogen and thrombin solutions to form the clot, as described by Porter and Hawn (1947).

At times, some of the tumor fragments planted according to the generally successful method still did not grow. Some of the factors which influenced the growth of these tissues in vitro can be summarized as follows:

1) The age of the tumor, i.e., the period of time between injection and harvesting.

2) The condition of the tumor, whether extremely bloody or necrotic.

3) The physiological state of tissue depending on the time allowed between the sacrificing of the chicken and the planting of fragments *in vitro*.

4) The size of the fragments. If the fragments are too large, they rapidly become necrotic and if too small, the cells may not survive the environmental change.

5) The number of tissue fragments. According to Zinsser and Schoenbach (1937), the medium may become too acid. Also, if too many fragments are present, it is difficult to form a fibrin clot.

6) The pH should be optimum for the type and species of tissues.

7) The temperature should also be at optimum. If tissues are allowed to remain too long at a temperature foreign to its natural environment, growth may be inhibited.

As the Charts indicate, tumor development was observed in some chickens and not in others inoculated from the same source. The outcome of avian inoculation depends on many factors, some of which are:

1) The age of the chicken; an older chicken is likely to be more resistant.

2) Natural susceptibility to lymphomatosis is found to be more prominent in some breeds than in others.

3) Immunity due to previous contact with lymphomatosis cases.

4) Number of tumor cells inoculated.

SUMMARY

SUMMARY

1. Maitland (suspended cell) type culture was found unsatisfactory for the cultivation of lymphomatosis tumor cells in vitro.
2. Ox serum ultrafiltrate used as a substitute for serum, did not support extensive or continuous cell multiplication in vitro.
3. Lymphomatosis tumor fragments cultivated by means of the plasma clot method, using serum ultrafiltrate instead of plain serum in the liquid nutrient did not produce tumors upon inoculation into animals.
4. Lymphomatosis tumor tissue was cultivated in plasma clot with serum, Hanks' Solution and embryo extract as liquid nutrient without loss of malignancy, as evidenced by tumor production upon inoculation into chickens and malignant characteristics observed in histological sections.
5. Tumors formed as a result of the inoculation of lymphomatosis tissue fragments cultivated in vitro were typical of those associated with Strain 12 lymphomatosis tumors.
6. Lymphomatosis tumor tissues were kept alive in vitro for a period of at least 139 days.
7. Serial transfers were made successfully from tissue culture to chicken, chicken to tissue culture, from tissue culture back to the chicken, etc., without loss of the characteristics of malignancy for a period of 160 days.
8. Normal spleen infected with lymphomatosis "agent" in vitro did not stimulate tumor formation upon inoculation.

9. Photomicrographs show that upon addition of lymphomatosis "agent" *in vitro* to normal spleen tissue, the growth of the fragments differentiated themselves from the normal noninfected spleen fragments, and took on a growth pattern similar to that observed in cellular outgrowth of tumor fragments.

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