IN VIVO AND IN VITRO TRANSFORMATIONS OF MOUSE TISSUES FROM A MURINE LYMPHOSARCOMA

DISSERTATION

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By

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The problem with which this investigation is concerned is that of determining the nature of events leading to the change of normal cells into malignant cells. The design of the study is multi-phasic: (A) to establish the presence or absence of an oncogenic virion, (B) to demonstrate by use of the electron microscopy any ultracellular alteration in malignant or transformed tissues, (C) to investigate the nature of the transforming agent in the murine lymphosarcoma, and (D) to employ various methods to demonstrate cellular transformations in vivo and in vitro.

The tumor line was produced by subdermal transplants of a 20-methylcholanthrene-induced lymphosarcoma tumor into isologous DBA/1J mice. The tumor has been maintained through more than four hundred passages. The mean survival time for the tumor-implanted mice was approximately eleven days.

Tumor, liver, spleen, and kidney tissues from the tumor-bearing mice were prepared for electron microscopy,
thin-sectioned, and observed for ultracellular alterations and for virus particles. Sections of the tissues were also prepared for light microscopy.

Single-cell suspensions of tumor, tumor liver, and tumor spleen were prepared. The cell suspensions were used in tissue culture preparations, diffusion chamber, and parabiotic chamber experiments for cellular transformations. Nuclei and mitochondria were isolated from the cell suspensions and implanted into DBA/1J mice.

The tissues observed by the electron microscope showed the mitochondria to be swollen and structurally deficient: fewer irregular or parallel tubular cristae, myelin-like figures, and pleomorphic. Some of the mitochondria had inclusions of dense and irregular bodies.

The nucleus of the tumor tissue repeatedly had invaginations of the nuclear membrane into the nucleoplasm, and in the older tumor tissue the nuclear membrane had become separated. In the necrotic area of the tumor, the nucleus was last to undergo alterations. The nucleolus in the tumor became enlarged as the tumor progressed.

In the tumor cells there was a noticeable lack of ergastoplasmic lamellae in the cytoplasm. Free ribosomes
and polysomal formations were abundant. The swollen cristae were disrupted into various sized vesicles.

The tumor could be passaged into the isologous DBA/1J mice by subdermal implantation of tumor liver, tumor spleen, tumor kidney, and single-cell suspension from each of these tissues. The single-cell preparations, when placed in diffusion chambers with twenty-five millimicron filters and implanted subdermally into mice, induced the formation of a lymphosarcoma. In parabiotic chambers, with normal cells in one chamber and tumor cells in the other chamber, separated by a filter, the normal cells became transformed and induced a lymphosarcoma in mice after implant.

When the isolated nuclei from tumor tissue were implanted into mice, a tumor developed. Treatment of the nuclei with nucleases and then implantation in mice cause a tumor only when the nuclei were treated with deoxyribonuclease.

It is concluded that the transforming and tumor-inducing agent in this investigation was not a virion, but an infectious ribonucleic acid genome or a segment of a viral genome which had become integrated into the genome of the mouse cells. The virion has lost its ability to form a protein coat; therefore it is not
demonstrable as a virion. But the ribonucleic acid is able to infect other cells and transform them from normal to neoplastic tissues.
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CHAPTER I

INTRODUCTION

This research, based on experiments with a murine lymphosarcoma, examines the nature of events leading to the change of normal cells into malignant cells. In the mouse, a system has been developed in which the malignant changes occur rapidly and fairly synchronously in both a homogeneous and a heterogeneous population of cells. Through this system, inquiry, employing both in vivo and in vitro experiments, has revealed that the presence of a virion is not necessary for cellular transformation. Further, ultrastructure of the tumor and fine structural alterations in the transformed tissue have been studied, and the results of the studies are reported.

Although the etiology of cancer has spawned numerous hypotheses, only those which have received further support from new studies have escaped obscurity. In the recent past, two main theories have guided investigators in their efforts to discover the key to the puzzling nature
of tumors: (A) the multi-etiologal theory that cancer can be caused by many extremely varied factors such as chemical substances (23), physical factors (101), and biological agents (72); and (B) the virus theory (38, 42, 72, 94).

This study explores the virus theory; and the fact that so much work has been done with the theory suggests its importance. The following discussion notes significant background information relevant to viral carcinogenesis and viral transformation.

Viral Carcinogenesis

The concept of the possible viral origin of tumors was first stated by Borrel (14). The first experimental evidence of the capacity of viruses to induce neoplastic processes was presented by Ellermann and Bang (29), who demonstrated the viral nature of fowl leukosis, and by Rouss (77), who discovered the virus capable of producing a chicken sarcoma.

Further development of the viral concept was slow. The most important contributions were the discovery of the Shope rabbit papilloma virus (85) and the Bittner
virus (11). Shope was the first to demonstrate the masked viral genome.

Interest in the role of viruses in the etiology of cancer has risen sharply, and many investigations have been devoted to the study of the problem (24, 37, 70, 85). Most investigators studying the tumor viruses considered that the action of the virion was similar to viruses inducing clinical infectious diseases, and that the acceleration of cell proliferation, as found in the neoplastic processes, was caused by the continuous presence of the virus in the affected tissue (71).

Zilbur (100) proposed the theory that the virus does not act as an infectious agent, but as a transforming one. The neoplastic process initiated by viruses comprised two phases, with the virus playing quite a different role in each of them. The first phase was a hereditary transformation of normal cells to tumor cells. The second phase was the reproduction of tumor cells, in which the virus did not play an important role, and this phase led to the clinically apparent disease.

Fraenkel-Conrat (33) and Gierer and Schramm (35) published almost simultaneously experimental data showing
that ribonucleic acid (RNA) of tobacco mosaic virus could cause disease in a certain percentage of trials. This was the first report of an infectious nucleic acid. Numerous experiments have shown that RNA of varying purity, obtained from the virus of Western equine encephalitis, poliomyelitis, West Nile encephalitis, West tick-borne encephalitis, stomatitis aphthosa, and mouse encephalitis can cause the corresponding diseases (4, 15, 16, 34, 47, 98). In these experiments, the extracted nucleic acids did not cause the infection, but induced the synthesis of virions within the cell, and that caused the pathological condition.

Hays, et al. (42) reported that the inoculation of salt suspensions of deoxyribonucleic acid (DNA) and RNA, from leukemic and non-leukemic organs of mice, into various strains of mice caused leukemia to develop. Latarjet, et al. (54) reported that it is possible to induce leukemia in AKR and C3H mice by means of nucleic acids (DNA and RNA) obtained from leukemic tissues of AKR mice. Lacour, et al. (50) observed similar results with RNA from Ehrlich's ascites tumor. Bielka and Graffi (10) prepared RNA from leukemia myeloid tissue and induced leukemia in mice. But efforts
to induce tumor with RNA from filtrates of Rous sarcoma proved negative (49).

Di Mayorca et al. (22) reported that DNA obtained from mouse embryonic tissue infected with polyoma virus had infective properties. Neither transformation nor cytopathic effects were observed if deoxyribonuclease had been added.

Graffi and Fritz (39) prepared DNA and RNA from polyoma tumors and showed a cytopathic effect on cultures of embryonic mouse tissue, and the medium from the culture induced multiple tumors in new born animals. Treatment of the samples with ribonuclease did not eliminate the activity of the RNA preparation.

Dmochowski et al. (25) found that cell-free extracts of tissue culture, inoculated with polyoma virus and phenol treated for deproteinization, caused parotid gland tumors in mice and subcutaneous sarcoma in hamsters. The nucleic acid preparation also caused cytopathic lesions in embryonic mouse tissue, and the material from the latter was carcinogenic.
Transformation by Viruses

The transforming capacity of a number of tumor viruses has been studied (93). Changes in the morphology of cells, in rate of cell metabolism and generation time, in antigenic composition, or in karyotype have been most often used as indices of transformation. Some of these changes may be accompanied by acquisition of neoplastic potential. It is these cells that in recent years have undergone intensive scrutiny, in efforts to study the mechanism underlying the conversion of non-oncogenic cells to those with oncogenic properties.

Some of the viruses found to illustrate a transforming capacity are SV40, Adenovirus, polyoma, and Rous sarcoma. Eddy, et al. (28), and Girardi, et al. (37) were first to report that SV40 could induce tumors when inoculated into newborn hamsters. These studies, as well as those reported later (6, 12), revealed the neoplastic lines to be fibrosarcomas from which viruses could be recovered only occasionally, and then only in small quantities. Subcutaneous inoculation of newborn hamsters with phenol-extracted deoxyribonucleic acid from SV40 virus has produced tumors (27), although the latent period was longer than that usually observed after inoculation of the complete virus. Treatment of the extract with DNAase rendered it non-oncogenic.
Hamster cells transformed by SV40 in vivo or in vitro were oncogenic when transplanted into young hamsters (74). Fewer than 100 cells were found necessary to produce tumors in 50 per cent of the animals. Simian cells have been transformed by SV40 (6, 31). The transformed cultures eventually stopped producing infectious viruses, but continued synthesizing an intranuclear tumor antigen, which indicated viral genome presence. Diderholm, et al. (21) reported on the transformation of rat and guinea pig kidney cells with SV40. Transformation was accompanied by appearance of predominantly epithelioid cells and the synthesis of SV40 tumor antigen within the cells. Introduction of larger numbers of the transformed cells ($10^8$ cells) into autologous, X-irradiated rats resulted in tumors, but untreated animals did not produce tumors.

Trentin, et al. (96) first reported that adenoviruses have oncogenic properties. They were concerned with pulmonary tumors induced in newborn hamsters following inoculation of adenovirus type 12. Trentin's results were confirmed by Huebner, et al. (46) and later by McLeod and Ham (61), who further demonstrated that adenovirus type 18 was
oncogenic for newborn hamsters. Although a large number of viruses were subsequently examined, only adenoviruses type 7 (36, 51), type 31 (74), and type 3 (44) were found to be oncogenic when inoculated into newborn hamsters.

McBride and Wiener (60) reported the only successful transformation of cells \textit{in vitro} by adenoviruses. The cells were derived from newborn hamster kidneys. As with the H-50 line of SV40-transformed cells (74), infectious virus could not be recovered, but cells did contain adenovirus-related antigens. Their tumor potential was not reported.

Latarjet (52) postulated that only a fraction of a viral genome was necessary for transformation and that irradiation of the virus would separate the tumorigenic property from the infective property, the former being more radiation-resistant than the latter. His theory has been proved correct for the polyoma virus (7, 8), for which approximately 50 per cent of the viral genome is necessary to transform cells of the young hamster kidney cell line (BHK 21).

Experiments with polyoma virus added to cultures of hamster and mouse embryo tissue showed that the transformation
of normal cells to tumor cells proceeds very quickly, and the formation of foci of transformed cells can be observed for several weeks. The cells transformed in vitro are identical to tumor cells obtained when animals are infected with polyoma virus (79, 97). The transformed culture cells change morphology and form foci of multilayered growth. If culture cells are implanted into the corresponding animals, a tumor is induced.

Carcinogenesis caused by the Rous sarcoma virus has been achieved by a number of investigators (40, 87, 90, 98). Temin (92), while working with Rous sarcoma virus, concluded that the production of the virus is not essential for carcinogenesis, and that conversion and carcinogenesis are not conditioned by the viral genome.

The neoplastic transformation of cells by Rous sarcoma virus was successfully reproduced in rat fibroblast culture. Rats, after having been inoculated with the transformed culture, developed tumors. These tumors were then transplanted into chicks by the cells, but not by the filtrates (87).

As a result of observations based on the widespread occurrence of RNA tumor viruses, two hypotheses have been
proposed: (A) the oncogene hypothesis (47) and (B) the protovirus hypothesis (94). The oncogene hypothesis states that the genetic composition of all vertebrates contains the DNA provirus for an RNA tumor virus. The provirus, in development or in carcinogenesis, is activated to make virus-specific products and virions. The provirus hypothesis states that in the germ-line of chordates there are contained regions of DNA which can evolve in various directions through DNA to RNA to DNA transfers in the somatic cells. This display of evolution is normally a part of embryonic differentiation, but an abnormal evolution might lead to the formation of the RNA tumor virus genome.

Tumor Cell Fine Structure

The fine structure of tumor cells remains as variable as that found in their normal homologues, and no specific or universal pattern of cancer has been found in electron microscope investigations (9). In routine diagnoses of tumors, histological, rather than cytological or ultrastructural, criteria prevail. The cancer cell should be referred to in the plural because of its polymorphism. In spite of the prodigious polymorphism, the characteristic
lesions encountered in tumor cells have been observed and interpreted.

The interphase nucleus of a tumor cell usually exhibited an enlarged and irregular shape. The nucleocytoplasmic ratio was increased, and the increase is attributed to the ploidy of the chromosome set, frequently encountered in tumor cells (18). The enlargement was caused by swelling under unphysiological conditions (nuclear edema) (9).

The nuclear membrane, inasmuch as fine structure is concerned, did not indicate any difference between normal and malignant cells (18). The only unusual feature repeatedly found in the membrane was reported by Bernhard (9) as deep invaginations of the membrane into the nucleoplasm. These invaginations were frequently in contact with the nucleolus. The other malformation of the nuclear membrane led to infolding or to cytoplasmic projections called "nuclear blebs" (3).

With the exception of the nucleolus, the nucleoplasm has been defined as the nuclear contents, including heterochromatic, euchromatin, and the interchromatic matrix
of the interphase nucleus. An increase or an irregular distribution of heterochromatin has been observed in many tumor cells (8). Margination of the chromatin has been shown to be accentuated and very pronounced in the nuclei of dying cells (18). In addition, perichromatin granules have been observed in increased numbers in the nucleoplasm of many tumors (67).

The increased size of the cancer cell nucleolus has been a rule with rare exceptions in classical pathology. The fine structural variations have been reported as numerous and extreme: dense, compact granules in nucleolar bodies, or a nucleolonema fibrillar structure without granules. Vacuoles, lipid inclusions, or inclusions of unknown composition have been shown in the nucleolus. Some of the altered nucleoli in tumor cells resembled those observed after protein deficiency in the rat liver nucleolus (9).

The mitochondrial laterations have been shown to be the most recurrent and striking anomalies found in the fine structural changes within tumor cells (9). But it has been reported that many tumors do have mitochondria
with number, shape, and ultrastructure that cannot be distinguished from mitochondria found in homologous normal cells (44). More often, the tumor cell has swollen and structurally deficient mitochondria: fewer irregular or parallel tubular cristae (55), concentric cristae (81), altered matrix, myelin-like figures, pleomorphic mitochondrial body shape or cup-shaped mitochondria (88). Tumor cell mitochondria at times exhibit various kinds of inclusions: glycogen (89), protein crystals (44), and dense, irregular bodies (88).

Cancer cells grow rapidly, and therefore need to produce greater quantities of proteins in comparison with embryonic cells, but a constant and specific feature for the ultrastructural substrate of protein synthesis has not been detected. The nucleolar apparatus, producing ribosomal precursors, displays hypertrophy, and polysomal formations have been seen more often than in normal homologues. Dalton (17) has shown that in benign adenoma, and in cancer cells, the cytoplasm may be packed with functional ergastoplasm. The same condition may also appear in milk-producing mouse mammary tumor, thyroid, and myeloma cells (17, 59).
Cell lines of mesenchymal tumors that have been maintained during many passages in vitro may have a large amount of ergastoplasmic lamellae; for example, Rous sarcoma cells and methylcholanthrene-induced tumor cell lines. Adenovirus 12-induced tumor, cultivated in vitro, has a decreased ergastoplasm, but many free ribosomes in the cytoplasm. The viral genome may influence the architecture of the tumor cell. For instance, the RAV virus maintained many ergastoplasmic lamellae, and the Rous virus induced partial disorganization (9).

The Golgi apparatus appears well developed in many tumors: exocrine organ tumors, mouse mammary tumors in which virus particles may appear, spontaneous or experimentally induced hepatomas, and Rous sarcoma. The multivesicular bodies in association with the Golgi apparatus have been found extremely hypertrophic in reticulosarcoma cells and myelomas (9, 69).

Abercrombie and Ambrose (1) have shown that certain strains of tumor cells display a loss of contact inhibition in tissue culture. They indicated that a generalized diminution of adhesiveness might be an important point in
malignant transformations. They also observed an increase in the negative charge in some tumor cell strains, and linked it with mucopolysaccharide production at the cell surface. Defendi and Gasic (19) have shown that hamster fibroblast transformed in vitro with polyoma virus produced considerable sialic acid. This observation has been confirmed by histochemistry and electron microscopy.

Oberling and Bernhard (70) have demonstrated that anaplasia is characterized by a low degree of differentiation in rapidly growing tumors. Malignant tissues gradually lost the architecture of the normal homologous tissue and their growth form tended toward the chaotic. Ultrastructurally, the cytoplasm was much less organized than usual. The mitochondria number varied, but had the tendency to decrease. Ergastoplasmic lamellae were rare or completely absent, as they were in the Golgi apparatus. The cytoplasm had a hydropic appearance similar to the cellular organization found in tumor regression. The nucleus had strong pleomorphic outlines.

Bernhard (9) posed a question: Do rapidly dividing cells have the time to build a complete cellular organization
which would enable the tumor cell to function normally? Even normal tissues, when grown in tissue culture and induced to rapid cell divisions, have exhibited anaplasia (dedifferentiation). Slowly growing tumors have been shown to be histologically complex, but cytologically organized. Thus the growth rate alone does not explain the total phenomenon of anaplasia.

The design of this study was multi-phasic: (A) to establish the presence or absence of an oncogenic virion, (B) to demonstrate by use of the electron microscope any ultracellular alterations in malignant or transformed tissues, (C) to investigate the nature of the transforming agent in the murine lymphosarcoma, and (D) to employ various methods to demonstrate cellular transformations in vivo and in vitro. The information reported in this investigation gives an insight into the nature of the causative transforming "factor" found to be present in the murine lymphosarcoma used in the study.
CHAPTER II

MATERIALS AND METHODS

DBA/1J pure-line male mice, four to six weeks old, obtained from Jackson Memorial Laboratory, Bar Harbor, Maine, were used to maintain the transplantable murine lymphosarcoma tumor used in this study. The original tumor was induced in the mice by painting on the dorsal unepilated interscapular skin a solution containing 0.6 per cent 20-methylcholanthrene in reagent grade benzene. The solution was applied with a number four camel's hair brush five times a week for nine weeks. After one week, epilation occurred at the painted site. By the sixth week, papillomas appeared. During the ninth week after the primary painting, squamous-cell carcinomas appeared (82).

The tumor line was produced by subdermally transplanting into an isologous DBA/1J mouse a lymphoid tumor which emerged in the 20-methylcholanthrene-painted mice. The tumor was maintained through one hundred ninety-two
passages by subdermal transplantation with a 12-gauge trocar needle of a section of tumor (2 to 3 mm in diameter) obtained from a tumor-bearing mouse one or two days before death. The mean survival time for the tumor-implanted mouse was approximately eleven days.

Electron Microscopy

Immediately after the mice were sacrificed, tissues were taken from the animals and fixed in 4 per cent paraformaldehyde (Eastman Chemical Company) in 0.2 M s-collidine (Sigma Chemical Company) buffer (pH 7.2), and placed in a refrigerator for 2-24 hr (78). Some of the tissues were fixed in 3 per cent glutaraldehyde (J.T. Baker Chemical Company) in 0.1 M cacodylate buffer (Fisher Scientific Company), pH 7.3, and placed in the refrigerator for 2-24 hr (78). Following the aldehyde fixation, the tissues were rinsed two times (5-10 min each) in 0.2 M s-collidine or 0.1 M cacodylate buffer (pH 7.2). The specimens were post-fixed for from one to two hours in 1.5 per cent osmium tetroxide (Fisher Scientific Company), in 0.2 M s-collidine or 0.1 M cacodylate buffer (pH 7.2), at 4-10 C. After the osmium post-fixation, the specimens
were rinsed two times (5 min each) in 0.2 M s-collidine or 0.1 cacodylate buffer (pH 7.2), and then dehydrated in ethanol or acetone (30, 50, 75, 95, and 100 per cent), and then embedded in Epon 812 (56).

Thin sections were prepared with a Porter-Blum MT-2 Ultramicrotome equipped with a diamond knife. The thickness of the sections was determined by interference colors (silver-gray to straw) to be 60 to 80 μm. The thin sections were collected on 200-mesh copper grids and stained with saturated uranyl acetate in 50 per cent ethanol for 5-10 min, and counterstained in lead citrate (63) for 3-5 min. The grids were coated with a thin layer of carbon in a Mikros Vacuum Evaporator for section stabilization in the electron beam. The specimens were examined in an RCA 3-G Electron Microscope fitted with a 45-micron objective aperture and operated at 50 Kv.

Thick sections were obtained from the same specimen blocks from which the thin sections were taken. The thick sections, approximately one micron thick, were removed from the water surface of the diamond knife by a single-bristle brush, and placed in a single drop
of water on a microscope slide. The water was evaporated by placing the slide on a hot plate at approximately 200°C. The sections were stained with Toluidine Blue 0 (Fisher Scientific Company) for 30 sec or with Paragon Stain (Paragon C and C Company) for 10 sec, rinsed in distilled water, dried, cover-slipped, and examined with a light microscope.

In each of the electron microscope specimen preparations, control tissues were processed simultaneously. This measure was taken to prevent misinterpretations of cellular abnormalities caused by improper fixation or embedding. Thus, any alteration exhibited in the tumor tissues was not an artifact, but was induced as a result of malignancy.

**Tissue Transplants**

A group of mice were implanted with tumor tissue, and on days two, four, six, eight, and ten after implant the mice were sacrificed by cervical dislocation. Liver, spleen, and kidney tissues (2 to 3 mm in diameter) were taken and transplanted subdermally into the axillary region of isologous DBA/1J mice. The animals were
observed daily for tumor progression. Tumor take and day were determined by an increase in size of the transplant tissue nodule.

**Cell Suspension Preparations**

Tumor tissue was taken aseptically from sacrificed animals from eight to ten days after tumor transplantation, and placed in a trypsinizing flask with 20 ml of the 0.25 per cent trypsin solution in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). A magnet stirring bar was used for gentle agitation of the solution until the tissue dispersed into single cells. The cell suspension was centrifuged for 5 minutes at 1000 rpm to form a cell pellet. The cells were washed two times in 10 ml of CMF-PBS. Viable cell number was determined by mixing 1.0 ml of cell suspension with 0.5 ml of a 0.5 per cent water solution of trypan blue, and the counting was done on a haemocytometer. After the viable cell count was made, the cell suspension was diluted to the desired cell number with CMF-PBS.

Tumor cell suspensions were also prepared aseptically by mincing the tissue over cracked ice with fine scissors.
using three changes of cold sterile CMF-PBS, totalling approximately 10 ml. The tissue suspension was aspirated into a 25-ml glass syringe and then inverted in ice for 10 to 20 min to allow larger tissue particles to settle out. A Swinney hypodermic adapter was attached to the syringe and the tissue suspension was forced through a wire mesh support screen. Viable cell counts were determined. The number of viable cells per cubic millimeter of suspension was ascertained and multiplied by 1000 to obtain number of viable cells per milliliter. Appropriate dilutions were made with CMF-PBS to obtain the desirable number of cells per milliliter for transplantation.

**Preparation of Cell-Free Extract**

Cell-free extract was obtained by placing minced tumor tissue in a trypsinizing flask with a magnetic stirring bar, and agitating at a moderate speed for 30 min. The suspension was centrifuged (Sorvall RC2B Centrifuge) at 3000 X g for 10 min, and the supernatant was recentrifuged at 8000 X g for 30 min. The supernatant was recentrifuged (Beckman Model L Ultracentrifuge) at 30,000 X g for one hr, using a Ti 50 rotor. One-ml
samples of the supernatants were injected subdermally into DBA/1J mice, which were observed periodically for tumor progression. The supernatants were incubated for one hour at 37 C with 100 mg/ml pancreatic deoxyribonuclease (DNAase) or 100 mg/ml ribonuclease (RNAase). The enzymes were removed by phenolic extraction, and the solutions were injected subdermally into mice which were observed for tumor progressions.

Preparation of Mitochondria

Mitochondria were isolated from tumor tissue by a modification of the procedure of Schneider and Hogeboom (80). The isolation medium (STEA) consisted of 0.25 M sucrose, 1.0 mM tris-HCL, 1.0 mM ethylenediaminetetraacetate, and 1.0 per cent bovine serum albumin.

The mice were sacrificed by cervical dislocation, and the excised tumors were placed in a beaker of cold STEA medium. The tissue was minced with sharp scissors, and the STEA medium was decanted, and the minced tissue was transferred to a tissue homogenizer. Approximately 10 ml of STEA medium per gram of original tumor were added to the vessel. The tissue was homogenized in the cold.
throughout the procedure. The homogenate was transferred to centrifuge tubes and centrifuged at 700 X g for 10 min in a refrigerated centrifuge to remove cell debris. After centrifugation, the supernatant was decanted through cheesecloth into clean centrifuge tubes, and the cell debris was discarded. The suspension was centrifuged at 900 X g for 10 min. The supernatant was decanted and discarded, and the mitochondrial pellet was resuspended in approximately half the volume of STEA used in homogenization. The suspension was centrifuged two times at 9000 X g for 10 min to remove microsomal and other cellular contamination. All operations in the procedure were carried out in the cold (1-4 C). The final pellet was resuspended in 0.5 ml STEA per gram of original tumor tissue. The mice were inoculated subdermally with 1.0 ml of the mitochondrial preparation. The control mice were injected subdermally with 1.0 ml STEA medium. The suspension was checked for purity by electron microscopy.

Preparation of Nuclei

Tumor tissue or liver from tumor-bearing mice (tumor liver) was removed from DBA/1J mice, weighed, and immediately placed in a cold medium (SM) of 0.25
M sucrose and 3.3 mM MgCl₂. The tissue was minced with sharp scissors, the SM medium was decanted, and the minced tissue was transferred to a tissue homogenizer (Potter-Elvehjem with teflon pestle). Approximately 5 ml of SM medium were added per gram wet weight of tissue. The tissue was homogenized and filtered through two layers of gauze, and the filtrate was centrifuged at 1000 X g for 10 min. The crude nuclear sediment was suspended in approximately 7.5 ml of 2.3 M sucrose and 3.3 mM MgCl₂ per gram of original tissue and centrifuged at 40,000 X g for one hr. The supernatant was decanted, and the nuclear sediment was suspended in 2 ml of SM medium per gram of original tissue. The suspension was centrifuged at 1000 X g for 5 min to remove contaminating erythrocytes. The pellet of nuclei was suspended in SM medium at a concentration of 35 X 10⁷ nuclei per ml.

All procedures were performed in the cold (1-4°C) under aseptic conditions. The nuclear preparation was checked for purity by electron microscopy. Mice were injected subdermally, by means of a 22-gauge hypodermic needle, with 0.5 ml of the nuclei suspension. The control mice were injected by the same method with 0.5 ml of SM medium.
Some control animals were injected with normal nuclei treated with the nuclease as in the test samples.

Chamber Implants

Diffusion chambers were constructed by a modification of Algire's method (5), by using Millipore lucite rings and 25 µm Millipore filters (Millipore Filter Corporation). The Millipore filters were glued to the lucite rings with Millipore chamber adhesive. The chambers were sterilized under ultraviolet light for 36 hr and were turned over approximately every 12 hr. The chambers were filled with a tumor cell suspension of $2 \times 10^6$ cells/ml in CMF-PBS. The cells were put into the chambers through a hole in the lucite ring by means of a 25-gauge needle fitted to a 2-ml syringe, and the opening was sealed with a lucite rod and adhesive. The chamber was inserted subdermally into the mouse through an incision made with scissors on the dorsal side above the base of the tail. After chamber transplantation, the incision was sealed with lanolin. Control mice were implanted with chambers containing CMF-PBS and CMF-PBS with normal tissues. The implanted mice were sacrificed at two-day
intervals, starting the fourth day after implantation. Liver, spleen, kidney, chamber tissues, and tissues in contact with the chamber were removed and placed at once in tissue fixative. The tissues were processed for electron microscopy. The viable cells in the diffusion chamber were counted.

Maintenance of Tumor Cells in Tissue Culture

Tumor was removed aseptically from a ten-day-transplanted mouse, minced with a sharp, sterile razor blade, rinsed two times in CMF-PBS, and placed in a solution of 0.25 per cent trypsin in CMF-PBS in a trypsinization flask with a magnetic stirrer. The mixture was allowed to agitate until the tissue was dispersed into single cells. The large particles were allowed to settle, and the single cell suspension was collected and filtered through cheesecloth to separate the fibrous tissue. The cell suspension was centrifuged at 600 X g for 3 min to separate erythrocytes from the suspension. The cell pellet was resuspended and washed two times in 10 ml of CMF-PBS. The cells were suspended in 10 ml of Eagle minimum essential medium (Difco) at pH 6.8, and a sample
of cells was diluted in CMF-PBS to give a suspension which was counted in a hemacytometer. Growth medium was prepared with Eagle's minimum essential medium (Difco) or McCoy's 5A medium (Grande Island Biological Company), supplemented with 20 or 30 per cent fetal calf serum, and maintained at pH 6.8. The cell suspension was diluted with the complete growth medium to give $10^6$ cells delivered in a volume of 6 ml into 4-oz flat prescription bottles. The bottles were sealed with silicon rubber stoppers to maintain a closed atmosphere, and incubated at 36 C. All procedures were performed under aseptic conditions, and sterile reagents were used.

Parabiotic Chamber

The parabiotic system (Bellco Glass Company) utilized contained two 150-ml chambers separated by a Millipore filter and held together by a bolted collar clamp. Each chamber had a suspended teflon-coated magnetic stirring mechanism. A Millipore filter with a mean pore size of 25 μm was used to separate the chambers. Each side of the chamber was filled with sterile water and autoclaved at 121 C for 15 min at 15 lb. pressure, assuring complete
sterilization without breaking the Millipore filter.
The water was aspirated from the chambers and replaced
with sterile growth medium, Eagle's minimum essential
medium or McCoy's 5A medium. Each medium was supplemented
with 20 per cent fetal calf serum and maintained at pH
6.8. Single-cell suspensions of tumor, normal liver, and
normal spleen tissues were prepared and counted. The
cells were added to the parabiotic chambers in a final
concentration of $10^6$ cells/ml, with tumor cells in one
chamber and either normal liver or normal spleen cells
in the opposite chamber. The parabiotic chambers,
containing cell suspensions, were incubated with continuous
stirring at 36 C. Cell samples were taken from both
chambers, viable cell counts were made, and 1.0 ml of the
sample was transplanted subdermally into DBA/1J mice.
Controls were injected subdermally with 1.0 ml of growth
medium.
CHAPTER III

RESULTS AND DISCUSSION

The transplantable murine lymphosarcoma used in these studies demonstrated a rapid proliferation of neoplastic growth. A small segment of tumor tissue (2 to 3 mm in diameter) implanted into an isologous host did not continue to develop, but degenerated and eventually became necrotic, and finally was rejected by the host (2, 20). Before the tissue reached total degeneration, an apparent induced transformation of host tissue cells surrounded the implant. The transformed cells developed into new, rapidly multiplying tumor cells to form a subcutaneous lymphosarcoma. The developing tumor is depicted in Figs. 1 and 2. Figs. 1a, 1b, 1c, 1d, and Fig. 2a show typical examples of subcutaneous lymphosarcoma tumor tissue after implantation of 2, 4, 6, 8, and 10 days, respectively. In Fig. 2b the animal has been shaved to expose the subcutaneous tumor nodule 10 days after transplantation. Excised tumors were placed in 10 per
Fig. 1—Photographs of DBA/1J mice bearing lymphosarcoma transplant (A) two days after implant, (B) four days after implant, (C) six days after implant, and (D) eight days after implant.
Fig. 2--Photographs of DBA/1J mice bearing lymphosarcoma transplant (A) ten days after implant and (B) external view of tumor nodule. Photographs (C) and (D) are excised tumors various days after implant.
cent formalin, fixed, and divided into sections. The resulting tumor implant is seen in the tumor mass as a white necrotic tissue in Figs. 2c and 2d. The implant was very soft when removed from the tumor and was separated from the surrounding cells by a capsular-like membrane. The host tissues seemed to seal off or reject the implant.

Anatomically, the mice showed an enlarged spleen, a motley-colored and enlarged liver, and an unusually light-colored kidney. The area surrounding the tumor was highly vascularized, but the implanted tumor tissue was devoid of capillaries. The lack of vascularization into the original implanted tumor tissue probably contributed to its necrosis. Folkman, et al. (32) have isolated a tumor factor which is responsible for angiogenesis. The tumor system used in this study was not examined for the presence of that factor.

In Figs. 2c and 2d, the comparative size of the excised tumor begins a rapid enlargement on approximately the sixth day after implant. On the sixth and subsequent days, the gross pathology of the internal organs became
obvious. At this point, evidence revealed that the most propitious time for successful transplantation of tissue from the various organs of tumor-bearing mouse into an isologous host in order to produce a tumor is on the sixth day or later after implant. Many tumor systems with longer durations for tumor development have been reported, but the shorter duration of this system creates a superior method for study. Observation on about the eighth day showed the animals beginning to appear leukemic, characterized by enlarged liver and spleen.

The fine-structural features of the murine lympho-sarcoma are shown in Figs. 3, 4, 5, 6, 7, and 8. Fig. 3 is an electron micrograph of tumor tissue taken 4 days after implant. The nucleus has a distinct nuclear envelope with only slight separation. The nucleoplasm is homogeneous and practically void of heterochromatin. The mitochondria are almost normal and show limited pleomorphism or enlargement. There is a marked lack of ergastoplasmic lamellae, but free ribosomes are abundant. The tumor cells are in close contact and do not have enlarged intercellular spaces.

Fig. 4 depicts tumor tissue from a mouse 6 days after transplant. The tissue differs from tissue in Fig. 3 in that
Fig. 3--An electron micrograph of tumor tissue four days after implant. X 16,500.
Fig. 4--An electron micrograph of tumor tissue six days after implant. X 16,500.
Fig. 5--An electron micrograph of tumor tissue eight days after implant. X 16,500.
Fig. 6--An electron micrograph of tumor tissue ten days after implant. X 16,500.
Fig. 7--An electron micrograph of tumor tissue twelve days after implant. X 16,500.
Fig. 8--An electron micrograph of the central necrotic area of tumor tissue. X 16,500.
there is a separation of the nuclear envelope and a slight increase in intercellular space. The cytoplasm is devoid of ergastoplasmic lamellae, but abundant polysomal formations are present.

Fig. 5 shows an increased separation between the tumor cells. The cytoplasm shows an increase in vesicles and a decrease in polysomal formations. The tissue was taken from an 8-day tumor after original transplant.

Tumor tissue taken from a 10-day mouse tumor implant is shown in Fig. 6, and from a 12-day tumor in Fig. 7. The nucleus shows irregular shapes and an enlarged nucleolus. The mitochondria are more pleomorphic than earlier figures show, and have a heterogeneous matrix.

In the development of the tumor, the nucleus became progressively more irregular, featured deep invaginations into the nucleoplasm, and formed nuclear blebs. In this study, heterochromatin was not evident, as reported by others (9, 69). On approximately the eighth day after implant, the nucleolus became noticeably enlarged and dark. According to reports, the nuclear membrane has not been known to manifest a difference between normal and
malignant cells (9). But practically all tumor cells in this study system demonstrated a separation of the nuclear membrane that has not been reported before. This separation is not considered an artifact, because control tissue prepared simultaneously did not exhibit nuclear membrane separation.

The mitochondria became progressively pleomorphic and distended. Their structural deficiencies were clearly defined: heterogeneous matrix, irregular body, and cristae shapes and swirls. Fewer mitochondria were present when the tumor cell was less differentiated. And as the tumor progressed, the mitochondria became increasingly degenerate.

Although there was a noticeable lack of ergastoplasmic lamellae in the cytoplasm, free ribosomes and polysomal formations were abundant. Swollen cisternae disrupted into various-sized vesicles.

The tumor implant in mice became increasingly necrotic, and autolytic processes took over. Fig. 8 shows tissue taken from a necrotic area in the implant. The nuclear membrane has become greatly separated and is characteristic of the tissue. Nucleolar lesions were not observed, and the nuclei were the last cellular organelle to undergo
degenerative changes. Cellular degeneration of the tumor implant mass proceeded from the exterior to the interior. Cytoplasmic alterations were evidenced in enlarged cristae, fewer polysomal formations, and expanded vacuolar spaces. The mitochondria were greatly swollen and showed signs of degeneration.

The nuclear membrane separation seen in Figs. 4 and 6 may be an indication of necrosis in the tumor mass, which is greatly expressed in Fig. 8. As the tumor mass enlarged, there was a decreased infiltration of capillaries toward the interior. This would cause cell starvation, leading to progressive deterioration and necrosis.

Fig. 9 is an example of an isolated tumor cell used in the cell suspension experiments. The cells were prepared by physical disruption of the tissue to produce single cell suspensions.

Fig. 10 is the representative appearance of a tumor cell grown in culture. Cells from cell suspension cultures, parabiotic chambers, and diffusion chambers gave the same appearance. The most prominent feature of the cells grown in vitro was the enlarged, darkened nucleolus. When grown in vitro, the mitochondria became
Fig. 9—An electron micrograph of a tumor cell from a single cell suspension. X 16,500.
Fig. 10--An electron micrograph of a tumor cell grown in tissue culture. X 16,500.
increasingly degenerate with time. Numerous myelin-like figures were found in many of the mitochondria.

In all investigations with the electron microscope, virus particles or virus-like particles were never positively identified in any of the specimens. The observations were made on several different tissues, subject to various treatments both in vivo and in vitro.

Table I gives the results for a lymphosarcoma developing in DBA/1J mice after transplantation of tumor tissue. The tumor implant tissue was a histologically diagnosed lymphosarcoma. The developing tumor was determined by palpitation and visual observations. Four days after the transplants were made, 34 per cent of the mice showed tumor development. After 6 days, 82 per cent of the animals had developed tumors, and by 10 days, 100 per cent of the animals had tumors. The mean survival time for the tumor-implanted mice was 11 days after transplant. The exact cause of death has not been determined, but several proposals may be considered: (A) toxic products produced by the tumor, (B) necrotic debris from the tumor causing renal failure, (C) metastasis of tumor cells to other organs, (D) a leukemic condition, (E) size of tumor,
TABLE I

INCIDENCE OF LYMPHOSARCOMA TUMOR RESULTING FROM TRANSPLANTS OF TUMOR IN DBA/1J MICE

<table>
<thead>
<tr>
<th>Days after transplant of tumor</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/50</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>17/50</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>41/50</td>
<td>82</td>
</tr>
<tr>
<td>8</td>
<td>49/50</td>
<td>98</td>
</tr>
<tr>
<td>10</td>
<td>50/50</td>
<td>100</td>
</tr>
</tbody>
</table>
and (F) viral or viral transformation of vital organs.

Tissues from various organs of the tumor-bearing mice were implanted into isologous mice. The organs selected were liver, spleen, and kidney.

Table II shows that 6 days after implant, 25 per cent of the recipients had developed a histologically diagnosed lymphosarcoma. Ten days after implant, 85 per cent of the animals had developed a malignant tumor. Three of the animals, after 10 days, did not have a tumor, but one of these mice had a nodule that developed and regressed. Tumor development, in these investigations, is defined as a terminal malignancy. Normal liver, transplanted into mice, did not produce a tumor, nor did any of the control animals develop tumors spontaneously.

Liver tissues from tumor-bearing mice were examined by electron microscopy. Four to five days after subdermal tumor implants in mice, tumor cells appeared in the liver, as seen in Fig. 11. Several more days of tumor progression revealed foci of several tumor cells, as shown in Fig. 12. The foci were not demonstratable by light microscopy when the tissues were stained with hematoxylin and eosin.
TABLE II

INCIDENCE OF LYMPHOSARCOMAS RESULTING FROM TRANSPLANT OF LIVER TISSUE FROM MICE RECEIVING LYMPHOSARCOMA IMPLANTS

<table>
<thead>
<tr>
<th>Days after transplant of liver tissue</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1/20</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5/20</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>11/20</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>17/20</td>
<td>85</td>
</tr>
</tbody>
</table>
Fig. 11--An electron micrograph of tumor liver tissue showing presence of tumor cell. X 11,000.
Fig. 12--An electron micrograph of tumor cell packet in tumor liver tissue. X 11,000.
Liver tissues used in the results of Table II were taken from animals with a 10-day tumor. A tumor of this duration had many tumor cell foci, which evidently developed into lymphosarcoma when implanted into isologous mice.

Table III gives the incidence of tumor development with spleen tissues taken from a tumor-bearing animal. A high percentage of animals developed tumors by the eighth day, 75 per cent as compared to 55 per cent found with liver tumor tissue (Table II). The spleen tissues gave a consistently high incidence of tumor, almost as high as that of the tumor tissues. The 10-day tumor spleen tissues, when examined microscopically, were pathologically diagnosed as having myeloid metaplasia. Normal spleen tissue did not produce a lymphosarcoma in any recipient animal.

When the spleen tissues were examined ultrastructurally, specimens showed that the tumor cell foci were present and resembled those in liver (Fig. 12). In Fig. 13, the foci are more numerous in the spleen, and this may have contributed to its high incidence of tumor production.
### TABLE III

INCIDENCE OF LYMPHOSARCOMA RESULTING FROM TRANSPLANT OF SPLEEN TISSUE FROM MICE RECEIVING LYMPHOSARCOMA IMPLANTS

<table>
<thead>
<tr>
<th>Days after transplant of spleen tissue</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2/20</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>7/20</td>
<td>35</td>
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<tr>
<td>8</td>
<td>15/20</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
<td>19/20</td>
<td>95</td>
</tr>
</tbody>
</table>
Fig. 13--An electron micrograph of tumor cell packet in tumor spleen tissue. X 11,000.
Cells resembling the Sternberg-Reed cell, Fig. 14, were frequently found in tumor spleen tissues. Large, multinucleated cells were found in the spleen 8 to 10 days after tumor implant.

Table IV gives production of tumors from tumor kidney implants. The kidney tissues appeared histologically normal when viewed by light and electron microscopy, but passage of the tissue produced a tumor in 70 per cent of the animals 12 days after implant. Two of the mice had regressive tumors. Kidney tissue from a normal mouse did not induce a tumor.

Tumor cell suspensions were prepared enzymatically (trypsin) from an excised tumor, diluted to the desired number of cells per ml, and injected subdermally into mice. Table V shows that $10^3$ cells/ml induced tumors in only 10 per cent of the animals. At higher concentrations of cells, $10^8$ cells/ml, 90 per cent of the mice developed tumors 16 days after transplant.

Tumor cell suspensions prepared physically gave consistently higher activity for tumor initiation than did enzymatically prepared tumor cell suspensions. The trypsin may have inactivated some of the transforming
Fig. 14--An electron micrograph of a Sternberg-Reed cell found in tumor spleen tissue. X 11,000.
### TABLE IV

**INCIDENCE OF LYMPHOSARCOMA RESULTING FROM TRANSPLANT OF KIDNEY TISSUE FROM MICE RECEIVING LYMPHOSARCOMA IMPLANTS**

<table>
<thead>
<tr>
<th>Days after transplant of kidney tissue</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>3/20</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>7/20</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>13/20</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>14/20</td>
<td>70</td>
</tr>
</tbody>
</table>
### TABLE V

**TUMORS PRODUCED BY CELL SUSPENSION IN DBA/IJ MICE FROM A TRYPsinIZED TUMOR**

<table>
<thead>
<tr>
<th>Number of tumor cells inoculated</th>
<th>Days after inoculation of cells</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^3) cells/ml</td>
<td>4</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0/10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0/10</td>
<td>10</td>
</tr>
<tr>
<td>(10^5) cells/ml</td>
<td>4</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3/10</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>5/10</td>
<td>50</td>
</tr>
<tr>
<td>(10^6) cells/ml</td>
<td>4</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4/10</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8/10</td>
<td>80</td>
</tr>
<tr>
<td>(10^8) cells/ml</td>
<td>4</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2/10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7/10</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>9/10</td>
<td>90</td>
</tr>
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</table>
activity. The cell suspension of $10^5$ cells/ml gave a per cent tumor development comparable to $10^8$ cells/ml trypsinized cell suspensions. When both cell suspensions were examined ultrastructurally, there were no observable differences revealed in their fine structure.

Cell-free preparations of tumor tissue were used to inoculate mice subdermally for induction of tumor. The suspension was centrifuged at 3000 X g for 10 min, and then 1.0 ml of the supernatant was injected subdermally into the animals. Tumor appeared in 48 per cent of the mice. The superatant, when centrifuged at 8,000 X g for 30 min and inoculated into mice, initiated tumors in 23 per cent of the animals. A third centrifugation of 30,000 X g and inoculation produced no tumors. When the solutions were treated with RNAase, tumor production was inhibited. But DNAase did not inhibit production. The nuclease treatments indicated that the transforming "factor" is RNA, or a ribonucleoprotein.

Isolated mitochondria suspensions from tumor tissues were injected subdermally into mice. After 6 months, no tumors were observed in the recipients. Investigations
of mitochondria-transferring virus and virosomes have been reported (48, 58).

Tumor nuclei, when transplanted into mice, produced tumors in 90 per cent of the animals 6 days after implant. Nuclei from livers of tumor-bearing mice were transplanted into normal recipients, and 85 per cent had tumors 8 days after implant.

The nuclei from the tumor disrupted, and the membrane-free sample was incubated with RNase or DNase. The suspension treated with RNase did not induce tumor formation, but the suspension incubated with DNase produced tumors in 83 per cent of the mice. On the other hand, liver nuclei gave an opposite effect. DNase treatment inhibited tumor production, whereas RNase did not. Tumors appeared in 56 per cent of the RNase-treated suspensions.

Tumor cell extracts, treated with heating to 60-70 C for 10-30 min, then treated with iodine or formalin solutions, and injected into mice, induced tumor in 55 per cent of the animals tested. If the treated solutions were further treated with RNase, the induction of tumor was inhibited. DNase did not inhibit.
Table VI shows that after 16 days, 65 per cent of the mice which had received diffusion chambers had tumors, and that the percentage increased to 80 per cent after 22 days. The tumor-cell suspension originally added to the chamber was $2 \times 10^6$ cells/ml. During the first 10 days, the viable count in the chamber decreased to $10^4$ cells/ml, but by the fifteenth day increased to $10^6$ cells/ml. The viable cells were found in close contact with the Millipore filters adjacent to the host's tissues.

The diffusion-chamber methods were developed to permit grafts or cells to survive and grow when separated from host tissues by means of porous but cell-impenetrable filters (82). In some of the investigations reported earlier, the average filter pore size covered a wide range (3, 61), but none were in the 25 µm range. Millipore filters used in this study would not allow cell-to-cell contact, a fact confirmed by electron microscopy. The average pore size of Millipore filter (25 µm) used in these experiments was less than the smallest known mouse tumor viruses (60-150 µm).

Liver and spleen tissues from tumor mice were examined by electron microscopy. The tumor was induced by diffusion
TABLE VI

DEVELOPMENT OF TUMOR IN DBA/1J MICE FROM DIFFUSION CHAMBERS CONTAINING LYMPHOSARCOMA CELL SUSPENSIONS

<table>
<thead>
<tr>
<th>Days after implant of diffusion chambers</th>
<th>Number of animals developing tumors</th>
<th>Per cent of animals developing tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1/20</td>
<td>5</td>
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<tr>
<td>12</td>
<td>5/20</td>
<td>25</td>
</tr>
<tr>
<td>16</td>
<td>13/20</td>
<td>65</td>
</tr>
<tr>
<td>20</td>
<td>14/20</td>
<td>70</td>
</tr>
<tr>
<td>22</td>
<td>16/20</td>
<td>80</td>
</tr>
</tbody>
</table>
chamber implants. Tumor cells like those found in Figs. 11, 12, and 13 were observed in the tissues. The chambers were impenetrable to the tumor cells; therefore the transforming "factor" diffused from the chamber to infect and transform cells. Where the transformation occurred is not known. Perhaps transformation was performed at a site adjacent to the chamber, and then the transformed cells metastasized to the soil tissue. It is even possible that the "factor" entered the circulation and transformed cells at the tissue site.

Tumor cells were cultivated in suspended-cell cultures through several passages. The primary cell inoculum was $10^6$ cells/ml, which increased to $2.6 \times 10^6$ cells/ml during the first 8 hr growth. Cells not transferred to fresh medium after 10 hr primary growth died within 18-24 hr. Cells transferred to new media after 10 hr growth survived to 60 hr before decreasing in viability. Cells carried through to the fourth transfer failed to survive for more than a few hours. The pH of the medium shifted rapidly toward the acid within the first few hours, which indicated a high metabolic rate. Later transfers did not give pronounced pH shifts.
Viable cell counts from the parabiotic chamber indicated an increase from $10^6$ cells/ml to $3 \times 10^6$ cells/ml within 12 hr after initial inoculum of cells. During the first 12 hr, normal liver cells increased to $1.2 \times 10^6$ cells/ml, and normal spleen increased to $1.4 \times 10^6$ cells/ml. After 24 hr, the viable cell count decreased in the liver cell suspension from $1.2 \times 10^6$ to $10^5$ cells/ml, and the spleen cell culture remained nearly the same at $1.2 \times 10^6$ cells/ml.

Cell samples of transformed liver or spleen cells ($10^6$ cells/ml) were inoculated subdermally into mice. The animals receiving 12-hr transformed liver cells or spleen cells developed tumors in 65 per cent of the recipients 10 days after transplant. Cell samples taken after 24 hr produced tumors in 45 per cent of the animals.

The Millipore filter separating the parabiotic chambers had an average pore size of 25 nm. The transforming "factor" was smaller than known tumor virions.

The experiments in this investigation have shown the transforming "factor" to be a RNA or a ribonucleoprotein of molecular size. RNA from purified avian and mouse
oncogenic viruses have approximately the same molecular weight. There seems to be very little difference between avian and murine RNA in regard to base composition, and there is no difference between those avian viruses; this confirms their close relationship (75, 76).

Investigations have revealed that heat and dimethyl sulfoxide (DMSO), two agents able to break hydrogen bonds between bases, can break the 65-70S RNA of Rous sarcoma virus (RSV) into pieces of similar size, $2.5 \times 10^6$ daltons; this suggested that the large RNA molecule consists of four (or possibly five) subunits held together by hydrogen bonds (13, 26, 65, 30).

Montagneir, et al. (65) found that the target size of the transforming capacity of RSV, in X-ray inactivation experiments, was about the same as that found in tobacco mosaic virus (TMV) infectivity (53). Moreover, the oncogenic capacity of RSV may be coded by a RNA subunit the size of TMV RNA, which is approximately $2.5 \times 10^6$ daltons.

Duesberg (26) reported 5-6 subunits by electrophoretic mobility for influenza virus RNA, and each subunit appeared to have a corresponding double-stranded replicative form.
Hence, if RNA subunits, obtained with DMSO or by some other means, replicate independently, and if each actually carries distinct information, one may expect to find recombination between RNA oncogenic viruses.

Viral RNAs have an interesting feature in their capacity to hybridize with cellular DNA. Temin (90, 91) reported that RSV-RNA hybridized with DNA from RSV infected cells. He concluded that viral RNA could be synthesized on a viral RNA-primed DNA template. Later investigations showed that RNA annealed equally well with DNA from transformed and normal cells (41). Harel, et al. (41) showed the hybridized RNA, consisting of a single piece about $7 \times 10^5$ daltons, with a high content of adenine. This finding demonstrated the existence of a segment of viral RNA, rich in adenine, that could anneal with homologous thymine-rich region(s) of cell DNA existing prior to infection.

Shibley, et al. (84) reported that leukemia could be induced in mice by nonneutralizeable suspensions of viral nucleoids obtained by ether extraction from plasma or viremic mice, and also from multiple in vitro passages.
smaller than mammary tumor virus (MTV) can carry the
total activity (64, 66, 68). These last data suggest,
even to a critical mind, that subviral components may
be actually infectious.

The results with a murine lymphosarcoma presented in
this investigation indicate that the transforming "factor"
from the tumor is an RNA. However, tumor liver or tumor
spleen indicated that DNA is the transforming agent.
One explanation may be that the genome of the virus is
incorporated into the tumor tissue as a provirus. The
provirus then produces a viral genome of RNA capable of
transforming other cells. The transformed cells have
RNA-dependent DNA polymerase to produce DNA complement
of the infectious RNA. But the transformed cells lack the
ability to incorporate the DNA into their genetic machinery;
thus an infectious DNA remains in the cells. For instance,
when the transformed tissue was used for transplants, the
surrounding tissue was transformed. The surrounding
transformed tissue then incorporated the infectious DNA
into its genome as a provirus capable of producing infectious
RNA as found in the tumor tissue.
Variations were neither found in nor isolated from any of the tissues of the tumor-bearing mice used for study. Perhaps, under certain conditions, the virus has lost its ability to form a protein coat; consequently, it would not be demonstrable as a virion. Therefore the transforming and tumor-inducing agent was not considered to be a virion, but an infectious RNA or a segment of RNA oncogenic viral genome.
CHAPTER IV

SUMMARY

A search was made to find an agent that maintains and induces a lymphosarcoma in DBA/1J mice and transforms cells in vivo and in vitro. The ultrastructures of the transplantable tumors and the transformed cells were examined for cellular alterations and the presence of virions. Virions were not demonstrable, and the transforming "factor" was determined to be an infectious RNA or a nucleoprotein. The agent was able to pass through Millipore filters (25 μ) employed on diffusion chambers and parabiotic chambers to transform cells in vivo and in vitro. When transplanted into normal mice, the transformed cell induced a lymphosarcoma.

This evidence indicated that it is not necessary to have a complete virion to transform a mammalian cell. Thus the "factor" in cell transformation may be an incomplete virion which has lost its ability to develop a protein coat.
LITERATURE CITED


