DETECTION OF VIRUS-LIKE PARTICLES FROM
RADIATION-INDUCED LEUKEMIA IN SWINE

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DETECTION OF VIRUS-LIKE PARTICLES FROM
RADIATION-INDUCED LEUKEMIA IN SWINE

By

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Chapter 1

INTRODUCTION

STATEMENT OF IMPORTANCE AND VALIDITY OF THE RESEARCH AREA

Leukemia, both experimental and spontaneous, is a well documented disease occurring in a wide variety of animals. Viruses are the etiological agents of feline, murine, and avian leukemia as well as of the avian leukosis disease complex (87). Studies of the murine leukemia and avian leukosis viruses have contributed considerably to our understanding of the complex relationship between the animal host and the intracellular oncogenic viruses. Most of the significant leukemia research has been conducted with laboratory mice, and the course of the disease has been related to human leukemia studies. Recent investigations however, suggest that mice are not as susceptible to the "hypothetical human leukemia virus" as other animals. Tissue culture studies indicate that leukemia viruses may be divided into two groups, one capable of infecting feline, canine, porcine, and human cells and the other group capable of infecting murine and avian cells (73, 122).

The failure, thus far, to demonstrate a viral etiology for human leukemia is partially due to technical difficulties in demonstrating the role of leukemia associated virus particles.

Inability to investigate directly the viral etiology of human leukemia has resulted in attempts to develop new techniques and to investigate additional animal models for the study of this disease.

In a 12-year study, Holzworth stated that 19% of cats necropsied were affected by neoplasms of the blood forming organs (58, 59). The transmission of feline leukemia by cell-free filtrates (72) has given a fresh impetus to the study of virus induced leukemias.

It is tempting to study only virus induced leukemia as a model since there are fewer variables than are to be found in radiation induced viral leukemia. Utilization of a more complex system possibly involving both radiation and leukemia viruses, may show how viruses are activated or induced to cause leukemia and tumors under natural conditions.

Therapeutic X-irradiation has been established as a potential and actual leukemogen in man. For example, routine obstetric radiography has been implicated as the causal agent for increasing the incidence of lymphocytic leukemia in children (130).

Radiation from certain deposited internal emitters also appears to be a potent leukemogenic factor. It is well known that bone seeking radionuclides are particularly effective in inducing leukemias and other malignant neoplasms in man and animals (22, 29, 53, 57, 63, 74, 103, 130).

Battelle's Pacific Northwest Laboratories (Richland, Washington) studies indicate that miniature swine display promise as a potential animal model for leukemia. When chronically ingested ^{90}Sr resulted in a variety of hemopoietic disorders (64). The spectrum of induced neoplasms included myeloid metaplasia, myeloblastic and granulocytic leukemia, and lymphoblastic leukemia. The prevalence of leukemia in various experimental groups ranged from a low of 4% in control animals to nearly 80% in animals exposed to ^{90}Sr in utero and later to 125 μCi of ^{90}Sr per day throughout their lifetime (Table 1.1).

Possibly the single most interesting result of the Battelle studies was the diversity of the hemopoietic disorders encountered. Aging mini-swine have an undetermined incidence of spontaneous granulocytic leukemia. This situation is similar to that of man and is different from that found in smaller laboratory animals.

A filterable and transmissible virus isolated from radiation induced leukemia in mice has been passed serially by cell-free filtrates (89, 52). This viral agent induces leukemia in susceptible non irradiated mice and rats. Holtzman rats have been reported to have a similar virus transmitted, radiation-activated leukemia (74). These findings have prompted me to examine several parameters in the leukemic swine including the search for oncogenic virus in the blood and other tissues.

Table 1.1
Hemopoietic Disorders in Swine Ingesting ^{90}Sr

^{90}Sr level ($\mu\text{Ci/day}$)	Gener- ation	No. at risks	Age at death (mo.)	Ave. rad dose to skeleton ($\times 10^3$)	Hemopoietic Disorders			
					Pathologic Diagnosis			
					Lymphoid neo- plasms	Stem cell neo- plasms	Myeloid neo- plasms	Myeloid meta- plasia
3100	Parent	5	12-13	7-10	--	--	--	2
625	Parent F_1^*	6	17-19	6-9	--	--	--	2
		28	3-34	2-10	1	--	1	15
125	Parent F_1, F_2	10	65-87	13-17	1	--	3	3
		32	50-92	3-6	2	--	1	3
5	Parent† F_1, F_2	--	--	--	--	--	--	--
		24	81	1	1	--	--	--
1	Parent‡ F_1, F_2	17	92-96	0.04	1	--	--	1
		59	52-82	0.2	4	--	--	1
0		48	103-105	--	--	--	2	--

* Removed from ^{90}Sr feeding at 3 months of age.

† Fed ^{90}Sr 31 months. Held until 105 to 108 months of age.

‡ Fed ^{90}Sr 8 to 22 months. Held until 90 to 107 months of age.

§ Number of live animals at onset of first disorder.

It could be argued that swine leukemia is not a spontaneous disease, but one caused by highly artificial conditions. Analysis of the information available from radiation-induced leukemia of mice and man, however, suggests that this system is not a laboratory artifact. For example it has been reported that the type of leukemia produced in mice or humans by irradiation is similar to the naturally occurring disease of each species (95). The only differences are an increased overall incidence of disease due to the action of the radiation or a decreased incubation period before the leukemia develops. Evidence in several species including monkey, swine, rats, mice and dogs indicates that ^{90}Sr , for example, does not elicit a distinct type of leukemia but enhances the occurrence of the leukemias most common in the species. In addition, animals with extremely low spontaneous leukemia appear to be more resistant to induction of leukemia by irradiation. It is, therefore quite likely we are dealing with swine leukemia occurring spontaneously in nature, even though the incidence has been enhanced.

The finding of a potential leukemia virus in miniature swine is significant for the following reasons:

1. If radiation induced leukemia of swine was shown to be caused by a virus, this would be an extension of the known etiological role of viruses in the leukemogenic processes of higher animals.

2. There is a need for better understanding of the relationship between the carcinogenic actions of radiation and viruses. My study was undertaken in an attempt to increase knowledge in this area.

3. Similarities of swine leukemias to human leukemias (types and relative occurrence of leukemias) is a factor which should be considered in any model system utilized for leukemia investigations.

4. Physiological and biochemical parameters already established for normal and leukemic swine is of value in studying therapy as well as the molecular etiology of leukemia (134).

5. Leukemia viruses of domestic animals may be related to the hypothetical human leukemia virus or may even contribute directly or indirectly to the human disease. It is thus essential to investigate the biological and pathogenic characteristics of these viruses.

6. Porcine leukemia viruses, if isolated and identified, may be used as helpers for the isolation of a hypothetical, defective human leukemia or sarcoma viruses.

7. The miniature pig may prove useful for the isolation of the hypothetical human leukemia or sarcoma virus.

8. The confirmation of a viral etiology in ⁹⁰Sr induced porcine leukemia would provide the first useful large animal model for the study of leukemia. It would provide an excellent model for

the further investigation of the etiology, epidemiology, progression, and therapy of leukemia. Since swine are physiologically and anatomically very similar to man, the study of leukemia in these animals may provide an excellent analog to human leukemia and therapeutics.

STATEMENT OF THE PROBLEM

X-rays and other types of ionizing radiation can elicit bone tumors, skin cancer, leukemia, and various types of malignant neoplasms in man. Similarly, a broad spectrum of experimental malignancies have been induced in animals by various forms of radiation.

Three theories are currently utilized to explain radiogenic cancer.

1. The Somatic Mutation Theory proposes that the radiation produces a mutation in the DNA of the cell which results in malignant changes to the cell and all its progeny.

2. The Virus Theory states that latent or occult viruses activated by any one of several mechanism are responsible for the leukemia.

3. The Hormonal Theory implicates imbalances of normal growth regulatory mechanisms as responsible for leukemia.

Only the Hormonal and Viral Theories have been conclusively established in experimental systems. Radiation damage to endocrine glands of experimental animals has been shown to produce a hormonal imbalance eventually leading to cancer. Under other experimental conditions radiation has caused leukemia as the result of an "activation" of leukemia viruses.

Whether or not these mechanism are involved in the induction of hemopoietic disorders of swine is not certain. It is the purpose of this study to initiate an investigation to determine if virus particles are present in the tissues of leukemic swine. In considering the problem the following hypotheses have been established:

1. Virus is present in the tissues and/or blood of leukemic swine.
2. The presence of virus can be detected if:
 - a) Both cytopathogenic effects and complete infectious virus are produced in susceptible cells, or
 - b) complete infectious virus without an overt cytopathic effect on the infected cells are produced in susceptible cells, and
 - c) some measurable or discernable changes in the infected cell (even if the virus is defective and does not produce complete infectious virus particles) are induced by the virus.
3. Productive viruses can be isolated, purified, and characterized.

4. Characterization of virus from leukemic swine will help in the selection of the most likely virus candidates for use in etiological studies.

It is important to note that this study deals only with the detection, isolation, and characterization of candidate viruses from leukemic swine. Procedures to implicate a given virus as the causative agent of radiation induced leukemia in the miniature pig, although currently being investigated, are not within the scope of this study.

DEFINITION OF TERMS

For a comprehensive review of the formation and composition of the blood, Darmady and Davenport (21) should be consulted. A brief outline of this subject is provided, (Fig. 1.1) to clarify terminology utilized and for the sake of orientation. The leukopoietic system, which is of primary concern here, consists of the myeloid series, lymphocytes, monocytes, and plasmacytes.

Leukemia is a generalized neoplastic disturbance of the entire leukopoietic system. The total mass of leukocytes, both in that system and in the blood, is usually increased to the detriment of normal hemopoietic elements, more particularly the bone marrow. Some investigators feel that normal control mechanisms are no longer operative, presumably because of various types of metabolic deficiencies or faults within the cell. A

Figure 1. Elements of the Blood as Produced by Bone Marrow and Reticuloendothelial System

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1. Bone Marrow

2. Reticuloendothelial System

new growth pattern could then become dominant due to temporary or permanent immunodeficiencies. Somatic mutation and immunodeficiency states probably play leading pathogenic roles with such factors as radiation, chemical insults, viral modification of genetic material, and heredity acting individually or in combinations as etiologic agents. The interaction of these various etiologic agents and pathogenic mechanisms probably determines the type of leukemia occurring. It is obvious then that leukemia is not a single disease but a group of diseases. Further, leukemia seems to comprise a number of different conditions due to different causes and mechanisms.

Diseases within the general classification of true leukemia and leukemia-like diseases to be discussed are: myeloproliferative disorders (including myeloid metaplasia and myelogenous leukemia), stem cell proliferative disorders and lymphoproliferative disorders. The definition of these diseases is based on histological, hematological, and chemical observations. The resulting definitions are mainly descriptive. The terms used have been accepted by a board of review composed of authoritative pathologists.

Myeloproliferative Disorders

Myeloid metaplasia. Myeloid metaplasia is an over proliferation of the cell lines comprising the myeloid tissue (e.g., basophils, eosinophils, neutrophils, etc.).

This proliferation usually occurs in extramedullary sites such as the liver, spleen, or lymph nodes. It occasionally involved the kidneys, myocardium and other tissues. Extramedullary lesions in our swine occurred most frequently in the intralobular areas of the liver and splenic red pulp. In addition to the afore-mentioned areas, the myocardium, adrenals, ovaries, and various other tissues often contained foci of myeloid cells. Proliferative lesions were not extensive in most instances.

The presence of a trilineage of marrow elements showing mature cells resulted in diagnosis as myeloid metaplasia. We often, however, found myeloproliferative lesions that were composed almost entirely of myeloblasts. Nearly specific cell types appearing histologically similar to leukosarcoma were present. Hematologic data revealed severe leukopenia and anemia with some circulating blast cells. Death in these animals was generally due to hemorrhage or microbial infection caused by deteriorated health.

There was a high degree of variability in the histological appearance of the marrow of swine with myeloid metaplasia. The marrow of some animals appeared normal. In a few animals the marrow was hypercellular, with a positive shift to blast cells and a lack of maturation. Finally in others, vascular damage, myelonecrosis and myelofibrosis with an increase in reticular fibers and collagen deposition were evident.

Myeloid metaplasia is included in the group of myeloproliferative disorders as recommended by Dameshek and Gunz (20), and Rappaport (114). This disorder is considered by most experts as a chronic myeloproliferative disease. However, disagreement as to whether myeloid metaplasia can transform to overt leukemia exists.

Myelogenous leukemia. The most frequently observed neoplastic disorder observed in the ^{90}Sr -fed swine was myelogenous leukemia. Histologically, the cell population was relatively homogenous and consisted of myeloblasts, promyelocytes and myelocytes. In the spleen, liver, and lymph nodes heterogenous populations were sometimes seen due to the accompanying extramedullary hemopoiesis in these tissues.

The infiltration by neoplastic cells in organs varied in each animal as might be expected, but infiltration of nearly every tissue in the body was found in one animal or another. The marrow consisted primarily of myeloblasts, but in several animals the marrow was overpopulated by immature reticulum cells.

The hemograms revealed chronic leukopenia was followed by clinical leukemia in over half the animals. The leukocyte count ranged from slightly above normal to more than $300,000/\text{mm}^3$. The leukemic cells were usually immature with myeloblasts and promyelocytes predominating in the most acute cases. Pronormoblasts, paramyeloblasts, and mitotic cells were sometimes observed. More

mature cell types, such as myelocytes, were rarely seen. While the most frequent observations were the acute myeloblastic leukemias, several animals had prolonged clinical disease terminating with a hematological picture of chronic granulocytic leukemia.

In the cases of chronic granulocytic leukemia, leukocyte alkaline phosphatase (LAP) levels progressively decreased. This is similar to observations of LAP levels in the spontaneous granulocytic leukemia of man.

In addition, a pronounced leukocytosis (up to $180,000/\text{mm}^3$) was observed. Tissue invasion by myeloid cells was widespread. Myelogenous leukemia found in control animals had a clinical course and hematologic pattern of chronic granulocytic leukemia.

Stem Cell Proliferative Disorders

These are neoplasms of undifferentiated cells resembling reticulum-like cells present in the peripheral blood. They had the appearance of a generalized neoplastic disease. Since these cells demonstrated no evidence of differentiation toward a recognizable cell line, they were considered to represent stem cell hemopoietic neoplasia.

Lymphoproliferative Disorders

Most of the lymphoproliferative lesions were typical lymphoblastic proliferations or a mixture of lymphoid cells with reticulum cells. Mitotic cells were abundant, and lymphoblastic forms were

predominant in the peripheral blood of animals with leukemia. The lymph nodes revealed proliferating lymphoblasts with scattered reticulum cells, and normal lymph node structuring was absent. Proliferations and infiltrations were apparent in the testes, myocardium, liver, adrenals and renal cortex. Typical "starry-sky" patterns could be found in the lymph nodes are due to a few phagocytic reticulum cells scattered among the neoplastic cells. The "starry-sky" patterns could be found in the lymph nodes. This is due to a few phagocytic reticulum cells scattered among the neoplastic cells. The "starry-sky" pattern is often seen in spontaneous lymphomas of animals. In addition, a mixture of neoplastic cells, including lymphoblasts, reticulum cells, and lymphocytes were present in the lymphoid tissues of the swine.

BASIC ASSUMPTIONS

It has been established that viruses are causative agents in leukemias of several animal species. The assumption is here made that a virus could be the etiological agent of leukemia of swine. It is further assumed that if either complete virus or viral genetic material is present in the cells of leukemic animals, they could be detected by employing various virological, immunological, and cell culturing procedures. If a virus is present in the leukemic swine it is also assumed the agent should possess biochemical, physical, and other properties similar to known leukomogenic viruses.

For the purpose of this study it is assumed that a progression of lesions occurs in the ^{90}Sr -fed swine, from metaplasia to neoplasia. This progression would naturally be affected by such factors as the dose level of radiation and the time until the death of the animal. Obviously, proof for this assumption is not available even though there are some pathological findings suggesting this to be the case (64). Therefore, in this study, animals suffering from myeloid metaplasia have been investigated under the assumption (however tentative) that they represent animals in a pre-leukemic state.

Finally, we are assuming that valid conclusions concerning the influence of radiation can be drawn, if radiation-"induced" forms of swine hemopoietic disorders differ in significant ways from those of the "spontaneous" tumors. On the other hand, we may be able to determine that radiation-induced leukemia does not differ significantly from "spontaneous" leukemia. In this instance radiation may only influence the frequency of hemopoietic disorders. In other words, radiation-induced tumors may represent only a quantitative rather than a qualitative difference.

Chapter 2

REVIEW OF PERTINENT LITERATURE

INTRODUCTION

The literal translation of leukemia is "white blood".

Although this is a misnomer, it does portray a situation occurring in certain types of this group of diseases. According to Dameshek and Gunz (20), the blood forming tissues and not the blood produce the abnormal cells. Their definition of leukemia is as follows:

"Leukemia may be defined as a generalized abnormal, neoplastic, self-perpetuating proliferation (slow or rapid) on one of the leukocytic tissues, often associated with abnormal white blood cell counts and eventually leading to anemia, thrombocytopenia, and death" (20).

Another definition of leukemia which helps to more accurately define the disease is that of Amromin (3).

"Leukemia is an almost invariably fatal group of diseases having in common an uncontrolled, purposeless, absolute increase of abnormal leukocytic elements at multicentric sites of origin, with or without a corresponding increase in the peripheral blood but with infiltration of other viscera" (3).

These definitions appear to have in common the idea that the leukemias are proliferative diseases and that the increase in leukocyte count is due to an excessive degree of proliferation.

This is in contrast to the hypothesis that the high leukocyte count is due to accumulation of certain leukocytes. The accumulative theory implies that the increase in leukocytes is due to a slowdown or an arrest of the maturation of the specific leukocytes. Similarly the increase in total leukocytes could be a result of an increased survival of certain types of leukocytes.

THEORIES OF CARCINOGENESIS

Over the years, a number of theories have attempted to explain how normal cells become converted to neoplastic or malignant cells. These theories can basically be summarized into two major categories.

The genetic theories involve some change in the genetic material present within the cell. The presence of new genetic material or the loss of old information is considered to be sufficient to convert the affected cell to the malignant or transformed state. The primary mechanisms by which the genetic changes take place are believed to be through somatic mutation or infection with a viral genome.

The epigenetic theories, on the other hand, state that the changes within the cell involve only phenotypic changes, and are the result of regulatory systems rather than modifications of the actual genotypic make-up of the cell. The primary mechanism of the epigenetic theories thus involve such ideas

as gene regulation resulting in embryonic arrest or activation of embryonic inducers. Factors which might participate in these types of reactions are histones, hormones, and any other compound which could interact with the nucleic acid in a manner resulting in the selective repression or derepression of a gene or series of genes.

Genetic Theories of Carcinogenesis.

Somatic mutation. This hypothesis is based primarily on the finding of chromosomal abnormalities in a chronic myeloid leukemia. In the majority of cases of this disease, there appear to be a very specific abnormality commonly referred to as the Philadelphia Chromosome (8, 108, 109, 141, 142). The hypothesis is also based on the fact that ionizing radiation and various alkylating agents, which are known to be potent leukomogens (or carcinogens) have also been shown to be capable of causing chromosome damage. Therefore, according to this concept leukemogenic (or carcinogenic) agents cause chromosome changes in the genetic make-up of the cell by either deletion of a gene or series of genes or by the permanent alteration of a gene and the product for which it codes. These somatic mutations would have significantly harmful effects if the mutant cell were selected for and the resultant cells eventually dominated the cell population.

The effect of dominance by a single cell type could be either beneficial, deleterious, or insignificant. One instance in which it would have detrimental effects would be in the cases of excessive proliferation and/or loss of function. When such a cell line arises, tumors, including various types of leukemias, result.

The hypothesis of somatic mutation does not have sufficient evidence for general acceptance. Unfortunately, the chronic myeloid leukemia in human beings appears to be somewhat unique since no other type of malignancy is known which consistently has an associated genetic abnormality. We are left with the problem of determining if the Philadelphia Chromosome abnormality is the triggering mechanism which caused the cell to develop the malignant properties ascribed to it, or if the production of the marker chromosome is a result of malignancy. In this final instance, the abnormal chromosome would have no relevance except as a convenient diagnostic tool for determining malignancy of the cell.

Viral carcinogenesis. Very simply, the theory of viral carcinogenesis would imply that neoplastic conversion of a cell is a direct result of virus infection. It is often implied that many, if not all, "leukemogens" may act through the mediation of occult viruses.

Needless to say, viruses have been shown to be causative agents in leukemia of many animal species, and this will be discussed in greater detail in a subsequent section. It should be noted that the properties of malignant and transformed cells have been explained in terms of structural changes on the cellular surface (55, 71, 101, 146). Oncogenic viruses have been shown to be necessary for the production and maintenance of transformation in vitro. Interestingly enough, the protein or proteins related to this transformation of cells appear to be dispensable for the production of infectious virus (55, 146).

The protovirus hypothesis. The protovirus theory formulated by Temin (131) combines various aspects of the somatic mutation and viral carcinogenesis theories. This hypothesis represents an attempt to formulate a comprehensive theory for explaining normal cellular differentiation as well as carcinogenesis. According to the hypothesis, the germ cells display the following modes of information-transfer: DNA to DNA; DNA to RNA; and RNA to protein. The germ cells are usually restricted primarily to these methods of information transfer, and this helps to preserve the stability of the germ cell line. On the other hand, somatic cells have an additional avenue of information transfer. These cells are capable of transferring information by a procedure involving the reverse transcription of RNA to DNA. This process is mediated by an enzyme called reverse transcriptase (9, 137).

The information transfer of RNA to DNA is postulated to be a normal cellular process which allows genetic variability, and thus a form of cellular evolution, to take place in somatic cells. Temin states that this provides a mechanism for producing stable genetic alterations in somatic cells (138). Temin's concept proposes possible mechanism by which normal differentiation of cells and tissues could take place.

One mechanism by which protovirus transfer could proceed in a normal cell is as follows: A portion of DNA in the first cell would serve as a template for the production of an RNA molecule. This RNA could be transferred to a second cell and DNA is produced as a result of interaction of the RNA with reverse transcriptase. This DNA then becomes integrated into the cell genome of the second cell in a location either adjacent to or distant from the homologous area of DNA. The second cell is now genetically different from the first cell. This process could then be repeated with the same DNA or a new DNA being produced in the same cell or a new cell. This procedure leads to gene duplication and amplification resulting in changes in amounts and kinds of genetic products. In addition, it is possible that the genetic information being transferred could become altered by the response of any of the components of the information transfer system (i.e., the RNA, the reverse transcriptase, or DNA) to environmental factors such as radiation or chemicals. These alterations could result in new genetic

messages (or capabilities). This would provide a mechanism by which a cell could undergo the neoplastic conversion without either the formation or the presence of an oncornavirus. All that is really necessary is for the cell to attain the information necessary for uncontrolled growth.

While the provirus theory provides a mechanism for the formation of virions it is possible for extraneous viruses to infect these cells and take a short-cut to neoplastic conversions. These viruses need only to enter the cell and make a new DNA, coded by the viral RNA, which contains the information required for neoplastic transformation of the cell. Such a mechanism would not have to go through the multiple cycles of DNA to RNA to DNA information transfer mentioned previously.

Epigenetic Theories of Carcinogenesis.

Hormones and humoral factors affecting differentiation and regulation. This area includes several factors which are concerned with the regulation of various cells in the hemopoietic system. It is indeed possible that leukemia is a neoplastic disease which is initiated in response to the regulator action of these factors. However, there is very little evidence to support such a hypothesis. Such compounds as erythropoietin, colony stimulating factor, the thymic humoral factor, thrombopoietin, chalone, antichalone, cortisone, growth hormones, and sex

hormones do play various regulatory roles in the hemopoietic systems of normal animals. Unfortunately, the levels present in leukemic animals do not explain the lack of regulation and organization present. Nonetheless, Metcalf (104) considers them to be of importance in some phases of leukemogenesis. The implication is that these factors may participate in the leukemogenic or carcinogenic process as co-factors. The mechanisms of this co-carcinogenesis were purported to be that these regulatory factors may cause: A) a total increase in the number of target cells, thus facilitating other leukemogenic factors such as viruses. B) an increase in mitotic activity of the target cells, elevating the possibility of somatic mutations, and finally C) modification of the phenotypic expression of the genetic information in the target cells (104). Evidence to support these theories is not available.

Oncogene theory of carcinogenesis. The oncogene theory was first proposed by Huebner, et. al. (69) and further explained by Huebner and Todaro (68). This hypothesis proposes that the cells of vertebrates contain the C-type viral genome as a part of their inherited genetic composition. The authors state that the C-type virus genome may play an important role in the early rapid proliferation during embryogenesis. The genome obviously being switched off during the final stages of differentiation and regulation. In this theory, the portion of the gene coding for the oncornavirus is referred to as the virogene.

The virogene is postulated to be carried in the cells in a provirus state, and the portion of the virogene that codes for the factors capable of transforming normal cells to tumor cells is called the oncogene. Various environmental factors, such as irradiation, hormonal imbalance, chemicals, aging, etc. are capable of derepression of the oncogene and/or virogene, resulting in virus production, tumor formation, or a combination of these two effects.

According to Huebner (68), in order to qualify as an oncogenic virus, the agent must meet the following requirements:

- 1) It must be transmitted vertically.....
- 2) It must be commonly switched off (the virogenic and oncogenic expression) in early life, being switched on spontaneously primarily in late life.
- 3) Genome expressions of all types must be subject to regulation by natural genes of host cells.
- 4) Certain genetic defects and exogenous factors apparently predispose to spontaneous derepression of the oncogene and virogene.
- 5) According to immunological theories of cancer-host interactions following tumor cell induction, the new tumor cells contain surface transplantation antigens and thus are inevitably subject not only to clonal selection but also to possible rejection mediated by immunological surveillance forces of the host (64).

Although the oncogene theory attempts to combine elements of the viral theory with epigenetic theories of carcinogenesis, it must still be classified as an epigenetic theory. The authors of this theory feel that cancer is a natural event resulting from the derepression of the oncogene and/or virogene. The basis of the

theory is that regulatory mechanisms are responsible for determining whether or not the oncogenic phenotype, present in the cellular genetic code is to be expressed or repressed.

PROPERTIES OF LEUKEMOGENIC VIRUSES

Classification.

The first virus-produced leukemia was reported in fowls by Ellerman and Bang in 1908 (32). Spontaneous leukemia was later discovered in laboratory mice, and in 1938 Englebreth-Holm and Fredrickson claimed cell-free transmission of the disease (33). However, subsequent attempts at transmission by these investigators as well as others failed to confirm this finding until Ludwig Gross (51) made the key discovery that transmission of the disease was enhanced in newborn mice. In spite of this breakthrough, progress in the investigation of leukemia (primarily with the avian and murine systems) continued at a slow pace. The assay system (which consisted of bioassay in susceptible animals), the complexity of the virus systems, and the natural presence of these viruses in the animal populations under study contributed to this slow progress.

It is no longer doubted that viruses cause leukemia in several other laboratory and domestic animals. The mechanisms of these virus-induced neoplasias is, however, still not understood. One can only speculate, based on experimental findings,

as to how these neoplastic conversions take place. In order to understand viral neoplasia, it is necessary to define and characterize the nature of the viruses which apparently play such an integral role in the mechanisms of these diseases.

The oncogenic viruses are usually classified into two broad categories based on nucleic acid composition. The oncogenic DNA viruses consists of several groups of agents including the adenoviruses, poxviruses, and papovaviruses. These viruses are known to cause tumors under laboratory conditions. In addition, the DNA viruses include the Herpes viruses, which are closely associated with several malignant neoplasms in man, including penile cancer, cervical cancer, Burkitt's lymphoma, Hodgkins disease, and nasopharyngeal cancer.

The oncogenic RNA viruses appear to have very similar biophysical, biochemical, and morphological properties, and consequently they have been classified into a single group. Several different names have been suggested for this group of viruses that include A-type, B-type and C-type particles. The names range from Thylaxovirudiae (4) and leukoviruses (38) to oncornaviruses (110).

The oncornaviruses are most commonly associated with mammary tumors (B-type particles), sarcomas, and leukemia (having both A-type and C-type particles present). Therefore, any C-type particles associated with leukemia and/or sarcoma of laboratory and domestic

animals, must be considered as candidates for causative agent. For this reason these viruses were a prime target in our search for a causative factor in the leukemic swine.

Structural Properties of Leukemia.

Recently Dmochowski (24) reviewed the morphology and development, including observed variations, of leukemogenic viruses. Infected cells often contain both type-A and type-C particles (11, 18). The type-A particles in leukemic animals are usually found either present in, or budding into the cistern of endoplasmic reticulum. The role of the A-type particle in leukemia is not known. It is not even certain that the A-type particle is a virus. One theory implies that the A particles represents an immature stage in the development of infectious C-type virus particle (24).

Mature C-type viruses are spherical in shape and are approximately 100nm in diameter. These viruses also contain a centrally located dense spherical nucleic internal component (140). The C-type particle in various stages of maturity is characteristically seen budding from the cytoplasmic membrane in close contact with the membrane or in vacuoles. The outermost ring of the C-type particle constitutes the lipid envelope and the spherical nucleoid (or core) is a helically structured nucleocapsid consisting of protein and RNA (119, 123).

Biological Properties.

Obviously, the most important biological property these viruses have in common is their oncogenic capability. Therefore, the primary basis for classification as an oncornavirus is the ability of these RNA containing viruses to cause tumors or leukemia. Oncornaviruses have been described in a wide variety of animal species. Although these viruses appear to have common characteristics and even common structures, there still appears to be a degree of host range specificity.

In the murine leukemia complex, even the genetic constitution of the host strongly influences the host animal's response to a leukemic virus. Specific genetic differences have been noted which account for the susceptibility and refractiveness of various mouse strains to the leukemogenic effects of leukemia viruses. For example, it has been reported that a gene closely associated with the complex histocompatibility (H-2) locus in mice appears to be the major determinant of the leukemic response to the Gross virus (91). The H-2 effect is also seen in the case of spontaneous leukemias in the AKR strain. These leukemias are known to be induced by the vertically transmitted Gross virus (92, 94).

Studies with the Friend virus shown that the H-2 chromosomal region governs a share of the erythroleukemia response to this virus. Interestingly, the evidence points towards some membrane phenomenon other than a receptor site function as a basis for the

H-2 effect (93). A separate component of the host response to infection with the Friend virus is governed by two genes independent of H-2, the Rgv-1 and Rgv-2 loci; these appear to influence the ability of the virus to proliferate in the host cells (94). Based on this type of information, one can see that genetic susceptibility apparently plays a very important role in the leukemogenic process.

In vitro production of infectious leukemia virus has been found to vary with the virus and target cell. The target cell of the species of origin or a closely related species are always superior with regard to virus synthesis. Conversely, cells of vastly different species cannot usually produce infectious virus, though all will permit replication of the viral genome. This inability to produce infectious virus appears to be due to variations in the membrane composition of the cell.

The radiation leukemia virus (RaLV) of Kaplan (89) has been studied in vitro and has been found not to replicate in mouse embryo cells (70, 41). It was, however, capable of rescuing and enhancing foci formation by a defective murine sarcoma virus concomitantly with replication of the RaLV (41). This finding indicates that the RaLV normally exists in a non-replicating form and requires the presence of an additional factor, such as infection with another virus, in order to replicate and produce infectious virus.

Like the RaLV, sarcomagenic viruses evidently cannot produce infectious virus in cell cultures (42). On the other hand, the leukemogenic viruses are capable of highly efficient virus synthesis (1). Based on these observations, the RaLV and sarcoma viruses are termed defective because they cannot produce infectious virus independently. However, sarcoma viruses can form foci of transformed cells in susceptible cell lines. The formation of these foci can serve a practical purpose in helping to quantitate the presence of leukemia viruses. Therefore, in vitro studies often deal with both leukemia and sarcoma viruses. The combination of these two closely related viruses allows easier detection and assay of the activities of both viruses.

Studies with Rous Sarcoma Virus conditional lethal mutants indicate that the induction and maintenance of the neoplastic state is under the control of viral genes. These conditional mutants are temperature sensitive (ts) with respect to foci-formation (cellular transformation). It was found that virus infection is necessary for the production of the transforming factor. This was proven by using the ts mutants and showing that altered cellular morphology and foci are present at permissive temperatures, while they disappear in cells grown at restrictive temperatures. The process of interconversion from transformed cells to "nontransformed" cells does not require DNA or RNA synthesis. However, cycloheximide stops recovery at permissive temperatures.

This indicates that a protein is directly involved in transformation and that transformation is apparently controlled at the translational level. The temperature-sensitive lesion apparently has some effect on the structural proteins of the cell but these effects are probably not directly caused by the transforming virus (55, 146).

In addition to showing that viral information is directly responsible for transformation, Vogt (146) has demonstrated that the transforming genes appear to be dispensable for the virus because production of complete infectious virus can be induced to proceed at the temperatures restricting foci formation (transformation). The interpretation of this is that, even though the transforming protein is coded for by the virus, the protein does not appear to be a structural component of the infectious virion.

Biochemical Properties of Oncornaviruses.

Oncornaviruses have several unusual metabolic pathways and products. These viruses have an infectious RNA genome which can be replicated through a DNA intermediate. This DNA is made during the first 8-12 hours after infection. Blockage of new protein synthesis, following infection, does not inhibit the formation of the DNA intermediate (139), indicating that the process is directed by an enzyme already present in either the cell or the infecting virus. It has been subsequently shown that the oncornaviruses contain within the virion a RNA-dependent DNA

polymerase. This enzyme, known also as reverse transcriptase, is involved in the formation of the intermediate DNA molecule (137, 9). The reverse transcriptase has been purified and the molecular weight was measured at 105,000. The enzyme clearly displays a preference for the 70S oncornavirus RNA as its template (13).

In addition to the reverse transcriptase and DNA polymerase activities for the normal synthesis of DNA, the leukemia viruses possess endonuclease and ligase activities. These enzymes provide the leukemia viruses with the capability of integrating virus-coded DNA into the host cell DNA (106).

Studies by Baluda (10) indicate that the virus DNA is indeed present in the nucleus of leukemia cells. He has also found that the virus-produced DNA is covalently bonded to the host cell DNA. As a result, in cells producing infectious virus, the viral RNA is apparently synthesized in the same manner as cellular RNA. Experimental evidence indicates that tumor cells in leukemic chickens contains an average of 5.8 DNA viral equivalents per cell compared to less than 1 DNA viral equivalent in cells from normal or resistant chickens. These data are consistent with the hypothesis that, in chickens, the amount of viral DNA (in terms of viral equivalents) per cell is directly correlated to the capacity of those cells to produce virus and induce spontaneous tumors (10).

After the early DNA synthesis is completed, the necessity of continued DNA transcription to RNA was demonstrated. The addition of Actinomycin D, which specifically inhibits DNA transcription, stopped most viral RNA synthesis. Thus virus production was halted (136, 145).

Other lines of evidence indicate that replication of the leukemia virus may occur by both the RNA to DNA cycle and RNA to RNA cycles. Biswal and Benyesh-Melnick, (14) have reported finding RNA-complementary strands of the viral RNA. Bonar et. al., (15) have also isolated a viral-coded RNA-dependent RNA polymerase capable of directing the RNA to RNA synthesis.

All oncornaviruses apparently contain a 60-70S RNA as the infectious component (10, 13, 5, 26, 50, 129). This large-size molecular weight RNA, representing infectious viral RNA, undergoes segmentation after infection. These two properties, a large (60-70S) RNA and the dissociation of an RNA into subunits upon infection, have only been described within the oncornavirus group.

Separation of several leukemia viruses into their major protein components indicated that except for small differences the feline, hamster, and rat viruses resembled the murine viruses. Each group of viruses had three major protein components compared with the four major components isolated from the avian leukemia

viruses. These viruses also contained two glycoprotein components (123) group specific (gs) proteins which were shown to be similar basic proteins in all of these leukemia viruses (2, 43, 46, 105, 110).

Schafer (123) has reported that in each of the leukemia viruses there was at least 5 detectable antigens. Three of these antigens appeared to be determinants related to species specificity; one antigen was a type-specific antigen and another was an antigen which displayed interspecies specificity.

The oncornaviruses which have been isolated and purified via density gradients had a bouyant density between 1.12 and 1.16 grams/cc (16, 105). This relatively low density was a reflection of the presence of considerable lipid in the viral envelope.

Finally, the nucleic acid of oncornaviruses was a single-stranded RNA with a molecular weight of about 12 million Daltons. This amount of RNA would provide the leukemia viruses with the capability to code for about 25 averaged-sized proteins (39).

RADIOGENIC LEUKEMIA

Ionizing radiation constitutes a known and proven leukemogen for man. Increase in childhood leukemias and other malignancies resulted from exposure to X-rays in utero (3, 97, 132). Leukemia has also resulted from the deposition of various radionuclides in bones and tissues of human beings. Individuals exposed to

atomic bomb blasts (53), as well as humans ingesting radium (57), have both shown significantly higher incidences of hemopoietic disorders. Interestingly, the results of several retrospective studies suggest that long-term irradiation over an extended period of time (102) is less leukemogenic than the short-term or instantaneous irradiation received by either atomic bomb survivors or patients with ankylosing spondylitis treated by radiotherapy (46, 49, 51, 54, 119, 126).

The aforementioned establishment of radiation as a known leukemogen should provide considerable impetus to future research in this area. The study of radiation-induced leukemia in experimental animals should provide an important tool for ferreting out and understanding leukemogenic mechanisms. Obviously, the assumption here is that all or many of the leukemogens have basic similarities in the process or mechanisms leading to the production of leukemia.

Various forms of radiation have been used to induce leukemia in animals. Indeed, the leukemogenic action of radiation has been described in rats (149, 82, 74), guinea pigs (17), dogs (22, 29), swine (103), and mice (75, 83). Of the animal systems described, only the radiogenic leukemia of mice has been studied in depth. The results indicate that viruses play an important role in the development of leukemia in mice exposed to radiation.

Role of Viruses in Radiation-Induced Leukemogenesis.

Virus-like particles have been reported to be present in leukemic animals of several species. C-type particles have been described in rat chloroleukemia (84, 148), murine leukemia (18, 23), and porcine leukemia (62).

Originally, most investigators felt that radiation-induced leukemia was produced through the direct method of somatic mutation. These investigators found that the size of the dose was not the sole factor determining the incidence of leukemia. The incidence could be increased by changing the rate of administration and the degree of fractionation of the dose (78). In addition, it was found that if part of the body was shielded the same dose of radiation did not induce leukemia (72, 73). These facts all refuted a direct-effect method such as somatic mutation.

The indirect action of irradiation suggested that viruses might be participating in the "radiation-induced" leukemias of mice. Radiation leukemia viruses (RaLV) were subsequently isolated and shown to be leukemogenic when transmitted to newborn mice via cell-free filtrates (49, 52, 84, 89).

It has been proposed that radiation and viruses interact to produce leukemia. The theory states that many, if not all leukemogens act through the mediation of occult viruses and

that the role of these leukemogens (whether radiation, chemical, or otherwise) is to produce injury to the bone marrow or reticuloendothelial systems. The leukemogens then may also act directly to allow the release of the occult viruses. It is the virus that directly induced the neoplastic conversion (80).

Large immature lymphoid cells have been described in the thymus of neonatal mice (88, 7). It has been speculated that these cells, which decrease rapidly after birth, are the target cells for leukemogenesis. A leukemogenic virus must infect one of these cells in order to fulfill its leukemogenic potential. Therefore, the effect of radiation or other "leukemogens" would be injury to the thymus and the subsequent regeneration of the thymus. This regeneration process would result in restoration of the immature target cells. These target cells would of course be available for infection with the RaLV and the subsequent neoplastic transformation (79).

Radiostrontium in Miniature Swine.

The chronic feeding of ^{90}Sr to Pitman-Moore strain miniature swine was initiated originally as a toxicity study. A total of 763 pigs, including parental, F_1 , and F_2 generations, have been fed ^{90}Sr at dose levels from 1 to 3100 $\mu\text{Ci/day}$ for their lifetime. In addition, 194 untreated litter mates were utilized as controls (Table 2.1).

Table 2.1

NUMBER OF SWINE ON ⁹⁰Sr
TOXICITY STUDY

<u>FEEDING LEVEL</u> <u>(μCi/DAY)</u>	<u>PARENTS</u>	<u>OFFSPRING</u>
3100	5	---
625	20	34
313	4	15
125	38	127
25	26	195
5	10	80
1	<u>24</u>	<u>195</u>
TOTAL FED	127	646
CONTROLS	78	116

Strontium-90 is known to compete with calcium for incorporation into the bone. This bone-seeking radionuclide has a half-life for 27 years, during which it emits beta rays having an activity of 0.54 MeV. Using thermoluminescent dosimetry (112, 147), the radiation dose rates in rads/day to the bone and soft tissue were calculated for each of the feeding groups (Table 2.2).

The bone-seeking nature of ^{90}Sr , as well as reports of bone neoplasia in humans, primarily radium dial painters (57) and in small laboratory animals by Finkel (40), resulted in a prediction that bone tumors would be the primary finding in the swine experiment. Eleven bone tumors have been found occurring in several animals (Table 2.3). These tumors however, were always found in leukemic animals.

Instead of producing large numbers of bone tumors, the chronic feeding of ^{90}Sr to Pitman-Moore strain has provided a model system for the induction of hemopoietic neoplasia (Table 2.4), while this effect has been observed in animals exposed as adults, a much higher incidence and shorter induction period occurs in those animals exposed to ^{90}Sr prenatally, by way of the colostrum, and during early pre-natal life. A broad range of histologic types of leukemia were observed with the majority being myeloid in origin. Lymphoid neoplasms

Table 2.2

^{90}Sr RADIATION DOSE RATES IN SWINE

^{90}Sr FEEDING LEVEL ($\mu\text{Ci}/\text{DAY}$)	DOSE RATES TO BONE (rad/day)		
	ORIGINAL ADULTS	FIRST AND SECOND GENERATIONS	
		FETUSES	ADULTS
1	0.064	0.004	0.072
5	0.32	0.02	0.36
25	1.6	0.1	1.8
125	8	0.5	11
625	40	2.5	-----

SOFT TISSUE DOSE RATES - 1/1000 OF BONE DOSE RATES

Table 2.3

BONE TUMORS IN ^{90}Sr SWINE

^{90}Sr LEVEL ($\mu\text{Ci}/\text{DAY}$)	NO. OF ANIMALS	NO. OF TUMORS	LOCATION
625	1	1	LEG
	1	1	JAW
125	1	3	SKULL RIB JAW
125	1	1	SKULL
125	1	3	SKULL UPPER JAW LOWER JAW
125	1	1	SKULL
125	1	1	SKULL
TOTAL	7	11	

Table 2.4

BLOOD DISORDERS IN ^{90}Sr SWINE

^{90}Sr LEVEL ($\mu\text{Ci}/\text{DAY}$)	GENERATION	DIAGNOSIS			
		LEUKEMIAS			NON-MALIGNANT DISORDERS
		LYMPHOCYTIC	STEM CELL	MYELOGENEOUS	
3100	PARENT	--	--	--	2
625	PARENT	--	--	--	2
	OFFSPRING	1	--	1	15
125	PARENT	1	--	3	3
	OFFSPRING	7	3	14	2
25	PARENT	--	--	2	--
	OFFSPRING	2	0	1	5
5	PARENT	--	--	--	--
	OFFSPRING	1	--	--	--
1	PARENT	1	--	--	1
	OFFSPRING	4	--	--	1
0		--	--	2	--
	TOTAL	17	3	23	31

have also occurred, primarily at lower radiation exposure levels, and in animals exposed in utero through the colostrum and post-weaning.

Hematologic evaluation indicates that the ^{90}Sr induces a chronic leukopenia, which transforms to a leukocytosis and blastic stage terminally.

In an attempt to understand the mechanism of the radiation induction of leukemia in swine, the problem has been examined from several view points. For example, it was observed that leukocyte alkaline phosphatase (LAP) values of swine showing clinical symptoms of granulocytic leukemia decreased from a score of 200 to a level of about 20 during the course of the disease (53). This mimics the decrease in LAP observed in humans with chronic granulocytic leukemia (99). Attempts were then made to correlate this observed decrease in LAP with cytogenetic changes. Unfortunately, the chromosome analyses of these groups to date have shown nothing analogous to the Philadelphia Chromosomal abnormality found in human beings. The breaks and other chromosome abnormalities seen in the chromosomes of the ^{90}Sr -fed leukemic animals appear to be attributable to radiation damage (64).

Studies of the mechanism of radiation leukemogenesis have indicated that among the different effects caused by X-irradiation, one important factor is the transient depression of the immune response of the host. Therefore, a preliminary investigation was initiated to ascertain whether the chronic feeding of ^{90}Sr affected the production of humoral antibodies. A decrease in the normal immunological mechanism was demonstrated by the decreased primary antibody response to the Brucella abortus vaccine in these animals. Leukocytes from animals similarly exposed to ^{90}Sr -fed animals failed to respond normally to stimulation with phytohemagglutinin (PHA). These studies also suggested an impaired immunological response in the ^{90}Sr -fed swine (64).

A series of investigations to evaluate the effect of internally deposited radionuclides on normal "cellular immune mechanism" (i.e., interferon, macrophage migration, and lymphocyte transformation) was initiated. This type of impaired immune responsiveness is more often associated with malignant disease.

The results of studies on the interferon production in the peripheral leukocytes of swine exposed to ^{90}Sr are shown in Table 2.5. Although the values for ^{90}Sr exposed swine appear to be slightly lower, these differences do not reflect a significant reduction in interferon titers in these animals (44).

Table 2.5

PRODUCTION OF INTERFERON FROM PERIPHERAL LEUKOCYTES

<u>ANIMAL NO. AND DESCRIPTION</u>	<u>INTERFERON TITERS *</u>	
	<u>NC VIRUS</u>	<u>INFLUENZA 'A'</u>
CONTROL - LEUKOCYTE CULTURES		
4018	<3	768
4022	<3	384
4152	<3	768
4153	<3	> 1536
4154	<3	384
125 μCi/DAY ^{90}Sr - LEUKOCYTE CULTURES		
4004	<3	384
4007	<3	192
3987	<3	192
3573	<3	768
3587	<3	384

* EXPRESSED AS RECIPROCAL DILUTION THAT INHIBITS 100 PFU OF VSV

A modification of the macrophage migration procedure was employed as an indicator of the delayed hypersensitivity responsiveness of these same animals. This allowed me to make some assessment of the effect of chronic ingestion of radionuclides on the immune mechanisms of swine. The results of a typical experiment (Table 2.6) indicates that chronic exposure to 125 μ Ci/day of ^{90}Sr results in an inhibition of delayed mechanisms of cellular immunity, as measured by the migration of these peripheral leukocyte in culture (44).

Regardless of the form of ionizing radiation or the experimental animal, the pathogenic mechanism of radiation-induced leukemia is not completely understood. Several possible mechanisms have been studied in an attempt to explain the development of leukemia subsequent to radiation exposure.

The transmissibility of certain radiation associated leukemias in mice, rats, and dogs naturally led us to search for an oncogenic virus as the inciting factor. Viruses morphologically similar to known leukemogenic viruses were demonstrated (41, 43, 59, 62). These findings led us to postulate that certain radiation-induced leukemias are caused by potentially leukemogenic viruses that are in some manner (whether by immune suppression or another mechanism) either "activated" or released from an occult state by the radiation insult. This concept

Table 2.6

IN VITRO MIGRATION OF PERIPHERAL LEUKOCYTE CULTURES FROM MINIATURE SWINE

ANIMAL GROUP	MIGRATION INDEX *			
	30 µg/ml PPD		200 µg/ml PPD	
	AVERAGE	RANGE	AVERAGE	RANGE
CONTROL - NOT IMMUNIZED	97	87-105	101	89-106
CONTROL - IMMUNIZED WITH FREUND'S COMPLETE ADJUVANT	59	49-65	30	26-39
125 µCi/DAY - NOT IMMUNIZED	91	82-99	97	94-99
125 µCi/DAY - IMMUNIZED WITH FREUND'S COMPLETE ADJUVANT	84	81-87	90	85-92

$$* \text{MIGRATION INDEX} = \frac{\text{MIGRATION AREA OF CULTURES IN MEDIUM CONTAINING PPD}}{\text{MIGRATION AREA OF CULTURES IN STANDARD MEDIUM}} \times 100$$

initially resulted in an examination of the tissues and body fluids of swine for these viruses. The findings of these studies comprise the experimental section of this thesis.

Chapter 3

MATERIALS AND METHODS

CELL CULTURE

Accepted guidelines for washing and care of all glassware used in the cell culture studies were followed (113). The detergent used was 7-X (Linbro Chemical Company, New Haven, CT).

Solutions and Media.

Unless otherwise stated, all solutions and media were stored at 2 to 4 C.

Antibiotics. A stock solution containing a mixture of 20,000 units potassium penicillin G (Chas. Pfizer and Co., New York) and 15,000 μ g streptomycin sulfate (E. R. Squibbs and Sons, New York) per milliliter in sterile deionized distilled water (DDW) was stored at -20 C. Five ml of this solution was added to each 1000 ml of medium to give concentrations of 100 units penicillin and 100 μ g streptomycin/ml of medium.

Amphotericin B (Fungizone) (E. R. Squibb and Sons, New York) was dissolved in sterile DDW at a concentration of 12,000 μ g/ml and stored at -20 C. This stock solution was stored at -20 C. For use, 3.3 ml was added to 1000 ml of medium to give a concentration of 40 μ g amphotericin B/ml.

Kanamycin sulfate (donated by Bristol Laboratories, Syracuse) was dissolved in sterile DDW at 20,000 $\mu\text{g/ml}$ and stored at -20°C . Five ml in 1000 ml of medium gave a final concentration of 100 μg of kanamycin/ml.

Unless otherwise stated, all cell culture media and salt solutions contained the penicillin and streptomycin mixture. Kanamycin and amphotericin B were only used for subculturing stock cells and were never added to medium used in experimental procedures.

Sodium bicarbonate. A stock solution containing 7.5% NaHCO_3 was prepared monthly and sterilized by millipore (Millipore Filter Corp., Bedford, MA) filtration.

Earle's balanced salt solution (EBSS) (31). The composition of EBSS is given in the appendix. This solution was purchased in powdered form from GIBCO (Grand Island Biological Company, NY). Ten-times concentrated stock solutions were prepared by dissolving 43.5 g of the powder in 5 liters of DDW. After proper mixing, the solution was sterilized by filtration.

For use as single strength EBSS, 10 ml of the stock solution and 3 ml of 7.5% NaHCO_3 were aseptically added to 87 ml of sterile DDW.

Hanks' balanced salt solution (HBSS) (56). Stock solutions for making HBSS were prepared by dissolving 49.5 of powdered HBSS mixture (GIBCO) in 5,000 ml of DDW giving a tenfold concentration of HBSS. The resulting solution was filter sterilized and dispensed into 8 oz. prescription bottles.

Single strength HBSS was prepared by adding 10 ml of the stock solution and 0.5 ml of 7.5% NaHCO_3 to 89.5 ml of DDW.

Phosphate buffered saline (PBS) (27, 28). PBS was prepared as follows:

Solution A

<u>Components</u>	<u>Amount</u>
NaCl	16.00 g
KCl	0.40 g
Na_2HPO_4	2.30 g
KH_2PO_4	0.40 g
DDW	1,500 ml

Solution B

MgCl_2	0.20 g
DDW	200 ml

Solution C

CaCl ₂	0.20 g
DDW	200 ml

These solutions were autoclaved for 20 min at 15 psi. After cooling to 5 C, the final solution was prepared by adding solution B and solution C in order to solution A, and then bringing the volume to 2 liters with sterile DDW. The resulting solution was dispensed into sterile 8 oz prescription bottles for storage.

A modification of PBS was also prepared by dissolving the components of solution A in 2,000 ml of DDW. The resulting solution was autoclaved for 20 min at 15 psi in sterile 8 oz prescription bottles. This solution referred to as PD was used as a balanced salt solution. The advantage of PD was the absence of Mg⁺⁺ and Ca⁺⁺ thus facilitating trypsinizing procedures and the action of versene.

Versene or EDTA. Dissolve 0.2 g ethylenediamine tetracetic acid (EDTA or versene) in 1,000 ml of PD and filter through a Seitz filter.

Collagenase. Collagenase was purchased from NBC (Nutritional Biochemical Corporation, Cleveland) in a sterile vial containing 50 ml of the enzyme. Five mg of collagenase was dissolved in 100 ml of sterile PD.

Trypsin. Dissolved 2.5 g of 1:300 trypsin (Schwarz Mann, Orangeburg, NY) in 1,000 ml of PD by agitation with a magnetic stirrer for 60 min at 20 C. The pH is adjusted to 7.8, the solution is filtered through a Seitz-filter, dispensed into sterile bottles and stored at 4 C. This solution was used for trypsinizing tissue to establish primary cell cultures. However, the stock trypsin solution was diluted 1:5 for use in subculturing of established cell cultures.

Serum. Fetal bovine serum was purchased from MIA (Microbiological Associates Inc., Bethesda, MD) and stored at -20 C. Serum was heat inactivated at 56 C shortly before use in preparing growth medium.

Agar. A modification of Dulbecco and Vogts' technique (28) was used for washing the Bacto-agar (Difco Labs., Detroit). One-half pound of this agar was washed 25 times in tap water, 10 times in DDW (using 5 liters/wash) and 3 times in acetone (2 liters/wash). The acetone was removed by filtering through a Büchner funnel. The agar was then dried under 3 desk lamps and collected when completely dry.

The agar was diluted in DDW to give twice the concentration of agar desired in the final overlay mixture. The agar solution was then autoclaved at 20 psi for 20 min and cooled to 42 C in a water bath immediately prior to use.

Crystal violet. A 1.4% solution of crystal violet was diluted 1:100 in tapwater for use in staining cell monolayers in the plaque assays and cell clones in the cloning efficiency studies.

Trypan blue. Four grams of trypan blue were dissolved in 100 ml of PBS. The solution was filtered through a millipore and stored at room temperature in sterile tubes.

Growth medium. NCTC 135 (34, 35, 36) was purchased in powdered form (GIBCO). This powder was stored at -20 C until utilized. Growth medium was prepared in the following manner:

<u>Order</u>	<u>Component</u>
1	4,500 ml of DDW
2	47 g of powdered NCTC 135
3	7.5 g NaHCO_3
4	50 ml of penicillin-streptomycin (pen-step) mixture
5	DDW to a total volume of 5,000 ml
6	1,000 ml fetal bovine serum (FBS)

The solution was mixed thoroughly using a magnetic stirrer and then filtered through a Seitz-filter into sterile bottles. This medium is referred to as either growth medium or NCTC 135-FBS 20.

Maintenance medium. Minimum essential medium (MEM) (30) was also purchased from GIBCO as a powder. This medium contained the following constituents:

<u>Order</u>	<u>Component</u>
1	4,500 ml DDW
2	49 g of MEM powder
3	11 g Na HCO ₃
4	50 ml pen-strep
5	DDW to a total volume 5,000 ml
6	100 ml FBS

After mixing thoroughly, the maintenance medium was filtered through a Seitz-filter and stored until use. The medium is hereafter referred to as MEM-FBS 2.

Cell Culturing Techniques.

Processing tissues and preparation of cell suspensions. The tissues were aseptically removed from freshly killed and exsanguinated monkeys. The organ or tissue was then trimmed in

order to obtain the portion desired which was then cut into small pieces (at least $1 \times 1 \text{ cm}^2$). The fragments were then washed in PD to remove the blood and then transferred to a trypsinizing flask. Then 50-70 ml of prewarmed trypsin was added and these tissue fragments and trypsin were agitated using a magnetic stirrer. After 10 minutes the supernatant was carefully removed and discarded. Fifty ml of fresh trypsin was added and allowed to stir for 15 minutes. The resulting supernatant contains a cell suspension which was collected in a 100 ml centrifuge bottle that contains approximately 50 ml of growth medium. The cell suspension was kept in an ice bath. The trypsinizing steps were repeated until the tissues were exhausted. The cell suspension was centrifuged at 600 rpm for 10 min. The supernatant was removed and the packed cells were resuspended in growth medium using approximately 50 ml for each 1 ml of packed cells. The cells were counted by adding 0.5 ml of the cell suspension to 0.1 ml of 0.4% trypan blue. After mixing well the viable cells were counted using a hemocytometer. The cell concentration was adjusted to 3×10^5 cells/ml and the cells seeded in flasks or onto slide cultures.

Collagenase treatment of cornea. The cornea was removed using aseptic techniques and the cornea was carefully trimmed to exclude the retina and white tissue around the cornea. The cornea was washed twice in PD containing antibiotics and then minced into pieces 1 mm square and placed in a trypsinizing flask. The pieces were washed twice with PD 50 ml of collagenase solution and gently agitated using a magnetic stirrer at 37 C for 60 min. The supernatant was removed and added to cold NCTC 109-FBS-10 centrifuged and cells were put into a flask. The pieces of cornea remaining in the trypsinization flask were then dipped in reconstituted chicken plasma (GIBCO) and placed in a Falcon flask (Falcon Plastics, Los Angeles). Then a drop of reconstituted chick embryo extract (GIBCO) was placed on the tissue pieces and these were allowed to set up for at least 15 minutes. Finally, growth medium was carefully added (with the flask upside down) in order to keep from disturbing the plasma clots.

When the cells in these clots were harvested, a trypsin solution was added and the clot was digested and removed. Then the cell monolayers were treated with a fresh trypsin solution and passed, employing the same procedure used with monolayer cultures.

Subculturing. All cell lines and cell cultures were cultured in presterilized plastic petri dishes (Falcon). The cells were grown in NCTC 135-FBS-20. Incubation was at 37.5 C in an atmosphere of 95% air and 5% CO₂. To prepare a cell suspension for transfer, the monolayered cell cultures were first washed several times with 10 ml of PBS, prewarmed at 37 C, and then trypsinized for 5 to 10 min at 37.5 C with 5 ml of prewarmed, diluted trypsin solution. Following this, the cells were gently pipetted back and forth several times to disperse any clumps, and 5 ml of NCTC 109-FBS-20 were added to the suspension. After thorough but gentle mixing, a viable cell count was performed using trypan blue and a hemocytometer. The suspension was then diluted with growth medium to the desired cell concentration.

Preservation of cells. One ml of sterile glycerin and 8.5 ml of growth medium were added to 0.5 ml of packed cells. The resulting suspension was mixed and 1 ml aliquots dispensed in 2 ml ampules. The cells were frozen using liquid nitrogen and the Linde BF-4 freezing apparatus.

Cytopathic effect in cell cultures. Whole blood specimens from use in virus isolation were processed immediately.

Heparinized blood was centrifuged for 20 min at 1,500 rpm and the plasma removed and added to an equal volume of PBS. One ml amounts of the blood cell suspension were added to various cell cultures. After 3 hours of incubation at 37 C, the cultures were washed with three changes of medium and replace with fresh maintenance medium. The cultures were observed daily using phase contrast microscopy for cytopathic changes, for 4 to 6 weeks, replacing the maintenance medium every 3 days.

Plaque assay procedure. A modification of the technique described by Dulbecco and Vogt (27) was employed. The general procedure is stated in the following section.

One million cells in volume of 5 ml of NCTC 135-FBS-20 were placed into 60-mm plastic petri dishes (Falcon). The dishes were incubated at 37 C until a nearly confluent monolayer developed. The growth medium was removed, and each monolayer washed with 3 ml of PBS. After removing the wash fluid, a 0.1 ml of a known dilution of the stock virus was added to each of the cell monolayers. The virus was diluted in MEM-FBS-2 serum (30), and all dilutions were made at 4 C. Virus adsorption was allowed to proceed at room temperature for 150 min. The agar overlay medium was added to the monolayers after removal of the inoculum with another washing using 3 ml PBS. In all experiments control cultures were treated identically except that the adsorption medium contained only a diluent similar to the virus-containing solutions.

To prepare overlay medium, equal volumes of washed Bacto-agar solution (1.3%) and double-strength MEM-FBS2, both held at 42 C, were mixed. Finally, 7.5% NaHCO_3 prewarmed to 37 C was added to the mixture to give a final concentration of 0.15%. Four ml of the resultant solution were added to each culture dish and allowed to cool and gel.

When the agar solidified, 4 ml of MEM-FBS2 were added to each culture as a supplement. The plates were incubated at 37.5 C in the air CO_2 mixture. The supplemental medium was removed and replaced with fresh medium every three days. After 12-14 days, the supplemental medium and the agar overlay was carefully removed and the remaining monolayers were stained using a modification of the technique of Holland and McLaren (58). Five ml of 75% ethanol were added to the monolayer to fix the cells. After 2 min, the alcohol was decanted and the cells were stained with crystal violet for approximately 20 seconds. The stain was decanted, and the cell layer rinsed with tap water. The plates were inverted and allowed to dry. Plaques appeared as transparent circular areas in a dark purple background.

Growth of cells in agar suspensions (98). A base layer was prepared by mixing (at 42 C) double strength MEM-FBS10 with equal volumes of 1% (in DDW) washed agar (27, 28). Approximately 7 ml of this mixture was then added to 60 mm petri dishes (Falcon Plastics) and allowed to set up.

The second layer contains the same double strength MEM-FBS10 mixed with an equal amount of 0.6% agar. To this solution a known number of cells were added (at 37 C), following mixing, this cell suspension was added on top of the base layer. The plates were incubated for 7-10 days at 37 C in an atmosphere of 5% CO₂ and humidified air. Colony counts were made with a stereomicroscope.

Mycoplasma cultures. A commercially prepared medium from GIBCO was used for the growth of mycoplasma.

CYTOLOGICAL AND HISTOLOGICAL PROCEDURES

Stains and Fixatives.

Bouins' solution.

750 ml saturated aqueous picuric acid

250 ml formalin

50 ml glacial acetic acid

Filter prior to use.

Carnoy's solution. Carnoy's was prepared by combining 1 part glacial acetic acid, 3 parts chloroform, and 6 parts ethanol (96-100%).

Neutral buffered formaldehyde (NBF). Formaldehyde was neutralized by the addition of powdered calcium carbonate and gentle shaking. BNF was prepared in the following manner:

100 ml of 36-38% neutralized formaldehyde solution

900 ml tap water

4.0 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

6.5 g anhydrous Na_2HPO_4

Acetic alcohol fixative.

25 ml glacial acetic acid

75 ml absolute methanol

Colchicine. Colchicine (Eli Lilly and Co., Indianapolis, IN) was purchased as a solution containing 0.5 mg/cc. This was diluted in DDW to give a final concentration of 1,000 colchicine in 5 ml; then, 0.25 ml of this was added to each 10 ml volume of medium and cells.

Phytohemagglutinin (PHA). Purchased commercially (Burroughs Wellcome & Co., London) as a freeze-dried material, the PHA was reconstituted with 5 ml DDW. For each 10 ml volume of medium and cells, 0.1 ml of PHA was utilized.

Giemsa staining.

stock A: 0.2M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

stock B: 0.2M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

Sixty-eight and one half ml of stock A was to 31.5 ml of stock B, and the pH was adjusted to 6.5. Five ml of this buffer was mixed with 5 ml of giemsa stain solution (Carolina Biological, NC) and 40 ml of DDW. The cells to be stained were placed in this final solution for 10 minutes. Following staining the slide was washed with DDW and blotted dry (these slides were mounted when necessary).

Kahle's fluid.

glacial acetic acid	3 ml
95% alcohol	45 ml
Formalin	18 ml
DDW	90 ml

Mc Ilvaine citric acid disodium phosphate buffer. Stock

solutions are preparing this buffer were made up as follows:

citric acid stock solution (0.1M)

citric acid 19.2 g

methanol (25%) 1,000 ml

disodium phosphate stock solution (0.2M)

Na_2HPO_4 28.4 g

methanol (25%) 1,000 ml

The buffer was finally prepared by combining 10 ml of the citric acid stock solution and 10 ml of the disodium phosphate stock solution.

Citric acid 1%.

citric acid	1.0 g
DDW	100 ml

Acetate buffer. Seventy-five ml of 0.2M glacial acetic acid was mixed with 25 ml of 0.2M sodium acetate, and the pH adjusted to 4.2 with either sodium hydroxide or acetic acid.

Purification of methyl green. One gram of methyl green was dissolved in 200 ml of acetate buffer. This solution was then washed in a separatory funnel with chloroform until all the violet color was removed.

Methyl green pyronin Y solution. One-tenth gram of pyronin Y was added to a 100 ml solution of 5% methyl green in acetate buffer (pH 4.2). After thorough mixing, the solution was ready for use. This solution was stored in a tightly sealed bottle at 4 C.

Schiff's reagent. This was prepared by adding 1 g of basic fuchsin to 200 ml of boiling DDW. The solution was then cooled to 50 C and filtered through Whatman filter paper. Twenty ml of 1N HCl was then added to the solution which was allowed to cool to room

temperature. Then 2 g of $K_2S_2O_5$ was added and the solution allowed to bleach for 24 hours. After 2 g of charcoal were added the solution was shaken for 1 minute and filtered through a coarse filter. The Schiff's is then ready for use. This solution was stored in an amber bottle.

Sulfurous acid solution. Add 5 ml 1N HCL to a solution of 0.5 g Na metabisulfite in 100 ml. This solution was prepared immediately prior to use because of its instability.

Fast green. Add 2 g of fast green powder to 100 ml of 95% ethanol.

Sodium periodate solution. One gram of sodium periodate was dissolved in 0.5 ml 70% HNO_3 and DDW was added to bring the final volume to 100 ml.

Staining Techniques.

Acridine orange staining procedure (66). The cells to be examined were grown on microscope slides in Leighton tubes. The slides were washed three times in PBS, and fixed in Carnoy's solution for 15 seconds. The cells were hydrated using an ethanol series of 95, 70, 50, and finally 30% solutions. The cells were then dipped in 1% citric acid solution, rinsed in DDW and placed in McIlvaine's citric acid disodium phosphate buffer (pH 4.95) for 5 minutes. Staining followed in a 0.01% acridine orange

solution (George Gurr Ltd., London) in McIlvaine's buffer (pH 4.95) for 5 minutes. The slide was rinsed in buffer for 5 minutes, mounted in buffer and examined using an American Optical Ultraviolet microscope.

Fuelgen staining technique (86).

<u>Procedure</u>	<u>Time</u>
fix slide in Kahles	30 min
70% alcohol	60 min
triethyl phosphate	30 sec
95% ethanol	30 sec
95% ethanol	30 sec
H ₂ O	30 sec
H ₂ O	30 sec
1N HCL	1 min
1N HCL (at 60 C)	10 min
1N HCL	1 min
Schiffs	10 min
fresh sulfurous acid solution	2 min
fresh sulfurous acid solution	2 min
fresh sulfurous acid solution	2 min
running tap H ₂ O	5 min
counterstain in Fast Green	5 min
95% ethanol	30 sec
95% ethanol	30 sec
triethyl phosphate	30 sec
xylene	3 min
xylene	3 min
mount slide	

Hematoxylin eosin staining technique (100). The cells were grown in monolayers on glass slides in Leighton tubes. After the designated time period, the slide was removed, washed three times in PBS and then processed as follows:

<u>Procedure</u>	<u>Time</u>
fixed in Bouin's solution	1 hr
95% ethanol	8-12 hr
Ehrlick Hematoxylin	60 min
H ₂ O	20 sec
rapid differentiation in 0.25%	
HCL in 75% ethanol	2-4 sec
H ₂ O	2-4 sec
H ₂ O	2-4 sec
70% ethanol	20 sec
eosin (1% in 95% ethanol)	2 min
absolute alcohol	20 sec
absolute alcohol	20 sec
xylene	30 sec
xylene	30 sec
mount	

Chromosome analysis of swine cell cultures. A confluent monolayer of cells was trypsinized and divided 1:2. Twenty-four to forty-eight hours later colchicine was added to a final concentration of 10 γ /ml. After four hours exposure to colchicine, the culture was treated in the following manner.

1. Wash culture three times with PD, then trypsinize after the cells are removed, add an equal volume of maintenance medium to the cell, and centrifuge at 800 rpm for 5 min.
2. Remove the supernate, but leave 0.4 ml of cells and medium in the centrifuge tube.
3. Add slowly at 37 C 0.5% sodium citrate to the 2.5 ml mark without disturbing the cells.
4. Incubate in water bath at 37 C for 20 minutes, then resuspend cells by tapping the centrifuge tube.
5. After an additional 5 minutes at 37 C, centrifuge the cells at 800 rpm for 5 min.
6. Remove the supernate again leaving 0.4 ml of fluid and cells, add acetic alcohol fixative to the 2 ml mark without disturbing the button.
7. After allowing the cells to fix for 30 minutes, centrifuge at 800 rpm for 5 minutes. Remove the fixative, add fresh fixative to the 2 ml mark, and mix the cells thoroughly.
8. Thirty minutes later centrifuge and remove the fixative as completely as possible, then add 0.5 ml of cold acetic alcohol fixative, mix the cells, and prepare chromosome slides.
9. The slides to be used must be properly prepared by washing with warm 7X solution using a cotton swab. The slide

was thoroughly rinsed in cold tap water and cells are immediately dropped onto the angled glass slide from a height of 6-10 inches.

10. The slides were allowed to air dry and then stained using giemsa.

Periodic acid leucofuchsin (PAS) method (90). Cells were grown in cell culture on microscope slides. Just prior to staining, the cells were washed three times with PBS to remove the medium and protein constituents. The slide was then placed in Kahles solution and the cells fixed for 30 minutes before staining.

<u>Staining Schedule</u>	<u>Time</u>
70% alcohol	60 min
50% alcohol	2 min
30% alcohol	2 min
30% alcohol	2 min
tap H ₂ O	2 min
DDW	2 min
1% sodium periodate solution	10 min
running tap water	10 min
schiff reagent	40 min
fresh sulfurous acid solution	2 min
fresh sulfurous acid solution	2 min
fresh sulfurous acid solution	2 min
running tap H ₂ O	2 min
30% alcohol	30 sec
70% alcohol	30 sec

95% alcohol	30 sec
95% alcohol	30 sec
absolute alcohol	30 sec
absolute alcohol	30 sec
xylene	3 min
xylene	3 min
mount	

Methyl green pyronin Y staining of RNA and DNA (135).

Again the cells were cultured on microscope slides for this staining technique.

<u>Procedure</u>	<u>Time</u>
Wash slide three times in PBS.	
fix cells in Kahles Solution	30 min
70% alcohol	60 min
triethyl phosphate	30 sec
95% alcohol	30 sec
95% alcohol	30 sec
DDW alcohol	30 sec
methyl green pyronin Y	60 min
DDW	5 sec
Blot dry with filter paper.	
75% tertiary butyl alcohol:25%	30 min
absolute ethanol with constant agitation	
xylene	5 min
xylene	5 min
mount slide	

ELECTRON MICROSCOPY

Reagents

Cacodylate buffer. This buffer consists of 0.1M solution of the sodium salt of cacodylic acid (Matheson Coleman & Bell, Cincinnati, OH) adjusted to pH 7.5 with 0.1 1N HCL.

$(\text{CH}_3)_2\text{AsO}_2\text{Na}$	1.60 g
DDW	100 ml

Ten ml of 2% OsO_4 is added to 10 ml of 0.2M cacodylate buffer giving the 1% solution used for fixing tissues and plasma pellets.

Glutaraldehyde. Glutaraldehyde was purchased (TAAB Labs., England) as a 25% aqueous solution. For fixing cell cultures and solid tissues, a 2% solution of glutaraldehyde was required for fixation. Therefore, 8 ml of glutaraldehyde stock was added to 92 ml of 0.1M cacodylate buffer.

Uranyl acetate. Two grams of uranyl acetate was dissolved in 100 ml H_2O and this solution adjusted to pH 3.8 with 1N HCL and filtered through coarse filter paper.

Lead citrate.

Lead nitrate $\text{Pb}(\text{NO}_3)_2$	1.33 g
sodium citrate	1.76 g
DDW (boiled)	30 ml

The solution was mixed for a full minute followed by intermittent shaking for 30 minutes. Eight ml of 1N Sodium hydroxide (carbonate free) was subsequently added and the solution was finally diluted to 50 ml with boiled DDW. The pH of the solution was then adjusted to 12.

Embedding material for electron microscopy.

Mixture A

Epon 812	62 ml
dodecyl succinic anhydride	100 ml

Mixture B

Epon 812	100 ml
methyl nadic anhydride	18 ml

Six parts of A were mixed with four parts of B. Finally, 0.15% of the catalyst DMP-30 was added, mixed thoroughly, and the Epon was ready to use for embedding.

Method of Fixation.

The animal was killed and the tissues removed as rapidly as possible in order to obtain tissues in a normal physiological state. The tissues were removed and placed immediately onto a chilled teflon slab and trimmed. Then they were quickly placed in a pool of prechilled fixative (2% glutaraldehyde in cacodylate buffer).

The tissue was minced using a razor blade into approximately 1 mm³ pieces. These tissue fragments were poured into a bottle of cold fixative and allowed to stand for 4 hours in an ice bath. The fixative was decanted and the tissue was washed three times with fresh cold (.1M) cacodylate buffer. When the final wash or buffer was removed the tissue was fixed a second time with the 1% osmic tetroxide for 1 hr under a hood. The tissue pieces were again washed three times with fresh cacodylate buffer and the specimens dehydrated and embedded in Epon.

The methods for fixing cell culture and virus preparations were similar to those for tissues with only a few exceptions. These samples were washed several times with PD prior to fixation in order to remove any proteins present in the external medium. This step, as well as others in the early procedure, required utilizing either a conventional or a preparative centrifuge in order to pellet the cells or viruses so the supernatants could be decanted.

Embedding Technique (96).

After the specimen has been properly fixed, it was then prepared for embedding by the following step by step procedure:

	<u>Time</u>
30% ethanol (ice bath)	10 min
50% ethanol (ice bath)	10 min
70% ethanol (ice bath)	10 min
95% ethanol	10 min
100% ethanol	10 min
100% ethanol	10 min
propylene oxide	10 min
1 part propylene oxide: 1 part embedding Epon with constant mixing	2 overnight
embedding Epon (in desiccator) with constant mixing	2 overnight

The samples were now ready for embedding and the Epon was dispensed into labelled BEEM (PELCO, Tustin, CA) capsules. The specimens were then properly oriented using a toothpick. The curing procedure (96) consists of a series of successive 12 hour steps (minimum incubation) at 35 C, 45 C, and 60 C. The specimens were removed from the oven and allowed to cool before trimming and sectioning.

Staining Procedure.

The grid containing the material to be stained was placed face down in a drop of uranyl acetate in a small covered dish. After 2 hours of staining, the grid was rinsed in DDW and blotted dry. The other side of the grid was stained in an identical manner.

The lead citrate staining was performed in a petri dish containing some sodium hydroxide pellets to remove CO_2 from the air. The grids were stained one at a time in this closed petri dish by floating the grid in a drop of lead citrate solution for 10 minutes. Following the staining these grids were rinsed in at least six different vials of fresh DDW, being sure to dip the grid at least 20 times in each vial.

SEROLOGY

Reagents

Alsever's solution (66).

dextrose	20.5 g
sodium citrate	8.0 g
citric acid	0.55 gg
NaCL	4.2 g
DDW	1,000 ml

Dissolve each compound in the order listed and dispense into 100 ml amounts. The solution was then autoclaved at 10 lbs. for 10 minutes. Each bottle of Alsever's was checked for contamination by culturing 1 ml in thioglycollate broth. The Alsever's was stored at 4 C.

Barbital (5,5-diethyl barbituric acid). Barbital of USP Grade was dissolved in hot ethyl alcohol until a saturated solution was obtained. The saturated solution was chilled and crystals allowed to form. These crystals were removed by filtration, and used for making buffers.

Sodium barbital (Na 5,5-diethyl barbituate). A solution of ethyl alcohol was saturated at room temperature with barbital, then with gentle stirring 0.2M NaOH (in alcohol) was added until the solution was alkaline. The solution was then cooled and the sodium barbital allowed to precipitate. The crystals were removed by filtration, dried, and used for making buffers.

Veronal buffer diluent.

Solution A

NaCl	83.8 g
NaHCO ₃	2.52 g
sodium barbital	3.09 g
DDW	1000 ml

Solution B

barbital	4.6 g
MgCl ₂ ·6H ₂ O	1.0 g
CaCl ₂ ·2H ₂ O	0.2 g
hot DDW	500 ml

These stock solutions were stored at 4 C until used.

A fresh 5X concentrate of the buffer was made up the day it was to be used by adding two parts solution A to one part solution B and one part DDW.

The working buffer was prepared by diluting the concentrate exactly five fold in DDW. The pH must be 7.3 to 7.4. Then bovine serum albumin (BSA) was added to give a final concentration of 1.1%. The solution was ready for use after sterilizing by filtration.

Borate buffered saline pH 9.0. This solution was prepared by diluting 80 ml of 1.5M NaCl, 100 ml 0.5M H_3BO_3 , and 24 ml of 1M NaOH to 1000 ml with DDW.

Erythrocyte storage for hemagglutination tests. The blood was collected in Alsever's solution (one part Alsever's to one part blood) under sterile conditions. The cells were washed four times with DGV (at 4 C). The first wash was in one volume blood cells to 2.5 volumes of DGV. Three volumes of DGV to one volume of red blood cells was utilized for the remaining washes.

Following each wash the cells were centrifuged for 15 minutes at 1500 rpm and 4 C. The DGV was subsequently removed and replaced with fresh DGV. After the last wash the cells were resuspended in three volumes of DGV. The cells were stored in DGV for 4 weeks without adverse affects.

Dextrose gelatin veronal (DGV).

Solution A

barbital	0.58 g
gelatin	0.60 g
hot DDW	250

Solution B

sodium barbital	0.38 g
CaCl ₂ (anhydrous)	0.02 g
MgSO ₄ ·7H ₂ O	0.12 g
NaCl	8.5 g
dextrose	10 g
DDW	600 ml

After cooling Solution A to room temperature, it was mixed with Solution B and brought to a total volume of 100 ml with DDW. The pH was between 7.2 and 7.4. The solution was dispensed in 50-100 ml fractions and sterilized by autoclaving for 10 minutes at 10 lbs. pressure.

Acid-Citrate-Dextrose (ACD).

sodium citrate	11.26 g
citric acid	4.09 g
dextrose	11.0 g
DDW to	500 ml

Autoclave for 10 minutes at 10 lbs. pressure.

Complement. Lyophilized guinea pig complement and the solution for restoring it to a liquid were purchased from GIBCO.

Hemolysin. Antisheep hemolysin, preserved with an equal amount of glycerol, was also obtained commercially from GIBCO.

Human reference serum. The reference serum with the group specific complement fixing antibodies against adenoviruses was purchased commercially from MAI. This serum was inactivated at 56 C for 30 minutes prior to being used in CF antigen determinations.

Erythrocytes for HA testing. These cells are made up to a 0.5% suspension by diluting with 0.9% NaCl.

Sheep erythrocytes. The sheep cells were collected in an equal volume of Alsever's solution under sterile conditions.

The sheep erythrocytes were washed with three volumes of VBD to each volume of cells in Alsever's and centrifuged at 600 g for 5 minutes. The supernatant fluid and the white cells were removed by suction. The cells were washed three more times with 10 volumes of VBD for each volume of cells. Finally the cells were resuspended in VBD (4 C) and centrifuged for 10 minutes at 600 xg for 10 minutes. The cells were subsequently diluted to give a 2.5% suspension in VBD. This concentration, when diluted 10 fold, has an OD of .450 at 490 nm.

Plaque Neutralization Tests.

Virus stock solutions were prepared in susceptible cell cultures and assayed using the plaque formation procedure. For use in neutralization studies, the virus was diluted to give approximately 200 plaque-forming units of virus in a volume of 0.1 ml of PBS.

Type specific antisera were inactivated at 56 C for 30 minutes and then serial two-fold dilutions of these antisera in PBS were carried out starting with a 1:2 dilution. Next equal volumes of the virus dilution and antiserum were mixed and allowed to set at room temperature for 60 minutes. One-tenth ml of the serum-virus mixture was then inoculated onto monolayers of susceptible cells. After a 150 minute incubation period, the cells were overlaid by agar to permit absorption of unneutralized virus. The neutralizing capacity of the serum was determined as that dilution of antiserum giving an 80% reduction in the number of plaques formed. The actual reduction index of the original antiserum was calculated by taking the dilution that gave an 80% reduction in the number of plaques and multiplying by two.

Procedure for Hemagglutination Test.

Hemagglutination titrations were performed in microtiter plates. Serial two-fold dilutions of the antigen in 0.05 ml DGV were carried out in each of the individual wells, then 0.05 ml

of either rat, monkey, or human O erythrocytes in the form of a 0.5% suspension were added to each well. The cells were allowed to sediment at 37 C for 1 hour. Following sedimentation, readings of the bottom patterns were made, and the highest antigen dilution showing complete agglutination was taken as the end point or hemagglutination antigen titer.

In the event that the adenovirus studied might belong to group III, the agglutination was enhanced by adding either antiserum against adenovirus type 1 or adenovirus type 6. This was done by using a 1:50 solution of the adenovirus antiserum (in DGV) as the diluent.

Preparation of Viral Antigens for Serological Testing.

A susceptible cell line was infected with a multiplicity of infection (MOI) of three plaque-forming units (PFU) per cell. The medium was changed at day 3 and the antigen was prepared by rapidly freezing and thawing of the culture four times. The cellular debris was removed by centrifugation at 2000 rpm for 15 minutes. The antigen preparation, used in both the hemagglutination and complement fixation tests, was stored at -70 C.

Typing Antisera.

Neutralizing type specific antisera prepared in rabbits against adenovirus Type 1, 2, 4, 5, or 6 was purchased from MAI. Hyper immune guinea pig serum against Herpes simplex was also purchased from MAI.

The neutralizing antisera against pseudorabies virus was graciously supplied by National Institutes of Health, Bethesda, Md 20014.

A typing antisera was prepared for the porcine adenovirus isolated from animal 1857 with myelogenous leukemia. HeLa cells were infected with a multiplicity of infection (MOI) of five PFU of the porcine adenovirus per cell. After 4 days, the culture (containing 3×10^8 cells) was frozen and thawed three times and the fluids pooled. This preparation was centrifuged at 2000 rpm for 15 minutes to remove the cellular debris. Next the adenovirus was sedimented by spinning at 40,000 rpm for 2 hours in L265B ultracentrifuge. The resulting pellets were collected and emulsified in 6 ml of complete Freund's adjuvant. Five rabbits were then given 3 weekly transmuscular injections of 1 ml of Freund's adjuvant adenovirus preparation. Fourteen days following the final injection the rabbits were exsanguinated and the serum collected.

All antisera was stored in -70 C in sealed ampules until ready for use.

Complement Fixation Procedure.

The complement fixation procedure is a well established technique and therefore will not be described in detail.

The hemolysin and complement titrations were carried out exactly as described in Viral and Rickettsial Infections of Man (65).

The solutions were adjusted to contain two units of complement and two units of hemolysin for these tests. The actual complement fixation experiment was performed in microtiter plates using the standard technique (125). A block titration was set up and serial two-fold dilutions of the antiserum and the antigen were set up in microtiter wells. The wells each containing 0.025 ml of diluted serum, diluted antigen, and complement.

If one of these components was left out, VBD was added to keep the volume constant. After 20 hours at 4 C, a 0.05 ml mixture of sheep erythrocytes and hemolysin was added to each well.

The tubes were then incubated for 1 hour at 37 C and the bottom patterns read for hemolysis.

BIOCHEMICAL AND PHYSICAL MEASUREMENTS

Reagents

Crystalline density gradient grade sucrose that was ribonuclease free was purchased from Schwarz Mann Bio Research, Inc., Orangeburg, NY.

Ribonuclease. Five times crystalline ribonuclease from bovine pancreas with a specific activity of 65 μ /mg was purchased from Calbiochem. RNA digestion was carried out by bringing the concentration of RNase to 1 μ g/ml in 0.15M NaCl. The solution was then incubated at 37 C for 20 minutes.

Ether Sensitivity of Viruses.

Ten milliliters of a known dilution of virus tissue culture fluid was mixed with 0.5 ml of pure anaesthetic ethyl ether (Mallinckrodt Chemical Works, St. Louis, MO) and placed in a screw cap tube sealed and placed at 4 C for 24 hours. The solution was then poured into a petri dish and allowed to evaporate at room temperature for 30 minutes. The titer was determined by plaque assay.

Metabolic Analog Studies.

A multiplicity of five virus plaque-forming units per cell of the virus to be studied was used to infect susceptible cells; then a known concentration of 5 Bromodeoxyuridine (BUdR) (Schwarz Mann) was added to the culture. After 50 hours, the virus in the supernatant fluid was assayed. The same experimental protocol was used in the thymidine reversal cultures except that thymidine was added in various concentrations and then the reversal effect was quantitated.

Zonal Centrifugation.

The Spinco Ti 15 zonal rotor having a capacity of 1665 ml was used for the initial concentration of C-type viruses from tissue culture fluids. The rotor was filled with 0.01M Tris H Cl, 0.1M NaCl and 0.001M EDTA, pH 7.4 (TSE buffer) assembled and accelerated to 5000 rpm; then 600 ml of 60% sucrose prepared in 0.05M sodium phosphate pH 6.8 was pumped to the edge of the rotor and stabilized against the rotor wall. The rotor was then accelerated to 35,000 rpm and 200 ml of virus containing fluid was added after 1 hour. The rotor was slowed to 5000 rpm and 200 ml 60% sucrose was pumped to the edge of the rotor wall. The 200 ml coming from the core was discarded and another 200 ml of culture fluid added again, displacing 200 ml of heavy sucrose and the rotor was again accelerated to 35,000 rpm for 1 hour. This procedure was continued for 8 to 10 times so that eventually one to two liters of culture fluid were processed. After the final sample was added, the centrifugation was allowed to continue for 3 hours. The rotor was then slowed to 5000 rpm and 63% sucrose was pumped to the rotor edge to recover the contents by displacement into 50 ml tubes. Fractions were collected for determination of radioactivity (when applicable) and refractive index as well as electron microscopic observation. Portions of each of the fractions containing virus were rebanded in sucrose gradients.

Estimation of RNA by the Orcinol Method.

RNA standard solution is prepared from yeast RNA (0.5 mg/ml) and diluted to give concentrations varying from 5-150 $\mu\text{g/ml}$.

The orcinol reagent is prepared by dissolving 100 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 100 mg of orcinol (Schwarz Mann) in 100 ml of concentrated HCL.

The RNA standard was mixed with an equal volume of the fresh orcinol preparation, allowed to set for 45 minutes in boiling water, cooled, and the UV absorbance recorded at 670 nm. By using several different dilutions, a standard curve was obtained for determining the RNA content of the unknown samples. The sample was tested for RNA by simply adding 1 ml of sample to 1 ml of the reagent heating, as described, and checking the optical density.

Radioactive Labeling Studies.

Labeled virus was produced by adding medium containing 20 $\mu\text{Ci/mmole}$ of tritium labeled uridine (Schwarz Mann; specific activity, 29.3 Ci/mmole) to infected PEK cell monolayers in roller bottles. The medium was collected every 12 hours for 48 hours and replaced each time by fresh medium containing the labeled uridine. The cells were removed from this media by low speed (10,000 $\times g$ for 15 minutes) and the supernates utilized in our experiments.

Radioactivity Determinations of RNA.

One-tenth milligram of BSA was added to the sample. Then trichloacetic acid (TCA) was added to give a 5% solution (in TSE buffer). The samples were maintained at 0 C for 30 minutes and the precipitate was collected on millipore filters 450 nm. These were washed with an additional 20 ml of cold 5% TCA prior to drying at 37 C overnight. The filters were subsequently placed in a scintillation vial with 1.0 ml scintillation fluid (without stabilizer) and counted in a Nuclear Chicago scintillation counter.

Chapter 4

RESULTS

With the aid of the electron microscope virus-like particles have been seen in plasma pellet preparations (Plate I) and tissues (Plate II) from miniature swine with lymphoproliferative and myelogenous disorders. These particles often appear to differ in morphology. However, this variance can often be explained by the treatment of the individual sample or the stage of maturation of the viruses.

While it is true that virus particles cannot be classified completely on the basis of morphology, it can be said that the viruses observed in leukemic swine resemble the C-type particles or oncornaviruses that cause leukemia in many domestic and experimental animals. The virus particles seen in plasma pellet preparations (Plates I, A and B) appear to be complete or mature and resemble the typical C-type particles. On the other hand, those in tissue sections often represent virus in different stages of maturation. In Plate II A, a dense mass resembling viroplasm together with what appears to be several virus particles in the process of maturation around the outside of the "viroplasm" can be seen. The partially disrupted cell in Plate II B shows several virus-like particles near the nucleus some of which appear to be in the process of budding.

PLATE I

A) Plasma pellet preparation from an animal with myeloid metaplasia. Type C particles a) are apparent throughout the preparation and occasional type B particles (b) are also in evidence.

B) The mature C-type viruses seen in plasma pellets from leukemic swine are spherical in shape and approximately 100 nm in diameter. These viruses contain a centrally located dense spherical nucleoid internal component. The outermost ring of the C-type particle constitutes the lipid envelope, and the spherical nucleoid core is a nucleocapsid consisting of protein and RNA.

PLATE II

A) Virus-like particles (arrows) appear to be forming next to a dense area (viroplasm) in the cytoplasm of a myeloblast from an animal afflicted with myelogenous leukemia.

B) An electron micrograph of a thin section of spleen obtained from a leukemic swine. Virus-like particles (arrows) seen in the cytoplasm adjacent to the nucleus.

PLATE II

X A) Particles (arrows) resembling viruses appear to be forming next to a dense area (viroplasm) in the cytoplasm of a myeloblast from an animal afflicted with myelogenous leukemia.

B) An electron micrograph of a thin section of spleen obtained from a leukemic swine. Virus-like particles (arrows) were seen in the cytoplasm adjacent to the nucleus.

A survey of 46 animals (Table 4.1) has revealed that of the 34 animals with either myeloproliferative or lymphoproliferative disorders, 21 demonstrated virus-like particles in their tissues or in plasma pellet preparations. Even more significantly, of the 19 animals with myelogenous or lymphocytic leukemia, 15 had virus-like particles present. In contrast, no virus-like particles were observed in preparations from control animals.

A number of diverse factors are important in the development of leukemia. It is, however, now known that viruses are the etiologic agent of leukemia in several laboratory and domestic animals. For this reason, our efforts have been directed toward the isolation and characterization of the viruses present in the experimentally induced leukemia of miniature swine.

One phase of the investigation sought to detect viruses by transmitting those viruses to cell lines in which they could produced cytopathic effects and infectious virus. If this was not possible, we utilized the electron microscope and various biochemical methods to determine if virus replication was present. In some instances where neither cytopathic effects nor virus synthesis were apparent, cell cultures and their growth characteristics were examined. Virus infected cells often

Table 4.1

Electron Microscope Screening for Virus-like Particles in the
Tissues and Plasma Pellets of Normal and Leukemic Swine

Pathological diagnosis	Number of animals observed	Number of animals having virus-like particles
Myelogenous leukemia	15	11
Lymphoproliferative disorders	4	4
Myeloid metaplasia	15	6
Control (non-leukemic)	12	0

displayed unique characteristics when grown in culture. This was particularly true if these cultures had undergone neoplastic conversion (transformation, as a result of a virus infection).

ISOLATION OF VIRUSES CAUSING APPARENT CYTOPATHOLOGY

Figure 4.1 gives a brief outline of the manner in which animals were usually sampled. However, isolation of viruses producing cytopathogenic effects (CPE) from some of these animals was made from frozen plasma, serum, or tissue homogenates prepared and stored at -70 C by other laboratory personnel prior to the initiation of these studies.

The purpose of this portion of the research was the isolation and in vitro replication of viruses present in the circulation or tissues of the animals at the time of sacrifice. In order to accomplish this goal, it was necessary to utilize a wide variety of indicator cells (Table 4.2) including, when possible, cell cultures from the homologous animal.

The animals in this study were classified into four groups based on standard hematological and histopathological diagnosis:

- 1) myelogenous leukemia
- 2) myeloid metaplasia (a myeloproliferative disorder)
- 3) lymphoproliferative disorders
- 4) controls (animals without hematological disorders)

Figure 4.1 Flow Chart for the Isolation of Viruses that Cause Cytopathic Effects in Cell Cultures.

Table 4.2

Established Cell Cultures Used as Indicator Cells
for Isolation of CPE Producing Viruses

Cell	Tissue origin	Source
Vero	African green monkey kidney	MAI
Hela	Human carcinoma of cervix	MAI
WI-38	Human embryonic lung	MAI
FL	Human amnion	MAI
KB	Human epithelial carcinoma	MAI
LLCMK ₂	Rhesus monkey kidney	MAI
BHK-21	Baby hamster kidney	MAI
Detroit-6	Human adult sternal marrow	MAI
Hep-2	Human carcinoma of larynx	GIBCO
PK-15	Porcine kidney	NADL*
PEK	Embryonic porcine kidney	NADL*
ST-128	Embryonic porcine kidney	NADL*

* National Animal Disease Laboratory, Ames, Iowa.

It was not possible to match the animals in each group (Table 4.3). The control group, however, contains both irradiated and unirradiated animals. This was necessary to help in evaluating and separating radiation effects from those related to the hematological disorders.

Primary cell cultures were prepared from kidney, spleen, lymph node, and lung tissues as well as leukocytes and bone marrow of normal and leukemic swine. Samples of those cultures were grown on microscope slides, then fixed and stained with H & E after 4, 8, 15 and 20 days of incubation. In these cultures, several different types of inclusions and other abnormalities were observed.

Vacuoles were in evidence in all cultures from leukemic swine (Plate III A). These vacuoles appeared between 8-15 days but generally shortly after each passage of the cell line. Similar patterns of vacuolization were seen in control cultures from radiation exposed swine (Plate III B). Although cultures from normal swine also showed some degree of vacuole formation, there were definite differences in numbers of cells containing vacuoles and time of appearance of vacuoles. For example, cultures originating from tissues of irradiated animals had larger numbers of vacuolated cells after 15 days, while those cells from normal animals did not develop significant numbers until the third passage in culture.

Table 4.3

Identification of Animals Utilized for the Isolation of
Cytopathogenic Viruses from Leukemic Miniature Swine

Pathological diagnosis	Animal no.	Sex	Age	Level of ^{90}Sr $\mu\text{Ci/day}$
Myelogenous leukemia	1857	F	1049	125
Myelogenous leukemia	1850	F	1076	125
Myelogenous leukemia	982	F	2046	125
Myelogenous leukemia	3128	F	1031	313
Myelogenous leukemia	3549	F	689	125
Myelogenous leukemia	977	F	2055	125
Myeloproliferative aleukemic	1257	F	1726	125
Myelogenous leukemia or multiple myeloma	4108	F	555	250
Lymphoproliferative	384	F	2789	25
Lymphoproliferative	1845	F	843	125
Myeloid metaplasia	820	F	2394	25
Myeloid metaplasia	405	F	3942	25
Myeloid metaplasia	380	F	3964	25
Myeloid metaplasia	396	F	3869	25
Myeloid metaplasia	980	F	2126	125
Myeloid metaplasia	1174	F	3174	25
Control	31	F	4476	0
Control	65	F	4360	25
Control	2685	F	4781	0
Control	83	F	4276	0
Control	801	F	3636	5
Control	984	F	2725	0
Control	863	F	3795	25

PLATE III

A) Primary cell culture from leukemic swine (15 days) induced by feeding ^{90}Sr . The vacuoles seen here were quite common in these animals. Hematoxylin eosin 870X.

B) Primary cultures (20 days) from normal swine that had been fed ^{90}Sr . Note vacuoles. Hematoxylin eosin 220X.

C) Primary culture (10 days) from an animal with myelogenous leukemia. Note large basophilic masses throughout culture. In some instances, these basophilic masses were transmitted to normal porcine cell lines and the transmissible agent was shown to be a mycoplasma. Hematoxylin eosin 220X.

D) A higher magnification of the basophilic masses observed in a 15 day cell culture from a leukemic swine. Hematoxylin eosin 560X.

Basophilic staining masses were observed in many of the cultures from leukemic animals. Low power examination of the cultures (Plate III C) shows several areas where this irregularity was evident. Upon examination of a single such mass under higher magnification (Plate III D), one may observe that the nuclei underlying these basophilic masses have rather bizarre shapes and are tightly clumped together resembling nuclei of a polykaryon. These basophilic inclusions varied in size and appeared to gradually progress from the involvement of a single cell up to large masses involving as many as 15 nuclei in a structure that appeared to be a syncytial cell. Several attempts were made to isolate a viral agent from these cells with no success. However, samples were eventually placed on mycoplasma medium and mycoplasma were isolated (Plate IV A). This agent was then added to normal porcine cultures where it eventually produced the same type of basophilic masses described earlier. Interestingly, these basophilic masses were not seen in cultures from control animals.

A third observation was the presence of basophilic inclusions in the cytoplasm of cultured cells (Plate IV B). In some instances these inclusions resembled a vacuole containing intensely staining basophilic granules. These granules had the appearance of a condensed nuclear chromatin network (Plate IV C). In early cultures, the size of these inclusions measured about

PLATE IV

- A) Mycoplasma isolated from cell cultures from a leukemic swine. These mycoplasma were often associated with cultures that displayed the basophilic masses described earlier. The dark round object at the bottom of the mycoplasma would eventually bud off and form another mycoplasma.
- B) Primary (10 day) culture from an animal with myelogenous leukemia. Note the basophilic inclusion which seems to contain some condensed chromatin-like material. 560X.
- C) Cultures from leukemic swine (25 days) note another stage of the basophilic inclusion. These inclusions were not transmittable but they were only observed in cell cultures of animals diagnosed as having either myelogenous leukemia or myeloid metaplasia. 560X.
- D) Basophilic inclusion. Note its appearance which resembles a cell nucleus. Closer examination, however, reveals that it lacks the morphological components usually seen in normal nuclei. 560X.

one-fourth the size of a nucleus. Later cultures often had inclusions as large as the nucleus (Plate IV D) or even larger (Plate IV B). These inclusions were not evident in secondary cultures, and neither virus nor mycoplasma were isolated that could transmit these inclusions to other cell cultures.

In addition to the inclusions, several nuclear abnormalities were noted in the cultures from the swine with hematological disorders. Four cell cultures from leukemic animals had cells (usually about 0.01%) with an enlarged abnormal nucleus. The nucleus was usually about 3-5 times the size of the other nuclei in the culture (Plate V A). These large nuclei remained characteristically stable in these cultures even up to the 10-15th passage.

Irregular nuclei with undulating nuclear membranes (Plate V B) were observed in five different cell lines from leukemic swine. These abnormally shaped nuclei were similar to nuclear aberrations having been reported in cell cultures infected with Rous sarcoma virus (88). The nuclear abnormality found in the swine cultures was neither transmissible nor stable in characteristic as these cells disappeared after only a few (2-5) passages.

PLATE V

A) Primary cultures from leukemic swine. Giant nuclei are demonstrated (arrow). These were found in several cultures, but were quite rare. This characteristic could not be transmitted to normal cell lines. Hematoxylin eosin 560X.

B) Cell lines from leukemic animals. The irregular shape of the nuclei (arrow) is noted. This characteristic was not transmissible to normal cell lines. Hematoxylin eosin 870X.

C) Fragmented nuclei (arrow) like those seen in this picture were present in about .1% of the cells. These were found to be typical of porcine cell lines, even those established from normal swine. Hematoxylin eosin 220X.

D) PK-15 cells 60 days after inoculation with fresh plasma from a leukemic swine. The rarefied zone (arrow) inside the nuclear membrane is present in about 5% of the cell population. Hematoxylin eosin. 560X.

The fragmented nucleus shown in Plate V C was first observed in cell cultures from leukemic animals. We later found this in all the control cultures as well. Even the stable porcine cell lines (PEK, St128, and PK-15) displayed a relatively high number of cells with fragmented nuclei. While this fragmentation appears to be very typically observed in porcine cell lines, the cause is not known.

Finally, after several passages corresponding to 60-90 days in culture, about 1% of the cells from most of the animals studies exhibited nuclear inclusions similar to those observed in advanced stages of herpesvirus, cytomegalovirus, or adenovirus infected cells. In addition, a stable porcine cell line (PK-15) inoculated with expended cell culture medium from the aforementioned cultures, plasma, or tissue homogenates began to develop similar inclusions (Plate V D). It is interesting to note that the cytopathology never progressed beyond this point i.e., no more than about 5% of the PK-15 cells or cell cultures from the animals showed infection. Even after subculturing these infected cells for up to 4 years, they continue to shed infective virus.

Table 4.4 lists the animals used in the search for cytopathic viruses and the cell lines in which successful isolations were made. When the indicator cells developed some detectable cytopathic changes, attempts were made to determine the identity of these agents. Assay procedures were initiated so that the viruses could be quantitated and used for experimental

Table 4.4

Results of Attempts to Isolate Cytopathogenic Viruses from Swine

Animal no.	CPE	Type of inclusion*	Isolated from	Cell line
Myelogenous leukemia				
1850	+	AHC	Plasma, cell culture	HeLa, Vero
1857	+	AHC	Plasma, cell culture	Vero, HeLa, KB, PEK
4108	+	AHC	Plasma, cell culture	Vero
3549	+	AHC	Plasma, cell culture	Vero
3128	+	AHC	Plasma, cell culture	Vero
1257	+	AHC	Plasma	Vero
982	+	AHC	Plasma	Vero
977	+	AHC	Plasma	Vero, KB, HeLa
Lymphoproliferative				
384	+	AHC	Plasma	Vero, KB, PEK
1845	+	AHC	Plasma	PEK, KB, HeLa
Myeloid metaplasia				
820	+	AHC	Plasma, cell culture	PK 15, PEK
1174	+	Rounded cells	Plasma, cell culture	PEK, Vero, HeLa
380	+	AHC	Plasma	Vero
396	+	AHC	Plasma	Vero
980	--	--	--	--
405	--	--	--	--

Table 4.4 (continued)

Animal no.	CPE	Type of inclusion*	Isolated from	Cell line
Control				
984	+	AHC	Plasma, cell culture	Vero
2685	--	--	--	--
987	+	AHC	Plasma, cell culture	Vero
801	--	--	--	--
65	--	--	--	--
987	--	--	--	--
83	--	--	--	--
31	--	--	--	--

* AHC = adenovirus, herpes, cytomegalo
 - = no CPE or inclusions that were transmissible

purposes. All of these agents were subsequently found to produce plaques in either VERO or PEK cells (Plate VI A). Electron micrographs of infected cells invariably contained electron dense D-type particles (Plate VI B). Finally the CPE produced in PEK or VERO cells was typical of that usually seen in adenovirus infected cells (Plate VI C).

Histological examination of infected cells was carried out in an effort to determine the type of nucleic acid possessed by the infecting viruses. The results of this study show that the inclusions within the cells contain high concentrations of double stranded DNA.

The deep green-blue inclusions (Plate VII A) in cells stained with methyl green pyronin Y are DNA, as are the purple inclusions (Plate VIII A) evident in cells stained with Fielgens. Using acridine orange (Plate VIII B, and C), the inclusions were determined to contain double stranded nucleic acid. The hematoxylin eosin stained cultures (Plate VII B and C) were included for comparison.

The presence of DNA was further confirmed in some of the first viral isolates by the use of a metabolic analog. The effects of various dilutions of 5 Bromodeoxyuridine (BUdR) on the replication of the virus isolate from animal 820 are shown in Table 4.5. BUdR was added to infected cell cultures in concentrations ranging from 10^{-4} M to 10^{-7} M. Virus growth was

PLATE VI

A) Plaque assay of porcine adenoviruses in VERO cells. The white areas (arrow) in the dark background represents a plaque caused by an adenovirus. These plaques were counted and thus the number of virus particles in a given volume could be determined. 2.27X.

B) Electron micrograph of adenoviruses isolated from leukemic swine and transmitted to a normal porcine cell line (PEK).

C) Embryonic porcine kidney cells fixed at 100 hours illustrate the nuclear inclusions developing after infection with adenovirus.

(a) Intranuclear inclusions showing a rarefied zone inside the nuclear membrane. (b) Inclusions with rarefied zone as well as formation of basophilic granules in the nucleus. (c) The nucleus and several basophilic inclusions in the nucleus are surrounded by halos or rarefied areas. (d) A cell showing the honeycombed control mass typical of adenovirus infections. Hematoxylin eosin 870X.

A

B

C

PLATE VII

A) VERO cells 8 days after infection with an adenovirus isolated from a leukemic swine. These cells are stained with methyl green pyronin Y and the dark green staining inclusions represent areas of high DNA content. 560X.

B) Vero cells sixteen days after infection with adenovirus type 2 (isolated from a leukemic swine). Note large number of cells with intranuclear inclusions (arrow). Hematoxylin eosin 220X.

C) Higher magnification of VERO cells infected with the adenovirus. These cells were stained 4 days after infection with hematoxylin eosin. 560X.

D) A cell culture of VERO cells four days after infection with adenovirus. These cells were stained with Periodic Acid Schiff's. The arrow points to a honeycombed nucleus. 560X.

PLATE VIII

A) VERO cells 6 days after infection with an adenovirus isolated from a leukemic swine. This culture was stained using the Fuelgen procedure and the dark purple areas (arrow) represent inclusions containing high amounts of DNA. 560X.

B) Acridine orange staining of adenovirus infected VERO cells showed typical adenovirus inclusions. 560X.

C) Intranuclear inclusions demonstrated by acridine orange staining. Observe the green area (arrows), representing double stranded nucleic acid is evident. 1020X.

D) Culture of embryonic porcine kidney cells transformed by the porcine type C virus. Note that the cells lie on top of one another in a random type of orientation. This is a typical characteristic of transformed cell cultures. 560X.

Table 4.5

Effect of 5 Bromodeoxyuridine on Viral Isolate 820

Concentration of BUdR	Virus titer PFU*
0	1.1×10^6
10^{-4} M	4.5×10^3
10^{-5} M	5.6×10^3
10^{-6} M	3.2×10^4
10^{-7} M	8.9×10^5

* PFU = Plaque forming units

substantially inhibited by 10^{-4} M while 10^{-7} M had very little effect. The effect of BUdR on the other viruses tested was similar (Table 4.6).

To prove that BUdR was inhibiting viral DNA synthesis, the ability of thymidine to reverse the inhibitory effect on the virus was determined. The addition of thymidine, which competes with the BUdR, was found to reverse efficiently the inhibition of viral synthesis (Table 4.7).

The viruses isolated from leukemic swine were shown to be relatively heat stable (Table 4.8) since storage at refrigerator temperatures for 5 months resulted in only a 10% loss of infectivity. Similarly the agents were not affected by ether (Table 4.9) suggesting the apparent absence of essential lipids. These and other characteristics of the viruses examined previously indicated that we were dealing with adenoviruses. To confirm this serologically, complement fixation tests were run using a human reference serum containing complement fixing antibodies against adenoviruses. All adenoviruses produce a group-associated soluble antigen detectable in this manner. Table 4.10 illustrates the design of a typical experiment used to titer complement fixing adenovirus group specific antigens. This specific test showed that the viral isolate from animal 1257 is indeed an adenovirus. The other isolates were similarly found to be adenoviruses.

3

Table 4.6

Effect of 5 Bromodeoxyuridine on Viruses
Isolated from Miniature Swine

Virus	Virus titer*	
	No drug	10^{-4} M BUdR
Newcastle disease virus	8.9×10^5	1.1×10^6
Herpes simplex	1.5×10^4	1.2×10^1
820 isolate	1.1×10^6	8.8×10^3
1174 isolate	2.2×10^6	9.8×10^3
1857 isolate	4.0×10^5	6.9×10^3
982 isolate	7.3×10^5	8.9×10^2
987 isolate	8.1×10^6	3.2×10^3
1850 isolate	7.0×10^5	2.2×10^2

* Virus titer is expressed as plaque forming units

Table 4.7

Effect of Adding Thymidine to Cell Culture with BUdR

Concentration of BUdR	Concentration of thymidine	Virus titer PFU*
0	0	2.2×10^6
10^{-5} M	0	4.3×10^3
10^{-5} M	10^{-4}	0.8×10^5
10^{-5} M	10^{-5}	5.3×10^5
10^{-5} M	10^{-6}	8.5×10^3

* PFU = plaque forming units

Table 4.8
Thermal Stability of Viruses Isolated from Swine*

Virus isolate	Treatment							
	4C (mo)		37C (days)			56C (min)		
	0	5	0	10	20	0	5	10
820	10 ^{6.0}	10 ^{5.8}	10 ^{6.0}	10 ^{4.2}	10 ^{0.7}	10 ^{6.0}	10 ^{3.1}	0
1857	10 ^{5.3}	10 ^{3.3}	10 ^{5.3}	10 ^{3.3}	10 ^{1.0}	10 ^{5.3}	10 ^{2.3}	0
1174	10 ^{6.1}	10 ^{5.4}	10 ^{6.1}	10 ^{3.9}	10 ^{0.5}	10 ^{6.1}	10 ^{3.7}	0

* Titrations performed in VERO cell cultures serial 10 fold dilutions were used with 8 petri dishes per dilution, 0.1 ml inoculum per plate, and a 180 min adsorption period. Virus titrations were calculated from plaque assay experiments and the titers are expressed in logarithmic form.

Table 4.9
Ether Stability of Viruses Isolated from Swine

Virus isolate	Virus titer*	
	Control	Ether treated
820	9.9×10^5	1.1×10^6
1174	1.7×10^6	1.4×10^6
1857	4.2×10^5	4.1×10^5
982	7.5×10^5	7.3×10^5
1850	6.2×10^5	5.9×10^5
987	7.1×10^6	7.2×10^6

* Expressed as plaque forming units

Table 4.10

Complement Fixation Test for Adenovirus
Antigens in Virus Isolate 1257

Antigen dilution	Human reference serum dilutions				
	1:16	1:32	1:64	1:128	No serum
1:2	4	4	4	4	0
1:4	4	4	4	4	0
1:8	4	4	4	4	0
1:16	4	4	4	4	0
1:32	4	3	3	2	0
1:64	1	1	0	0	0
1:128	0	0	0	0	0
No antigen	0	0	0	0	--

4 = no hemolysis
 3 = 25% hemolysis
 2 = 50% hemolysis
 1 = 75% hemolysis
 0 = complete hemolysis

Since there are over 35 known adenoviruses, a series of hemagglutination experiments were in order since the adenoviruses can be separated into four groups based on their ability to hemagglutinate rat, rhesus monkey or human type O erythrocytes. Since group III adenoviruses only given complete agglutination (of rat erythrocytes) in the presence of antiserum against a heterotypic adenovirus, two additional series were run utilizing rat blood cells in the presence of either type 1 or type 6 adenovirus antiserum.

The results of an experiment with virus 1850 (Table 4.11) are typical of those found with the other agents. A minimal amount of hemagglutination was found in the rat cells without the added antiserum while a more consistent and typical hemagglutination of rat erythrocytes was observed in the wells that contained added heterotypic adenovirus antiserum.

Table 4.12 summarizes the data obtained using all of the viruses isolated. This chart also allows a comparison of the complement fixing antigen, the hemagglutination and the virus infectivity titers.

The adenoviruses isolated from my swine all belong to Group III. Final identification of the viruses was based on neutralization of the viruses with type specific antiserum.

Table 4.11

Hemagglutination Titration of Adenovirus Isolated From Animal 1857

Antigen dilution	Erythrocytes				
	Human O	Rhesus	Rat	Rat and anti- adenovirus 1	Rat and anti- adenovirus 6
1:4	--	--	+	++	++
1:8	--	--	+	++	++
1:16	--	--	<u>+</u>	++	++
1:32	--	--	--	++	++
1:64	--	--	--	<u>+</u>	++
1:128	--	--	--	--	--
1:256	--	--	--	--	--
1:512	--	--	--	--	--
1:1024	--	--	--	--	--

0 = no agglutination
 + = questionable agglutination
+ = partial agglutination
 ++ = agglutination

Table 4.12

Hemmagglutination Titration of Cytopathic Viruses from Swine

Viral isolates	Tissue source	HA titer* with erythroocytes from				
		Rat and anti-adenovirus 6	Rat and anti-adeno 1	Rhesus	Human 0	Rat
Myplogenous leukemia						
1850	Vero	32	32	--	--	8
1857	PEK	64	32	--	--	8
4108	Vero	16	16	--	--	4
3549	Vero	16	16	--	--	4
3128	PEK	32	32	--	--	4
1257	PEK	32	32	--	--	8
982	Vero	32	16	--	--	--
977	Vero	8	16	--	--	4
Lymphoproliferative						
384	Verc	8	8	--	--	--
1845	Verc	16	16	--	--	4

Table 4.12 (continued)

Viral isolates	Tissue source	HA titer* with erythrocytes from				
		Rat and anti-adenovirus 6	Rat and anti-adenovirus 1	Rhesus	Human 0	Rat
Myeloid metaplasia						
820	PEK	64	64	--	--	8
1174	Vero	64	128	--	--	16
380	Vero	16	16	--	--	8
396	Vero	16	8	--	--	--
Control						
984	Vero	512	512	--	--	16
987	Vero	256	256	--	--	16
Control	Vero	--	--	--	--	--
Control	PEK	--	--	--	--	--

* Titer expressed as reciprocal of highest dilution of virus in 0.5 ml of saline showing complete agglutination.

The results of these experiments are compiled in Table 4.13. The majority of the viruses isolated appear to be a previously unidentified adenovirus, which is probably of porcine origin rather than human and will thus be referred to as porcine adenovirus type A (PAd-A). In addition to PAd-A, two human serotypes were isolated from these animals including adenoviruses types 2 and 4.

Adenoviruses were isolated from all of the animals having hematological disorders and approximately half of the control animals. The adenovirus study was carried one step further with the serological screening of the part of the herd at Battelle for adenovirus antibodies. This study revealed that there was no antibody against adenoviruses in the serum (stored from 3 months to 5 years) of leukemic animals. It was noted, however, that neutralizing antibody titers were detectable in fresh serum from 16 to 20 animals with hematological disorders, or if they were tested prior to onset of clinical symptoms. By contrast only 30 of 83 (36%) control animals had detectable neutralizing antibodies against any of the viruses isolated.

Table 4.13

Serological Identification of Adenoviruses Isolated from Miniature Swine

Antisera Reciprocal of Dilution that Inhibits 100 PFU.*

Virus isolates	Adenovirus					Porcine type A	Cyto- megalo	Pseudo- rabies	Herpes simplex
	Type 1	Type 2	Type 4	Type 5	Type 6				
Myelogenous leukemia									
1850	--	--	--	--	--	64	--	--	--
1857	--	--	--	--	--	64	--	--	--
4108	--	--	--	--	--	256	--	--	--
3549	--	--	--	--	--	32	--	--	--
3128	--	--	--	--	--	32	--	--	--
1257	--	--	--	--	--	64	--	--	--
982	--	--	--	--	--	128	--	--	--
977	--	--	--	--	--	64	--	--	--
Lymphoproliferative									
384	--	--	--	--	--	--	--	--	--
1845	--	--	--	--	--	256	--	--	--
Myeloid metaplasia									
820	--	64	--	--	--	--	--	--	--
1174	--	--	32	--	--	--	--	--	--
380	--	--	--	--	--	32	--	--	--
396	--	--	--	--	--	16	--	--	--
Control									
984	--	256	--	--	--	--	--	--	131
987	--	518	--	--	--	--	--	--	--

* PFU = plaque forming units

GROWTH CHARACTERISTICS OF CELL CULTURES

Cell cultures were prepared from the organs and tissues of normal and leukemic swine for the purpose of comparing these cells as long term cell cultures. The assumption was that the abnormal characteristics contributing to leukemic or neoplastic disease process in vivo may be more evident in cultured cells.

The first observation was that porcine cells from normal animals generally did not grow as well as those from swine suffering from either myeloproliferative or lymphoproliferative disorders. After only two to three passages in culture, cells from normal swine had fibroblastic characteristics (Plate IX, A) and rarely did the culture continue to propagate past the fourth or fifth passage. In contrast, cell cultures from animals with hematological disorders (Plate IX, C) had many cells with epitheloid features. With each subsequent passage in culture, these cells continue to develop morphological and growth characteristics consistent with those of transformed cells. For example, the cells from animals with hematological disorders usually grew either in clumps of multilayered cells (Plate X, A) or in sheets of cells displaying frequent foci of unrestricted growth as the cells continued to grow in piles several cell layers deep (Plate X, B). When these cultures were viewed under low power microscopy (Plate X, C and D), these cells

PLATE IX

A) Cell culture from kidney of a normal pig. Large fibroblast are evidence these cells are essentially dead since they will not divide and often cannot be passed. Phase contrast 280X.

B) Cell culture from the kidney of a pig receiving ^{90}Sr but without histologically detectable hematologic disorder. Again this culture is dying out. Phase contrast 290X.

C) This cell culture from the kidney of a swine with myeloid metaplasia shows both fibroblast and epitheloid-like elements. This cell line was eventually established as a long term cell culture. Phase contrast 280X.

D) Cell culture from the kidney of a swine with myelogenous leukemia. The cells appear healthy after the fifth passage and in fact later became established as a long term cell culture. Phase contrast 280X.

PLATE X

- A) Stabilized cell culture obtained from kidney tissue of a miniature pig with myelogenous leukemia (these cells grow in multilayered colonies with numerous mitotic figures indicating at least a degree of transformation). Phase contrast 280X.
- B) Stabilized cell lines originally cultured from an animal diagnosed with myeloid metaplasia. This culture had foci, such as the one shown, of unrestricted growth where the cells grew several layers thick. Phase contrast 280X.
- C) Cell culture from a leukemic swine photographed at low power to show the unusual growth patterns of these cells in culture. Phase contrast 100X.
- D) Again the unusual growth characteristics of a cell culture established from an animal with myelogenous leukemia. Phase contrast 100X.

appeared transformed. The formation of foci of piled up cells seen growing in random orientation indicated that these cells had apparently lost normal contact inhibitory mechanisms.

Several of the cell lines were karyogrammed at various stages to determine if any stable or consistent chromosomal characteristics could be associated with the changes in growth characteristics. All of the cells were karyogrammed within the first three passages in culture and all showed a normal porcine chromosome picture. The normal karyotypes of cell cultures from male and female swine are shown in Plates XI and XII respectively. The modal number of chromosomes is 38 but the frequency of cells having a certain number of chromosomes is given in Table 4.14. This table illustrates some of the observable differences in the chromosome frequency distributions of these cells after various stages of culture. The long term cell lines PC977 (25 passages), PC2685 (21 passages), P₃K (19 passages) and P₂K (23 passages), all have undergone radical changes in chromosome numbers since they were originally karyotyped and counted. The modal chromosome numbers have dropped from the 38 chromosomes, considered to be normal, to modal numbers of 31 or 33.

Karyotyping of the cell lines that had undergone shifts in modal numbers revealed that each of the cell lines had lost several acrocentric chromosomes. A karyotype representative of the P₃K (820) cells with 33 chromosomes (Plate XIII) has

PLATE XI

Normal karyotype from a miniature swine. The total number of chromosomes is 38. The pair of chromosomes marked (a) are the sex chromosomes and this particular karyotype is of a male swine.

p_k
 $n=38$

PLATE XII

Karyotype from a leukemic female miniature swine. This karyotype still contains 38 chromosomes after the second passage in cell culture. This was seen in all cell cultures from leukemic animals however, after 10 passages in culture the cells from leukemic swine had lost several chromosomes.

p, k
 $n=38$

Table 4.14
Chromosome Frequency Distribution

PC 977-25 (ML)											
Chromosomes	28	31	32	33*	34	35	36	64	66	67	68
Cells	2	18	25	43	3	2	1	1	3	1	1
PC 2685-21 (Control)											
Chromosomes	27	28	29	30	31*	32	33	60	61	62	64
Cells	2	5	1	3	76	2	3	1	2	3	2
P ₃ K-19 (MM)											
Chromosomes	29	30	31	32	33*	34	35	64	66	73	--
Cells	1	2	13	11	61	5	1	2	3	1	--
P ₂ K-23 (ML)											
Chromosomes	27	28	29	30	31*	32	33	34	58	60	64
Cells	2	1	3	3	43	20	19	1	1	1	3
PEK-10 (Control)											
Chromosomes	21	26	34	35	36	38*	39	73	74	76	--
Cells	1	1	6	2	18	52	5	1	1	3	--
P ₉ K-10 (Control)											
Chromosomes	34	35	36	37	38*	39	62	69	--	--	--
Cells	2	2	6	1	80	5	2	2	--	--	--
PK3549-3 (ML)											
Chromosomes	25	27	28	30	31	36	37	38*	39	52	60
Cells	2	1	1	4	5	20	2	52	2	1	1
Chromosomes	68	72	74	76	93	--	--	--	--	--	--
Cells	2	2	4	2	1	--	--	--	--	--	--
PK3128-3 (ML)											
Chromosomes	21	26	31	35	36	37	38*	39	40	65	73
Cells	1	1	3	5	2	19	51	5	2	1	1
Chromosomes	74	76	--	--	--	--	--	--	--	--	--
Cells	3	6	--	--	--	--	--	--	--	--	--

* Modal chromosome number

PLATE XIII

Metaphase karyotype of P₃K cell after 19 cell passages in cell culture. These cells only have 33 chromosomes with nine missing acrocentric chromosomes (a). In this cell there are also four extra metacentric chromosomes (b). Whether these arose from duplication of other metacentrics, combining of acrocentrics or both could not be determined.

p_3k
 $n=33$

← a

← b

nine missing acrocentric chromosomes and the acquisition of four "new" metacentric chromosomes. It was not possible to determine if the added metacentric chromosomes represented duplication of existing chromosomes or the translocation and combination of acrocentric chromosomes.

A cell line from an animal with myelogenous leukemia (P_2K or 1857 has a modal chromosome count of only 31 chromosomes (Plate XIV). Here six acrocentric chromosomes and two metacentric chromosomes are missing. The additional large metacentric chromosome in these cells may represent the joining of two large acrocentric chromosomes at the centromere.

The PC977 cell line was originally established from the cornea of a normal embryonic piglet. This cell line was cultured in the presence of plasma from a leukemic animal (977) for several weeks and subsequently was cared for in the same manner as other long term cultures. The representative karyotype from this animal has a modal count of 33 chromosomes, and upon examination (Plate XV) this number apparently resulted from the loss of four acrocentric and one metacentric chromosomes.

Another aliquot of the embryonic porcine cornea cells was established in the presence of plasma from a normal pig (2685) and the resulting cell line was designated PC2685. This cell line had a modal count of 31 chromosomes as a result of losing nine acrocentric and adding two metacentric chromosomes (Plate XVI). Interestingly, this cell line (PC2685) and the PC977 cell line originally came from the same culture.

PLATE XIV

Metaphase karyotype of P₂K cell after 23 passages in cell culture. The cell contains 31 chromosomes. Apparently six acrocentric (a) chromosomes, and two metacentric (c) chromosomes are missing. There appears to be an extra large metacentric chromosome (b) which may represent the joining of two large acrocentric chromosomes.

p_2k
 $n=31$

← a

← b

PLATE XV

Karyotype of PC₉₇₇ cell line after 25 passages in cell culture.

The total modal number of chromosomes in this culture is 33.

The cell here is representative of the cells with 33 chromosomes.

There are one missing metacentric chromosome (a) and four missing acrocentric chromosomes (b).

PC₉₇₇

n=33

149

PLATE XVI

Metaphase karyotype of PC₂₆₈₅ cell line after 21 passages. There are a total of 31 chromosomes as a result of nine missing acrocentric chromosomes (a) and two additional metacentric chromosomes (b). This cell line was originally identical to PC₉₇₇ with the only different treatment being this porcine cornea (PC) cell line had plasma from 2685 added to it.

pc₂₆₈₅

n=31

67 88 88 88 88 88

88 88 88 88 88 88

0 88

88 88

← a

← b

The cell cultures from normal swine appeared to be more difficult to propagate in cell culture. Therefore, the plating efficiencies, or the ability of a single cell to give rise to a colony of cells, of several cell lines, were examined. It is apparent from the data (Table 4.15) that there is some difference between the plating efficiencies of cell lines from normal and leukemic animals. Most of the cell lines from animals with hemopoietic disorders showed relatively high plating efficiencies ranging roughly from 30-60%. The exceptions to this are the kidney cultures from animals 3128 and 3549. These cells gave plating efficiencies of only 14-16%.

Third passage cell lines from normal animals in our experiment generally had low plating efficiencies ranging from 0.5% to 15%. However, the stable cell lines used (PK-15, PEK, and PC2685) yielded relatively high plating efficiencies. These results cast considerable doubt as to the value of drawing any significant conclusions from the results of the plating efficiency studies. Therefore, further studies of the growth characteristics were initiated.

Various dilutions of the cells were placed in a soft agar-medium mixture to determine if the cells would continue to grow and multiply. Cell division of normal cells was usually restricted by the surrounding agar (Plate XVII, A), but transformed cells did not display normal contact inhibitory mechanisms.

Table 4.15
Plating Efficiency of Cell Cultures from Miniature Swine

Animal number and diagnosis	Source of cell line	Number of cells/0.1 ml	Number of cell clones formed*		Plating efficiency† %
			0.1 ml/plate	0.2 ml/plate	
1857 (P ₂ K) ML†	Kidney	45	28.1	61.2	56.2 - 68.0
977 (PC977) ML	Normal porcine cornea	105	30.2	64.0	28.8 - 30.5
83 (P ₈ K) control	Kidney	300	17.4	27.3	5.8 - 4.6
31 (P ₉ K) control	Kidney	100	12.8	29.0	12.8 - 14.5
820 (P ₃ K) MMs	Kidney	52	30.8	52.3	59.2 - 50.3
3128 ML	Kidney	550	76.8	165.0	14.0 - 15.0
3128 ML	Lung	270	132.1	TNTC	49.0
3549 ML	Kidney	610	89.2	192.1	14.6 - 15.7
3549 ML	Lung	272	137.2	TNTC	50.4
65 Control	Lung	570	2.8	15.4	.49 - 1.4

Table 4.15 (continued)

Animal number and diagnosis	Source of cell line	Number of cells/0.1 ml	Number of cell clones formed*		Plating efficiency† %
			0.1 ml/plate	0.2 ml/plate	
65 Control	Kidney	630	30.5	33.2	4.8 - 2.6
31 Control	Lung	400	1.8	14.4	.45 - 1.8
2685 (PC2685) control	Normal porcine cornea	88	19.2	41.3	21.8 - 23.5
PK-15 Control	Kidney	110	36.0	68.0	32.7 - 30.4

* Cell clones represent the number of cells that grew into clones containing more than 20 cells during 10 days of the experiment.

† Plating efficiency is the number of clones that are present divided by the number of cells added to each plate.

‡ ML = Myelogenous leukemia

§ MM = Myeloid metaplasia

PLATE XVII

A) PEK cells grown for 16 days in semisolid agar medium.

The cells have not divided, however, they were found to be viable when removed and grown in normal medium. This is the reaction expected from normal cells. Phase contrast 560X.

B) A stable cell line from a leukemic swine. This colony grew from a single cell placed in semisolid agar medium 21 days earlier.

These cells have lost all contact inhibitory mechanisms, one of the criteria utilized to classify a cell as transformed.

Phase contrast 560X.

A

B

Consequently these cells continued to grow and divide in the agar suspension. This continued multiplication of cells resulted in the formation of clumps or colonies of cells in the agar (Plate XVII, B) which could be seen easily and counted with a stereomicroscope.

The ability of several cell lines from normal animals to continue to grow and multiply was severely restricted in the agar suspension. However PK-15, St-128, and PC 2685, the stable cell lines, were still as capable of forming colonies in the agar suspension as the cell lines from leukemic animals (Table 4.16).

The cell lines from this study were subsequently re-examined by electron microscopy to determine if there was any correlation between the unusual growth characteristics of apparently normal cells and the presence of C-type particles. The results (Table 4.17) indicated that while there was still considerable virus in the cell lines from leukemic animals (Plate XVIII, A and B), there was not any C-type viruses apparent in the cells from normal animals. Therefore the study did not show any relationship between the presence or absence of virus-like particles and the ability of the cultured cells to continue unrestricted cell division in the agar suspension.

Table 4.16

Growth in Agar Suspension of Cell Cultures from Miniature Swine

Animal number and diagnosis	Source of cell line	Number of cells/0.1 ml	Number of macroscopic colonies*		%
			0.1 ml/plate	0.2 ml/plate	
1857 (P ₂ K) ML†	Kidney	45	15.3	26.0	28-34
977 (PC977) ML	Normal porcine cornea	105	29.4	56.7	31-36
83 (P ₈ K) control	Kidney	300	0	0	0
31 (P ₉ K) control	Kidney	100	0	3.0	0-2
820 (P ₃ K) MM‡	Kidney	52	18.8	34.2	31-36
3128 ML	Kidney	550	28.6	66.2	5-6
3128 ML	Lung	270	78.3	161.0	27-30
3549 ML	Kidney	610	26.8	25.6	2-4
3549 ML	Lung	272	30.7	41.9	11-15
65 Control	Lung	570	0	0	0
65 Control	Kidney	630	0	0	0
31 Control	Lung	400	0	0	0
2685 (PC2685) control	Normal Porcine cornea	80	21.0	37.2	26-23
FK-15 Control	Kidney	95	11.4	24.9	12-26
PEK Control	Kidney	107	3.4	8.6	3-4

* Colonies counted measure 0.1-0.5 mm in diameter, this is after about 10 days.

† ML = Myelogenous leukemia

‡ MM = Myeloid metaplasia

Table 4.17

Electron Microscopic Survey for C Type Particles in
Cell Cultures Prepared from Tissues of Miniature Swine

Cell line	Tissue of origin	C type particles
P ₂ K (1857)	Kidney	Present
P ₃ K (820)	Kidney	Present
PC977	Cornea	Present
P ₈ K (83)	Kidney	Absent
P ₉ K (31)	Kidney	Absent
PC2685	Cornea	Absent
3128	Kidney	Present
3128	Lung	Present
3549	Kidney	Present
3549	Lung	Present
65	Kidney	Absent
65	Lung	Absent
31	Lung	Absent
PK-15	Kidney	Absent
PEK	Kidney	Absent

PLATE XVIII

A) Type C particles (arrows) present in a stabilized cell line established from an animal diagnosed as having a myelogenous leukemia.

B) Electron micrograph of PEK cells inoculated with fresh plasma from a swine with myelogenous leukemia the particles (arrows) about 100 nm in diameter.

PLATE XVIII

- A) Type C particles (arrows) present in a stabilized cell line established from an animal diagnosed as having a myelogenous leukemia.
- B) Electron micrograph of PEK cells inoculated with fresh plasma from a swine with myelogenous leukemia the particles (arrows) about 100 ^{nm} ~~mm~~ in diameter.

ISOLATION AND CHARACTERIZATION OF NON-PATHOGENIC VIRUSES

Both adenoviruses and C-type particles were usually present in the tissues and plasma pellets of swine with hematological disorders. Therefore separation of these agents became necessary in order to study the C-type particles individually.

Stable cell cultures from leukemic swine simultaneously producing both adenovirus and C-type particles were grown in roller bottles, and the expended medium was harvested. The C-type particles were concentrated using a 60% sucrose interface in a Ti 15 zonal rotor as described in materials and methods. This cushion was sufficient to stop the C-type particles while allowing the adenoviruses to go through to the rotor wall. The sample was subsequently removed by adding 60% sucrose from the outer wall. The samples were collected and those containing C-type particles (as determined by EM and UV absorption) were banded in linear sucrose gradients to assure purity (Plate XIX, A). The virus preparation obtained was then used to infect PEK cells. Attempts were then made to prove that these cells would replicate the C-type porcine isolate. The first step involved the electron microscope, and pictures were obtained that showed budding C-type particles in these cells (Plate XIX, C). The presence of budding virus is one stage of maturation and an indication that the cells are actively replicating virus. Identical determinations were

PLATE XIX

A) The internal components of the C-type virus particles isolated from leukemic swine are readily apparent in this picture (arrow).

B) Virus-like particles from leukemic swine, after isopycnic banding following continuous-flow centrifugation. Chemical analyses were performed on these preparations following isopycnic banding experiments. Particles measure 90-100 nm in diameter. The "tails" displayed by some particles (a) are probably due to osmotic factors. The internal components are readily visible in some particles (b).

C) Type C particles budding from the membrane of a embryonic porcine kidney cell line infected with a virus preparation obtained from a Hanford miniature pig with myelogenous leukemia.

attempted utilizing cell cultures from 22 animals, 15 of which had some type of hematological disorder. The results of these attempts to observe and isolate type C virus from cell cultures are summarized in Table 4.18.

Labeling studies using culture medium containing ^3H labeled uridine was then added to normal PEK cells, PEK cells inoculated with preparations from cells without apparent C type virus particles, and PEK cells inoculated with the type C virus. The culture medium from these cells was collected and processed using the Ti 15 zonal rotor.

The resulting virus enriched virus enriched preparations were identified by EM and UV adsorption. The virus was further purified by banding in sucrose gradients. Aliquots of the gradients were removed and examined using electron microscopy, UV absorbance, and by scintillation counting to locate the labeled uridine and virus. Figure 4.2 illustrates the finding with the control cultures. There was no virus present in these samples showing that the control PEK cells were not producing any RNA virus. The infected cells on the other hand showed bands of absorption and radioactivity (Figure 4.3). Electron microscopy has also revealed that these bands coincided with the presence of virus particles (Plate XIX, B). This study indicated quite clearly that the PEK cells are capable of replicating the porcine type C virus. Identical experiments were

Table 4.18

Identification of Animal Cell Cultures Utilized for the Isolation
of Non-Cytopathogenic Viruses from Leukemic Miniature Swine

Pathological diagnosis	Animal No.	C type virus purified and studied	C type virus isolated	Level of ^{90}Sr $\mu\text{Ci/day}$
Myelogenous leukemia	1857	Yes	Yes	125
Myelogenous leukemia	1850	--	Yes	125
Myelogenous leukemia	982	--	No	125
Myelogenous leukemia	3128	Yes	Yes	313
Myelogenous leukemia	3549	--	Yes	125
Myelogenous leukemia	977	Yes	Yes	125
Myeloproliferative aleukemic	1257	--	No	125
Myelogenous leukemia or multiple myeloma	4108	Yes	Yes	250
Lymphoproliferative	384	--	Yes	25
Lymphoproliferative	1845	--	Yes	125
Myeloid metaplasia	820	Yes	Yes	25
Myeloid metaplasia	405	--	Yes	25
Myeloid metaplasia	380	--	No	25
Myeloid metaplasia	396	--	No	25
Myeloid metaplasia	980	--	No	125
Myeloid metaplasia	1174	--	No	25
Control	31	--	No	0
Control	65	--	No	25
Control	2685	--	No	0
Control	83	--	No	0
Control	801	--	No	5
Control	984	--	No	0
Control	863	--	No	25

Figure 4.2
 UV ABSORPTION PROFILE OF (^3H) UdR LABELED SUPERNATANT
 FROM PORCINE EMBRYONIC KIDNEY (PEK) CELL LINE
 AFTER CENTRIFUGATION THROUGH A PREFORMED GRADIENT

Figure 4.3
 UV ABSORPTION PROFILE OF (^3H) UdR LABELED PORCINE
 VIRUS AFTER CENTRIFUGATION THROUGH A
 PREFORMED GRADIENT

carried out on the type C viruses isolated from five different animals. The results of these experiments are summarized in Table 4.18.

The average buoyant density of the porcine C viruses from several experiments were estimated to be about 1.11 g/cc. The viruses prepared in this manner appeared fairly homogenous (Plate XIX, B) and therefore suitable for nucleic acid preparations. The purified virus from several experiments were pooled and then pelleted to remove any extracellular nucleic acid. The nucleic acid component of the virus was then extracted from the virus by the phenol-SDS method and precipitated from the aqueous solution with ethanol. The absorbance spectrum of the nucleic acid is shown in Figure 4.4. It can be determined from the 240:260 and 280:260 ratio that this preparation was relatively free of protein contamination.

The DNase digestion of a portion of this sample caused no observable change in absorbancy at 260 nm or 280 nm but RNase caused a rapid increase at 260 nm and a decrease at 300 nm. In addition the amount of RNA was estimated using the orcinol method (124), and it was found that usually 100-180 μ g of viral RNA was produced with this procedure.

Figure 1.1
**ABSORBANCE OF THE PORCINE VIRUS NUCLEIC ACID
AS A FUNCTION OF WAVE LENGTH**

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PEK cell cultures infected with the porcine RNA virus were maintained by serial passage of the cells. Before the 15th passage, the infected cells grew in orderly monolayers (with fibroblastic elements) and appeared unchanged even though the electron microscopy and radioactive labeling studies showed that the cells were actively producing virus. After the 23rd passage, these same cultures had taken on many of the characteristics of transformed cells. Table 4.19 compares the growth characteristics of normal PEK cells with those of the apparently transformed PEK cultures. The apparent loss of contact inhibitory mechanisms is well illustrated in Plate VIII, D. The transformed cells can be seen growing as a focus of multilayered cells with random orientation instead of the well ordered monolayer culture seen with normal PEK cells.

The essential difference between these cells and ordinary cells, which occasionally grow in sheets two layers thick, is the complete randomness by which the transformed cells are distributed in all three dimensions. This appearance is typical of irregular growth transformation.

Table 4.19

Morphological and Growth Characteristics of
Normal and "Transformed" PEK Cells

Cellular characteristic	PEK cells	PEK cells after "transformation" with porcine type C virus
Morphology, cells	Fibroblastic	Epitheloid elements predominant
Chromosomes	Chromosomes stable at 38 2% polyploidy	Increased aneuploidy 6% polyploidy
Plating efficiency	20%	37%
Growth in agar suspension	0-4%	15-20%
Morphology, culture	Growth in monolayers	Loss of contact inhibi- tion, growth random with no patterns or orientation

Chapter 5

DISCUSSION

The early cytopathology observed in cell cultures from leukemic swine have some counter-parts in other animal species.

The basophilic cells reported are also seen associated with cell cultures infected with Rous Sarcoma viruses (25), as are nuclei with undulating or wavy membranes (88). Quite possibly the basophilia is due to an increase in the concentration of ribosomes (47, 117). In addition the small basophilic cells could represent growth of myeloblasts or lymphoblasts growing in association with fibroblasts. The intimate contact between these cell types and the need of an association with fibroblasts for the successful culture of lymphoblasts is well documented (113, 127, 128, 143). This interaction usually involves either an adherence of the lymphoid or myeloid cells to the surface of the fibroblasts (peripolesis) or an actual engulfment of these cells by the fibroblasts (emperipolesis). These types of reactions explain a number of our observations with the primary cultures from leukemic swine.

Heavily vacuolated cells have also been described in RSV infected cells (130). However, our data indicates that the degree of vacuole formation observed in the porcine cultures is probably more of a radiation effect.

Finally the significance of the mycoplasma isolations is not clear at this time. These organisms have been isolated from numerous human patients with leukemia and other forms of cancer, but they are usually regarded as opportunistic agents that are present because of the debilitated condition of the host. It was not within the scope of this research to investigate these agents further.

DNA containing viruses have been isolated from miniature swine suffering proliferative hematologic disorders including myelogenous leukemia, lymphocytic leukemia and myeloid metaplasia. Based on the experimental evidence the agents all appear to belong to the adenovirus group. These isolates could well be opportunistic passenger viruses present in a viremic state as a result of the animals debilitated condition. The alternative hypothesis that these agents may play a role in the leukemogenesis of swine chronically exposed to ^{90}Sr by daily ingestion should also be considered.

The DNA nature of the virus itself does not exclude this agent from the group of leukemia producing viruses. A DNA virus has been found to be the causative agent of Marek's Disease (12). In addition, it is well known that several DNA viruses are capable of transforming cells in vitro. For example, one of the adenoviruses isolated in these studies (adenovirus type 2) is capable of transforming rat cells in vitro (45) and adenovirus 7 has been reported to cause lymphoma in hamsters (85).

It is known that adenovirus can produce tumors by inoculation into certain species of animals. Infection with these agents can cause anyone of three types of interaction. In certain cells, the virus causes a lytic interaction due to the cytocidal changes taking place in the cell as the result of virus replication. In other cells the virus causes an abortive interaction that results in formation of virus specific antigens but not to complete virus. Finally the infected cells may transform into morphologically altered cells which are able to undergo unrestricted multiplication. This final reaction corresponds to tumor formation in vivo.

Among the known human adenoviruses some are classified as weakly oncogenic and others are classified as highly oncogenic. The basis of these classifications is the length of the latent period and the efficiency in tumor production.

Adenoviruses have also been isolated from human patients with leukemia and other forms of cancer. However, numerous attempts to detect antigens, which should be present if these adenoviruses are causing cellular transformation, have all been unsuccessful. Therefore, it is generally accepted that the adenoviruses are opportunistic passenger viruses.

The possibility that the adenoviruses are playing some role in the disease process has not been fully investigated.

It is quite possible that these agents participate as some sort of helper in the synthesis of the porcine type C virus, which is more probably the leukemogenic agent.

There is some evidence to support this possibility since it has been reported that 3T3 cells transformed by a DNA virus produced low but reproducible levels of the murine leukemia virus gs antigen. It should be mentioned that the murine leukemia virus has not been isolated from this system (1). However, the apparent interaction suggests that the oncogenic DNA viruses could function by activating previously repressed oncogenes in the cells as a process in the infective cycle. The action of the DNA tumor viruses would then merely be similar to the role of radiation or other carcinogens. These agents, if Huebner is correct, all derepress the C-type RNA virus information considered to be indigenous to the cells (68).

The fact that these two types of viruses could interact with one another is further supported by the report that in vitro transformation of rat cells by various adenoviruses, was greatly accelerated by infecting the rat cells with C-type RNA virus prior to infection with an adenovirus (68).

The second aspect of the study has dealt with a comparison of the characteristics of cell cultures from normal and leukemic swine. The intention of this investigation was to determine

if correlations existed between the pathological diagnosis and the presence of virus, the presence of virus and cellular transformation, and the pathological diagnosis and cellular transformation.

C-type particles were observed only in cell cultures from leukemic animals and normal cultures inoculated with C-type virus. However, both cell cultures containing C-type particles and cell cultures without detectable C-type virus showed characteristics of transformed cells. These cells developed aneuploidy and displayed relatively high cloning efficiency as well as foci of clumped and multilayered cells in culture. These cells were also able to form colonies in soft agar.

The absence of observable C-type particles in the stable porcine cell lines could be attributed to a concentration factor. If this is not the case, active replication of the virus is not essential for most porcine cell cultures to develop colonies in semisolid agar. This is not unusual since other viruses, both DNA and RNA, have been reported to transform cells in a nonproductive manner; that is, without active production of virus by the transformed cell (67, 81, 133). Similarly many long term cell lines, from normal animals undergo some degree of transformation (i.e. infinite growth transformation) without producing virus-like particles (47, 61).

After approximately 15 cell passages in vitro, cells from leukemic animals and one normal animal showed radically altered chromosomal characteristics. The cells became sub-diploid (a form of aneuploidy) with modal chromosome numbers dropping from 38 (normal for swine) to as low as 31. This again is not unusual and it has been well established that nearly all cell lines which have undergone infinite growth transformation (either spontaneous or induced) are karyotypically abnormal (37, 105, 118). The four cell lines in this study displaying aneuploidy all have a type of marker present, in that these cell lines can be distinguished by their respective karyograms. It appears that aneuploidy does have constraints and certain chromosomes (in this case most of the metacentric chromosomes and at least three of the acrocentrics) must be maintained. In other words, while the progression of aneuploidy appears to proceed by somewhat different pathways in these cells the process of chromosomes loss does not progress at random. Based on our observations with these cell cultures, it appears possible that cells in culture might be selectively eliminating those chromosomes not functional to the single unit (or cell) for survival. This might in fact be related to the derepression of the oncogene since repression of the ability for unlimited or unrestricted growth and other growth regulatory factors is really only essential in multicellular organisms. Once the cell is growing as a single

unit and competing for its existence rather than cooperating, regulatory controls would become a hindrance and thus impart a selective disadvantage to cells retaining such controls. In spite of this speculation it remains questionable whether there is any casual relationship between the development of aneuploidy and the transformation process (116, 144).

The varying degree of sensitivity of porcine cell cultures to contact inhibition is difficult to assess. The stable cell lines used were capable of forming foci of aggregated cells and colonies with a high population of viable cells in soft agar. Only the PEK cell line and the cultures from animals 83, 65, and 31 appeared to be affected by cell density and contact inhibitory mechanisms. These apparently are the only non-transformed cell lines.

The majority of the porcine cell lines investigated showed some properties of transformed cells. Our studies further indicate that virtually all porcine cell lines that can be passed in culture more than 15 times undergo some degree of spontaneous transformation.

Let us assume that loss of contact inhibition (i.e. ability of cells to grow and divide in agar suspensions) is associated with neoplasia and the oncogene theory of Huebner and Todaro is correct. The expression of the oncogene without an expression of the virogene could then be the factor responsible

for enabling the cells to continue growth and cell division in the agar suspensions. The findings of Sanford et al. (120, 121) indicate that the repression of the virogene in normal animals may be altered by the in vitro manipulation of the cells resulting in the expression of the virogene. Our results suggest the possibility that in vitro manipulation of cell cultures may derepress the oncogene without allowing the expression of the virogene. On the other hand, these studies suggest that expression of the virogene does result in cellular transformation or the expression of the oncogene.

Finally the percentage of cells from leukemic animals producing C-type particles is extremely high. Several studies indicate that cell cultures from these leukemic animals may produce the C-type virus more readily than tissues from other species. This finding, if completely substantiated, means that swine would have certain advantages as a model system in the study of leukemia.

Although the control swine that have been examined do not appear to have C-type virus-like particles in their tissues, these particles have been observed in most of the leukemic swine studied. In addition some of the animals showing myeloid metaplasia also have had C-type particles.

The virus particles which are found in lymph node, spleen, kidney, liver, and blood plasma appear morphologically indistinguishable from the oncornaviruses causing leukemia in several animal species (87, 109). The particles are the same size and show the same type of replication and maturation as the infected cell. The porcine type C viruses are usually been budding through the cytoplasmic membrane in close contact with cellular membranes or within the vacuoles of an infected porcine cell. This budding process, which is frequently observed is considered to be a normal process in the maturation of an oncornavirus (19, 24).

Some investigators have reportedly observed C-type particles in long term cultures of "normal" porcine cell lines. The cells used in my experiments, however, did not have apparent C-type particles present prior to infection. The fact that C-type particles have never been observed in primary or secondary cultures from normal swine (6) is of particular interest to these studies since I did find C-type particles in primary and secondary cultures from 8 of 10 leukemic swine. Virus from four of these animals was subsequently isolated, purified, and transmitted to one of several stable porcine cell lines which were found to support replication of these viruses in vitro.

All the RNA tumor viruses described to date have similar biophysical, biochemical and morphological properties. My experimental results indicate that the porcine viral isolate possess properties that are similar to those of the known oncogenic RNA viruses (Table 5.1).

In addition, to these properties that are common to the porcine isolates and the oncornaviruses, it has been reported that the oncornaviruses have several unusual metabolic pathways and products. For example, these viruses have an RNA dependent DNA polymerase (reverse transcriptase) which is involved in the production of a DNA that is coded for by the viral RNA (9, 137). All oncornaviruses similarly contain a 60-70S RNA as the infectious component. This large size molecular weight RNA undergoes segmentation after entering the cell. These two properties, a large (60-70S) RNA and the disassociation of the viral RNA into subunits upon infection, have only been described within the oncornavirus group (5, 10, 13, 26, 50, 129). Future studies with the porcine viruses from leukemic swine will be directed towards determining if the porcine isolates possess these important properties. The finding of the aforementioned features would almost certainly indicate that these porcine viruses are involved in the radiation induced leukemogenic process. Even without this information, the porcine type C viruses are excellent candidates for etiological agents of swine leukemia. This conclusion is based on the properties

Table 5.1
**COMPARISON OF PROPERTIES OF THE VIRUS ISOLATED FROM
 LEUKEMIC SWINE AND THE RNA TUMOR VIRUSES**

<u>PROPERTY</u>	<u>PORCINE VIRUS</u>	<u>RNA TUMOR VIRUSES</u>
PARTICLE SIZE	80-100 nm	80-100 nm
MORPHOLOGY: SPHERICAL PARTICLE HAVING A DENSE INTERNAL COMPONENT SURROUNDED BY AN ENVELOPE	YES	YES
VIRUS DENSITY	1.09-1.13 SUCROSE	1.16-1.18 SUCROSE
RNA SIZE		60-70S
REVERSE TRANSCRIPTASE		YES
ONCOGENIC IN <u>VIVO</u>		YES
TRANSFORM CULTURES IN <u>VITRO</u>	YES	YES
SITE OF MATURATION BUDDING FROM CELL MEMBRANE	YES	YES

of the viruses described in this research including the ability of these viruses to transform porcine cells in vitro. However, the actual leukemogenic and/or oncogenic potential of these viruses in swine remains to be determined.

CHAPTER 6

SUMMARY

A survey of 46 miniature swine has revealed that of 34 animals with either myeloproliferative or lymphoproliferative disorders, 21 had demonstrated virus-like particles in their tissues or in plasma pellet preparations. Even more significant, was the finding that 15 of 19 animals with myelogenous or lymphocytic leukemia, had virus-like particles present. In contrast, no virus-like particles were observed in preparations from the 12 control animals.

Cytopathogenic adenoviruses have been isolated from both normal and leukemic swine. These agents are probably only opportunistic passenger viruses, but they may interact with type C viruses to enhance the leukemogenic process.

Long term cell cultures from normal and leukemic swine displayed characteristics of transformed cells. The transformation always occurred if type C viruses were present. However, some cell cultures were apparently able to "spontaneously" transform in the absence of the C type particles.

A type C virus has been isolated from 10 animals with myeloproliferative or lymphoproliferative disorders and five of these virus preparations have now been purified and examined from several biophysical and biochemical parameters. These porcine viral isolates all have properties similar to those of known oncogenic RNA viruses. Porcine embryonic kidney (PEK) cells infected with the porcine type C virus become transformed in vitro indicating that these agents have an oncogenic potential. The cells transformed by the porcine viral isolates undergo a change in morphology from a fibroblastic type of cell to a cell culture in which epitheloid elements predominate. In addition, the cells show a loss of normal contact inhibitory mechanisms. The cells grow as foci of multilayered cells with random orientation instead of the well ordered monolayer culture seen with normal PEK cells. Finally, the transformed cells show both increased aneuploidy and polyploidy.

Based on the findings reported in this thesis, the porcine type C virus is now considered as a candidate for classification as an oncornavirus.

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