

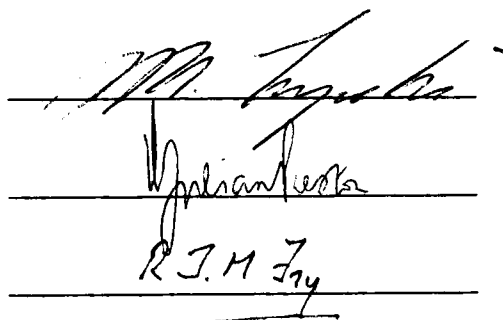
To the Graduate Council:

I am submitting herewith a dissertation written by Stephen Paul Ethier entitled "The Influence of Ionizing Radiation and 7,12-Dimethylbenz(a)anthracene on the Expression of Mammary Ductal Dysplasia in Mice." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Radiation Biology.



Robert L. Ullrich, Major Professor

We have read this dissertation
and recommend its acceptance:



The block contains three handwritten signatures, each written over a horizontal line. The first signature is 'M. J. ...', the second is 'William ...', and the third is 'R. J. M. Fry'.

Accepted for the Council:



Vice Chancellor
Graduate Studies and Research

THE INFLUENCE OF IONIZING RADIATION AND
7,12-DIMETHYLBENZ(A)ANTHRACENE ON
THE EXPRESSION OF MAMMARY DUCTAL
DYSPLASIA IN MICE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Stephen Paul Ethier

December 1982

3065033

It is with great love and respect that I dedicate this work to my
parents, Conrad and Eleanor; and to my wife Beth.

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ABSTRACT

These studies were undertaken to determine if altered growth potential of mammary epithelial cells could be detected in outgrowths derived from monodispersed mammary cells of virgin female BALB/c mice previously exposed to ionizing radiation or 7,12-dimethylbenz(a)anthracene (DMBA).

Monodispersed mammary cells were obtained by enzymatic dissociation of mammary tissues of 12-week-old virgin female BALB/c mice. Twenty-four hours prior to cell dissociation, donor animals were exposed to either γ -ray irradiation or DMBA, while control donors were untreated. Mammary outgrowths were then derived from the donor cells by injecting either 10^5 or 10^4 cells into the gland free mammary fat pads of three-week-old virgin female BALB/c mice. In the initial studies all outgrowths were removed 10 weeks after injection of cells. The outgrowths that resulted were examined at the whole mount and histological level and were classified as having a normal ductal architecture or as having ductal dysplasia or alveolar adenosis. Outgrowths exhibiting ductal dysplasia were further classified as having mild or severe epithelial hyperplasia. The data indicated that treatment of donor animals with either γ -radiation or DMBA could result in an increased incidence of ductal dysplasias over control levels. Further, the incidence of lesions observed in all groups was influenced by the number of cells used to derive the outgrowths in that lesions were more frequent in outgrowths derived from 10^4 rather than 10^5 cells.

The findings of these experiments indicate that acquisition of altered growth potential by mammary cells that results in expression of ductal dysplasia occurs soon after carcinogen treatment and that deriving mammary outgrowths from dissociated cells results in enhanced expression of these lesions.

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CHAPTER I

INTRODUCTION

It is both interesting and ironic that the prevalence of the family of diseases that are collectively referred to as cancer is in many ways the result of the advances in life style and technology which have taken place in the last century. Undoubtedly, humans have been dying of cancer for milenia, however the prevalence of these diseases has grown to large proportions only recently. This increase in the occurrence of cancer is related to modern technology both in that these advances have resulted in greater exposure of human populations to agents which cause or accelerate the onset of this disease, and also in that the improvements which have been made in our life style have drastically increased our lifespan. Thus, because cancer is a slowly progressing disease, which has its most devastating effect in older populations, we have only recently given ourselves the luxury of dying of cancer.

Since cancer is so closely related to modern technology it is only fitting that modern science find ways to deal with these diseases. There can be no question that science has responded to the challenge but clearly the answers are coming very slowly.

One of the biggest stumbling blocks to cancer research is inherent in the complex nature of the disease itself. Truly, cancer is many different diseases which can be caused by agents which differ in their properties about as much as they possibly can (e.g. viruses, chemicals, and radiation). Yet the various types of neoplastic diseases share many

important characteristics and this has led to the general belief that cancer induction could be described by a single, albeit general, model. The elucidation of the mechanisms of carcinogenesis, however, has proved to be a formidable goal. One reason for the slow progress in our understanding of cancer development is that this disease has as its hallmark an aberration in the normal mechanisms of cell and tissue growth regulation. Thus, in order to understand the nature of this aberration it is first necessary to understand the normal mechanisms by which tissues maintain themselves. Unfortunately, these mechanisms are very complex and we are just now beginning to understand some of the most basic control mechanisms. For these reasons, the various models of carcinogenesis that have emerged from experimentation involving viruses, chemicals and radiation have had to rely upon operational terms to describe the process. Thus, terms such as hits and targets, initiation and promotion, fixation of transformation, etc. have been advanced to describe various aspects of carcinogenesis. Although the work from which these terms were derived has provided valuable information regarding cancer development, the limited scope of these operational terms has made it difficult to synthesize the information that has been obtained with different model systems into an acceptable general model of carcinogenesis.

One area of carcinogenesis research in which further work is needed and which can contribute to the effort to obtain a unified model of carcinogenesis is the area concerned with the biology of neoplastic progression. In recent years an impressive amount of information has accumulated from work with several model systems including the mouse

skin model system, mouse mammary tumor system and the rat liver system that indicate that cancer development occurs in a sequential stepwise fashion. These findings have sparked research aimed at isolating and studying cell populations which have properties that are intermediate between normal and neoplastic. This is important because acquiring a better understanding of the biological changes associated with cancer development will help to give biological significance to operational terms and this will provide a common vocabulary for the various disciplines of carcinogenesis research.

The work which is the subject of this dissertation hopefully contributes to this effort by providing some new information concerning the biology of cells soon after exposure to carcinogens and also by providing an improved model system for the study of neoplastic progression.

CHAPTER II

LITERATURE REVIEW

Role of the Mammary Tumor Virus in Mammary Cancer Development in Mice

Any discussion of the factors which influence breast cancer development would not be complete without at least a brief discussion of the murine mammary tumor viruses (MuMTV). MuMTVs are a family of retroviruses, that is, they possess an RNA genome which can direct the synthesis of a complementary DNA template via a virally encoded reverse transcriptase. The viral DNA so synthesized can then integrate into the genome of the infected cell from which point it can direct the synthesis of viral specific gene products which ultimately results in the development of progeny viral particles.

The role which these viruses play in mammary tumorigenesis in the mouse has been the subject of intensive investigation for many years. These studies are of great importance for two principle reasons. First, viruses are good molecular probes, thus they can be used to provide important mechanistic information concerning cancer development at the most basic level. Second, there is a substantial volume of evidence indicating the presence of MuMTV like particles, nucleotide sequences and antigens in human breast tissues and breast cancer cells and this evidence has recently been reviewed as part of an excellent monograph on the mammary tumor viruses (Zotter et al., 1981).

Discovery and characterization of milk transmitted MuMTVs. It has been known for nearly 50 years that certain stocks of laboratory mice

are particularly susceptible to the development of mammary cancer. Genetic studies, performed at the Jackson Laboratories in the 1930's, provided data which suggested that an extrachromosomal factor of maternal origin was responsible for the observed high incidences of mammary cancer in these mice (Jackson Memorial Laboratory, 1933). Later Bittner (1936) and co-workers demonstrated that this extrachromosomal factor was transmitted from mother to offspring via the milk. Thus, newborn mice of stocks which normally developed high incidences of mammary tumors, when foster nursed on mothers of low mammary cancer incidence stocks, would develop far fewer mammary tumors. These workers further characterized the milk factor as an infectious agent which, when purified in cell free extracts and inoculated into low mammary cancer incidence mice, would result in the development of many mammary tumors (Bittner, 1941, 1942, 1945). It was not until many years later however, that this infectious agent was shown to be a virus when electron microscopic examination of cell free extracts and mammary tumors revealed the presence of particles which ranged in size from 30 μ m to 130 μ m (Graff et al., 1949; Passey et al., 1950; Porter and Thompson, 1948).

That MuMTV was the primary etiological agent responsible for the development of spontaneous mammary tumors in mice which bore the virus was conclusively demonstrated by Smith (1966) who observed that 11 years after the development of the high mammary cancer incidence subline C3H/StWi that these mice stopped developing mammary tumors and that they also no longer had virus particles in their milk. Further work demonstrated that a high spontaneous mammary tumor incidence could be

reestablished in these mice by foster nursing them on C3H/AnWi mothers which had viral particles in their milk.

These studies, which clearly demonstrated that the MuMTV was responsible for the neoplastic transformation of mammary epithelial cells, sparked a barrage of work aimed at understanding the mechanisms underlying this most important host-virus interaction.

Some of the earliest information regarding the mechanisms by which MuMTV induces mammary tumors was provided by Michalides and co-workers (1976). These workers studied the integration of MuMTV specific DNA sequences into the genome of normal and malignant cells of C3H and RIII mice, both of which carry highly oncogenic milk born MuMTVs. Their work demonstrated that malignant mammary cells from these mice contained several more copies of MuMTV specific DNA per genome than did either normal mammary cells or liver cells taken from the tumor bearing animals. This important finding has been confirmed by others (Morris et al., 1980; Smith, 1966) and has been interpreted to indicate that replication and re-integration of the MuMTV-DNA is an important factor in virally-induced neoplastic transformation.

Subsequent studies have demonstrated that host genetic factors can significantly influence the action of MuMTVs. For example, C3H mice carry a MuMTV (designated MTV-S) which is highly oncogenic in this strain and which results in the development of mammary tumors in greater than 90% of the infected animals. BALB/c mice do not carry a milk born MuMTV and have a low spontaneous incidence of mammary tumors, however introduction of MTV-S into these mice by foster nursing results in the development of a high incidence of mammary tumors (Zotter et al., 1981).

In contrast, introduction of MTV-S into C57BL mice in the same manner does not result in the development of mammary tumors (Andervant, 1944). Bentvelzen et al. (1970, 1978) has interpreted this difference to indicate the existence of a host regulatory gene, the product of which can suppress the expression of the MuMTV. He has further postulated that mice which are not susceptible to the oncogenic effects of MTV-S, such as the C57BL, possess this gene in its wild type form, while susceptible mice such as the BALB/c and C3H, possess a defective form of the gene.

Discovery and characterization of endogenous MuMTVs. In addition to the highly oncogenic milk transmitted forms of the MuMTV, all mice carry within their genome endogenous forms of the MuMTV which are passed from generation to generation in proviral form via the germ line. The oncogenic capacities of these endogenous MuMTVs vary greatly!

The existence of endogenous MuMTVs was discovered quite accidentally as the result of experiments in which attempts were made to derive substrains of low mammary cancer incidence mice by foster nursing newborn C3H mice on mothers which were free of the milk born virus. Several workers reported that the C3Hf mice so derived developed spontaneous mammary tumors with an incidence of 21 to 52% (Boot and Muhlbock, 1956; Heston, 1958; Heston and Deringer, 1952; Pullinger and Iverson, 1960) in breeding females, and that these tumors developed late in the life of the animals. Electron microscopic examination of hyperplastic alveolar nodules and mammary tumors which arose in these mice revealed the presence of both B-type and intracytoplasmic A-type viral particles which are characteristic of MuMTV (Pitelka et al.,

1960). These particles were isolated by several groups and were found to have low oncogenic activity when inoculated into BALB/c mice (Hageman et al., 1968). These findings indicated the presence of a MuMTV that was distinct from the highly oncogenic MTV-S which is transmitted via the milk in these mice. This MuMTV variant has been designated MTV-L (synonyms are NIV = nodule inducing virus and MTV-1 which refers to the proviral gene) and DNA sequences homologous to this virus have been detected in all tissues of C3Hf mice. This has been taken as further evidence for the presence of MTV-L within the germ line. Van Nie and Verstraeten (1975) have demonstrated that MTV-L behaves as a single dominant gene with regard to the induction of tumors and the synthesis of viral antigens which, although present in very low concentrations, can be detected in the milk of these mice. Bentvelzen et al. (1978) confirmed this finding and also provided evidence which indicates that the expression of this form of the MuMTV is also modified by a regulator gene. In these experiments Bentvelzen observed that the progeny of the cross C3Hf $\times$ C57BL did not develop mammary tumors. Thus, just as MTV-S was ineffective at inducing tumors in C57BL mice, the expression of MTV-L was also suppressed in the hybrid presumably by the presence of a gene from the C57BL parent.

A second mouse strain which carries an endogenous form of the MuMTV which has oncogenic potential was characterized in 1965 by Muhlbock (1965). This strain, designated GR, carries an endogenous MuMTV which results in a greater than 90% incidence of mammary tumors in which viral particles can be detected. Further, foster nursing these mice on low mammary cancer incidence mothers does not alter the incidence of

spontaneous mammary tumors (Bentvelzen and Daams, 1969; Van Nie et al., 1977). This proviral form, designated MTV-2, has also been demonstrated to behave as a single dominant gene with regard to tumor induction (Van Nie et al., 1977). The GR mouse carries multiple proviral (up to 12 copies per genome) forms of this virus, however recent studies have demonstrated that only one form is responsible for the induction of mammary tumors (Michalides et al., 1981).

These studies demonstrate that MuMTVs that are transmitted solely via the germ line can have significant oncogenic potential. Further, the mammary tumors which are induced by endogenous viruses, like those induced by the exogenous forms, all have been shown to exhibit B-type particles and to contain multiple copies of viral specific DNA within the genome of the tumor cells. These similarities are of great importance for two reasons. First, unlike other highly oncogenic retroviruses, MuMTV does not possess a cancer gene and thus an unambiguous marker for neoplastic potential of MuMTVs has not been discovered. Second, there are several strains of mice which do not develop high incidences of spontaneous mammary tumors but which carry endogenous MuMTVs. Many of these mouse strains are susceptible to mammary tumor induction by a variety of agents and this has aroused great interest in putative carcinogen-virus interactions as mechanisms for carcinogen induced mammary tumors. The mouse strain which has been most often used for these studies has been the BALB/c and consequently a great deal has been learned about the endogenous MuMTV carried by these mice and the influence which carcinogen treatment has on its expression.

The endogenous MuMTV in BALB/c mice. The endogenous MuMTV that is carried by BALB/c mice has been termed MTV-0 (named by Bentvelzen who feels that the importance of this virus in BALB/c mammary tumorigenesis has been underemphasized, hence 0 = overlooked). Data have been reported by several workers which suggest that this virus has the potential for mammary cancer induction. For example, B-type particles have occasionally been detected in spontaneous mammary tumors which arise in old mice of this strain as well as in milk of retired BALB/c breeders (Bentvelzen, 1980; Hageman et al., 1972). These workers have purified these particles and have found them to have high oncogenic activity when inoculated into young BALB/c mice. Activation of MTV-0 has been reported by Bentvelzen (1974) who demonstrated that bone marrow cells from lethally irradiated mice, when injected into nonirradiated animals could result in the development of mammary tumors in which B-type particles were detected. Links et al. (1977) have reported activation of MTV-0 in vitro by treatment of baby mouse kidney cells with either 3-methylcholanthrene (MCA) or bromodeoxyuridine (BrdU). Treatment of these cells with these agents resulted in the appearance of altered foci of cells in which MuMTV particles could be detected. Purification and inoculation of these particles into young BALB/c mice resulted in the appearance of mammary tumors. Thus, under some circumstances MTV-0 can be activated and when active appears to have oncogenic potential. It should be emphasized at this point, however, that in each case in which oncogenic activity of MTV-0 has been demonstrated the synthesis of complete viral particles has also been observed.

Ionizing radiation has also been shown to activate endogenous MuMTVs in vivo. In both 020 and X/GF mice, treatment with x-rays and urethane resulted in the development of mammary tumors in which oncogenic B-type particles were detected (Goldfedder, 1972; Timmermans et al., 1969).

Role of MTV-0 in hormone and carcinogen induced mammary tumorigenesis. One characteristic of the BALB/c mouse strain is its low incidence of spontaneous mammary tumors. These mice are, however, rather susceptible to the induction of mammary tumors by a variety of agents. Thus, the discovery of MTV-0 in BALB/c mice and the finding that this virus has the potential for oncogenic activity led to speculation that this virus may be involved in the induction of mammary tumors following carcinogen treatment.

The two biological properties of oncogenic MuMTVs which are consistently expressed in neoplastic mammary cells of virus bearing mice are the presence of mature viral particles amongst the tumor cells and the amplification and re-integration of MuMTV specific DNA within the tumor cells. Mature viral particles have not been detected in chemical carcinogen induced mammary tumors of BALB/c mice (Bentvelzen, 1974). Further, the number of copies of MTV-0 specific DNA present in the cells of these tumors has not been found to be increased over that found in non-neoplastic BALB/c mouse cells (5 to 6 copies per genome) (McGrath et al., 1978; Michalides et al., 1981a).

Although these findings provide strong evidence that MTV-0 is not directly involved in carcinogen induced mammary tumorigenesis in BALB/c mice, several groups have investigated the possibility that MTV-0 is

involved in a more subtle manner. To do this, the transcription and translation of the MTV-0 genome after carcinogen treatment has been investigated.

Enhanced levels of MTV-0 specific RNA have been consistently measured in carcinogen induced mammary tumors in BALB/c mice, however the degree of enhancement varies considerably and appears to depend, in part, on the mode of tumor induction. For example, Michalides et al. (1979) reported a 10-fold increase in the level of MTV-specific RNA in cells of hormonally induced mammary tumors compared to that found in normal mammary tissues of hormonally treated mice. Similar findings have been reported by a number of other groups (Dudley et al., 1978; McGrath and Jones, 1978; McGrath et al., 1981). In contrast, Marcus et al. (1981) reported no differences in the levels of MTV-specific RNA in mammary tissues and tumors from hormonally treated BALB/c mice. Several groups have reported detecting low levels of MTV-RNA in mammary tumors induced by exposure of BALB/c mice to DMBA or x-radiation (Butel et al., 1981; Dudley et al., 1978; Dusing-Swartz et al., 1979; Pauley et al., 1979; Varmus et al., 1973). In contrast to these findings are results reported by Michalides et al., (1979) which indicated a nearly 2000-fold increase in MTV-RNA levels in cells of mammary tumors induced by combined treatment with x-irradiation and urethane. This is particularly interesting in that the levels of MTV-RNA that were detected in mammary tumors from mice treated with either of these agents alone was quite low, yet the tumor incidence observed in these animals was not different from that found in the animals treated with both agents. Thus, although all of these data indicate that MTV-expression

occurs during non-virally induced mammary carcinogenesis in BALB/c mice the evidence does not indicate that MTV-expression is necessary for induction or maintenance of the transformed state.

Although transcription of the MTV-0 genome is consistently found in carcinogen induced mammary tumors of BALB/c mice, translation of the RNA sequences into viral specific gene products is only occasionally observed. The presence of two viral antigens, a 28,000 dalton protein (p28) and a 52,000 dalton glycoprotein (gp52), which are present in high levels in mammary tumors induced by MTV-S, have been studied in tumors of BALB/c mice. McGrath and Jones (1978) and Smith et al. (1981) have detected low levels of p28 in every tumor which they assayed, while other workers have reported that p28 is not present in BALB/c mammary tumors (Dusing-Swartz et al., 1979). These differences reflect the different techniques which have been employed to study this protein. The viral glycoprotein gp52, on the other hand, is only rarely expressed in mammary tumors of these mice (McGrath and Jones, 1978).

The extensive investigations into the role of MTV-0 in carcinogen induced BALB/c mammary tumorigenesis have not yielded any convincing evidence that the virus is an important etiological factor. However, the possibility cannot be ruled out entirely, particularly in light of recent advances made with certain leukemia inducing retroviruses which, like MuMTVs, appear not to have oncogenes, but rather exert their oncogenic activity by a mechanism involving transposition of the virus and subsequent down stream promotion of cellular genes that are normally not expressed (Hayward et al., 1981). Thus, the possibility still exists that carcinogens can effect MuMTV proviruses in a manner which

either induces or allows transposition of the virus. This possibility remains to be investigated more thoroughly.

Induction of Mammary Tumors in Experimental Animals by Chemical and Physical Carcinogens

At about the same time that an extensive research effort was being undertaken to understand the viral etiology of mouse mammary tumors, other workers began studying the influence of carcinogenic chemicals on the development of mammary tumors in mice. In 1941 Englebrath-Holm (1941) published the first account of a carcinogenic effect of a chemical on mouse mammary tissue. In this study it was found that painting of Little's dilute brown mice with a 0.5% solution of MCA once weekly for 23 weeks resulted in the development of mammary tumors in 100% of the animals which survived the treatment. A few years later Kirshbaum et al. (1946) reported similar findings following treatment of NH mice (NH mice are without the milk agent) with MCA. The mammary tumors reported in this study were predominantly adenoacanthomas and were histologically similar to tumors which arose spontaneously in these mice. These workers also demonstrated that the tumors were preceded by lesions which, at the whole mount level resembled hyperplastic alveolar nodules, which are usually found in mammary tissues of mice bearing the MuMTV. Histological examination of these lesions, however, revealed them to be ductal lesions exhibiting substantial squamous metaplasia.

The first report of mammary tumor induction in mice following oral administration of a chemical carcinogen was published by Andervant and Dunn (1953). In this work 9 mg of MCA was administered to DBA/2 mice as

weekly 1 mg doses and this treatment resulted in a mammary tumor incidence of 32%.

The experiments described above demonstrated clearly that mouse mammary tissue was susceptible to the carcinogenic effects of polycyclic aromatic hydrocarbon carcinogens, particularly MCA. However the data obtained from these experiments were difficult to interpret because the dosages which were needed to observe reasonable incidences of mammary tumors were quite toxic and resulted in the early death of many of the animals. This led several workers to attempt to devise methods of inducing mammary tumors without the detrimental side effects observed in earlier work.

A novel approach was employed by Bonser (1954) who implanted bees-wax pellets impregnated with MCA into the inguinal mammary fat pads of female IF mice. This method allowed exposure of the mammary tissue to the carcinogen while avoiding many of the systemic effects of the carcinogen. When these pellets were allowed to remain in place for varying time periods ranging from 4 to 16 weeks the incidence of mammary tumors observed increased with increasing exposure time and ranged from 11% to 50%.

In 1962 Biancifiori et al. (1962) reported that other carcinogens, particularly 7,12 dimethylbenz(a)anthracene (DMBA), were carcinogenic to mammary tissue. Subsequently other workers demonstrated that oral administration of DMBA resulted in moderate to high incidences of mammary tumors in virgin mice of several strains including the BALB/c (Butel et al., 1981), C3H/StWi (Smith et al., 1978) and the BD2F (Medina et al., 1980). Recently the dose response relationship for the

induction of mammary tumors in BALB/c mice following oral administration of single and multiple doses of DMBA has been reported (Ethier and Ullrich, 1982). The results of these studies indicate that mammary tumor induction is dose dependent over a relatively narrow dose range and that the plateau of the dose response curve occurs at a dose which is some 10 to 20 times lower than the doses which had been previously used in mouse mammary tumorigenesis experiments involving DMBA (Figure II-1). Further, the data indicate that increasing the dose of DMBA above that which yields the maximum incidences of mammary tumors serves to progressively shorten the time of appearance of these tumors.

The role of hormones in chemical carcinogenesis of mouse mammary tissue. In many of the early experiments involving chemical carcinogen induction of mouse mammary tumors several workers observed that mammary tumors appeared more frequently in carcinogen treated mice that were allowed to breed following exposure, than in animals that were maintained as virgins (Biancifiori and Cashera, 1963). These findings were confirmed and extended in a number of experiments in which pseudopregnancy was induced in mice following carcinogen administration. In these experiments induction of pseudopregnancy resulted in a dramatic increase in the number of mammary tumors which developed in response to carcinogen administration (Biancifiori and Cashera, 1962; Biancifiori et al., 1959; Biancifiori et al., 1962; Marchant, 1961; Ranadive et al., 1960). Thus, it became apparent that hormones played a significant role in the induction and expression of mammary tumors in mice.

Indeed, the importance of hormones in mammary tumorigenesis was emphasized further by results of experiments which were reported by

Figure II-1. Dose Response for the Induction of Mammary Tumors in Virgin Female BALB/c Mice by DMBA.

The solid line curve represents the data for the observed incidences of mammary tumors and the broken line curve represents the age adjusted tumor incidences. The age adjusted tumor incidences are what would be expected in the absence of life shortening that is caused by the toxic and carcinogenic effects of DMBA on other tissues.

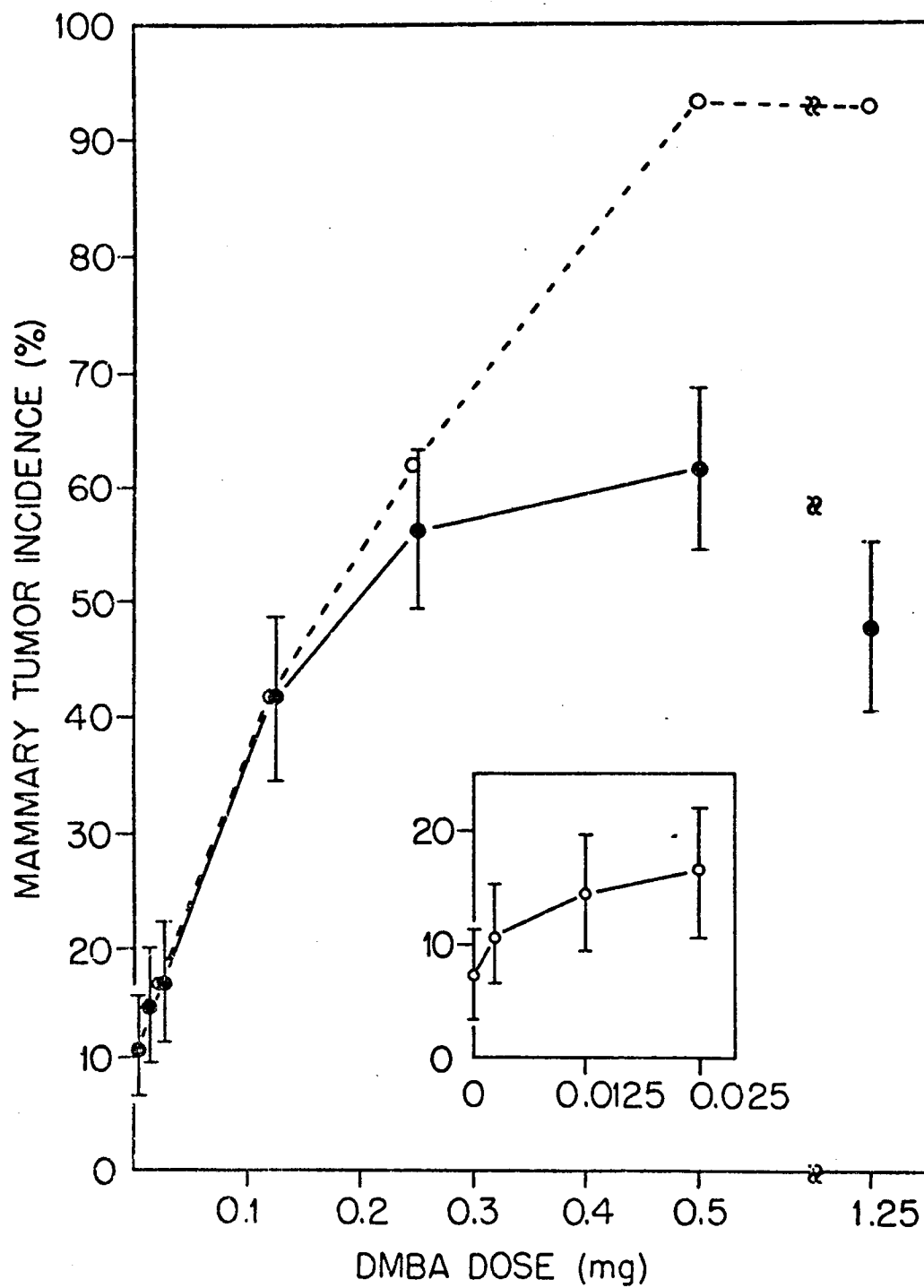


Figure II-1

Mühlbock and Boot (1959) who showed that hormonal stimulation alone, accomplished by ectopic transplantation of pituitary glands, was sufficient for induction of mammary tumors in various mouse strains. Susceptibility to this "hormonal carcinogenesis" however varied widely among the different strains. For example, implantation of 20 pituitary isografts into C3Hf mice resulted in the development of mammary tumors in 100% of the mice, while 200 such isografts when implanted into C57BL mice only yielded 2 mammary tumors in 8 mice.

Later Leibelt and Leibelt (1961) reported that a single pituitary isograft could enhance the incidence of spontaneous mammary tumors in DBA/2F mice and McGrath and Jones (1978) reported similar findings for BALB/c mice.

Hormonal stimulation via pituitary isografts also markedly enhances mouse mammary tumorigenesis following chemical carcinogen administration. Haren-Ghera (1961, 1962, 1963) demonstrated that in LAF mice, hormonal stimulation via a pituitary tumor or pituitary isograft could increase the final incidence of MCA induced mammary tumors from 9% to 35%. Further, this effect was only observed when the hormone treatment was begun prior to carcinogen treatment. Medina (1974a,b) extended these findings to the BALB/c mouse strain and for other mammary carcinogens, including DMBA and urethane. Medina's findings also indicated that hormone treatment must be done prior to carcinogen administration in order for the effect to be observed.

When the pituitary gland is transplanted to a site in which it is no longer under the influence of the hypothalamus it secretes prolactin constitutively (Mühlbock and Boot, 1959). Thus the observations that

pituitary isografts influence the expression of spontaneous and carcinogen induced mammary tumors in mice provided compelling evidence for an important role of prolactin in mammary tumorigenesis. Subsequent observations made under a number of experimental conditions have provided further evidence for the importance of this hormone in mammary tumorigenesis. First, suppression of prolactin synthesis either surgically or via administration of ergot alkaloids dramatically reduces the incidence of mammary tumors following carcinogen administration (Welch and Gribler, 1973). Second, removal of steroid hormones by ovariectomy and/or adrenalectomy also results in an inhibition of mammary tumorigenesis in mice. Susceptibility can be restored to these mice by administration of estrogens (Bern and Nandi, 1961). Importantly, these surgical procedures result in a drastic reduction in prolactin secretion which is restored upon treatment with estrogens. If, however, estrogen therapy is coupled with drug induced inhibition of prolactin secretion, mammary tumors do not develop (Chen and Meites, 1970). Thus, it appears that the role of steroid hormones in mammary tumorigenesis is mediated at least in part via their effects on prolactin secretion.

Although prolactin is of considerable importance in mammary tumorigenesis, high levels of the hormone are not required for the development of tumors. Indeed, several workers have attempted to correlate blood and pituitary prolactin levels with susceptibility to mammary tumorigenesis; however, no consistent pattern has been established (Medina et al., 1977; Sinha et al., 1979).

Despite the obvious importance of hormones, particularly prolactin in mammary tumorigenesis, very little has been learned concerning the

mechanism by which these hormones influence neoplastic development.

This is at least partly due to the pleiotropic effects which prolactin has on mammary tissue. Prolactin not only affects growth of mammary epithelium but is also involved in various aspects of mammary gland function including the induction of milk protein synthesis, lipid metabolism and maintenance of ion fluxes within mammary epithelial cells (Shiu and Friesen, 1980). Further complicating matters are the various multiple hormone interactions which are involved in mammary gland physiology, and which are at present, not clearly understood (Topper and Freeman, 1980). A detailed discussion of the influence of hormones on mammary gland physiology is beyond the scope of this work; however this subject has been presented recently in several excellent reviews (Benerjee, 1976; Cowie and Tindal, 1971; Meites et al., 1972; Shiu and Friesen, 1980; Topper and Freeman, 1980).

Despite the lack of a detailed understanding of the role of hormones in mammary gland function, various hypothesis have been advanced to explain the role of hormones in mammary tumorigenesis. Two such hypotheses have been advanced recently and are of particular interest because while their similarities demonstrate what is reasonably well understood about carcinogenesis, their differences point clearly to gaps in our understanding of how factors such as hormones can influence neoplastic development.

One hypothesis, presented recently by Welch and Nagawasa (1977) postulates that prolactin is involved in carcinogenesis at two levels in a manner analogous to the two stage skin carcinogenesis model. According to this model the influence which prolactin has on the

metabolic capabilities of mammary cells renders them susceptible to carcinogens such as DMBA by facilitating both metabolism of the carcinogen and the subsequent fixation of the transformation event. In the second phase prolactin stimulates mitotic activity of the transformed cells either directly or via the ovary and this results in the development of a tumor.

Nandi (1978) has also advanced a hypothesis for the action of hormones in mammary tumorigenesis. This hypothesis also envisions two phases and in fact Nandi's hypothesis suggests that hormones act in the first phase to facilitate initiation of transformation. In the second phase, however, Nandi proposes that hormones affect normal mammary epithelial cells directly and that the effect on normal cells is what ultimately results in expression of the transformed cells. In support of this hypothesis Nandi cites evidence which suggests that (a) normal cells have a finite division capability while neoplastic cells have infinite division capability (Daniels et al., 1968) and (b) that the presence and architecture of normal cells inhibit expression of neoplastic cells (DeOme et al., 1978). Thus, Nandi suggests that a key controlling factor in expression of neoplasia is the ratio of normal to altered cells. Hormones stimulate division of normal cells which, because of their finite division capability, results in a shift in the ratio of normal to altered cells and this ultimately allows expression of neoplastic cells.

Unfortunately, the evidence which is available to support either of these hypothesis is circumstantial and as yet there is very little

evidence to indicate a mechanism for the role of hormones in mammary tumorigenesis (Nandi, 1978).

Induction of mammary tumors in mice by ionizing radiation. There is much less information available concerning the carcinogenic effect of ionizing radiation of mouse mammary tissue than is available for chemical carcinogens such as MCA and DMBA. This is at least partly due to the fact that ionizing radiation is rather inefficient at inducing mammary tumors in mice. One of the first reports of mammary tumor development in mice following radiation exposure was published by Lorenz and co-workers (1951). These workers noted that mammary tumor development was usually associated with the development of ovarian tumors all of which contained some granulosa cell elements. This observation led to speculation that these mammary tumors were not directly induced by the action of the radiation on the mammary epithelium but rather developed via an indirect mechanism involving the endocrine imbalance which resulted from ovarian tumor development.

Several years later Ullrich and Storer (1979) published dose response data for the induction of a variety of neoplasms, including mammary and ovarian tumors in BALB/c mice following γ -ray irradiation. These data however do little to resolve the question of whether mammary tumors were induced via a direct or indirect mechanism in that the dose range over which mammary tumor induction was dose dependent was not very different from the dose range over which ovarian tumor induction was dose dependent. Specifically, the data indicate that at doses greater than 50 rad, the incidence of ovarian tumors stopped increasing with increasing dose as did the incidence of mammary tumors. These authors

postulated that the incidence of ovarian tumors did not increase beyond that found at 50 rad following higher doses because of excessive cell killing of the target cells in the ovary. This interpretation suggests an alternate hypothesis for the coincidence of mammary tumor induction and ovarian tumor induction following radiation exposure. That is, that mammary tumor development, like the normal maintenance of mammary tissue, requires a functional ovary and not necessarily a neoplastic one. Thus high doses of radiation effectively kill the ovary and this renders the mammary tissue refractory to neoplastic change. Data recently obtained by Ullrich from experiments in which BALB/c mice were exposed to 200 rad of γ -ray irradiation as partial body exposures support this view. In this experiment the anterior three pair of mammary glands of one group of animals (i.e., the ovaries were shielded) were exposed to radiation while the posterior two pair of glands of a second group were irradiated. Among the 48 mice irradiated with the ovaries shielded 10 mammary tumors developed while only three tumors developed in animals in which both mammary tissues and ovaries were exposed (R. L. Ullrich, personal communication). A similar finding has also been reported by Michalides et al. (1979) who reported a mammary tumor incidence of 45% in BALB/c mice exposed to 200 rad of x-irradiation with the ovaries shielded.

Thus, although these findings indicate that ionizing radiation is not an extremely potent carcinogen for mouse mammary tissue they do indicate that this agent can act directly on the mammary epithelium and result in neoplastic change.

Histopathology of mouse mammary tumors. In 1953 a book entitled *The Physiopathology of Cancer* (Homburger, 1953) was published in which one chapter was dedicated to mammary tumors in mice. This chapter, which was written by Thelma Dunn, contains a thorough and detailed histological description of mammary tumors that are observed in mice and this work has become the reference standard (Homburger, 1953, p. 38). For a detailed review of this subject the reader is referred to this work.

The majority of mammary tumors which appear in mice can be classified as either adenocarcinomas or adenoacanthomas. Adenocarcinomas are generally classified as one of three types. Type A adenocarcinomas are very well differentiated tumors in which the predominant appearance is one of numerous tightly spaced acini lined by small cuboidal epithelial cells. Most importantly these tumors are highly uniform in appearance. The type C adenocarcinoma (or fibroadenoma) is also of uniform appearance. These tumors consist of multiple cysts which are surrounded by an edematous appearing stroma. This particular type of tumor is relatively rare in carcinogen treated mice and has most often been observed in old virus free hybrid mice. The third type of adenocarcinoma, the type B, is the form most often reported in both virally and carcinogen induced mammary tumors. Unlike types A and C, which are highly uniform in appearance, type B adenocarcinomas are highly variable and are without a single predominant feature. In type B adenocarcinomas the cells are of glandular epithelial origin and exhibit characteristics such as acinar structures, cysts which may contain papillary projections, and dense areas consisting of chords of

epithelial cells. The hallmark of this tumor type is nonuniformity and these tumors can exhibit all of the above characteristics within a single section.

The second major category of mammary carcinomas which appear in mice are the adenoacanthomas. These tumors are distinguished from adenocarcinomas by the presence of large epidermoid areas consisting of keratin producing squamous cells. These epidermoid areas are responsible for the production of keratin pearls which are the most distinctive feature of these tumors. Adenoacanthomas have been observed to arise spontaneously in some mouse strains (Kirschbaum, 1949) including the BALB/c (Deringer, 1965). However they have been most often detected in experiments in which virus free mice have been exposed to high doses of chemical carcinogen in the presence of exogenous hormone stimulation. In this regard it is interesting to note that carcinogen dose seems to have some influence on the development of adenocarcinomas versus adenoacanthomas in that while high doses of DMBA yield many adenoacanthomas in BALB/c mice (Medina, 1974a), low doses of this agent result almost exclusively in the induction of adenocarcinomas (Ethier and Ullrich, 1982). Although the mechanism which underlies the development of adenocarcinomas versus adenoacanthomas has not been established, Slemmer (1974) has presented data which suggest that adenocarcinomas arise from transformation of alveolar or ductal mammary epithelial cells while adenoacanthomas arise from transformed myoepithelial cells.

In addition to these major tumor types Dunn has also described other types of mammary tumors including neoplasms classified as

carcinosarcomas, sarcomas and of course a category of miscellaneous tumors. Since these tumor types have not been observed in studies in our laboratory they will not be discussed further here. Figures II-2 through II-5 present the types of mammary tumors which have been observed most often in carcinogenesis studies performed in this laboratory with BALB/c mice along with their classification according to Dunn.

Chemical and physical induction of mammary tumors in rats. The study of mammary tumorigenesis in the rat has also been widely used as a model for human breast cancer development. The rat mammary tumor system has been a popular model for several reasons. First, mammary tumors can be readily induced in rats by exposure to chemical carcinogens (Huggins et al., 1959, 1961) and by ionizing radiation (Shellabarger et al., 1957). Second, the spectrum of histological types of mammary tumors which are observed in rats resembles that seen in humans. Third, a fairly high proportion of the mammary tumors observed in rats are hormone dependent (i.e., cannot grow in the absence of ovarian hormones) and this also is similar to the situation in human breast cancer.

Studies using the rat mammary tumor system have yielded important mechanistic information regarding DMBA induced mammary tumorigenesis. In some of the early investigations with this system several workers noted that the incidence of mammary tumors which occurred in response to carcinogen administration was strongly influenced by the age at which the animals were exposed (Dao, 1969; Huggins et al., 1959; Nagawasa, 1981). This finding led to the investigation of the kinetics of mammary tissue in relation to the age of the animals. Nagawasa et al. (1974)

Figure II-2. Mammary Adenocarcinoma Type A.

Note that this tumor is both well differentiated and highly uniform in appearance.

Figure II-3. Mammary Adenocarcinoma Type B.

Note that this tumor is highly variable and has some areas that appear well differentiated while other areas consist of chords of cells.

Figure II-4. Mammary Adenocarcinoma Type C.

Note the abundant stroma and the dialation of the acini.

Figure II-5. Mammary Adenoacanthoma.

Note the squamous metaplasia and the abundance of cornified cells and keritan.

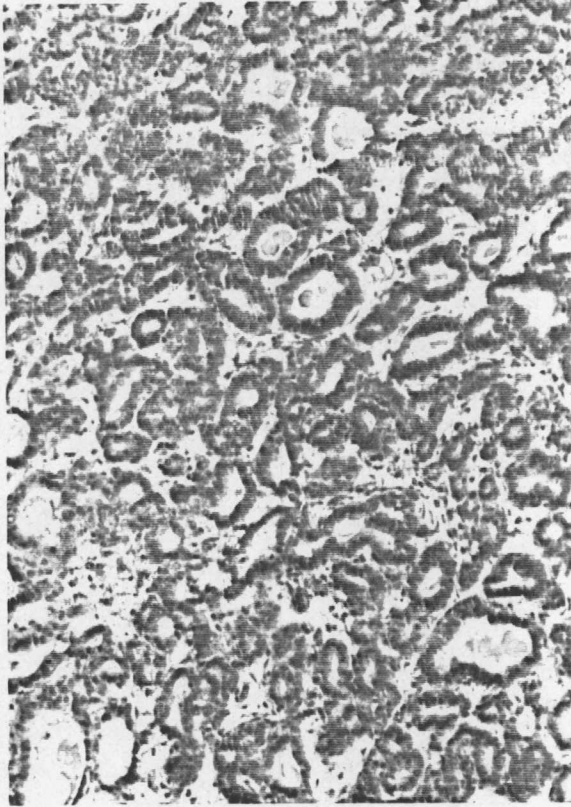


Figure II-2

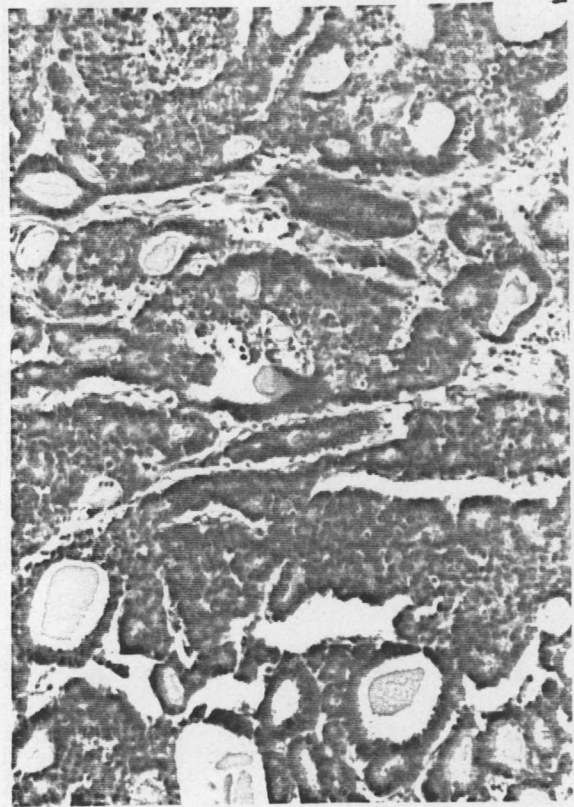


Figure II-3

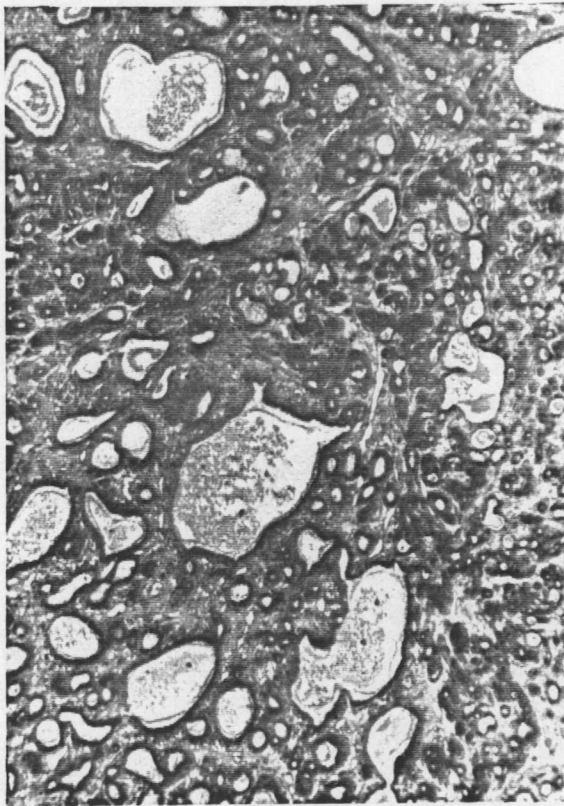


Figure II-4

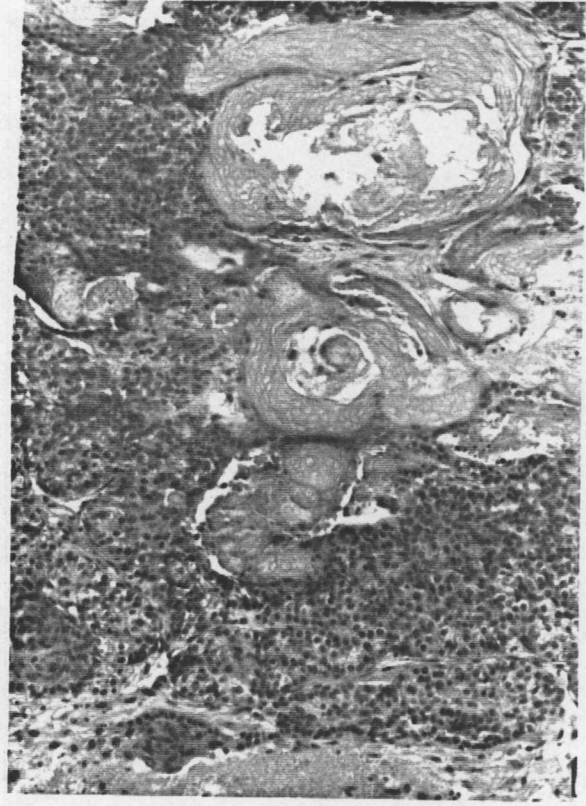


Figure II-5

measured ^3H -thymidine incorporation into mammary cells of Sprague-Dawley rats at various ages and found that DNA synthetic activity within the mammary gland (as measured by the labeling index) began to rise rapidly when the animals were about 30 days of age and continued to rise until reaching a peak value at 50 days of age. The high labeling index which was measured at day 50 persisted through day 70 after which time it decreased rapidly. This finding indicated a close correlation between age related changes in mitotic activity within the mammary gland and susceptibility to DMBA induced tumorigenesis. Other workers have performed similar studies, the results of which confirmed those of Nagawasa (Sinha and Dao, 1980; Schad and Janss, 1980).

Some time later Sinha and Dao (1980) reported the results of experiments which further indicated the importance of high levels of mitotic activity for mammary tumorigenesis but which also indicated that DMBA itself could influence this activity. In this work 200 day-old Sprague-Dawley rats were exposed to DMBA either intravenously or by direct application to the mammary tissues. As expected, the animals which received the DMBA intravenously did not develop mammary tumors; however those animals which received direct applications of the carcinogen developed a high incidence of mammary tumors. Further experimentation revealed that direct application of DMBA resulted in a dramatic increase in the mitotic activity within the mammary tissue and this effect was not observed in animals given the carcinogen systemically.

The general agreement of the importance of high levels of DNA synthetic activity during carcinogen administration led to further investigations aimed at understanding physiological factors which

influenced the kinetics of mammary tissue and hence susceptibility to carcinogenesis.

Several workers have attempted to demonstrate a correlation between a specific stage of the estrus cycle and susceptibility to carcinogenesis. DNA synthetic activity is highest during the diestrous stage of the estrus cycle and Young et al. (1970) and Lindsey et al. (1981) have reported that administration of DMBA during this phase results in the highest incidence of mammary tumors. Nagawasa et al. (1976), however, found that rats are most susceptible to DMBA during proestrus. These conflicting results are difficult to reconcile in that in the studies of Young et al. (1970) and Lindsey et al. (1981) DMBA was administered via gastric instillations, while in Nagawas's work the animals were exposed intravenously.

Russo and co-workers have performed elegant studies on the kinetics of Sprague-Dawley rat mammary tissue during various physiological states and their findings have provided further insight into the factors which influence susceptibility to tumorigenesis. These workers demonstrated that the mammary glands of rats which are most susceptible to mammary tumorigenesis (50-70 days of age) consist mainly of ducts which are capped by relatively undifferentiated structures that are referred to as terminal end buds (TEB). Old virgin rats, which are intermediate in susceptibility, contain far fewer TEBs which have been replaced by more well differentiated alveolar buds (AB). Finally, parous rats, which are resistant to induction of mammary tumors, have mammary glands which are without TEBs and consist entirely of alveolar buds (Russo and Russo, 1978a,b). Kinetic studies of the various mammary gland structures

indicated that cells within TEBs had a higher labeling index and a shorter cell cycle time than cells in alveolar bud structures of old virgin or parous animals. Further, cells within ABs of parous animals had a considerably longer cycle time than did cells in similar structures of old virgin animals (Russo and Russo, 1980a). Thus susceptibility to mammary carcinogenesis appears to be strongly influenced by the degree of differentiation within the mammary gland and this, of course, is under the influence of hormones. Russo has further suggested that these findings provide a mechanism for the protective effect of early first pregnancy for breast carcinogenesis which is well known in humans and has been observed in rats (Russo and Russo, 1979, 1980b).

Yet another factor which appears to play a role in age-related changes in susceptibility to mammary tumorigenesis is the ability of mammary cells to metabolize DMBA or MCA to the ultimate carcinogenic form as well as the cell's ability to remove DMBA-DNA adducts. Greiner et al. (1980) showed that mammary cells of 50-day-old rats metabolize MCA more efficiently than mammary cells of older animals. Tay and Russo (1981a,b) extended these findings and showed that metabolites of DMBA bind to DNA of mammary cells of 50 day old rats approximately 1.5 to 2 times greater than to DNA of cells of old virgin or parous animals. They further demonstrated that cells from parous animals removed DMBA-DNA adducts more completely and at a faster rate than cells from young virgin or old virgin animals.

In addition to induction by carcinogenic chemicals rats are susceptible to mammary tumorigenesis following exposure to ionizing radiation. Shellabarger and co-workers (1957) have described the

development of mammary tumors in Sprague-Dawley rats following exposure to x-rays and Vogel (1969) has performed similar studies with neutron irradiation. These studies indicated that ionizing radiation can profoundly influence the development of mammary tumors in these animals, however the data are difficult to interpret mechanistically because of the relatively high spontaneous incidence of mammary tumors that is characteristic of this strain. Shellabarger et al. (1980) have analyzed the development of these tumors over the entire lifespan of irradiated and non-irradiated rats. The mortality corrected cumulative prevalence of fibroadenomas in non-irradiated rats is 80% and the data for irradiated groups indicates that radiation exposure results in a forward shift in time of the spontaneous incidence. The relatively small number of adenocarcinomas observed in the study precluded a similar statistical analysis for these tumors separately, however statistical analysis of the data for all mammary neoplasms (including fibroadenomas and adenocarcinomas) also indicated that the dose dependent increase in tumor incidence following radiation exposure was due to earlier development of spontaneous neoplasms (Shellabarger et al., 1980).

Induction versus acceleration of neoplasia is very difficult to resolve experimentally, and it is uncertain what role acceleration of spontaneous neoplasia may play in chemical carcinogenesis of mammary tissue in rats. A study of the influence of DMBA on mammary tumor development in Sprague-Dawley rats similar to that performed by Shellabarger et al. (1980) for radiation has not been done, however very recent data presented by Holtzman et al. (1982) do suggest that the mechanism of action of radiation and chemicals may be different. They

demonstrated that exposure of Sprague-Dawley rats to radiation during pregnancy did not reduce the incidence of mammary carcinomas relative to that observed following exposure to age matched virgin animals. This is in contrast to data from similar studies with chemicals which indicate that mammary tissue is refractory to DMBA induced tumorigenesis during pregnancy (Sinha and Pazik, 1981).

Evidence that ionizing radiation plays a role in initiation of mammary tumorigenesis in rats has been presented by workers using Wistar-Furth rats. These animals have a lifetime spontaneous incidence of mammary tumors of approximately 17% and the majority of these tumors are benign fibroadenomas (Kim et al., 1960). Yokoro and co-workers (1977, 1980) reported that exposure of these rats to x-ray, γ -ray or neutron irradiation alone did not alter the incidence of mammary tumors in these animals, however when radiation treatment was followed with prolactin stimulation, accomplished by grafting a prolactin secreting pituitary tumor to the irradiated animals, many tumors developed. Further, the majority of the mammary tumors observed in these studies were adenocarcinomas. An important observation from these experiments was that delaying the time between radiation treatment and prolactin stimulation did not effect the yield of mammary tumors even when 7 months elapsed between administration of the two agents.

Thus, it would appear that ionizing radiation has the potential both to initiate the carcinogenic process as well as accelerate the development of tumors.

The Importance of Preneoplastic Lesions in the Progression of Mammary Neoplasia

An area of research which has become the subject of a good deal of investigation in recent years is concerned with factors which can influence the progression of cells from the normal to the neoplastic state. In order to study neoplastic progression, experimental systems are required which allow one to detect intermediate stages of carcinogenesis, and in this regard the study of preneoplastic lesions has been of first importance. Preneoplastic lesions are abnormalities which occur within tissues, the cells of which are not neoplastic themselves but which have a higher probability of progressing to neoplasia than do cells of normal tissue. The study of preneoplastic lesions is of great importance in cancer research for two reasons. First, identification of discrete, morphologically identifiable tissue abnormalities which have a high probability of yielding a malignant tumor can have important prognostic and diagnostic significance. The identification of preneoplastic lesions which occur in human skin, breast, colon and uterine cervix has already become of prime importance for cancer diagnosis. Second, preneoplastic cells, being intermediate between normal and neoplastic cells, can be used to study the important early events in carcinogenesis since these cells have acquired fewer of the ancillary biological changes that are characteristic of neoplastic cells.

The rat and mouse mammary tumor systems are particularly amenable to the study of lesions which are precursors of mammary carcinoma.

Indeed, a good deal of what is known about the biology of preneoplastic cells has come from work with this model system.

Hyperplastic alveolar nodules (HAN) in MuMTV⁺ mice. It has been known for many years that mammary carcinoma in mice is preceded by discrete hyperplastic lesions. In fact, the first description of the lesion which is now known to be the precursor of MuMTV induced mammary cancer was reported in 1906 and then again in 1911 by workers who studied the morphology of mammary tissues in mice which developed high incidences of spontaneous mammary tumors (Haaland, 1911). The lesions observed in these studies were termed hyperplastic alveolar nodules (HAN) because morphologically they appeared as discrete focal areas of lobuloalveolar growth within otherwise ductal mammary tissue. Some time later Fekete (1938) and Huseby and Bittner (1946) noted that the incidence of HAN in these mice correlated with the development of spontaneous mammary tumors and consequently postulated that HAN were precursors to mammary carcinoma. It wasn't until 1959 however that K. B. DeOme and co-workers (1959) conclusively demonstrated the preneoplastic significance of HAN. In order to carry out these experiments a novel method for the study of growth of mammary tissue was developed. In this method the rudiments of inguinal mammary glands of 3-week-old virgin mice were removed surgically by excising a small portion of the mammary fat pad. Clearing of the mammary tissue in this fashion left within the animal the remainder (about 3/4) of the mammary fat pad which was then free of host mammary tissue. The gland free mammary fat pad so derived provided a perfect site for the isologous transplantation of small pieces of mammary tissue the growth of which

could then be easily studied. DeOme used this method to test the growth potential of different types of mammary tissue. In one experiment, either normal or HAN tissue were identified in situ in MuMTV-S⁺ mice by injecting the animals with trypan blue prior to surgery. The mammary tissues so identified were excised and cut into 0.5 mm pieces and then transplanted into gland free mammary fat pads of 3-week-old syngeneic mice. When pieces of normal ductal tissue were transplanted, outgrowths exhibiting a ductal architecture developed which grew to fill the mammary fat pad within 10 weeks of transplantation. When pieces of HAN tissue were transplanted in a similar fashion, the outgrowths which developed in virgin hosts exhibited lobuloalveolar morphology (architecture which is normally observed only in pregnant animals) and these outgrowths also filled the fat pad within 10 weeks of transplantation. Of great importance in these studies was the observation that the lobuloalveolar outgrowths which developed from HAN progressed to overt neoplasia with a much greater frequency than did the outgrowths that were derived from normal ductal tissue. Thus HAN were demonstrated to be a definitive high risk lesion in that although HAN or HAN derived outgrowths were not neoplastic themselves they possessed a higher than normal probability of progressing to overt mammary tumors.

This very important finding prompted further experimentation, the results of which has provided some of the best information available concerning the biology of preneoplastic cell populations. One such study was undertaken because it had been observed in in vitro studies that there was a great difference in the proliferative capabilities of neoplastic and normal cells. Daniel et al. (1968, 1975) used the fat

pad transplantation method to measure the in vivo lifespan of HAN and normal mammary tissue. For this experiment several different HAN outgrowth lines were developed. These HAN lines, along with normal ductal tissue, were transplanted as 0.5 mm pieces every 10 weeks at which time the degree of outgrowth development was assessed and the tissues passaged into gland free fat pads of new host mice. The data from these studies indicated that normal ductal tissue could be transplanted and would continue to grow for about 8 transplant generations. In contrast, all of the HAN outgrowth lines that were developed continued to proliferate (at the same rate) for over 30 transplant generations. In other, similar studies, DeOme et al. (1961) made the important observation that the frequency with which a given HAN line yielded mammary tumors was a stable characteristic of that line which was maintained from transplant generation to transplant generation. The tumor potentials of the different HAN lines varied widely, however, and did not correlate with the growth rate of the line. These findings have been interpreted to indicate that nodule transformation results in cells with an enhanced growth potential and an altered response to hormones and that neoplastic transformation is a separate event which takes place within populations of HAN cells. Thus, the alteration which results in nodule transformation yields cells that are more likely to undergo neoplastic transformation than are normal cells.

In studies performed more recently DeOme et al. (1978) have provided evidence which indicates that expression of the preneoplastic phenotype is controlled to some degree by the presence and architecture of normal mammary cells. Prior to this work several lines of evidence indicated

that MuMTV induced transformation was a rare event which occurred long after infection by the virus. For instance, HAN and tumors appear in numbers which are small relative to the total number of infected mammary cells. Further, the tumors which develop in these animals appear a relatively long time after infection which occurs at birth. Finally, transplantation of pieces of normal ductal tissue from MTV-S⁺ mice into gland free fat pads of syngeneic mice only rarely results in the development of HAN or tumors. DeOme and co-workers postulated an alternate hypothesis to explain these observations. They postulated that virally induced transformation was not a rare event but that expression of transformation was inhibited by the presence and architecture of normal mammary cells. In order to test this hypothesis a modification of the fat pad transplantation technique was developed in which a single cell suspension of mammary cells obtained by enzymatic dissociation of the mammary tissues, was injected into gland free mammary fat pads. Using this approach these workers injected monodispersed mammary cells from MTV-S⁺ donor animals of ages well below that at which HAN are normally detected. The outgrowths which developed from these dissociated cells exhibited a high frequency HAN, and this finding was consistent with DeOme's hypothesis.

The fat pad transplantation method has also been useful in elucidating the role of hormones in mammary tumorigenesis. Medina et al. (1970a) used this technique to determine if hormones acted at the level of nodule transformation by the virus, or if hormones facilitated expression of transformation. Medina demonstrated that prolonged hormone stimulation via pituitary isografts of MTV-S⁺ mice increased the

frequency and decreased the time of appearance of HAN in these mice. Further, when pituitary isografts were given to virus free mice which were bearing HAN derived outgrowths the hormonal stimulation decreased the latent period for tumor development but did not increase the frequency of tumors derived from the HAN line. These data suggested that hormones could facilitate nodule transformation by the MuMTV and could accelerate the rate of neoplastic progression but that hormones themselves did not induce neoplastic transformation. Support for this conclusion was provided later by DeOme et al. (1978b) who, using the cell dissociation method, demonstrated that outgrowths derived from the cells of young MTV-S⁺ parous mice (MTV-S⁺ parous mice have a significantly higher incidence of spontaneous mammary tumors than do virgin mice) exhibited significantly more HAN than outgrowths derived from cells of age matched virgin mice. These findings were interpreted to indicate that there were substantially more inapparent nodule transformed cells in the mammary tissue of parous than virgin animals and thus that hormones probably played some role in MuMTV induced nodule transformation.

The discovery of the hyperplastic alveolar nodule in the mammary tissues of mice which developed a high incidence of spontaneous mammary tumors was one which led to some very significant findings about the biology of neoplastic progression. Experiments performed with this system indicated that overt neoplasia could be preceded by a non-neoplastic lesion, the cells of which were more likely to progress to neoplasia than normal cells, that these pre-neoplastic cells acquired the ability to proliferate indefinitely in vivo before and apparently

separate from the time they acquired neoplastic potential and that normal cells can influence the expression of carcinogen altered cells.

Hyperplastic alveolar nodules in MuMTV⁻ mice. Not long after the demonstration of the preneoplastic nature of HAN in MTV-S⁺ mice, the question was asked whether mice which were free of the virus would develop similar lesions in response to treatment with carcinogenic chemicals? The answer to this question was provided by two separate groups who demonstrated that administration of chemical carcinogens, when given under the proper hormonal conditions, did yield HAN in the mammary tissues of the exposed mice (Biancifiori and Caschera, 1962; Faulkin, 1966). These findings prompted Medina to attempt to derive chemical carcinogen induced HAN outgrowth lines analagous to the MuMTV induced HAN lines. For this work Medina used BALB/c mice because they are free of both the milkborne MTV-S and the NIV. To induce HAN in these mice the animals were given pituitary isografts at 6 weeks of age (to induce lobuloalveolar differentiation of the mammary tissues) and then exposed to MCA once weekly for 3 to 6 weeks. Following completion of the carcinogen administration protocol the pituitary isografts were removed to allow the mammary tissues to regress to the ductal architecture. Whole mount examination of mammary tissues which were treated in this fashion revealed the presence of alveolar nodules which had not regressed following removal of the pituitary isografts and thus had the same altered hormone responsiveness as MuMTV induced HAN. Medina, using this protocol to induce HAN, and using the fat pad transplantation method described earlier, developed the D-series of carcinogen induced nodule outgrowth lines. These lines exhibited many

of the same characteristics as the virally induced HAN lines. First, they could all be passaged indefinitely in vivo. Second, the tumor potential of individual lines was a stable characteristic of the line, yet there was a great deal of variability in the tumor potential of different lines (Medina and DeOme, 1968, 1970).

These findings indicated that HAN could be induced by both the oncogenic MuMTV as well as chemical carcinogens. The evidence, from work performed on virally induced HAN, indicated that nodule transformation and neoplastic transformation were separate events. With the availability of MuMTV free mouse strains, and non-virally induced HAN, experiments could be performed to determine if either the virus or chemical carcinogens were also involved in the second step, i.e., transformation from nodule to neoplastic. In order to determine if the MuMTV could induce neoplastic transformation of nodule transformed cells pieces of the nodule outgrowth line D1 were transplanted into the gland free mammary fat pads of BALB/cf C3H mice (BALB/c mice foster nursed on C3H mothers and thus infected with MuMTV-S). Transplantation of mammary tissue into these mice resulted in infection of the nodule outgrowth with the MTV-S and this resulted in an increase in the tumor incidence derived from the D1 outgrowths from 8.3% to 83.8% (Medina and DeOme, 1968). Similarly, when pieces of the D1 nodule line were transplanted into gland free mammary fat pads of C3Hf mice (C3H mice foster nursed on virus free mothers and thus only carrying the NIV) the nodule outgrowths were infected with the NIV and this also resulted in an enhanced tumor potential of the D1 line, however to a lesser degree than that found after infection with MTV-S (Medina and DeOme, 1969). In other

experiments BALB/c mice bearing D1 nodule outgrowths were exposed to MCA. Here, as was the case for the MuMTV, carcinogen administration enhanced the tumor potential of the nodule outgrowth lines (Medina and DeOme, 1970; Medina et al., 1970b). Finally, D1 nodules were transplanted into pituitary isograft bearing virus free BALB/c mice to determine if hormones could induce neoplastic transformation. The results of these experiments indicated that prolonged hormone stimulation decreased the latent period for tumors derived from the D1 nodule outgrowth line without increasing the tumor potential of this line (Medina et al., 1970a).

All of these experiments served to characterize a unique model system of neoplastic progression which allowed one to separately study early stages in carcinogenesis that result in the emergence of cell populations which are likely to progress to neoplasia, and the development of overt tumors from these preneoplastic cell populations.

Since the development of this model system, several groups have studied various biological characteristics of HAN in order to gain a better understanding of factors which occur prior to neoplastic transformation. Guillino (1977) studied the ability of HAN to induce angiogenesis in the rabbit iris. These studies demonstrated that the low tumor potential D1 HAN line could induce neovascularization with about the same efficiency as that of normal mammary tissue, while the moderate tumor potential D2 line (30% tumor incidence) induced neovascularization with an efficiency that was intermediate between normal and neoplastic mammary tissue. This suggests that some HAN tissues possess a second characteristic normally associated with

neoplasia, i.e., the ability to induce angiogenesis. Medina et al. (1975) demonstrated that the mean latent period for mammary tumors derived from the D1 nodule outgrowth line is 481 days yet these tissues will grow in ovariectomized mice as long as the surgery is performed at least 4 weeks after nodule transplantation. Thus, nodule tissues also acquire hormone independence (the ability to grow in ovariectomized animals) prior to neoplastic transformation.

Carcinogen induction of non-HAN mammary dysplasias in mice. Because HAN are of such importance in the progression of MuMTV induced mammary tumors and because of the ease with which these lesions could be detected and studied, a large fraction of the work which involved detection of early lesions following chemical carcinogen administration of mice centered around this lesion. However other types of mammary dysplasias have been detected in chemical carcinogen treated mice. Some of these lesions bear some resemblance to HAN. For instance, Faulklin (1966) described lesions which he referred to as alveoli along ducts (alveolar adenosis) and noduloids in mice treated with hormones plus MCA. These lesions bore morphologic resemblance to HAN but differed either in their size or their location along the duct. Medina (1976a) described a lesion which was termed a keratinizing nodule (KN). This lesion also exhibited a lobulalveolar morphology in an otherwise ductal mammary gland but in addition had areas of epidermoid differentiation and keratin production. It was hypothesized that this lesion gave rise to adenoacanthomas; however transplantation of KN into gland free mammary fat pads yielded outgrowths which exhibited a ductal architecture and rarely gave rise to a tumor.

In addition to these alveolar type lesions, mammary dysplasias of ductal origin have been reported in several different mouse strains following chemical carcinogen administration, however their importance in mouse mammary tumorigenesis had not been recognized until very recently. This is largely because these lesions occur following carcinogen administration to virgin animals and, as has been discussed earlier, most experiments done with mice have employed protocols in which carcinogens were administered during hormonal stimulation of the mammary gland.

The first report which suggested that mammary tumors which developed in carcinogen treated virgin mice were preceded by ductal dysplasias was published by Bonser (1956). In this work female IF mice were exposed to the carcinogen by implanting beeswax pellets impregnated with MCA into the inguinal mammary fat pads of these mice. A serial sacrifice study of these animals revealed the presence of ducts which exhibited intraductal and intra-acinus epithelial hyperplasia soon after treatment. Bonser noted that these lesions progressed with time and ultimately gave rise to mammary carcinoma.

In 1974 Medina (1974a) reported the occurrence of ductal hyperplasias in DMBA treated BALB/c and C57BL mice. This finding led to further investigation which indicated that in virgin BALB/c or C57BL mice treated with MCA, DMBA or urethane, ductal hyperplasias, and not HAN, were the predominant lesions detected and that their incidence correlated with the incidence of tumors. Further, histological analysis of these tumors indicated that they arose within or adjacent to areas exhibiting the ductal hyperplasias. An interesting finding from these experiments was that ductal lesions observed in carcinogen treated

BALB/c mice were morphologically different from those observed in C57BL mice. In BALB/c mice the lesions were end bud hyperplasias and were characterized by ducts which gave rise to numerous abnormal end bud like structures. Histologically, these end bud like structures showed marked epithelial hyperplasia with cells which proliferated into and often occluded the ductal lumen. In contrast, C57BL mice developed lobular hyperplasias which were characterized by numerous and hyperplastic terminal ductules (Medina and Warner, 1976; Medina, 1976b; Smith et al., 1980). Ductal hyperplasias also have been detected in carcinogen treated C3H/StWi mice (Smith, 1980).

These findings suggested that lesions, different from the HAN, were of importance in the pathogenesis of mammary tumors in mice. More direct evidence for the preneoplastic significance of these lesions, which was also provided by Medina (1979), served to reinforce this view. In this work BALB/c mice and mice of the hybrid strain C57BL $\times$ DBA F₁ were exposed to doses of DMBA and urethane that were known to induce high incidences of ductal hyperplasias and tumors. Several months following exposure, pieces of dysplastic mammary tissue were identified in situ and transplanted into gland free mammary fat pads of syngeneic mice. Using this method Medina developed several in vivo lines of mammary tissue which exhibited varying degrees of ductal dysplasia. These lines were similar to HAN lines in that they progressed to mammary carcinoma within approximately 7 transplant generations (tissues were passaged every 10-12 weeks), however they were unlike HAN lines, in that those tissues that did not progress to carcinoma lost their growth potential within approximately the same time. These findings

strongly suggest that mammary carcinoma in mice can be preceded by ductal dysplasias. The data, however, should be viewed with caution because of the method with which the lesions were detected in situ. Unlike HAN, ductal dysplasias are difficult to detect in situ and for this reason the atypical ductal areas were identified by looking for areas within the mammary fat pad which exhibited a high degree of vascularization. This, coupled with the long times between exposure and assay for ductal lesions (8-10 months) suggests the possibility that the ductal tissue that was transplanted may have contained overtly neoplastic cells. Thus, although these findings clearly indicate that chemical carcinogen induced mouse mammary tumors arise from ductal structures, and not HAN, the question as to whether ductal hyperplasias represent a stable intermediate condition between normal and neoplastic remains open.

Preneoplastic lesions in rats. The demonstration of the importance of HAN in both virus bearing and non-virus bearing mice stimulated research aimed at determining if similar lesions were precursors to mammary carcinoma in the rat. Although structures resembling HAN have been known to occur in mammary tissue of carcinogen treated rats since 1953 (Tuba et al., 1953), there is still considerable controversy as to the preneoplastic significance of this lesion. In fact several reports have provided data which indicate that these lesions have little if any preneoplastic significance in the rat. For example, in DMBA or x-ray treated rats of several strains the incidence of HAN increases with age, however the incidence is similar in rats which develop tumors and in those that do not and thus HAN incidence does not correlate with tumor

incidence (Beuving et al., 1967a,b; Faulkin et al., 1967). Transplantation of HAN detected in Sprague-Dawley, Fisher and Wistar rats into gland free fat pads of syngenic hosts yields alveolar outgrowths in virgin animals which escape from ovary dependence soon after transplantation, and in these respects closely resemble HAN found in mice (Beuving, 1968, 1969a,b). However the data of Beauving et al. (1967a,b) and Sinha and Dao (1977) indicate that these lobuloalveolar outgrowths do not progress to mammary tumors. In contrast Beauving (1968) found that 8 of 37 HAN outgrowths derived from DMBA treated Lewis rats progressed to mammary carcinoma. Support for this finding has recently been provided by Rivera et al. (1980) who found that when HAN from carcinogen treated Lewis rats were maintained and serially passaged in organ culture that the tumor potential of these cells was greatly enhanced. That these HAN can acquire oncogenicity with time in organ culture was subsequently confirmed by Maiorana and Guillino (1975) who showed that primary rat HAN had little or no ability to induce angiogenesis in the rabbit iris, but acquired an ability to do so following several in vitro passages.

There is a large body of data which indicates that mammary carcinoma in rats arise from the ducts and that these tumors are preceded by ductal dysplasias (Middleton, 1965). Fisher et al. (1975) have performed a serial sacrifice study with DMBA treated Wistar Furth rats. They reported the development of ductal lesions which occurred within weeks of carcinogen treatment. These early lesions were characterized by epithelial hyperplasia within the terminal duct structures. The cells which made up these lesions were large and had prominent nucleoli and many mitotic figures were observed. Analysis of the chromosomes of

these cells did not reveal any differences compared to cells of normal mammary tissue. Lesions which appeared at later times after carcinogen treatment exhibited more advanced hyperplasia and the cells within these lesions had chromosome abnormalities similar to those observed in neoplastic cells.

Haslam and Bern (1977) and Purnell (1980) have observed similar lesions in DMBA treated Lewis rats. Both groups observed that the ductal dysplasias occurred in terminal ductal structures and their incidence correlated closely with the observed tumor incidence.

The lack of preneoplastic significance of HAN found in rats, coupled with the observation of the ductal origin of mammary tumors in these animals, have led several workers to suggest that a significant difference exists in the pathogenesis of mammary tumors in these two species. However close scrutiny of the available data indicate that just the opposite is true. For example, chemical carcinogen induced HAN in mice are preneoplastic only when they are induced by chemical carcinogen treatment combined with exogenous hormone stimulation. Without hormone stimulation HAN are rarely observed in MuMTV free mice. Rather, mammary tumors observed in mice exposed to carcinogen alone appear to arise directly from the ducts. Thus mammary tumor development in rats and mice appear to progress through similar developmental stages.

Precursor lesions in human breast carcinoma. One of the reasons for studying the development of atypical lesions which occur in mammary tissue prior to the development of overt carcinoma is to facilitate the early diagnosis of breast cancer in humans. The demonstration of the existence of true preneoplastic lesions in rodent mammary tissues

prompted human pathologists to search for similar lesions in human breast biopsy specimens. These studies have led to a number of interesting findings which have recently been reviewed in detail (Squartini, 1981; Wellings, 1980; Wellings et al., 1975) and can be summarized here as follows:

- 1) The terminal ductal lobular unit (TDLU) is the site of origin of a variety of human breast disorders including fibroadenoma, fibrocystic disease, ductal and lobular carcinoma in situ and infiltrating ductal and lobular carcinoma.
- 2) TDLUs from breasts removed because of the presence of mammary carcinoma often exhibit a variety of atypias and in many cases the atypical lobules exhibit epithelial hyperplasia.
- 3) The cells within hyperplastic atypical lobules occasionally exhibit anaplasia and there appears to be a continuum between intraductal hyperplasias in which the cells appear uniform and those with sufficient anaplasia for their classification as carcinoma in situ.

These findings have led to the general agreement among human pathologists that intraductal hyperplasias occurring in the TDLU are the predominant precursors to human mammary carcinoma.

Comparison of the morphological and histological characteristics of mammary ductal hyperplasias which have been observed in rodents to those lesions of the TDLU which appear to be important precursors of human breast cancer reveal remarkable similarity. For this reason the study of the induction of these lesions in rodents and their progression to carcinoma can provide information which can be of great importance for

the treatment and eventually for the prevention of malignant neoplasia of the human breast.

Rational and Scope of the Dissertation

The evidence, which has been presented in the preceding section, demonstrates clearly that non-virally induced mammary carcinoma in both rats and mice arise from the ductal structures and are often, if not always, preceded by ductal dysplasias. An important question which remains to be answered regarding these ductal dysplasias, is whether they represent a stable preneoplastic condition the expression of which is under the influence of cells which have not yet progressed to become neoplastic, or whether these lesions represent simply an early phase of neoplasia. This difference has significance both for the understanding of the biology of neoplastic progression, and for the usefulness of detection of preneoplastic lesions in diagnosis of breast cancer in humans. If the first possibility is correct then ductal dysplasias are analogous to the virally induced HAN. Such a finding would provide further evidence to indicate that the acquisition of biological characteristics associated with neoplasia occurs in a stepwise fashion, with the ability to grow continuously, and to invade other tissues occurring later in the process.

HAN, which are involved in the pathogenesis of MuMTV induced mammary tumors in BALB/c mice, represent a true preneoplastic condition. One characteristic of these preneoplastic lesions is that their expression is to a large extent under the influence of normal tissue regulatory mechanisms. Thus, one way to address the question of whether ductal dysplasias are the chemical/physical carcinogen analog of HAN is

to create an experimental situation analagous to the infection of mice by the MuMTV and ask the question whether expression of ductal dysplasia is under similar regulatory influences. Exposure of BALB/c mice to low doses of DMBA, below 0.25 mg per mouse, results in the development of mammary tumors nearly all of which occur in the second year of life (Ethier and Ullrich, 1982). Further, exposure of these mice to low doses does not result in any detectable ductal abnormalities until approximately 40 weeks after treatment (S. P. Ethier, unpublished observations). Thus, one of the aims of this research is to test the hypothesis that enzymatic dissociation of mammary cells from mice treated with low doses of DMBA at 12 weeks of age and injection of these cells into gland free mammary fat pads of syngeneic hosts will result in outgrowths exhibiting ductal dysplasia.

The second aim of this work is to address the question of whether ionizing radiation can have a direct effect on the growth potential of mammary epithelial cells. In this regard, the cell dissociation assay is again useful in that the growth potential of mammary cells from irradiated donors can be studied in non-irradiated host mice whose ovaries are healthy. To a large extent the growth of the mammary cells from irradiated donors will be compared to that from donors treated with DMBA, the carcinogenic action of which is unquestioned.

CHAPTER III

METHODS OF PROCEDURE

Animals

In these experiments, germ-free-derived, specific-pathogen-free virgin female BALB/cAnNBd mice, which were bred and maintained in the Biology Division barrier facility, were used. The animals were housed 8 per cage and were fed Purina Laboratory chow and water ad libitum.

Carcinogen Exposure

Donor mice were exposed to either γ -ray irradiation or DMBA when they were 12 weeks old. DMBA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in stripped corn oil (Eastman Kodak Co., Rochester, N.Y.) and administered to unanesthetized mice by gastric intubation via a 20-gauge needle ending in a silver-soldered bulb. γ -Ray irradiation was carried out with a 2000-Ci ^{137}Cs source at a dose rate of 32.8 rads/minute. During the irradiation, the animals were kept in plastic tubes and rotated in the beam. Details of the irradiation procedures have been published (Ullrich and Storer, 1979). Control donors were either sham irradiated or untreated.

Enzymatic Dissociation of Mammary Tissues

Mice that were used as donors of mammary tissue were first anesthetized with Diabotol (Diamond Laboratories, Inc., Des Moines, Iowa). Mammary tissues were exposed by making a midventral incision starting between the inguinal mammary glands and extending anteriorly to just

below the mandible while taking care not to cut into the peritoneal or thoracic cavities. The mammary tissues so exposed were then removed free of lymph nodes and other extraneous tissues. The tissues were then carefully minced with razor blades on an inverted glass petri dish until cut into individual pieces that were about 1 mm in diameter. Proper mincing of the mammary tissues was found to be of crucial importance to the number and quality of the cells obtained in the final preparation. Mincing the tissues by slicing them, rather than chopping or tearing, resulted in the best cell preparations. The minced tissues were then suspended in an enzyme preparation consisting of 0.1% collagenase (107 units/mg; Millipore Corp., Freehold, N.J.) and 0.1% hyaluronidase (255 units/mg; Sigma Chem., St. Louis, Mo.) at a concentration of 20 ml/g of tissue and incubated at 37° in a shaking water bath that oscillated at 80 cycles/minute for 90 minutes. Next, the enzyme preparation was centrifuged at 1000 rpm for 5 minutes. This centrifugation resulted in two distinct layers, a fatty layer which floated on the top of the supernatant and a pellet. Both the fatty layer and the pellet were transferred to the second enzyme preparation which consisted of 0.5% pronase (56,300 PuK/g; Calbiochem., LaJolla, CA) at a concentration of 10 ml/g of tissue. The enzyme preparation was incubated at 37° for 30 minutes in the shaking water bath that oscillated at 40 cycles/minute. Next, the preparation was centrifuged at 1000 rpm in a 50 ml centrifuge tube for 5 minutes. After this spin both the fatty layer and the supernatant were discarded. The pellet was then completely resuspended in fresh media and transferred to a 15 ml centrifuge tube. The preparation, which at this point consisted of dissociated cells as

well as a large amount of digested connective tissue and muscle, was then washed three times by centrifuging and resuspending in fresh media. The bulk of the pellet that resulted from these centrifugations was made up of connective tissue and muscle and the dissociated cells formed a small layer on top. In order to obtain a preparation that was free of non-cellular material, after each wash the cells only were gently resuspended using a pasteur pipette and the rest of the pellet was allowed to remain intact. After the final wash, the cells were filtered through 20 μ m nitex and counted on a hemocytometer. Viability of the cells was determined by trypan blue exclusion and was always greater than 90%. Enzymatic dissociation of mammary tissues from an individual 12-week-old virgin female BALB/c mouse, according to this protocol, resulted in cell preparations that consisted of 4 to 7 million cells.

Clearing of the Host Mammary Fat Pad

Host animals were 3 to 4-week-old virgin female BALB/c mice that weighed between 12 and 17 grams. The mammary tissues in the inguinal fat pads of mice at the above stated ages and weights, although growing have not yet progressed beyond the lymph node which thus provides a useful marker for removing the mammary rudiments (Figure III-1). The host animals were anesthetized with Diabuto1 (Diamond Laboratories, Des Moines, Iowa) and pinned to a cork board. The inguinal mammary glands were exposed by making an incision along the ventral midline that extended from the level between the nipples of the inguinal mammary glands anteriorly to the base of the xiphoid process (Figure III-2). Next, left and right incisions were made, each of which extended from the posterior border of the first incision, laterally on a line midway

Figure III-1. Whole Mount of an Inguinal Mammary Gland of a 3-Week-Old Female BALB/c Mouse.

Figure III-2. Clearing of the Mammary Fat Pad: Midline Incision.

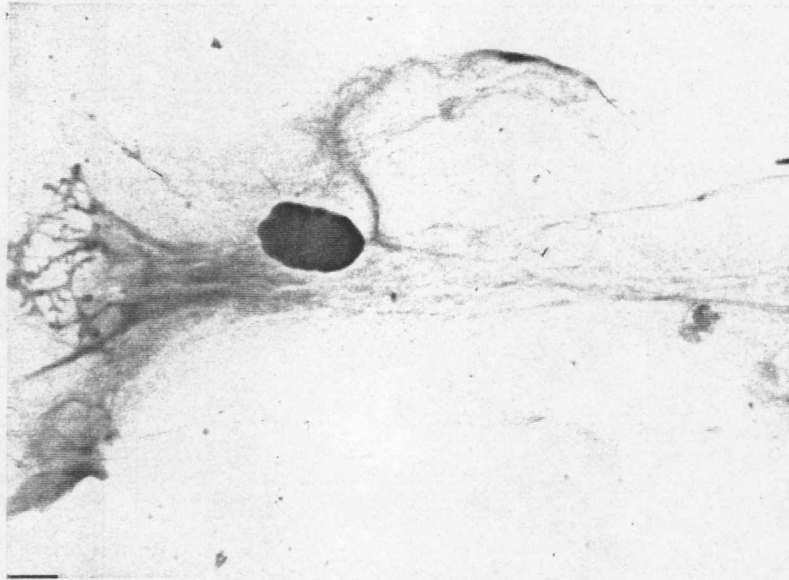


Figure III-1

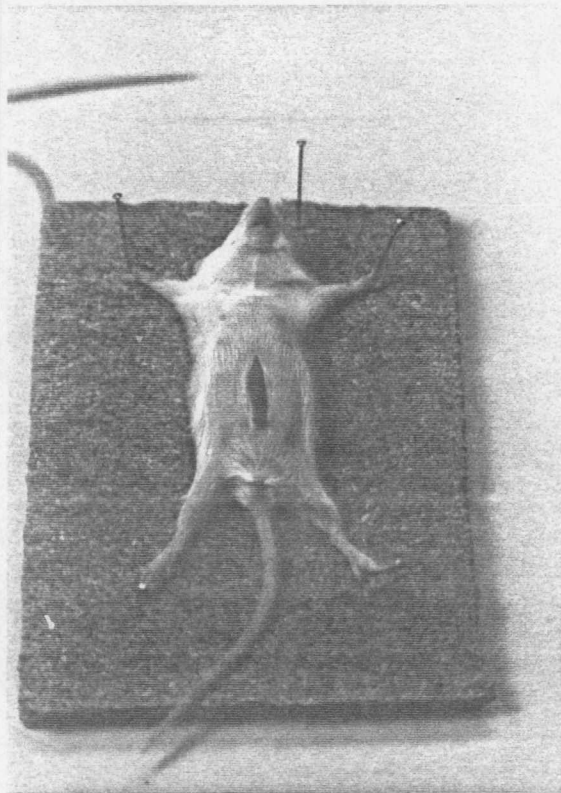


Figure III-2

between the fourth and fifth nipples (Figures III-3). The fourth mammary glands were then exposed by blunt dissection of the skin away from the body. Once exposed, the nipple and primary duct were destroyed using an electrocauterizer. The gland was then carefully dissected away from the skin and the connection with the fifth mammary gland was severed. Next, the portion of the fourth mammary fat pad, containing the mammary rudiments was removed by cauterizing just below the lymph node (Figures III-4, 5). This procedure left approximately 3/4 of the fat pad which was without host mammary tissue in the animal.

The cleared mammary fat pads so derived were then injected with 10 μ l of the cell suspension via a 27-gauge needle attached to a Hamilton syringe (Hamilton Co., Reno, Nev.) (Figure III-6). Following injection of the cells the wound was closed with autoclips (Clay-Adams, Parsippany, N.J.).

Preparation of Mammary Outgrowths for Whole Mount and Histological Examination

At varying times after the injection of cells, depending on the experimental protocol, the host animals were killed and both inguinal fat pads were removed along with a piece of the third mammary gland. A piece of the third mammary gland was taken in order to compare the morphology of the outgrowth to that of the host mammary tissue. The tissues were spread on Tissue Tek capsules and fixed in 100% ethanol: glacial acetic acid (3:1) for 24 hours. Following fixation, the tissues were transferred to 70% alcohol for 15 minutes, washed in distilled water for 5 minutes and then transferred to alum carmine stain for 24 hours (for details on preparation of alum carmine stain see Banerjee,

Figure III-3. Clearing of the Mammary Fat Pad: Lateral Incision.

Figure III-4. Clearing of the Mammary Fat Pad: Blunt Dissection to Expose Inguinal Mammary Glands.

Figure III-5. Clearing of the Mammary Fat Pad: Removal of Mammary Rudiments.

Figure III-6. Injection of Dissociated Cells Into Cleared Fat Pads.

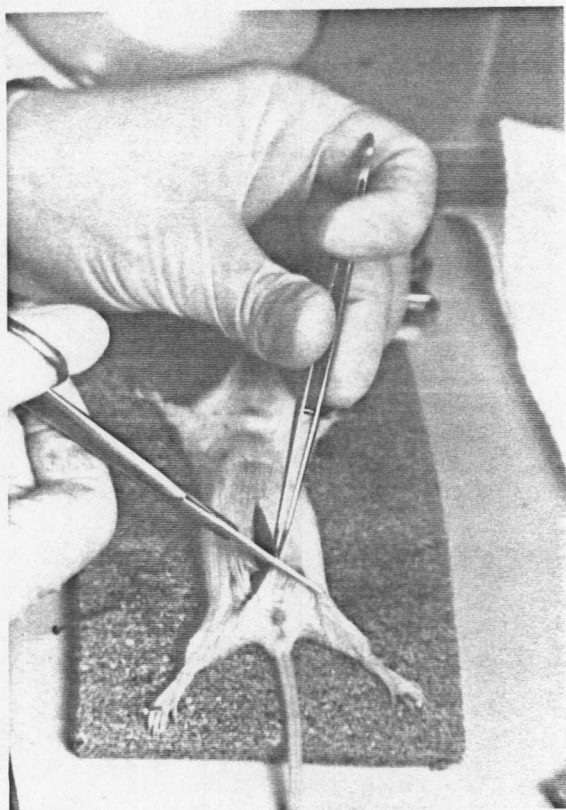


Figure III-3

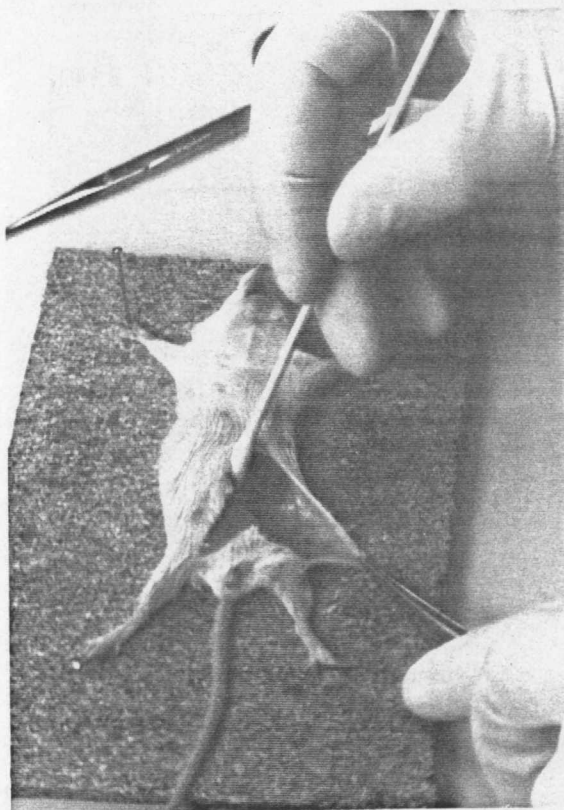


Figure III-4

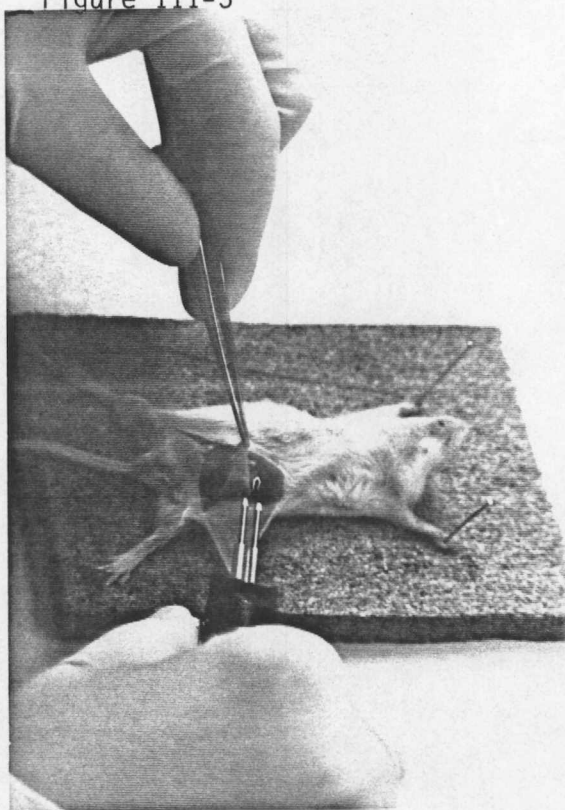


Figure III-5

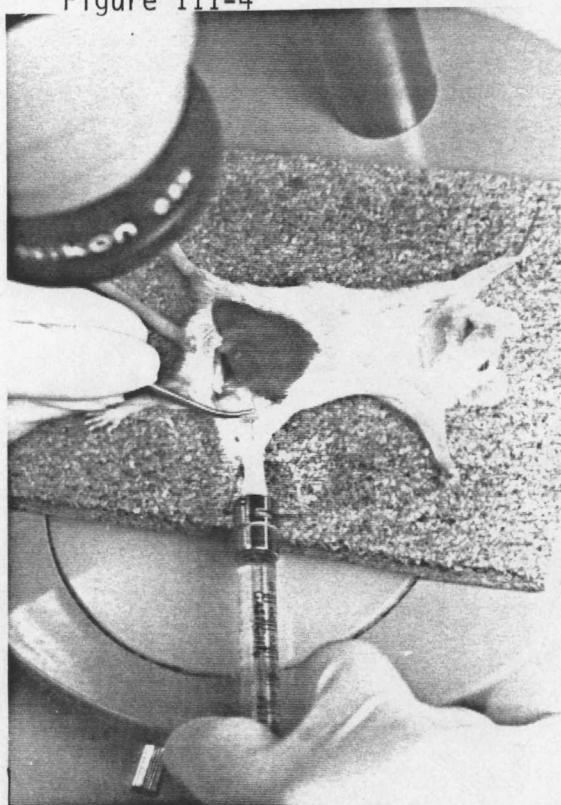


Figure III-6

1976). After staining, the tissues were rinsed in 70%, 95% and 100% alcohol for 15 minutes each and then cleared in toluene for one hour. Stained mammary tissues of individual mice were stored in liquid scintillation vials with 3 ml of methylsalicylate. For histological examination of the tissues, alum carmine stained mammary tissues were either embedded in paraffin or methacrylate. 5 μ m sections were cut from paraffin mounted tissues and were stained with hematoxylin and eosin. 1 μ m sections were cut from methacrylate mounted tissues and these sections were stained with methylene blue and basic fuchsin.

CHAPTER IV

DEVELOPMENT OF MAMMARY OUTGROWTHS FROM MONODISPERSED CELLS

The experiments that constitute the heart of this research project rely on a technique that allows the development of mammary outgrowths from a monodispersed suspension of mammary epithelial cells. Given the importance of this technique to the research project, an experiment designed to investigate the development of these outgrowths from dissociated cells was warranted. Therefore studies were carried out in which gland free mammary fat pads were injected with monodispersed mammary cells and were examined at regular intervals. In this experiment gland free mammary fat pads were injected with either 10^5 or 10^4 monodispersed mammary cells. In order to monitor the development of the outgrowths samples of host mice were killed at 1, 3, 7, 10, 14, 21 and 28 days after injection of cells. Examination of whole mounts of mammary fat pads obtained from this study indicated that the development of the outgrowths could best be described as occurring in three stages, each of which exhibited at the whole mount level, distinct and easily recognizable structures.

The first phase of outgrowth development was characterized by the presence of a spherical, amorphous, darkly staining structure. This structure, shown in figures IV-1 and 2, was nearly always observed in fat pads injected with 10^5 cells and occurred approximately 1 week after injection of cells. Similar structures were visible approximately two weeks after injection of 10^4 cells. At the histological level these

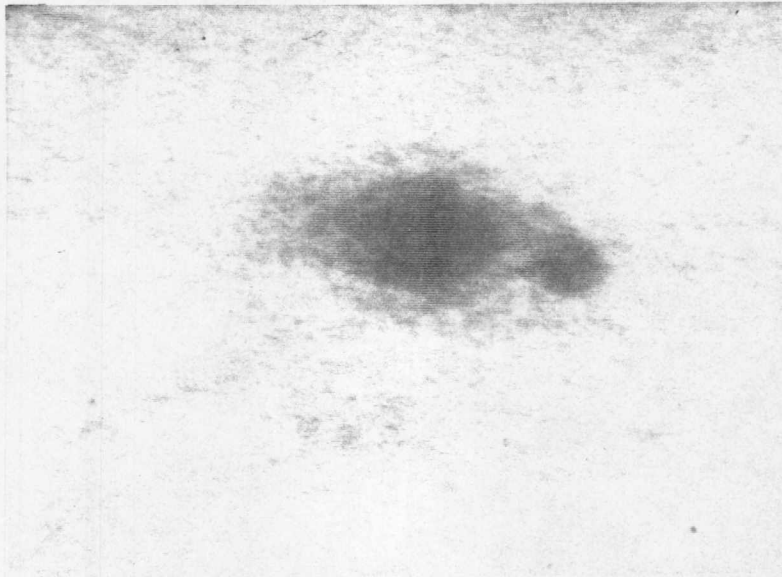


Figure IV-1 Whole Mount of Early Amorphous Structure.

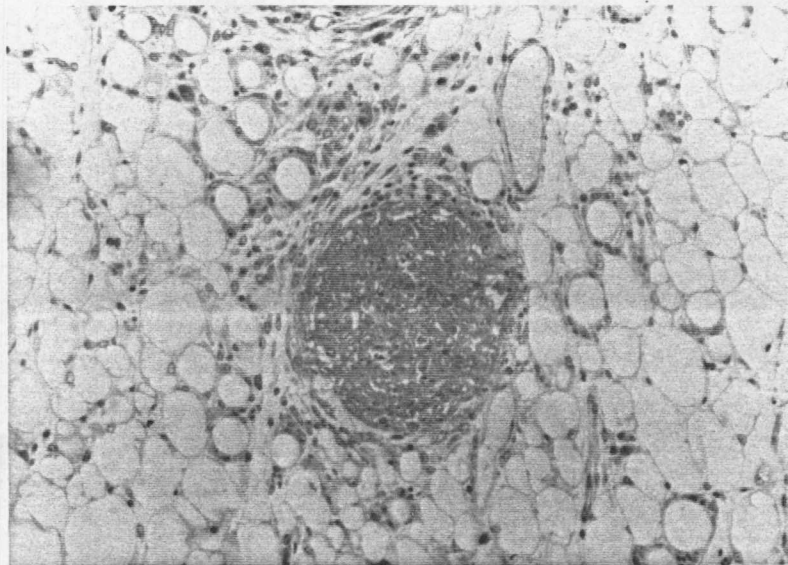


Figure IV-2 Histological Section of Early Amorphous Structure.

structures were nearly solid balls of epithelial cells. The cells were highly variable in size and shape, and had large prominent nucleoli (Figure IV-2).

The second stage of outgrowth development that was readily recognizable occurred one to two weeks after injection of 10^5 cells and two to three weeks after injection of 10^4 cells. In this stage small outgrowths were observed that had short ducts, which were capped by large spherical end bud-like structures. The end buds that were visible at this stage were characterized by a darkly staining crescent shaped area at the bottom of the end bud (Figure IV-3). Histological examination of these early outgrowths revealed that the short ducts were lined by small, darkly staining cuboidal epithelial cells. In contrast, the cells within the primitive end buds appeared large and pale and resembled the cells seen in the solid mass of cells described above. The dark crescent shaped area that was visible in these end buds at the whole mount level, consisted of several layers of these large non-uniform cells that lined the bottom of the end bud (Figure IV-4). Occasionally, small outgrowths were observed which appeared to be intermediate between these first two stages and one such outgrowth is shown in figures IV-5 and 6. The outgrowth shown in the figures had two primitive end bud structures that were not quite full with cells, yet had not yet progressed to the stage in which the cells formed the crescent shaped area in the bottom of the end bud. Note, however, that even in this outgrowth the short duct that connected these two structures was lined by highly uniform cuboidal epithelial cells.

Figure IV-3. Whole Mount of Early Outgrowth With Crescent End Buds.

Note the dark staining crescent shaped area at the bottom of each end bud.

Figure IV-4. Histological Section of Crescent End Buds.

The cells in the early end buds are large and irregular and have abundant cytoplasm compared to the cells that line the ducts.

Figure IV-5. Whole Mount of Early Outgrowth With Morphological Characteristics Intermediate Between Stages 1 and 2.

Figure IV-6. Histological Section of Outgrowth With Morphological Characteristics Intermediate Between Stages 1 and 2.

The cells within the end bud are similar to those seen in crescent end buds; however no lumen has yet formed, thus no crescent shaped area is visible. Note also that even in this early outgrowth the cells lining the short duct are highly uniform.



Figure IV-3

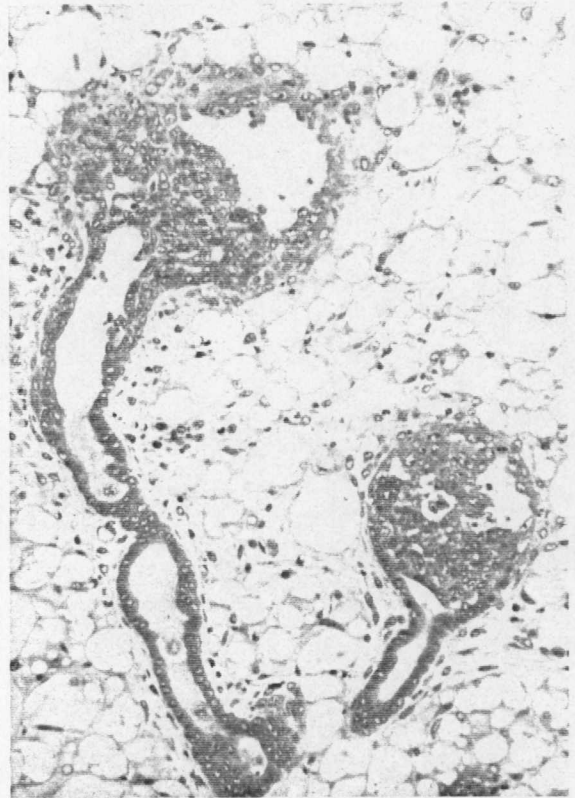


Figure IV-4



Figure IV-5

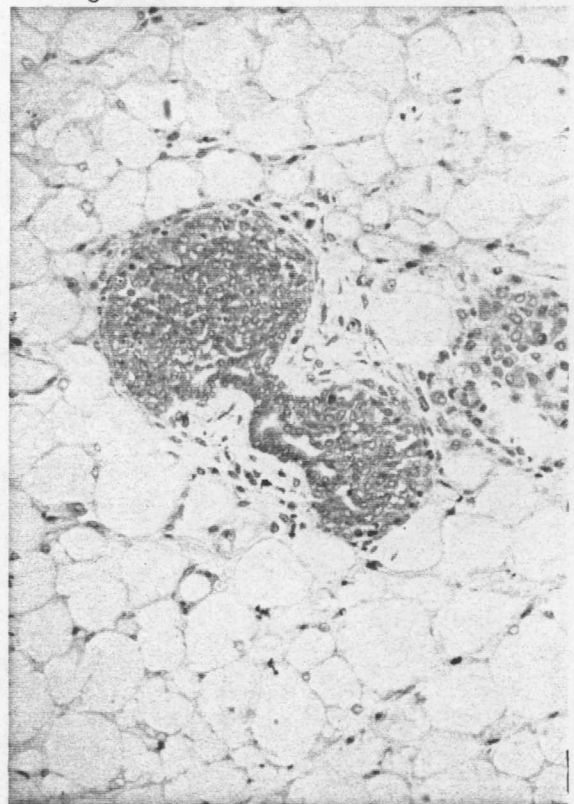


Figure IV-6

The third readily recognizable stage in the development of the outgrowths occurred a few days after the development of the crescent type end buds. In this stage the ducts had elongated considerably, however the most noticeable change was that the ducts no longer exhibited crescent type end buds but rather were capped by smaller club shaped end buds that closely resembled those found in normally growing intact mammary tissue. At the whole mount level these end buds were lined on their distal border by a thin, dark staining ring (Figure IV-7). Histological examination of these end buds revealed that the dark staining ring consisted of several layers of uniform darkly staining glandular epithelial cells. Directly above these cells, and separated from them by a small space, was a single layer of larger, more pale staining cap cells (Figures IV-8 and 9). The cap cell layer has been shown to be of importance in breaking down the stroma and thus allowing duct elongation (Silberstein and Daniel, 1982). Once these ring type end buds developed, the only further change that occurred within the outgrowths was duct elongation which continued until the ducts reached the edges of the fat pad, at which time growth stopped and the end buds disappeared (Figure IV-10). Disappearance of the end buds also occurs in intact mammary glands once the fat pad is filled.

The results from this experiment indicate that the first structure that forms after injection of dissociated cells is that which ultimately becomes the end bud. Further, it appears that ducts arise from the end buds and that this process begins soon after formation of the primitive end bud structure. This finding is of importance because it suggests that development of normal mammary outgrowths depends on proper formation

Figure IV-7. Whole Mount of Early Outgrowth With Ring Type End Buds.

Figure IV-8. Histological Section of Normal Ducts and a Ring Type End Bud.

Figure IV-9. Higher Magnification of Ring Type End Bud.

In this section the difference between the small darkly staining glandular epithelial cells and the more lightly staining cap cells that line the outer border of the end bud can be seen.

Figure IV-10. Whole Mount of Fully Developed Outgrowth in Which End Buds Have Regressed.

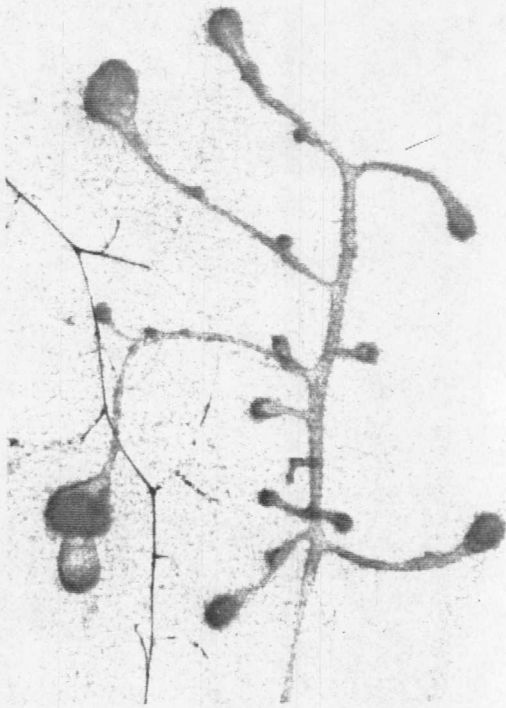


Figure IV-7

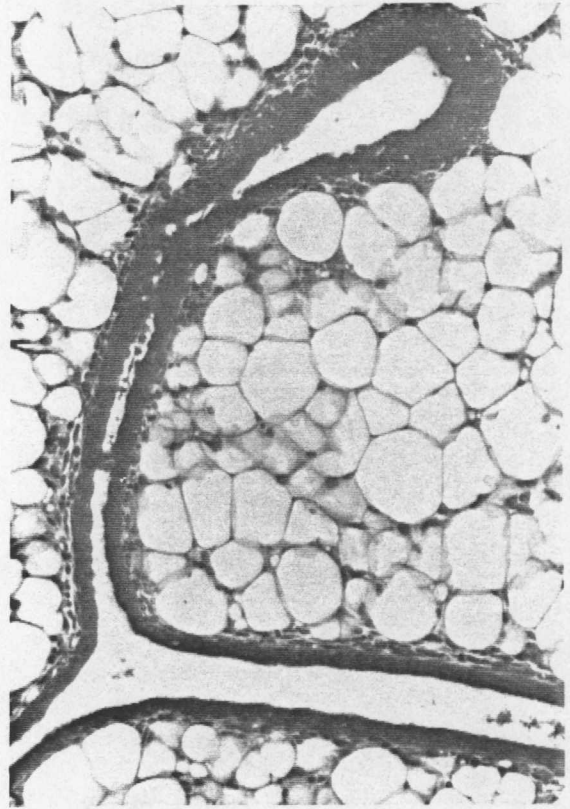


Figure IV-8



Figure IV-9

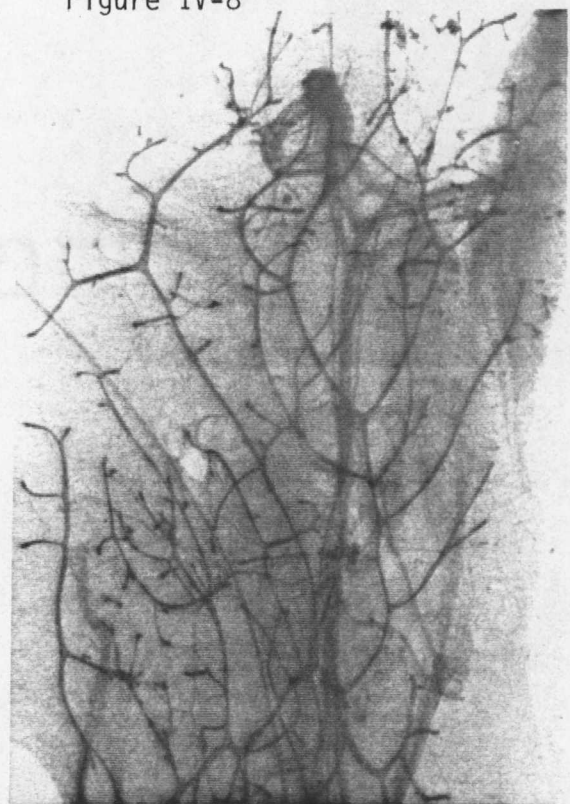


Figure IV-10

of the end buds. From this it follows that abnormal outgrowth development could be the result of abnormal end bud formation. This possibility is supported by the observation of a small number of outgrowths that appeared to have abnormal end buds and irregular branching patterns that were observed just three weeks after injection of 10^4 cells. Examples of such outgrowths are given in figures IV-11 and 12.

The results of this experiment also indicate that crescent type end buds represent a normal stage in the early development of outgrowths from dissociated cells. This finding is significant because occasionally outgrowths that were examined 8 to 10 weeks after injection of cells were found to have crescent type end buds at the ends of some ducts and not the ring type end buds that are normally found on outgrowths at this stage of development. These outgrowths will be discussed further in the next chapter; however the finding that crescent type end buds represent a normal stage in outgrowth development may facilitate interpretation of the significance of these end buds in more well developed outgrowths.

Figure IV-11. Whole Mount of an Early Abnormal Outgrowth.

In this outgrowth duct elongation does not appear to be occurring, rather the primitive end buds have remained very close together and appear overly hyperplastic.

Figure IV-12. Whole Mount of an Early Abnormal Outgrowth.

In this outgrowth the side branches appear not to be growing and abnormal end buds appear to be at the ends of these branches.

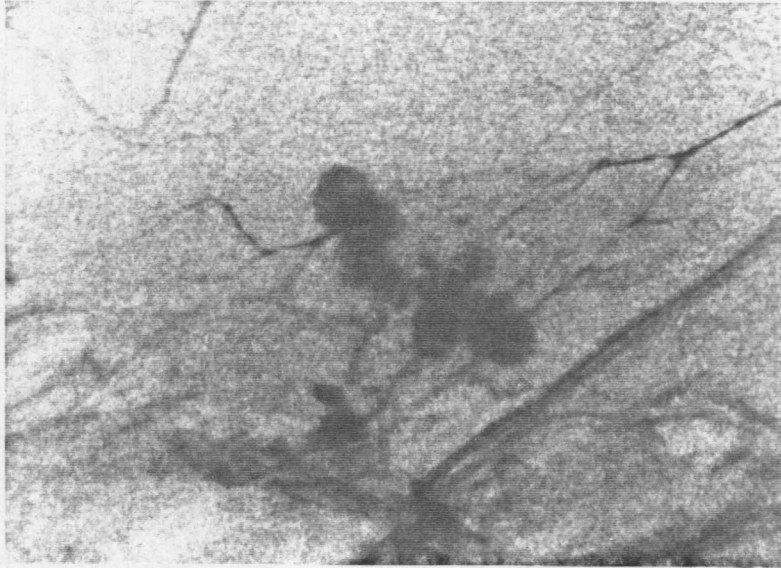


Figure IV-11

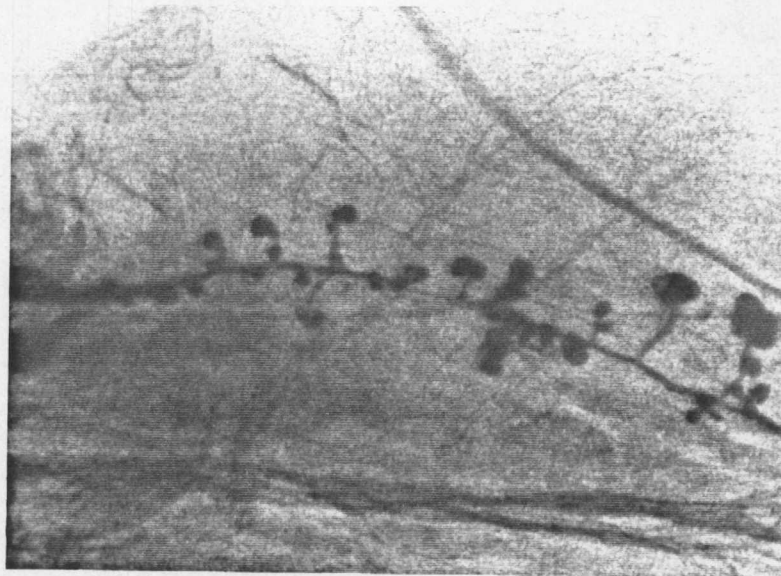


Figure IV-12

CHAPTER V

MORPHOLOGICAL AND HISTOLOGICAL DESCRIPTIONS OF NORMAL MAMMARY OUTGROWTHS AND DUCTAL DYSPLASIAS

Over the course of these experiments well over two thousand mammary outgrowths derived from monodispersed mammary cells were examined. By far the majority of these outgrowths were normal. However mammary outgrowths exhibiting a variety of abnormalities were also observed. In this chapter an attempt will be made to describe the various abnormalities that have been observed in these experiments in a logical manner and to organize them in such a way as to allow comparisons to be made between the lesions that were observed in these studies and those which have been detected in intact mammary tissues following administration of chemical carcinogens.

In order to describe the various mammary lesions that have been observed in these experiments it is first necessary to describe the morphology of the normal mammary outgrowths that resulted from the injection of dissociated cells. These outgrowths were similar to normally developed host mammary tissue in many respects. The outgrowths consisted of ducts that branched dichotamously at regular intervals and terminated by sending off two or three terminal ductules. When these outgrowths were in the process of growing, the terminal ductules were capped by end buds as is shown in Figure A^{*}-1. At the whole mount level, the end buds were club shaped structures that were lined on their outer border by a thin dark staining ring and were identical to end buds found

* Figures labelled A appear in the appendix.

on normally growing intact mammary tissue. A histological description of these end buds was given in the previous chapter (Chapter IV, p. 67 and Figure IV-6, 7). When the outgrowths were still growing, the density of the ductal growth was somewhat less than that of the host mammary tissue, but by the time the outgrowths had filled the fat pad the density of the mammary outgrowths was indistinguishable from that of the host tissue.

The abnormal mammary outgrowths that were observed in these studies were of two general types; one in which the outgrowths exhibited abnormalities in the ducts and end buds, and one in which focal areas of lobuloalveolar growth developed in the absence of hormonal stimulation. The former were classified as ductal dysplasias and the latter as alveolar adenosis.

Ductal Dysplasias

Lesions that were classified as mammary ductal dysplasias had abnormalities both in their branching pattern as well as in the cellularity of the ducts and end buds. The abnormal branching pattern that was observed most frequently was an abnormality in the number and branching of the terminal ductules which instead of consisting of two or three ductules consisted of many ductules (up to 10 and sometimes more) that were very closely spaced. These lesions were further classified into two groups: (a) ductal dysplasia with mild hyperplasia and; (b) ductal dysplasia with severe hyperplasia, based on the degree of epithelial hyperplasia associated with the lesion.

Ductal dysplasia with mild hyperplasia. Lesions that were classified as having mild hyperplasia were those in which the normal shape of the end buds could still be recognized and in which a lumen could still be seen, but which had a clear overabundance of cells. The lesions that were classified as having mild hyperplasia often exhibited distinctive morphological characteristics that were an exaggeration of the normal condition. The most commonly observed lesion that was classified as mild hyperplasia was one which consisted of one or more abnormal terminal structures that had terminal ductules capped by hyperplastic end buds. One such lesion is shown in figure A-2. As is shown in the figure, the hyperplastic end buds were of normal size but were distinguishable from normal end buds by a much thickened ring of cells along the distal border. Histological examination of these ring type hyperplastic end buds suggested that this thickening was the result either of hyperplasia of the glandular epithelial cells or of the cap cells. In figures A-3 and 4 histological sections of the hyperplastic end buds shown in Figures A-2 are given. In this lesion the hyperplasia appeared to be of glandular epithelial origin. The hyperplastic epithelium consisted of uniform, darkly staining cells in which mitotic figures were relatively abundant. Note, however, that despite the extent of hyperplasia in these end buds, the cells still formed a smooth luminal surface. Note also that above the hyperplastic epithelium and separated from it by a small space, a single layer of cap cells can be seen. In contrast Figures A-5 and 6 show end buds in which the hyperplasia appeared to be of cap cell origin. In this lesion the hyperplastic cells were large and pale staining and had more cytoplasm

than was normally seen in the glandular epithelial cells. Further, the glandular epithelial cells formed a single layered ring of smaller, more darkly staining cells that were directly beneath the hyperplastic cap cells.

A second type of lesion which was classified as mild end bud hyperplasia is shown in figures A-7 and 8. This lesion had several abnormal terminal structures that were capped by large cystic looking end buds. A histologic section of this lesion is shown in figure A-9 and reveals end buds that were lined by a single layer of cells and which appeared to be filled with secretory material. Occasionally in these lesions cystic end buds were observed which also exhibited epithelial hyperplasia (Figure A-10). In these abnormal end buds, as in those described previously, hyperplasia of both the glandular epithelial cells and of the cap cells could be observed.

In addition to lesions that were characterized by exaggerated forms of the normal end bud, lesions were detected that were characterized by the presence of primitive end bud structures that were normally seen only in early outgrowths derived from dissociated cells (see Chapter IV). These end buds have been designated as crescent type because they contain a mass of epithelial cells which lay on the bottom of the end bud. At the whole mount level this mass of cells appeared as a darkly staining crescent shaped structure. Although these end buds were normally observed on early outgrowths that developed within approximately two weeks of the injection of 10^4 cells, occasionally outgrowths were observed 10 weeks after injection of cells which had grown to reasonable sizes but which still had ducts that were capped by crescent type end

buds and two such lesions are shown in figures A-11 and 12. The end buds on these outgrowths were very similar to those observed in early outgrowths. In general, they consisted of large, irregular cells that exhibited more pale staining cytoplasm than was normally seen in glandular epithelial cells. Further, these irregular cells often formed bizarre patterns of proliferation into the lumen (Figure A-13). A significant difference between these crescent type end buds and other hyperplastic end buds (and normal ones as well) was that in the latter, the glandular epithelial cells could be readily distinguished from the cap cells. In early outgrowths in which crescent type end buds were normally found, it was not possible to make this distinction because all the cells within the primitive end bud structure appeared large and irregular. In more well developed outgrowths that exhibited crescent type end buds it was also not possible to distinguish cap cells from glandular epithelial cells. Thus, crescent type end buds that were found in outgrowths 10 weeks after injection of cells were similar morphologically and histologically to those observed in early outgrowths (2-3 weeks post-injection) derived from dissociated cells.

Ductal dysplasia with severe hyperplasia. Lesions that were classified as having severe hyperplasia were those in which the hyperplastic epithelium completely occluded the lumen of the end buds and terminal ductules and thus distorted the size and shape of these structures. The lesions shown in Figures A-14 through A-17 were the most common type of severe lesions observed in these studies and were characterized by severe hyperplasia of the terminal ducts and end buds. At the histological level these structures were completely occluded by

chords of uniform appearing epithelial cells which appeared to wind through the lumen. In some cases, however, the hyperplastic cells appeared variable in size and shape and were less well organized. In these hyperplastic structures a cribriform pattern was often observed.

A second type of severe lesion that was occasionally observed was characterized by end buds which at the whole mount level appeared very large and opaque (Figure A-18). A histological section of one such end bud is shown in figures A-19 and 20). This particular end bud was also occluded with cells but in this case the cells formed several papillary structures which projected into the lumen. The cells within the papillary projections were clearly of glandular epithelial origin but were rather non-uniform and contained many mitotic figures.

A third type of severe lesion that was detected in these studies was one in which the hyperplasia was so extensive that at the whole mount level neither ducts nor end buds could be recognized. An example of this type of lesion is shown in figures A-21 and 22. At the histological level this lesion was characterized by several closely spaced terminal ductules that were arranged in a lobular configuration. These terminal ductules were occluded with cells which often exhibited bizarre patterns of proliferation within the lumens. The lesion shown in Figure A-21, appears particularly severe and closely resembles lobular carcinoma in situ.

Alveolar Adenosis

In addition to lesions of ductal origin, mammary lesions have also been detected that were characterized by lobuloalveolar differentiation and these outgrowths were classified as having alveolar adenosis. At

the whole mount level these lesions appeared as discrete areas of extensive alveolar growth. In every case in which alveolar adenosis was observed, the lesion was associated with normal ductal growth (Figure A-23). Histologically these lesions appeared as discrete lobules that consisted of many ductules lined by a single layer of cuboidal epithelial cells (Figures A-24).

Summary

The mammary ductal lesions that were observed in these studies varied widely in severity and yet a few common characteristics were shared by nearly all of the lesions. The most striking feature of the vast majority of the lesions was epithelial hyperplasia. This hyperplasia was nearly always found in the terminal ductules and end buds of these outgrowths. The cytological characteristics of the cells that constituted these hyperplasias were similar in lesions which exhibited both mild and severe hyperplasia. In addition, in nearly all of the lesions that were observed in these studies, more than one type of hyperplastic structure was observed and indeed many of the more severe lesions exhibited all of them. Thus, there is every indication that the diversity in the lesions that were observed reflects a difference not of quality, but of degree and that there was a continuum in the hyperplastic ductal lesions that ranged from mild hyperplasia to severe hyperplasia and perhaps even to carcinoma in situ.

Insofar as one hypothesis that was tested in these experiments was that the cell dissociation assay could be used to detect mammary ductal dysplasias similar to those that are the precursors of mammary carcinoma in mice, the range of lesions that was detected was what one would have

expected. However, there was one particular type of lesion, the type that exhibited crescent type end buds, that had not been reported previously and which may be peculiar to the cell dissociation assay.

Crescent type end buds are not the precursors to normal end buds in the developing mammary gland, but do precede normal end bud development in mammary outgrowths derived from dissociated cells. The presence of these end buds on outgrowths examined 10 weeks after injection of cells suggests that they were the result of an alteration in a normal program of multicellular organization and differentiation. Thus, there may be a basic difference between lesions that exhibited crescent type end buds and those that were characterized by epithelial hyperplasia.

CHAPTER VI

FACTORS INFLUENCING THE EXPRESSION OF MAMMARY DUCTAL DYSPLASIA

In this chapter data will be discussed that have been obtained from a series of experiments designed to provide information concerning the factors that influence the expression of ductal dysplasia in outgrowths derived from monodispersed mammary cells. In the initial studies that were performed using the cell dissociation assay the principle factor that was examined was the influence of carcinogen treatment of the donor animals on the development of outgrowths and the expression of ductal dysplasia. In these experiments donor animals were exposed to either γ -ray irradiation or DMBA 24 hours prior to cell dissociation. (A twenty-four hour interval was chosen to allow sufficient time for metabolic activation of the DMBA.) Monodispersed mammary cells were then prepared from the mammary tissues of the donor animals and outgrowths were derived by injecting inocula of 10^5 cells into the gland free fat pads of the host mice. In these first experiments it was observed that when inocula of 10^5 cells were injected into the fat pads outgrowths developed in nearly every fat pad injected and these outgrowths contained very dense ductal growth. This dense ductal growth was usually the result of development of two or three individual outgrowth units, however many more smaller outgrowth units could be seen in the center of the fat pads. These observations indicated that 10^5 cells was clearly many more cells than was necessary to derive mammary outgrowths. For this reason, experiments were performed to

examine the influence of cell dose on the development of outgrowths and the expression of ductal dysplasia. To do this, mammary outgrowths were derived by injection of 10^4 cells from control and carcinogen treated donors into gland free fat pads of host mice.

In all of these experiments the host animals were killed and the mammary tissues processed for whole mount examination 10 weeks after the injection of cells. Ductal lesions that were observed in these outgrowths were classified either as ductal dysplasia with mild hyperplasia (type A lesions) or as ductal dysplasia with severe hyperplasia (type B lesions) according to the criteria described in Chapter V.

Detection of Ductal Dysplasias

Mammary ductal dysplasias were detected in outgrowths derived from the cells of both treated and nontreated donor animals. The frequency of occurrence, as well as the severity of these lesions, was influenced both by carcinogen treatment of the donor animals and by the number of donor cells which were injected into the host fat pads and these data are given in Table 1.

The outgrowths that were derived from 10^5 control cells did not exhibit any ductal lesions. In contrast, ductal dysplasias which exhibited both mild and severe hyperplasia were observed in outgrowths derived from 10^4 control cells. As can be seen from Table 1, the majority of the lesions that were observed in outgrowths derived from non-treated control cells were type A.

Exposure of donor mice to 100 rad of γ -ray irradiation prior to cell dissociation resulted in an increased frequency of ductal dysplasia over control levels and this increase was observed in both 10^5 and 10^4

TABLE 1

FREQUENCY OF DUCTAL DYSPLASIAS IN MAMMARY OUTGROWTHS 10 WEEKS
AFTER INJECTION OF 10^5 AND 10^4 MAMMARY CELLS

Group	Cell Dose	Frequency of Ductal Dysplasias (%)		
		Type A	Type B	Total
Controls	10^5	0 (0/153) ^a	0 (0/153)	0 (0/153)
γ -irradiation (100 rad)	10^5	1.6 ± 0.9 (3/186)	1.6 ± 0.9 (3/186)	3.2 ± 1.2 (6/186)
DMBA (0.075 mg)	10^5	0 (0/94)	0 (0/94)	0 (0/94)
DMBA (0.25 mg)	10^5	0.6 ± 0.6 (1/154)	0.6 ± 0.6 (1/154)	1.3 ± 0.9 (2/154)
Controls	10^4	4.3 ± 1.4 (9/207)	1.0 ± 0.7 (2/207)	5.3 ± 1.5 (11/207)
γ -irradiation (100 rad)	10^4	10.6 ± 2.9 (12/113) ^c	0.9 ± 0.9 (1/113) ^b	11.5 ± 3.0 (13/113) ^c
γ -irradiation (25 rad)	10^4	2.9 ± 2.0 (2/68) ^b	5.9 ± 2.9 (4/68) ^c	8.8 ± 3.4 (6/68) ^b
DMBA (0.075 mg)	10^4	7.8 ± 1.8 (17/219) ^b	4.1 ± 1.3 (9/219) ^c	11.9 ± 2.2 (26/219) ^c
DMBA (0.25 mg)	10^4	6.3 ± 2.7 (5/80) ^b	1.3 ± 1.3 (1/80) ^b	7.5 ± 2.9 (6/80) ^b

^aNumbers in parenthesis, number of lesions/number of classifiable outgrowths in the group.

^bNot significantly different from controls.

^c $p < 0.05$ versus control.

cell derived outgrowths; however the frequency of these lesions was substantially higher in the latter group. Interestingly, in outgrowths that were derived from 10^5 cells an equal number of type A and type B lesions were observed while in 10^4 cell derived outgrowths all but one of the lesions detected were type A. Outgrowths were also derived from 10^4 cells of donors exposed to 25 rad of γ -ray irradiation. In this group, as in the 100 rad group, radiation treatment resulted in an increased incidence of lesions over control levels.

Qualitatively similar results were obtained from outgrowths derived from cells of donor animals treated with DMBA prior to cell dissociation. When donor mice were exposed to 0.075 mg of DMBA, no lesions were detected in any 10^5 cell derived outgrowths. When outgrowths were derived from 10^4 cells of similarly exposed donors, however, both type A and B ductal dysplasias were detected. Similarly, when outgrowths were derived from cells of donors exposed to 0.25 mg of DMBA a low frequency of ductal lesions were detected in 10^5 cell-derived outgrowths and a substantially higher frequency of these lesions were seen in 10^4 cell derived outgrowths. In this case, however, the incidence of lesions was not significantly different from that found in controls.

Cell number not only influenced the frequency of ductal dysplasias but also affected the number of donor animals that had cells which gave rise to lesions and these data are presented in Table 2. When outgrowths were derived from injection of 10^5 cells only a small fraction of the donors had cells which yielded lesions. In contrast, when outgrowths were derived from 10^4 cells nearly all of the donors, including the controls, had cells which yielded some type A lesions in

TABLE 2
 FREQUENCY OF POSITIVE DONORS IN CONTROL AND
 CARCINOGEN TREATED GROUPS

Group	Cell Dose	Type A	Type B
Control	10^5	0/9 ^a	0/9
γ -radiation (100 rad)	10^5	2/7	3/7
DMBA (0.075 mg)	10^5	1/6	0/6
DMBA (0.25 mg)	10^5	1/6	1/6
Control	10^4	5/7	1/7
γ -radiation (25 rad)	10^4	2/3	2/3
γ -radiation (100 rad)	10^4	7/8	1/8
DMBA (0.075 mg)	10^4	6/8	7/8
DMBA (0.25 mg)	10^4	3/3	1/3

^aNumber of donors that had cells which yielded lesions/
 total number of donors in the group.

the outgrowths which resulted. Further, in two of the exposure groups, namely the 0.075 mg DMBA and the 25 rad γ -ray groups, a high percentage of the donors had cells that yielded the more severe type B lesions.

The results of these experiments indicated that mammary ductal dysplasias could be detected in outgrowths derived from monodispersed mammary cells and that the incidence of these lesions was increased by exposure of the donor animals to either γ -radiation or DMBA. In addition, the data from these experiments indicated that the expression of these lesions was enhanced in outgrowths derived from 10^4 rather than 10^5 cells.

There were at least two possible explanations for the cell dose effect that was observed in these experiments. The first possibility was that development of outgrowths from 10^4 cells was substantially different from the development of outgrowths from 10^5 cells and that this difference was one that allowed greater expression of altered growth potential in outgrowths derived from the smaller number of cells. The second possibility was that the cell dose effect was not real but rather was an artifact of the experimental protocol that was used. This was possible because in these experiments animals bearing mammary outgrowths were killed 10 weeks after injection of cells regardless of whether the outgrowths were derived by injection of 10^4 or 10^5 cells. At 10 weeks following injection of 10^5 cells nearly all of the outgrowths had reached the edges of the fat pads and had stopped growing and thus end bud structures were only rarely observed in these outgrowths. In contrast, a fairly large percentage of outgrowths derived from 10^4 cells had not reached the edges of the fat pads and were still growing 10 weeks after injection of cells and thus many end bud structures were

observed in these outgrowths. Since the lesions that were observed in these studies were almost always end bud hyperplasias it was possible that regression of normal end buds, which occurred when the outgrowths filled the fat pads, was accompanied by regression of the atypical end bud structures as well. If this were the case then the comparatively low frequency of lesions that was observed in 10^5 cell derived outgrowths was really the result of the time at which these outgrowths were examined. To investigate this possibility an experiment was performed in which outgrowths were derived from 10^5 cells of control and DMBA treated donor animals and examined either 4 1/2 or 10 weeks after injection of cells, and from 10^4 cells of similarly treated donors which were examined either at 10 or 16 weeks after cell injection. These times were chosen because the extent of development of outgrowths derived from 10^5 cells 4 1/2 weeks after injection of cells is similar to that of 10^4 cell derived outgrowths 10 weeks after cell injection. Similarly, outgrowths were full and resting by 10 and 16 weeks after injection of 10^5 and 10^4 cells respectively. The data from this experiment are given in Tables 3 and 4. These data indicate that the extent of growth of 10^5 cell derived outgrowths 4 1/2 weeks after injection of cells resembled that which was observed in 10^4 cell derived outgrowths 10 weeks after transplantation. Yet despite the fact that many of the outgrowths were still growing when the animals were killed, ductal lesions were not observed in any of the outgrowths derived from control cells and only 1 lesion in 92 outgrowths was detected in outgrowths derived from the cells of DMBA treated donors. Thus these data indicate that ductal lesions were not expressed in outgrowths derived from 10^5

TABLE 3

FREQUENCY OF DUCTAL DYSPLASIA IN 10^5 CELL DERIVED OUTGROWTHS
4 1/2 AND 10 WEEKS AFTER INJECTION OF CELLS

Group ^a	Time (wks)	Frequency of Ductal Dysplasia (%)			Full outgrowths ^b (%)
		Type A	Type B	Total	
Controls	4 1/2	0 (0/75)	0 (0/75)	0 (0/75)	84.0
Controls	10	0 (0/77)	0 (0/77)	0 (0/77)	97.4
DMBA (0.075 mg)	4 1/2	1.1 $\pm$ 1.1 (1/92)	0 (0/92)	1.1 $\pm$ 1.1 (1/92)	68.8
DMBA (0.075 mg)	10	0 (0/94)	0 (0/94)	0 (0/94)	100

^aFor each group, outgrowths examined at 4 1/2 and 10 weeks after injection of cells were derived from the cells of the same donor animals.

^bpercent of outgrowth which filled greater than 3/4 of the fat pad.

TABLE 4

FREQUENCY OF DUCTAL DYSPLASIA IN 10^4 CELL DERIVED OUTGROWTHS
10 AND 16 WEEKS AFTER INJECTION OF CELLS

Group ^a	Time (wks)	Frequency of Ductal Dysplasia (%)				Full Outgrowth ^b
		Type A	Type B	Total		
Controls	10	6.5 ± 2.8 (5/77)	1.3 ± 1.3 (1/77)	7.8 ± 3.1 (6/77)		58.1
Controls	16	0 (0/91)	0 (0/91)	0 (0/91)		93.4
DMBA (0.075 mg)	10	10.1 ± 3.4 (8/79)	1.3 ± 1.3 (1/79)	11.4 ± 3.6 (9/79)		50.6
DMBA (0.075 mg)	16	0 (0/91)	1.1 ± 1.1 (1/91)	1.1 ± 1.1 (1/91)		92.3
γ-irradiation (100 rad)	10	12.5 ± 5.2 (5/140)	0 (0/140)	12.5 ± 5.2 (0/140)		33.3
γ-irradiation (100 rad)	16	0 (0/52)	0 (0/52)	0 (0/52)		82.6

^aFor each group, outgrowths examined at 10 and 16 weeks after injection of cells were derived from cells of the same donor animals.

^bpercent of outgrowths which filled greater than 3/4 of the fat pad.

cells to the extent that they were in 10^4 cell derived outgrowths regardless of the time at which they were examined. These data do not, however, rule out the possibility that some lesions regress when the outgrowths fill the fat pad. The data given in Table 4 address this question and indicate that lesions that were expressed in 10^4 cell derived outgrowths 10 weeks after injection of cells had regressed by 16 weeks in that no lesions were detected in outgrowths examined at this time.

Although the data presented in Table 4 indicates that lesions regressed when the outgrowths filled the fat pads, it should be emphasized that in some experiments lesions were observed that were detected in full fat pads and which occurred in groups in which the normal outgrowths were full and resting (Figures VII 1-3, page 118). These persistent lesions were always extensive, and were most often severe type B ductal dysplasias. Unfortunately it was not possible to determine what fraction of the ductal lesions were persistent based on morphological criteria because in many cases outgrowths in which severe lesions were detected had not filled the fat pad. Further studies will be required to understand the difference between persistent and regressing lesions.

The data indicate that the expression of ductal dysplasia in outgrowths derived from monodispersed mammary cells is influenced by several factors which include carcinogen treatment of donor animals, the number of cells used to derive the outgrowths and the developmental state of the outgrowth at the time of observation.

The findings of a true cell dose dependence for the expression of ductal dysplasia in cell dissociation derived outgrowths suggested

the possibility that the enhanced lesion frequencies that were observed in some of the carcinogen treated donor groups might have been the result of the cytotoxic rather than the carcinogenic action of the agents. This was possible in that significant cell killing would have reduced further the number of cells which gave rise to the outgrowths, and this could have resulted in an increased lesion frequency. In Table 5 data for the percent takes, percent classifiable takes, and the percent of the outgrowths that filled the fat pad 10 weeks after the injection of cells for several exposure groups are given. These parameters provide a good comparative indicator of the toxicity of the various treatments. These data demonstrate that the viability of the cells derived from the radiation treated donors of both dose groups was substantially less than that for the controls. In contrast, exposure of donor animals to either 0.075 mg or 0.25 mg of DMBA appeared not to have resulted in appreciable toxicity.

These data indicate that the lesions that were observed in outgrowths derived from DMBA treated donors were induced by the carcinogen in that a significant frequency of lesions were observed in these groups following doses that did not result in measurable toxicity. On the other hand, even the relatively low doses of radiation that were used in these studies appeared to result in appreciable toxicity and this may have influenced the lesion frequencies observed in these groups.

In order to address this question more directly an experiment was performed in which the toxicity of the radiation treatment was compensated for by injecting a sufficient excess of cells so as to derive outgrowths from the same number of viable cells from control and

TABLE 5

RELATIVE VIABILITY OF MAMMARY CELLS FROM CONTROL AND CARCINOGEN TREATED DONORS
AS INDICATED BY DEVELOPMENT OF 104 CELL DERIVED OUTGROWTHS

Group	Takes (%)	Classifiable Takes (%)	Full (%)
Controls	87.7 (221/252) ^a	82.1 (207/252) ^b	56.6 (125/221) ^c
γ-irradiation (25 rad)	77.1 (74/96)	70.8 (68/96)	36.5 (27/74) ^d
γ-irradiation (100 rad)	82.3 (130/158)	71.5 (113/158)	38.5 (50/130) ^d
DMBA (0.075 mg)	91.7 (233/254)	86.2 (219/254)	50.6 (118/233)
DMBA (0.25 mg)	95.8 (92/96)	83.3 (80/96)	55.4 (51/92)

^aNumber of outgrowths with recognizable ductal structures/total number of fat pads injected.

^bNumber of outgrowths which filled at least 1/4 of the fat pad/total number of fat pads injected.

^cNumber of outgrowths which filled greater than 3/4 of the fat pad/number of takes.

^dp < 0.05 versus control.

radiation treated donors. To do this experiment data were used that had been obtained recently in this laboratory and which indicate that the dose dependent survival of cell dissociation derived mammary cells is an exponential function of radiation dose with a D_0 of 190 rad (Adams and Ullrich, 1982). Thus outgrowths were derived from 10^4 viable radiation treated cells by injecting 2×10^4 cells from animals exposed to 100 rad of γ -radiation and 10^5 cells from 600 rad treated donors into the gland free fat pads. The data obtained from this experiment are given in Table 6 and indicate that compensation for the cytotoxic action of the radiation in this manner resulted in outgrowth development and lesion frequencies similar to those seen in control outgrowths derived from 10^4 cells.

These results are consistent with the observation of a cell dose effect for the expression of lesions. The data should not, however, be taken to indicate that radiation does not induce lesions in a manner similar to that of DMBA. There are several lines of evidence that suggests that radiation induces ductal dysplasia. For example, lesions have not been observed in control outgrowths derived from 10^5 cells, however both type A and type B lesions were detected in outgrowths derived from 10^5 cells of donors exposed to 100 rad of γ -rays (Table 1, page 83). Further evidence that radiation induces ductal lesions was obtained from the experiment in which outgrowths were derived by injection of 10^5 cells from donors exposed to 600 rad of γ -rays. Although this cell dose was chosen to yield 10^4 viable cells, the data for the percent classifiable outgrowths and the percent of the outgrowths that filled the fat

TABLE 6

EFFECT OF CELL REPLACEMENT ON OUTGROWTH DEVELOPMENT AND LESION FREQUENCY

Group	Cell Dose	Classifiable Outgrowths (%)	Frequency of Ductal Dysplasia (%)			
			Full (%)	Type A	Type B	Total
Controls	10^4	82.1	56.6	4.3 ± 1.4	1.0 ± 1.0	5.3 ± 1.5
γ -irradiation (100 rad)	10^4	71.5	38.5^a	10.6 ± 2.9^a	0.9 ± 0.9	11.5 ± 3.0^a
γ -irradiation (100 rad)	2×10^4	89.6	67.7	3.5 ± 2.0	0	3.5 ± 2.0
γ -irradiation (600 rad)	10^5	94.7	88.8^a	4.5 ± 2.2	1.1 ± 1.1	5.6 ± 2.4

^ap < 0.05 versus controls.

pads (Table 6) indicate that 10^5 cells clearly overcompensated for the toxicity of the treatment. Yet, despite the extent of outgrowth development in this group, lesions were still observed and these lesions were detected in outgrowths which had filled the fat pads. Given that lesions have never been detected in control outgrowths that had filled the fat pads, it is quite likely that the lesions observed in this group were not merely the result of the toxicity of the treatment but rather were induced in a manner similar to that of DMBA.

Isolation of Cells That Yield Mammary Ductal Dysplasia

There is a great deal of circumstantial evidence that indicates that carcinogen induced mammary carcinoma in mice are preceded by ductal dysplasias. As discussed in Chapter V, the ductal lesions that were detected in these studies resemble those putative preneoplastic lesions. However, in order to unequivocally demonstrate that ductal lesions are the precursors of mammary neoplasms and to identify which type of lesions are most likely to yield these tumors, it is necessary to isolate the lesions or the cells that yield them and test their growth potential independently. In an attempt to do this, two sets of experiments were carried out. In the first set of experiments attempts were made to identify ductal lesions in situ and to transplant these tissues into gland free mammary fat pads of host mice to test their growth potential. In the second set of experiments, in vitro methods were used to isolate cells with altered growth potential.

Identification of ductal dysplasia in situ. In order to identify mammary tissues in situ, animals bearing cell dissociation derived mam-

mary outgrowths were given an intraperitoneal injection of 0.4% trypan blue. Twenty-four hours later the animals were killed and the mammary outgrowths were removed and examined under a dissecting microscope. When areas of suspected ductal dysplasia were detected they were excised, cut into 1 mm pieces and transplanted intact into gland free fat pads of host mice. Using this procedure several outgrowths lines were started from intact pieces of cell dissociation derived outgrowths that were originally derived from control and carcinogen treated donors. Every 10 to 12 weeks the tissues were passaged in vivo by transplanting 1 mm pieces of the new outgrowths into gland free fat pads of new host mice.

Based on results of similar experiments performed with virally induced HAN, it was expected that transplantation of intact pieces of abnormal mammary tissue would yield entire outgrowths that exhibited the same abnormality. In these experiments, however, no such abnormal outgrowths were detected. Rather, all of the outgrowths that were derived from intact pieces of mammary tissue had a normal ductal architecture that only occasionally exhibited subtle abnormalities in branching patterns. These outgrowth lines also behaved like normal mammary tissue with regard to the duration of their growth potential in that the outgrowths became progressively less able to fill the fat pads with each passage and could only be maintained for about 6 or 7 transplant generations.

There are several possible explanations for the disappointing results that were obtained from this experiment. First, the bulk of this work was done before some of the factors that influence lesion

expression were understood as well as they are now. For this reason many of these experiments were not performed under conditions that would have maximized the likelihood of detecting lesions in situ. For example most of these experiments were performed with cells of donors treated with low doses of radiation. Further, the outgrowths that were examined often had developed to the point where they had filled the fat pad and stopped growing. Second, in many cases it was very difficult to observe the ducts in the outgrowths because of the amount of fat that was present in the inguinal fat pads. Thus some lesions may have gone undetected. Third, even if abnormal ducts or end buds were transplanted there is no way of knowing how many of the cells that made up a particular lesion were truly abnormal. Thus it was possible that abnormal areas were transplanted which gave rise to fairly normal appearing mammary outgrowths. The last two factors in particular point to important disadvantages in this method of testing the growth potential of suspected mammary lesions in that one can never be sure of the morphology of the piece of tissue that was transplanted. This is particularly important given the range in the severity of ductal lesions that have been observed in these experiments.

In vitro methods for isolating carcinogen altered cells. In the second set of experiments aimed at isolating mammary cells with altered growth potential, an in vitro method was used that has been used previously to isolate preneoplastic tracheal epithelial cells. In this method cells that have acquired altered in vitro growth potential are selected by plating cells from the donor animals at low density and attempting to grow them in a medium that does not support the growth of

normal cells. Thus cells that have acquired an enhanced capacity for in vitro growth form epithelial foci (EF) which are detectable 4 weeks after the cells are seeded into culture. Terzaghi and Nettesheim (1978) have reported that enhanced capacity for in vitro growth is a characteristic of preneoplastic tracheal epithelial cells. Thus, these experiments were performed in an attempt to isolate the cells that yield preneoplastic mammary ductal dysplasia in vivo.

In these experiments mammary tissues from control and DMBA treated BALB/c mice (0.075 mg DMBA per mouse) were enzymatically dissociated under aseptic conditions and the cells were seeded into culture at varying cell densities. From each donor animal, 4 dishes each were plated with 10^3 , 5×10^3 , 10^4 , 5×10^4 and 10^5 cells and the plates were examined 4 weeks later for the presence of proliferating EF. The dishes that were plated with cells from control animals did not exhibit any EF regardless of the initial plating density. EF were detected, however, in several of the dishes that were plated with cells from DMBA treated donors. The dishes that exhibited significant numbers of these EF were those that were plated initially with 5×10^4 cells or more. The cells in one of the EF positive plates grew to confluency and was passaged. Soon after passage of the cells, fibroblasts appeared to take over the culture, however three distinct foci of epithelial cells also grew in this dish. The fibroblasts were selectively scraped from the dish and this allowed the epithelial cells to form a monolayer of hexagonal cells. These cultures are still growing and have now been in culture for over 9 months.

Following the detection of EF in dishes seeded with mammary cells from DMBA treated donors, a second experiment was carried out that was designed to repeat the first experiment and also to determine if EF could be detected in dishes seeded with mammary cells from radiation treated donors. In this experiment donor animals were either untreated, exposed to 0.075 mg of DMBA, 100 rad of γ -ray irradiation, 20 rad of fission neutron irradiation or 50 rad of fission neutron irradiation. The cells from each donor animal were seeded into four 60 mm tissue culture dishes at a density of 5×10^4 cells per dish. As in the previous experiment, no EF were found in dishes that were plated with cells from control donors, while all dishes plated with cells from DMBA treated donors exhibited several EF. Although most of the EF detected in the DMBA dishes ultimately senesced, one dish had cells which continued to proliferate for 6 months in culture before senescing and one dish had cells that are still growing and have been in culture for nearly 7 months. In contrast, EF were not detected in any dishes that were plated with cells from radiation treated donors.

The results that have been obtained from these experiments are in good agreement with results that have been obtained with the EF assay using tracheal epithelial cells. In both cases EF have been detected in dishes plated with cells from DMBA treated donors and have not been detected in similar dishes started from radiation treated donors. Insofar as the tracheal epithelial cells that have been isolated using the EF assay have been shown to be preneoplastic (Terzaghi and Nettesheim, 1979), the results of these experiments are encouraging and

suggest that the EF assay may provide a valuable means for isolating carcinogen altered mammary epithelial cells.

In summary, these studies provided information which indicated that the cell dissociation assay can be used to detect subtle abnormalities in the growth potential of mammary epithelial cells at very short times after exposure to carcinogen. The finding that cells possess altered growth potential long before the potential is normally expressed and the finding of the cell dose dependence for the expression of ductal lesions should be useful in the planning of future experiments and should provide some further clues as to the mechanisms involved in the progression of neoplasia.

CHAPTER VII

DISCUSSION

The experiments that are the subject of this dissertation were originally designed to test a two-fold hypothesis. First, that the acquisition of altered growth potential by mammary epithelial cells that occurs following administration of DMBA and which results in the expression of mammary ductal dysplasia occurs soon after carcinogen treatment. Second, that the expression of the altered growth potential is influenced by the presence and architecture of normal mammary cells. Essentially this hypothesis proposes that expression of carcinogen induced mammary ductal dysplasias is regulated in a manner similar to that of virally induced HAN. If this is the case, and the above stated hypothesis is correct, then the use of the cell dissociation technique to derive mammary outgrowths should result in enhanced expression of ductal dysplasias in these outgrowths as it does for the expression of virally induced HAN. The results that have been obtained from this work are consistent with this hypothesis and can be summarized as follows:

1. Use of the cell dissociation technique to derive mammary outgrowths from monodispersed cells results in enhanced expression of mammary ductal dysplasia
2. These ductal dysplasias resemble, at the whole mount and histological level, ductal lesions that have been associated with the pathogenesis of breast cancer in mice, rats and humans

3. Expression of ductal dysplasias in cell dissociation derived outgrowths is influenced by the number of cells used to derive the outgrowths and is enhanced in outgrowths derived from 10^4 rather than 10^5 cells
4. Many more donor animals have cells that give rise to lesions than would be expected based on the incidences of mammary tumors that result from exposure of BALB/c mice to doses of radiation or DMBA that were used in these experiments
5. Many of the ductal dysplasias observed in cell dissociation derived outgrowths regressed when the outgrowths filled the fat pads, while a small fraction of the lesions persisted.

Similarity of Cell Dissociation Derived Ductal Dysplasia to Those Found in Mice, Rats and Humans

One of the most interesting aspects of the results of these experiments concerns the similarity of the lesions observed in the mammary outgrowths to ductal dysplasias that have been associated with the pathogenesis of mammary carcinoma in carcinogen treated mice and rats as well as to high risk lesions that have been associated with breast cancer development in humans.

In mice, the morphological and histological characteristics of mammary dysplasia that precede the development of overt carcinomas in two mouse strains have been described by Medina (1976b). At the whole mount level the lesions observed in carcinogen treated BALB/c mice exhibited abnormalities in the branching pattern of ducts, particularly terminal ductules, which often violated the normal growth spacing regulatory mechanisms. In addition to appearing dysplastic at the whole

mount level, these lesions exhibited varying degrees of epithelial hyperplasia at the histological level. The hyperplasia ranged from mild infoldings or papillary projections of epithelial cells to severe hyperplasia in which chords of epithelial cells occluded the ductal lumen. In some abnormal ducts and end buds hyperplasia was observed in which the cells exhibited a cribriform pattern. Many of the hyperplasias consisted of uniform appearing cells while some of the more severe lesions exhibited atypias to an extent that warranted their classification as carcinoma in situ. In carcinogen treated C57BL $\times$ DBA F₁ mice, lobular hyperplasias were observed. These lesions consisted of numerous closely spaced terminal ductules that were filled with uniform appearing epithelial cells.

Ductal abnormalities similar to those found in mice have also been observed in a variety of rat strains following administration of DMBA. The earliest lesions that have been observed in Wistar-Furth and Lewis rats after DMBA treatment were characterized by mild terminal ductal hyperplasia in which the ductal lumens were filled with uniform appearing epithelial cells. These lesions appeared to progress to more severe abnormalities in which the hyperplastic ducts and end buds were distended with cells, many of which exhibited moderate degrees of atypia. Finally, neoplastic lesions were seen which appeared to arise from the previously hyperplastic areas (Fisher, 1975; Haslam and Bern, 1977).

As was discussed in Chapter V, the lesions that were observed in cell dissociation derived outgrowths were nearly all ductal dysplasias. These lesions were characterized by abnormalities in the branching

patterns of the ducts, particularly the terminal ductules, and usually exhibited epithelial hyperplasia. Further, the cytological characteristics of the lesions varied considerably. Some lesions were observed in which the hyperplasia consisted of densely packed uniform appearing epithelial cells, while in others the cells exhibited a cribriform pattern. In several of the more severe lesions, bizarre patterns of cellular proliferation into the lumen were observed which consisted of epithelial cells that exhibited a high degree of atypia. Thus the lesions that were detected in cell dissociation derived outgrowths resembled, both at the morphological and histological level, lesions associated with the pathogenesis of breast cancer in rats and mice.

The lesions that were found in mammary outgrowths also resembled ductal abnormalities that have been associated with the development of human breast cancer and these lesions have recently been described by Wellings (1980). Wellings reported that human mammary dysplasias nearly always consisted of abnormalities in the terminal ductal lobular unit (TDLU) which were abnormal in both the number and branching pattern of terminal ductules and in which varying degrees of hyperplasia were observed. Wellings described two distinct forms of atypical lobules (AL) and these were designated as types A and B (ALA and ALB). ALA were lesions of the terminal ductules and were organized into a grading system based on the morphology of the abnormal TDLU, the degree of hyperplasia within the terminal ductules and the cytological characteristics of the hyperplastic cells. Grade 1 lesions were those in which the TDLU was greatly enlarged and the individual terminal ductules distended but in which no epithelial hyperplasia was evident. Grade 2

lesions were those in which mild hyperplasia was associated with the abnormal TDLU. These abnormal terminal ductules exhibited several extra layers of uniform epithelial cells. Grade 3 lesions were characterized by more extensive hyperplasia in which the terminal ductules were filled with uniform appearing epithelial cells. Lesions that were classified as grade 4 had hyperplasia to such an extent that the terminal ductules were distended with cells. The cells within these lesions often exhibited a cribriform pattern. Finally grade 5 lesions were those in which the hyperplastic cells exhibited marked atypia and were thus classified as carcinoma in situ.

ALB were lobular dysplasias. These lesions were characterized by the presence of an increased number of terminal ductules which were often occluded by epithelial cells. When the hyperplastic cells within the ALB's exhibited atypia they were classified as lobular carcinoma in situ.

The evidence that associates these human dysplasias with the development of breast carcinoma is by necessity circumstantial and is threefold: (a) these lesions are more common in cancer associated breasts than they are in breasts taken from random autopsy samples; (b) the incidence of these lesions increases with age and they persist after menopause while normal lobules atrophy; (c) the lesions can be arranged in morphological sequence that leads to and includes carcinoma in situ.

The range of ductal lesions that was described in human breast tissues is so similar to what was detected in the cell dissociation derived mammary outgrowths that these lesions can easily be graded

according to the scheme proposed by Wellings. For example the lesion shown in Figure A-7 could be classified as ALA grade 1. This lesion exhibited an atypical lobule in which little or no hyperplasia was observed. The lesion shown in Figure A-2 is characterized by moderate hyperplasia and thus fits the description of ALA grade 2. Examples of lesions that exhibit the characteristics of ALA grades 3 and 4 are shown in Figures A-14 and 16. These lesions had significant hyperplasia and the cells exhibited a cribriform pattern. The lobular dysplasia shown in Figure A-21 bears marked resemblance to lesions classified as ALB, and in fact several pathologists have examined this lesion and have classified it as lobular carcinoma in situ.

Importance of the time required for the expression of ductal dysplasia. The ductal dysplasias that were observed in cell dissociation derived outgrowths clearly resemble lesions that appear to precede mammary carcinoma in several species. However, there is an important difference between the experiments reported here and those in which ductal lesions were detected in intact mammary tissues of carcinogen treated mice. This difference concerns the time required after carcinogen administration for expression of ductal dysplasia. In the work of Medina (1976b) virgin female BALB/c mice were exposed to varying doses of DMBA starting at 8 weeks of age. These animals were killed when they reached 10 months of age and their mammary tissues were removed and examined. The results of this experiment indicated that administration of 3.0 or 4.0 mg of DMBA resulted in incidences of ductal dysplasia of 47% and 53% and of mammary tumors of 26% and 37%, respectively. When, however, mice were given 2.0 mg of DMBA the incidence of

dysplasias and tumors at 10 months of age was 14% and 5%, respectively. The decrease in the incidence of dysplasias and tumors that was observed in the animals treated with 2.0 mg of DMBA was not because of a difference in the tumorigenic effectiveness of this dose compared with that for a 3.0 mg exposure but rather was because of differences in the latent period for the expression of mammary tumors following administration of the two doses. Evidence to support this interpretation of Medina's data has recently been obtained and indicates that the lifetime incidence of mammary tumors in BALB/c mice treated with 2.0 or 3.0 mg of DMBA is approximately the same with the only difference being in the latent period. The time required for expression of mammary tumors following 3.0 mg is shorter than that following administration of 2.0 mg (Éthier and Ullrich, 1982). Thus, at 10 months of age animals that had been treated with 2.0 mg of DMBA had simply not yet developed dysplasias or tumors. This suggests that neoplastic cells may arise either concomitantly or shortly after the expression of ductal dysplasia.

When BALB/c mice are treated with low doses of radiation or DMBA such as those that were used in the cell dissociation experiments, the mammary tumors that develop nearly all occur in the second year of life. Thus, based on Medina's findings, one would not expect to find ductal dysplasias in mammary tissues of these mice until long after carcinogen treatment. The results of a small experiment performed in this laboratory in which mammary tissues of BALB/c mice were examined at regular intervals following treatment with 0.25 mg of DMBA support this view. The data obtained from this study indicated that ductal dysplasias did not develop until between 30 and 40 weeks after treatment

(unpublished observations). This finding is consistent with data for the latent period for mammary tumor development following this dose which indicate that tumors begin to appear approximately 30 weeks after exposure (Ethier and Ullrich, 1982).

When one takes all of this information into account, the finding of ductal dysplasias in cell dissociation derived outgrowths 10 weeks after injection of cells that were obtained from donor animals exposed to low doses of radiation or DMBA 24 hours prior to dissociation indicates that the potential for expression of ductal dysplasia is acquired soon after administration of carcinogen. The results also suggest that control mechanisms exist that can, for a relatively long time, inhibit the expression of altered growth potential and this finding is of central importance to the original hypothesis that was tested in these experiments.

Factors Influencing the Expression of Ductal Dysplasias

The expression of ductal dysplasias in cell dissociation derived outgrowths appeared to be influenced by several factors. One of these factors was carcinogen treatment of the donor animals which in several cases resulted in enhanced incidences of lesions over control levels. The data did not indicate a dose dependence for the induction of these lesions. This is not surprising however, given the many factors that have been found that appear to influence both outgrowth development and lesion frequency. Further work will be required to understand how carcinogen dose affects lesion induction and expression.

A second factor that had a profound effect on lesion expression was the number of cells that were used to derive the outgrowths. When inocula of 10^5 cells from non-treated donor animals were injected into gland free fat pads, no lesions were detected in the outgrowths that resulted. In contrast, lesions were consistently observed in outgrowths derived from 10^4 cells from control donors and although the majority of these lesions were not severe, two type B ductal lesions were observed. Similarly, the lesion frequencies obtained in outgrowths derived from cells of carcinogen treated donor animals were always higher in groups in which the outgrowths were derived from 10^4 rather than 10^5 cells.

The finding of a cell dose dependence for the expression of ductal dysplasia is consistent with findings of other workers. An influence of cell dose on expression of neoplastic transformation of 10T 1/2 cells in vitro has been reported by a number of groups (Haber et al., 1977; Han and Elkind, 1979; Terzaghi and Little, 1976). These workers found that expression of transformed foci is influenced by the number of cells that are initially plated such that if cells are not plated at a sufficiently low density no transformed foci are expressed. One interpretation of these findings is that the cells must undergo a certain number of rounds of cell division in order to express the altered phenotype. The data of Haber et al. (1977) however suggest that in addition, a more complex interaction between normal and altered cells is involved in determining whether altered cells express the transformed phenotype. The finding of a cell dose dependence is also consistent with a hypothesis that has recently been proposed by Nandi (1978b). This hypothesis attempts to explain how normal cells influence the expression of nodule transformed cells as HAN and thus why deriving

mammary outgrowths from dissociated cells can result in enhanced expression of HAN. Central to this hypothesis is the assumption that the basic difference between normal and carcinogen altered cells is that the former has a limited capacity for cell division while the latter has an unlimited division capability. With this assumption, these workers proposed that the factor that is of greatest importance for the expression of nodule transformed cells is the ratio of normal to altered cells. When this ratio is high the altered phenotype is not expressed. Further, anything that results in a decrease in this ratio results in greater expression of the altered cells. Thus, as time goes on normal cells exhaust their division capability while abnormal cells continue to divide and thus the ratio is naturally shifted as the animals get older. The age dependent increase in the expression of HAN and tumors in MuMTV infected mice is consistent with this hypothesis. This hypothesis has also been proposed to explain why cell dissociation results in enhanced expression of nodule transformed cells in that deriving outgrowths from dissociated cells requires that a large number of cells undergo many divisions over a short period of time and this results in a more rapid shift in the ratio of normal to altered cells and hence enhanced expression of HAN. If carcinogen altered mammary cells that have the potential to express ductal dysplasias behave in a manner similar to nodule transformed cells then the finding of a cell dose dependence would be consistent with this hypothesis because in order for an outgrowth to develop from 10^4 rather than 10^5 cells, the cells have to undergo even more divisions and thus the ratio of normal to altered cells would be decreased even more rapidly. Thus, this hypothesis also

emphasized the importance of the number of rounds of cell division as well as an interaction between normal and altered cells in expression of the altered phenotype.

Although it is possible that expression of ductal dysplasia is influenced by the number of rounds of division which the cells undergo, the situation may be more complex. There is some evidence to suggest that the ductal lesions that were observed in the mammary outgrowths were abnormal from the beginning of outgrowth development and therefore did not have to undergo a certain amount of proliferation before expressing their abnormal growth potential. For example, many of the dysplasias (particularly type B lesions) observed in 10^4 cell derived outgrowths exhibited little, if any normal growth. In other experiments, outgrowths were observed as early as three weeks after injection of cells that appeared to have ductal abnormalities (Chapter IV, p. 70). Thus, it appears that at least some outgrowths exhibited abnormal growth potential before a great deal of proliferation had taken place. For these reasons, the cell dose dependence may not be totally explained on the basis of the number of divisions needed to yield an outgrowth.

There is one yet another aspect of the data that was obtained in these experiments that should, at least, cause one to look with caution on Nandi's hypothesis to explain the cell dose effect. That aspect concerns the frequency with which ductal dysplasias were observed in outgrowths derived from 10^4 cells of non-treated donor animals. If the expression of all ductal dysplasias are the result of expression of potentially neoplastic cells, then the data obtained for the frequency

of lesions in outgrowths derived from 10^4 control cells (Table 1, p. 83) and the number of donor animals that gave rise to these lesions (Table 2, p. 85) would indicate that virtually all 12-week-old virgin female BALB/c mice possess many (perhaps thousands) potentially neoplastic cells nearly all of which do not ever express their potential. Although this possibility cannot be ruled out entirely, data obtained from a long term study in which outgrowths derived from 10^4 dissociated cells were allowed to remain in the host animals for 1 1/2 years after injection of cells, make it unlikely. In this experiment no mammary tumors were detected in any of 96 outgrowths derived from 10^4 dissociated cells of control animals. Based on Medina's data which indicate that mammary tumors develop soon after expression of ductal lesions, one might have expected at least a few tumors to develop from these outgrowths if indeed the lesions observed in control outgrowths are the result of expression of preneoplastic cells.

There may be an alternate explanation for the relative abundance of lesions observed in outgrowths derived from 10^4 control cells. This alternate hypothesis proposes that there is some lower limit to the number of cells required for the development of normal mammary outgrowths. Thus, occasionally slightly abnormal outgrowths may develop that result directly from the development of outgrowths from too few cells. Evidence to support this hypothesis has been provided by Adams and Ullrich (1982) who demonstrated that as the cell dose is decreased the percent of fat pads in which outgrowths develop also decreases and

this indicates that at sufficiently low cell doses the cells lose the ability to form outgrowths altogether. If this possibility is correct, then two mechanistically distinct types of lesions were observed in these studies: one type that was the direct result of deriving mammary outgrowths from inocula of 10^4 cells, and a second type that resulted from the action of the carcinogen on the growth potential of the mammary cells. This possibility has implications for the suitability of Nandi's hypothesis for explaining the cell dose effect because if lowering the cell dose taxes the cells ability to yield normal outgrowths then this may also explain why carcinogen induced mammary dysplasias are more likely to be expressed in outgrowths derived from the lower cell dose. Admittedly, this possibility is highly speculative, however, the relative abundance of lesions in control outgrowths certainly makes further investigation warranted.

Frequency of Positive Donors in Control and Carcinogen Treated Groups

One of the very interesting findings from these experiments concerns the number of donor animals that had cells that gave rise to lesions. The data for the frequency of positive donors for the various exposure groups were given in Table 2 (page 85) and are particularly interesting when compared to the lifetime incidences of mammary tumors that one would expect from exposure of virgin BALB/c mice to the doses that were used in these experiments (Table 7). Comparison of the data in this way suggests that the alteration that results in the acquisition of altered growth potential occurs with a frequency that is much greater than that for true neoplastic transformation. This finding is certainly consistent with the findings of other workers that indicate that

TABLE 7

INCIDENCE OF MAMMARY TUMORS IN DMBA
OR RADIATION TREATED VIRGIN FEMALE
BALB/C MICE

Dose	Tumor Incidence (%)
Control	7.5
γ -radiation (25 rad)	11.7
γ -radiation (100 rad)	11.1
DMBA (0.075 mg)	22.9
DMBA (0.25 mg)	56.3

carcinogenesis is a multistage process and that the events that are important in the early stages are not rare.

The most well-known example of this phenomenon comes from work done with the mouse skin model system. Work performed with this system indicates that doses of DMBA can be administered to mouse skin that are so low that no tumors develop. If, however, one follows a single subthreshold dose of carcinogen with tumor promoting agents, then many tumors develop (Boutwell, 1964). This finding was instrumental in the development of the two stage theory of carcinogenesis and led to the definition of the stages known as initiation and promotion. The information that has been obtained from experiments with this model system indicate that initiation is not a rare event.

There is also evidence that has been obtained from work with the mouse mammary tumor system that indicates that the initial steps in virally induced tumorigenesis occur much more frequently than do the development of mammary tumors. Much of this work has already been discussed, and indicates that nodule transformation occurs with a much higher frequency than neoplastic transformation which presumably takes place within a population of HAN cells.

Some of the more recent evidence which indicates that the early stages in carcinogenesis are not rare events has been obtained with a different model system, the rat tracheal epithelial system. Terzaghi and Nettesheim (1979) have demonstrated that administration of low doses of DMBA to rat tracheas results in epithelial cells that acquire an enhanced ability to grow in vitro. Thus these cells can be selected by plating them at low density and maintaining them in a medium that does

not support the growth of normal cells. They have further demonstrated that the cells isolated in this fashion do not have tumor potential shortly after isolation in vitro but acquire the ability to form carcinomas with time in culture. The findings from experiments performed with this system were of importance for the understanding of neoplastic progression in two respects. First, the results indicated that one of the earliest alterations that could be observed and which appeared to be the result of exposure to DMBA was an enhanced capacity for cell division, particularly in vitro, and this characteristic was acquired prior to the acquisition of the neoplastic phenotype. Second, preneoplastic cells with enhanced in vitro growth capacity could be isolated from 100% of tracheas exposed to doses of DMBA that resulted in incidences of tumors of only 10%.

Given the results of experiments performed with epidermal cells, mammary cells and tracheal cells, the finding of a higher frequency of donors that yielded ductal dysplasias than would be expected based on tumor incidences observed following the respective doses of carcinogen should not be surprising and indeed is consistent with a large body of evidence regarding the early events in carcinogenesis.

The Fate of Ductal Dysplasias in Cell Dissociation Derived Outgrowths

One finding from these experiments that was rather unexpected concerned the fate of the lesions detected in the mammary outgrowths. Many of the lesions observed in 10^4 cell derived outgrowths which were examined 10 weeks after injection of cells appear to have regressed by 16 weeks after injection of cells (see Table 4, p. 89) and thus appeared to behave like normal end buds. Although the data given in Table 4

indicate that most lesions do regress, it is important to emphasize that some lesions did persist. For example, the ductal dysplasias shown in Figures VII-1,2 were detected in outgrowths derived from 10^5 cells of donors treated with 100 rad of γ -radiation examined 10 and 15 weeks after cell injection. These lesions are within outgrowths that completely fill the fat pad. Similarly, the lesions shown in Figures VII-3,4 were also detected in full outgrowths and were in groups in which the normal outgrowths were also full and resting when they were examined. Thus, clearly some lesions did not regress when the outgrowths filled the fat pad. As can be seen from these figures, lesions that persisted were usually quite severe. Unfortunately it is not yet possible to predict the fate of the cell dissociation derived ductal dysplasias based on morphological criteria because severe lesions were detected in 10^4 cell derived outgrowths that had not yet filled the fat pad (see Figures A-14 and 20).

Although these findings were unexpected they were remarkably similar to findings that have been reported independently by two groups working with separate models for rat liver carcinogenesis. Both groups have found that hyperplastic liver nodules, which result from carcinogen treatment and which are thought to be precursors of hepatocellular carcinoma, can regress under certain circumstances.

In one model, developed by Farber and co-workers (1980a,b), rats are given a single feeding of the carcinogen diethylnitrosamine followed by chronic exposure for seven days to 2-acetylaminofluorine (2-AAF). The hypothesis for this particular model of liver carcinogenesis is that the basic change which occurs in carcinogen initiated hepatocytes is one

Figure VII-1. Whole Mount of a Full Outgrowth, Derived From a 100 Rad Treated Donor, Exhibiting Severe Ductal Dysplasia.

Figure VII-2. Whole Mount of a Full Outgrowth, Derived From a 600 Rad Treated Donor, With Ductal Dysplasia.

Figure VII-3. Whole Mount of a Full Outgrowth, Derived From a 100 Rad Treated Donor, Exhibiting Severe Ductal Dysplasia.



Figure VII-1

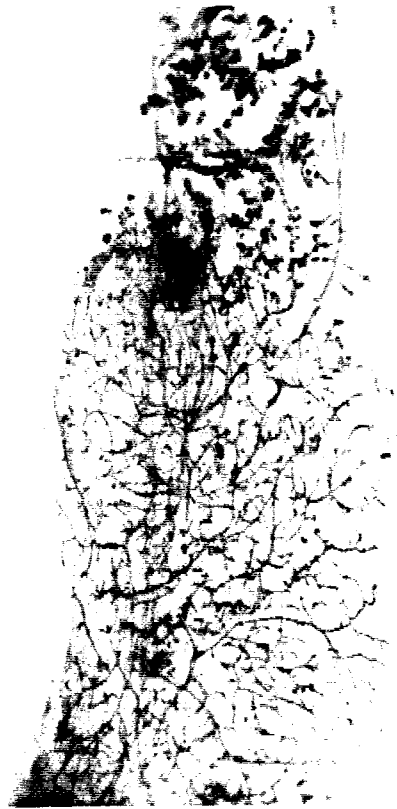


Figure VII-2

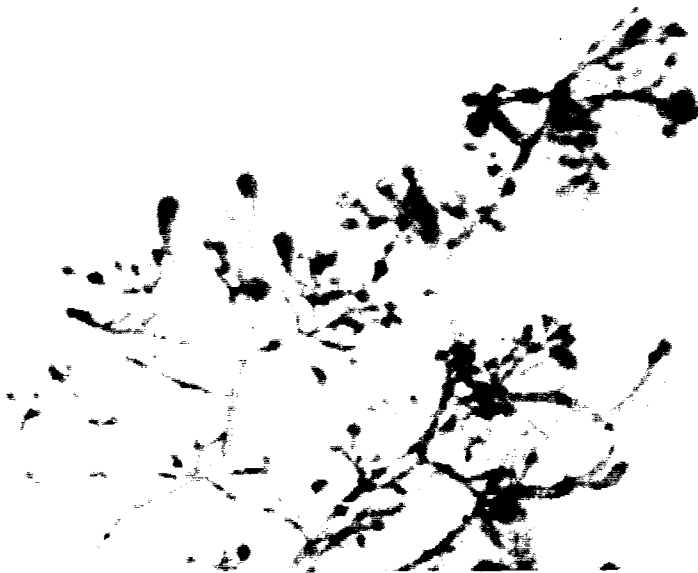


Figure VII-3

that makes the cells resistant to the growth inhibitory effects of high doses of a toxic agent which in this case is 2-AAF. Thus, this treatment induces proliferation of carcinogen altered cells and this results in the development of many hyperplastic foci (approximately 10^3 per liver) that exhibit a number of abnormal phenotypes. These hyperplastic nodules can be detected by histochemical means approximately 4 weeks after the end of treatment. By 8 weeks after treatment however, most of the hyperplastic foci (greater than 90%) remodel and are indistinguishable from normal liver. The term remodel rather than regress is appropriate here as it has been shown that if one treats animals that bear remodeled hyperplastic foci with a liver damaging agent that the lesions will reappear and this indicates that the cells did not return to the completely normal state. Although the hyperplastic nodules that are observed four weeks after treatment are indistinguishable from one another some of the lesions do persist and it appears that, at least in some cases, neoplastic lesions arise from within the persistent hyperplastic foci.

Similar findings have been reported by Becker (1982) from experiments in which a different liver model system was used. These workers found that exposure of rats to the carcinogen 2-AAF for a single three-week feeding cycle did not result in any detectable changes in the liver and tumors did not develop. If, however, animals were exposed to two or three similar exposure cycles, than hyperplastic nodules developed in the liver which were similar in their histochemical features to those observed by Farber (1980a,b). Also, like the nodules observed by Farber, these lesions remodeled after administration of the carcinogen was

discontinued. When the animals were treated with this agent for 4 feeding cycles, the hyperplastic foci that resulted were similar to those observed after treatment for two or three cycles; however these lesions did not remodel when carcinogen treatment was discontinued but rather appeared to progress to hepatocellular carcinomas.

Thus, the findings from work done with the liver model system indicate that lesions which appear to be involved in the pathogenesis of neoplastic disease can regress under certain circumstances. Perhaps, then, some of the ductal dysplasias observed in the cell dissociation derived outgrowths were similar to preneoplastic nodules seen in liver, since the mammary lesions were induced by very low doses of carcinogen and were observed a relatively short time after treatment.

Synthesis and Directions for Future Work

Over the years many different hypotheses have been advanced that attempt to explain the factors that are important in the development of cancer. As the level of sophistication in methods to study neoplastic progression has improved, the hypotheses that have come from them have become more detailed. More importantly, as model systems which make use of different cell types have been developed, some basic principles regarding neoplastic progression have emerged and these unifying principles are of first importance to the understanding of cancer progression. Thus it appears that exposure of cells to a carcinogenic agent results in a change in some cells which is complete within a very short time after exposure. These carcinogen altered cells then can remain dormant or can proliferate and yield readily detectable, but non-neoplastic tissue abnormalities (HAN, ductal dysplasia, papilloma,

hyperplastic liver nodule, etc.). Finally, neoplastic cells can emerge that ultimately give rise to a tumor; however, neoplastic cells appear to arise very late in the process and thus a long time after carcinogen treatment. Carcinogenesis then appears to be a multistep process.

The results obtained from the cell dissociation experiments make a significant contribution to what is known about these basic principles of carcinogenesis. The finding of ductal dysplasias in mammary outgrowths derived from cells of donor animals treated with low doses of DMBA or radiation indicates that the potential for expression of these abnormalities is acquired soon after carcinogen treatment and long before they would normally be expressed. The frequency with which these lesions were observed and the number of donor animals having cells which gave rise to the lesions indicate further that the acquisition of this altered growth potential occurs more frequently than one might expect based on the tumor incidences that result from exposure to the doses that were used.

The results obtained from this work also raised some interesting questions that remain unanswered at this point. Some of these questions are directly concerned with the experimental method itself. For example, elucidation of the mechanism for the cell dose effect for the expression of lesions will require further investigation. Further work will also be required to determine if the fate of ductal dysplasias can be predicted on the basis of morphological and histological criteria. Finally, although the ductal dysplasias that developed in the mammary outgrowths bear remarkable resemblance to lesions which appear to

precede mammary carcinoma in rats and mice, the progression of cell dissociation derived ductal dysplasias to mammary tumors has still to be demonstrated.

The results obtained from these studies also will allow other more general questions regarding the biology of neoplastic progression to be addressed. One of these questions concerns the rate limiting step for the expression of neoplasia. The finding of ductal dysplasia in cell dissociation derived outgrowths 10 weeks after exposure to low doses of radiation or DMBA, coupled with the results of other workers which indicate that ductal dysplasias usually arise in close temporal proximity to neoplastic lesions, suggests that an important step in determining the final incidence of tumors is concerned with the factor which allows or inhibits expression of these altered cells.

Another question that can be addressed concerns the mechanism for the late acquisition of the neoplastic phenotype. The finding that neoplastic cells arise relatively late in the carcinogenic process has prompted Farber (1980) to postulate a second "rare event" and Potter (1982) to suggest a second initiation step which must occur in order for neoplastic cells to emerge from a population of preneoplastic cells. For the mammary system these hypotheses are consistent with the general agreement that in MuMTV infected mice neoplastic cells arise from populations of (preneoplastic) HAN cells. There is, however, an alternate hypothesis which has not been given as much consideration. That is, once carcinogen altered cells have progressed to the point where there is some escape from the tissue growth regulatory mechanisms

they can give rise to a variety of different types of lesions, some of which are abnormal but non-neoplastic and others of which are neoplastic. Thus, it is possible that putative preneoplastic lesions and neoplastic lesions arise from a "common ancestor" and not necessarily one from the other.

The cell dissociation assay can be used to perform experiments relevant to these two important questions. These experiments should seek to determine if mammary tumors that arise from cell dissociation derived ductal dysplasias do so in close temporal proximity to the expression of lesions, as they do in the intact animals, or if the tumors arise with a latent period that is more characteristic of the original dose of carcinogen that was administered. If the former possibility is correct then this would indicate that the rate limiting step is involved with mechanisms that allow expression of carcinogen altered cells. Further, this finding may also allow one to determine, by careful histopathological examination and cytological grading of the lesions observed, if neoplasms arise from preneoplastic lesions or whether neoplasia is one of a number of options that carcinogen altered cells may have. If the second possibility is correct, then strong evidence would be obtained for the existence of true preneoplastic lesions and the requirement for a second initiation-like event for the development of a neoplasm.

Finally, the preliminary results that were obtained with the EF assay indicate that this method may be useful for isolating carcinogen altered cells shortly after treatment. This is important because if indeed preneoplastic cells can be isolated in this way then experiments

can be performed to test directly the hypothesis that expression of altered cells is influenced by the ratio of normal to altered cells. Further, this method may allow experiments to be performed that might identify the types of lesions that are at highest risk to progress to neoplasia.

In conclusion, the experiments that have been discussed in this dissertation make a significant contribution to the growing body of knowledge regarding the development of cancer both through the information that has been gained from these studies and also through the questions that have been raised and the possibilities for future investigation that have resulted from them.

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APPENDIX

Figure A-1. Whole Mount of an Outgrowth Exhibiting Normal Ring Type End Buds.

Figure A-2. Whole Mount of an Outgrowth Exhibiting Ductal Dysplasia With Mild Hyperplasia.

Figure A-3. Histological Section of Lesion With Mild Hyperplasia.

Figure A-4. Histological Section of End Bud With Mild Hyperplasia.

In this section a clear distinction can be seen between the hyperplastic glandular epithelial cells and the cap cells which form a normal single layered ring around the end bud. Note also that despite the hyperplasia a smooth luminal surface is maintained. Mitotic figures are numerous in this lesion.

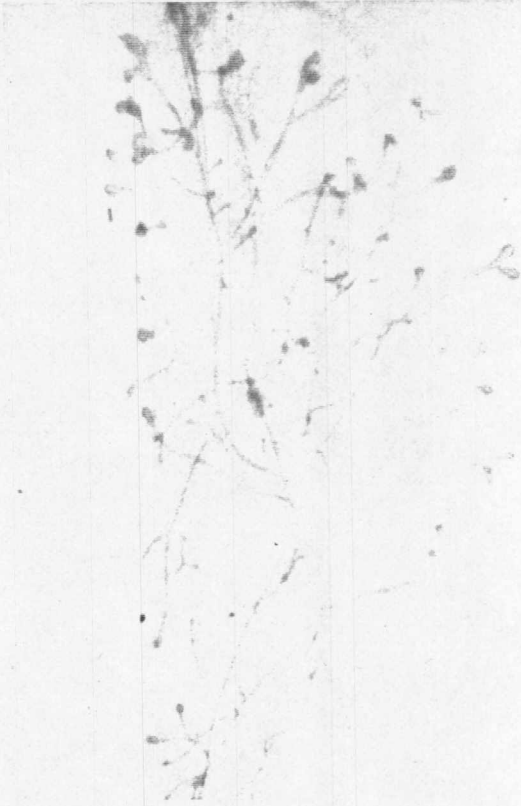


Figure A-1

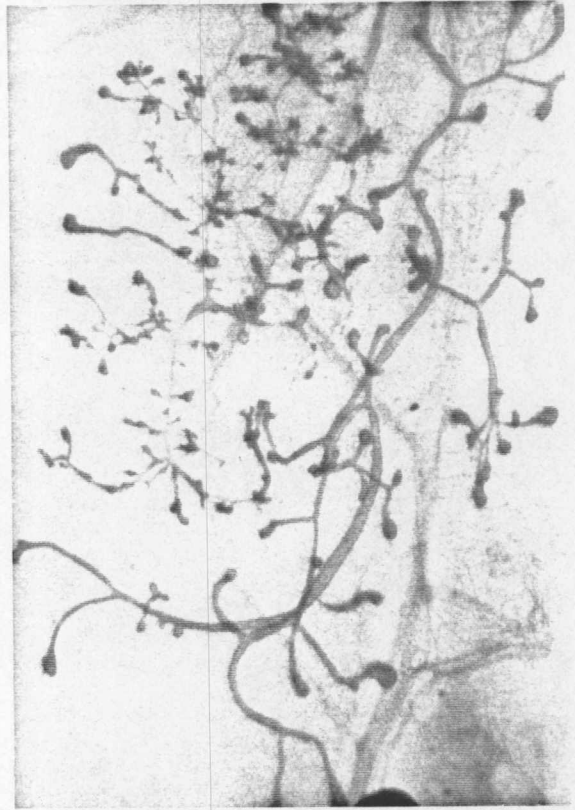


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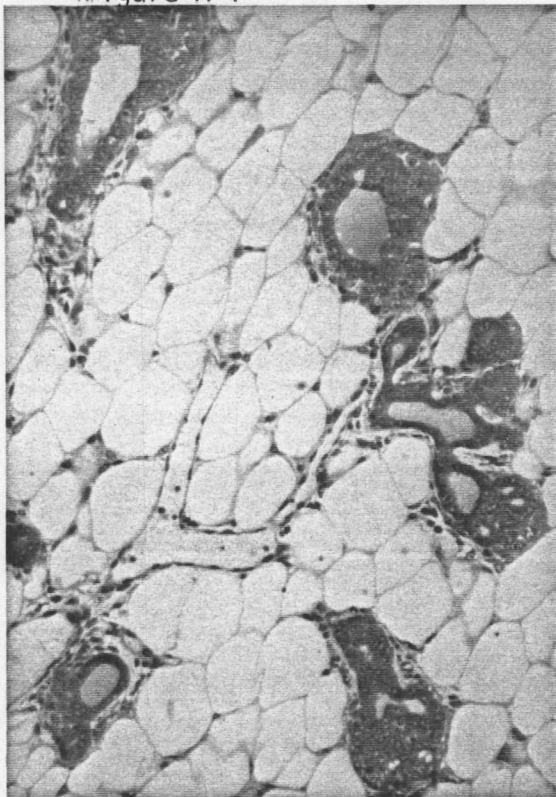


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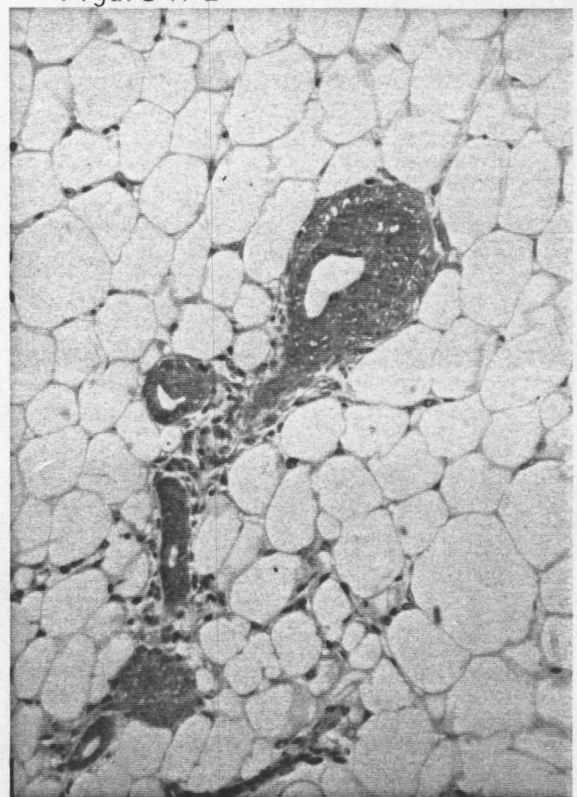


Figure A-4

Figure A-5. Whole Mount of an Outgrowth Exhibiting Ductal Dysplasia With Mild Hyperplasia.

Figure A-6. Histologic Section of Abnormal End Bud Exhibiting Hyperplasia of the Cap Cells.

In this lesion the hyperplastic cells are large and non-uniform. Further, a separate single layer of cap cells is not present.

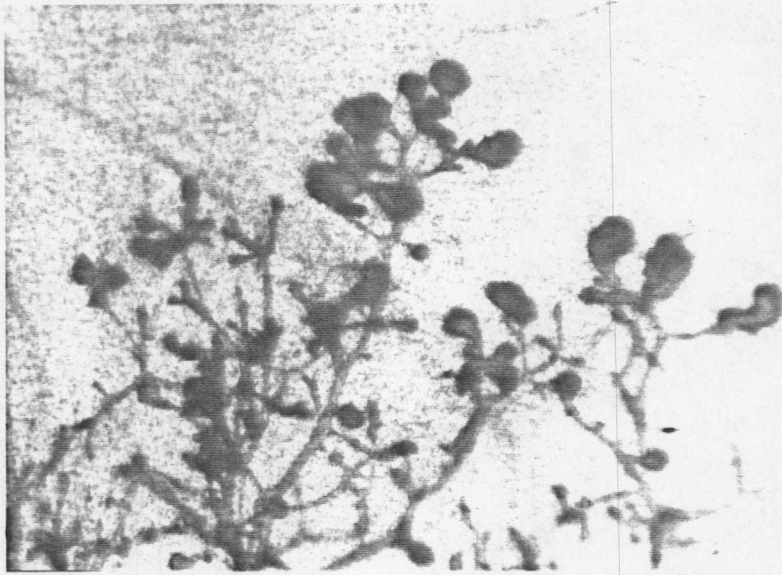


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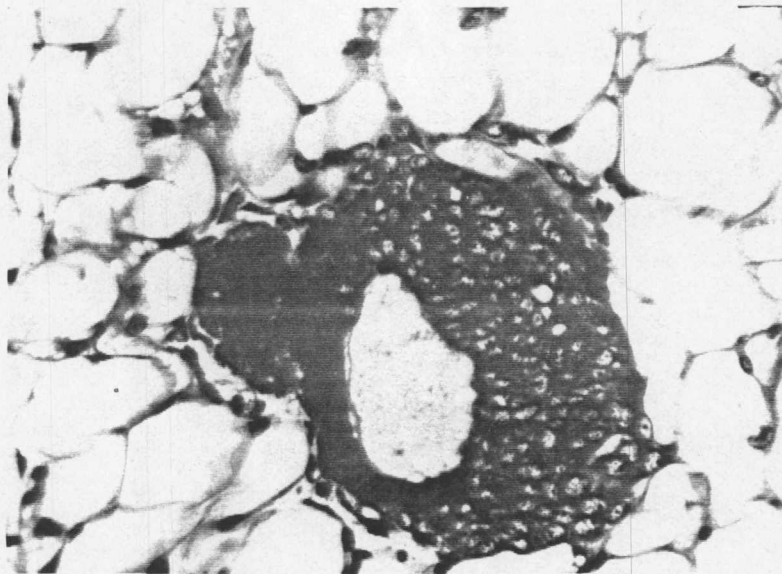


Figure A-6

Figure A-7. Whole Mount of Outgrowth Exhibiting Cystic End Buds.

Figure A-8. Higher Magnification of Cystic End Buds.

Figure A-9. Histological Section of Cystic End Buds Without Hyperplasia.

Note that the ducts appear distended and are several times their normal size. Although in this lesion there does not appear to be any hyperplasia of the glandular epithelial cells, the cap cell layer appears to be thickened in two of the cystic structures.

Figure A-10. Histological Section of Cystic End Bud With Mild Hyperplasia.

Here also the end bud is many times its normal size and in addition exhibits hyperplasia which is clearly of glandular epithelial origin.

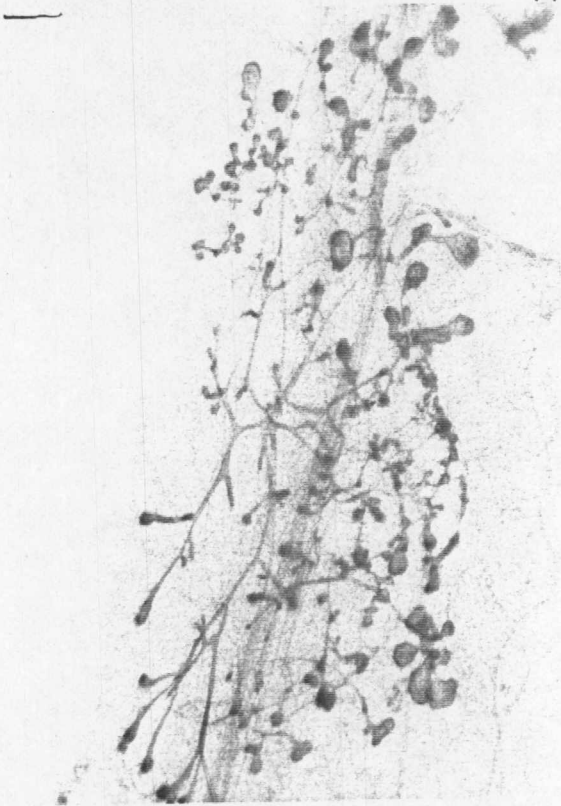


Figure A-7



Figure A-8

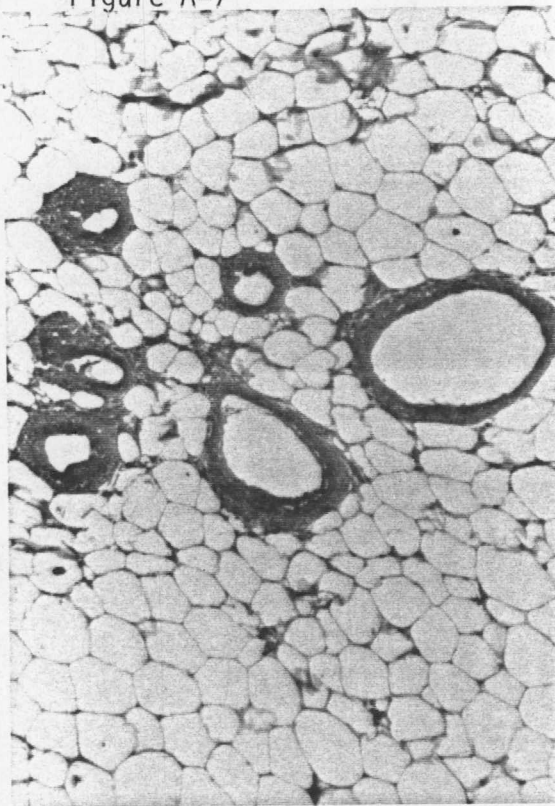


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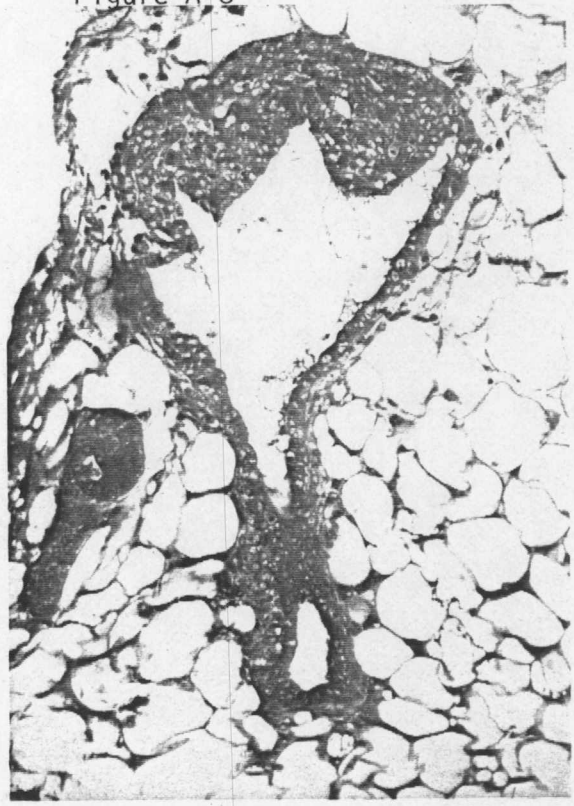


Figure A-10

Figure A-11. Whole Mount of a 10-Week Outgrowth Exhibiting Crescent Type End Buds.

Figure A-12. Whole Mount of a 10-Week Outgrowth Exhibiting Crescent Type End Buds.

Figure A-13. Histological Section of Lesion Exhibiting Crescent Type End Buds.

Here, as in crescent type end buds observed early in outgrowth development, the cells are large, pale and non-uniform. Further, no distinction between glandular epithelial cells and cap cells can be made.

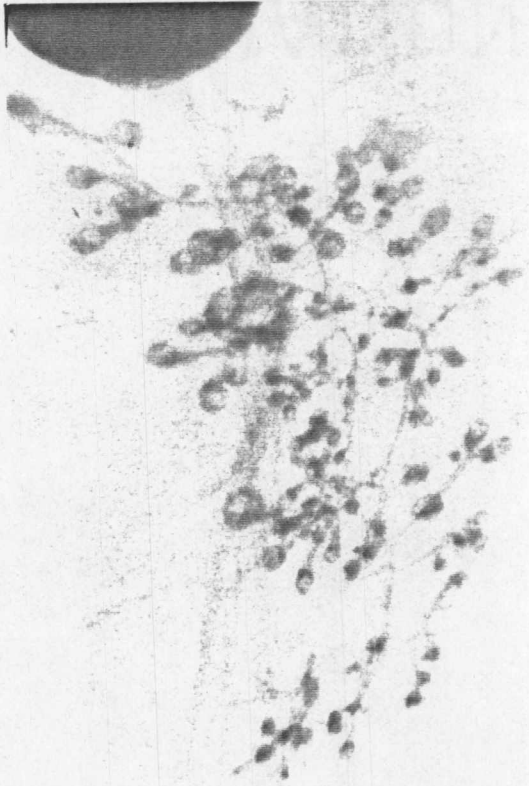


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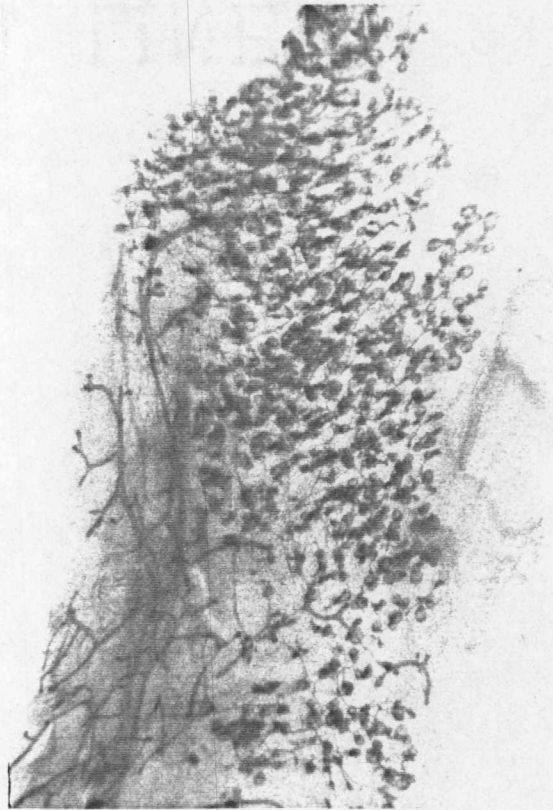


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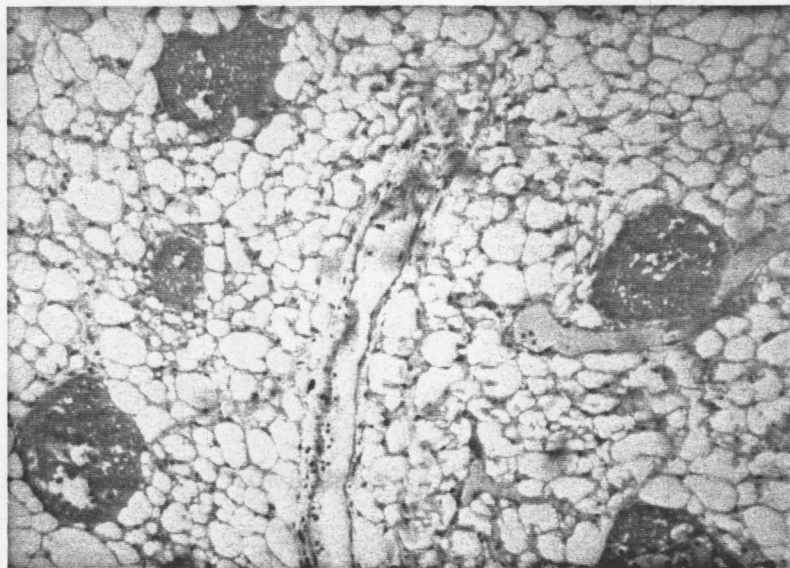


Figure A-13

Figure A-14. Whole Mount of Ductal Dysplasia With Severe Hyperplasia.

In this lesion the hyperplasia is so extensive that the lumens of the terminal ducts and end buds cannot be seen.

Figure A-15. Histological Section of Severely Hyperplastic Lesion.

The ducts and end buds are occluded with chords of epithelial cells.

Figure A-16. Whole Mount of Ductal Dysplasia With Severe Terminal Ductal Hyperplasia.

Figure A-17. Histological Section of Hyperplastic Terminal Ductule.

The hyperplastic epithelium in this lesion exhibits a cribriform pattern.

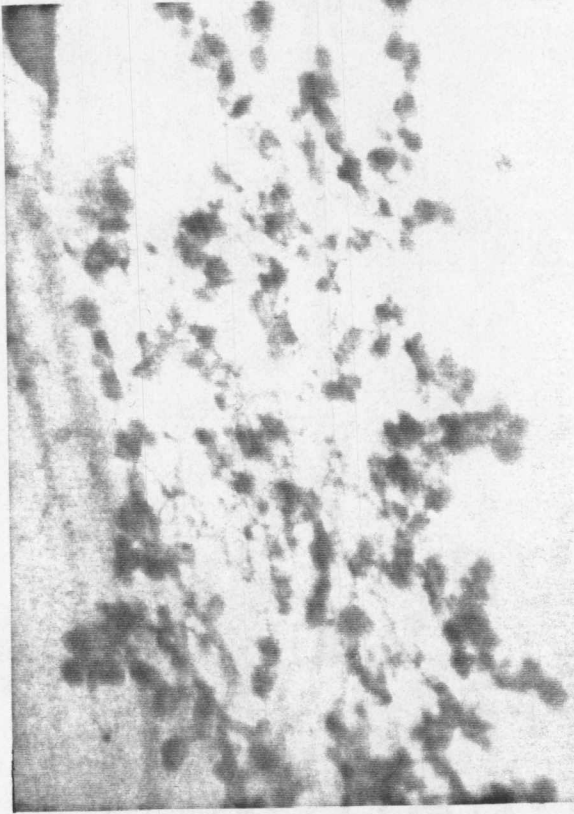


Figure A-14

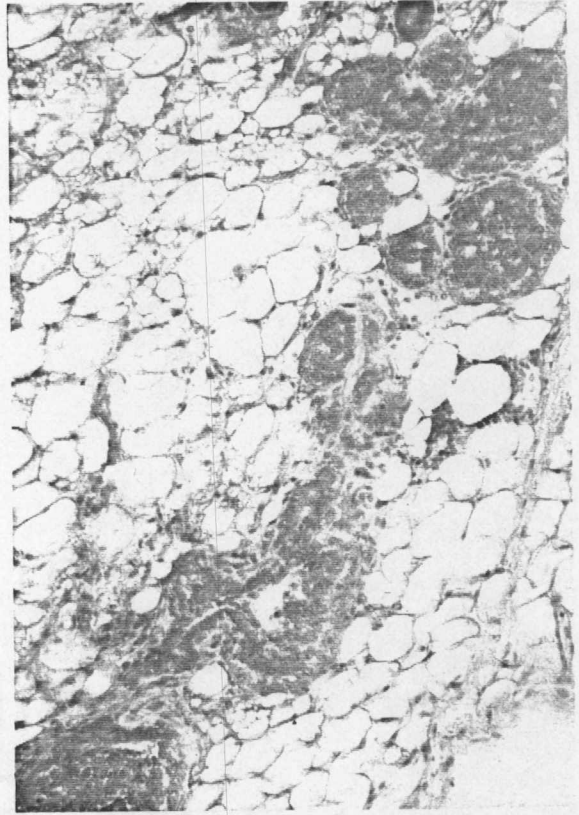


Figure A-15



Figure A-16

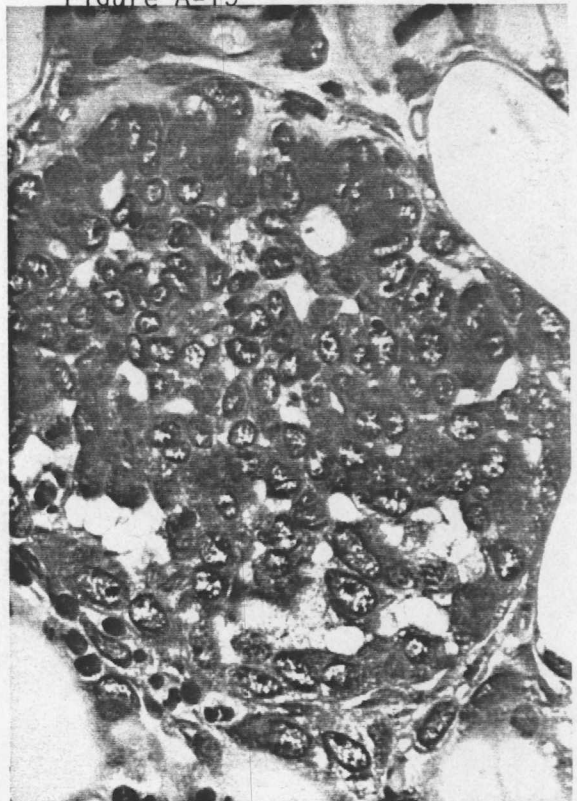


Figure A-17

Figure A-18. Whole Mount of an Outgrowth With Large Opaque End Bud.

Figure A-19. Histological Section of Large Opaque End Bud Showing Papillary Projections.

Figure A-20. Higher Magnification of End Bud With Papillary Hyperplasia.
Note the abundance of mitotic figures in this lesion.

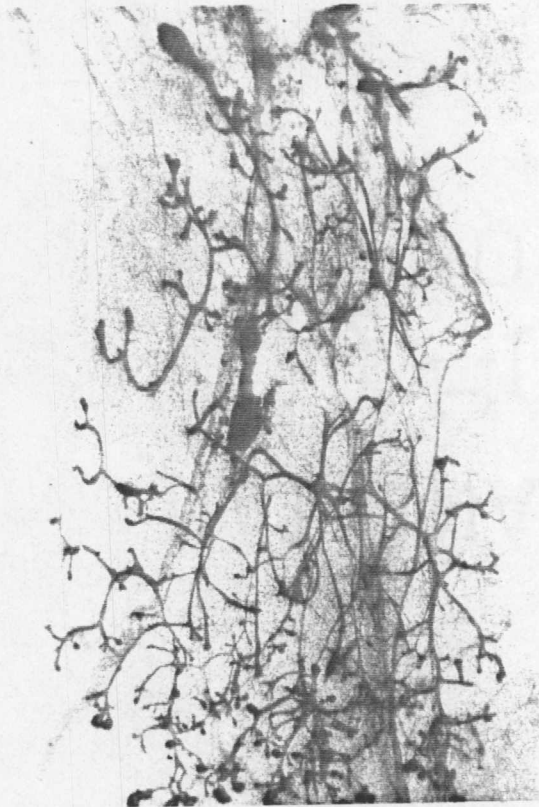


Figure A-18

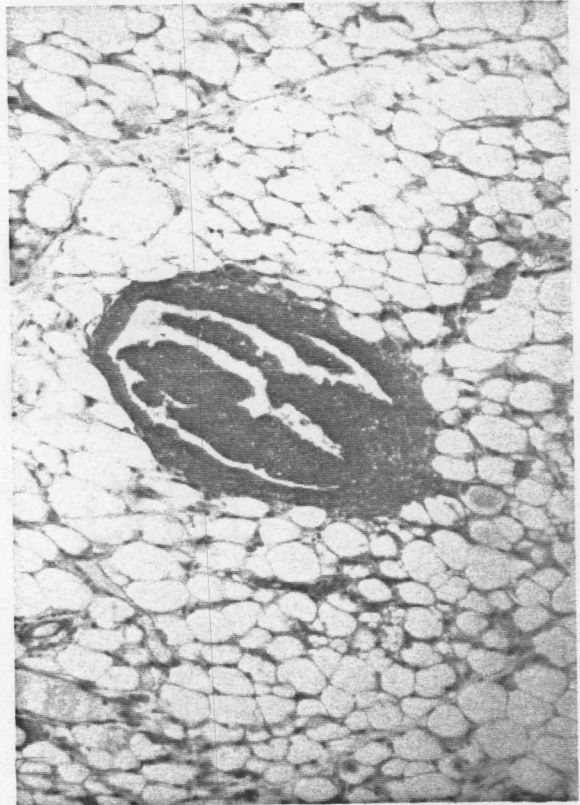


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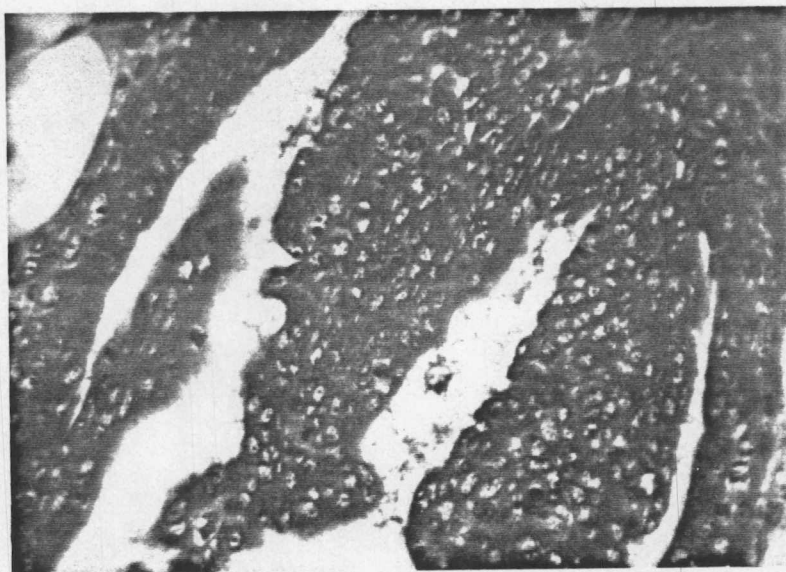


Figure A-20

Figure A-21. Whole Mount of an Outgrowth With Severe Terminal Duct and Lobular Dysplasia.

Figure A-22. Histological Section of Outgrowth Exhibiting Lobular Differentiation and Severe Hyperplasia.

Figure A-23. Whole Mount of an Outgrowth Exhibiting Lobular Differentiation.

In this outgrowth the lobular area was surrounded by normal ductal growth.

Figure A-24. Histological Section Showing Lobular Differentiation Without Hyperplasia.

This lesion is classified as alveolar adenosis.

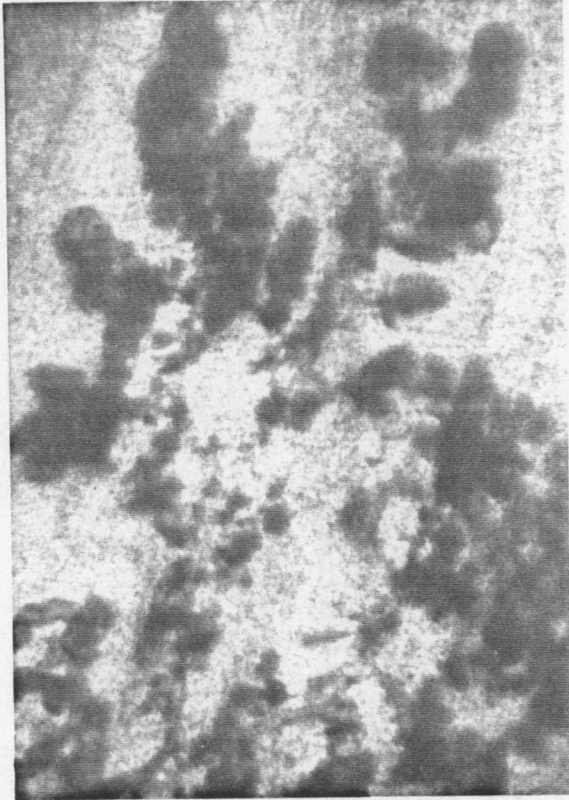


Figure A-21

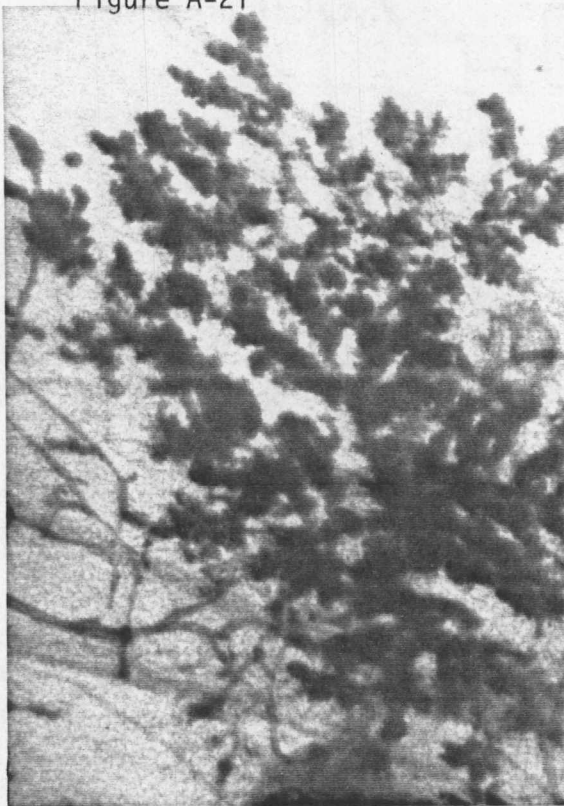


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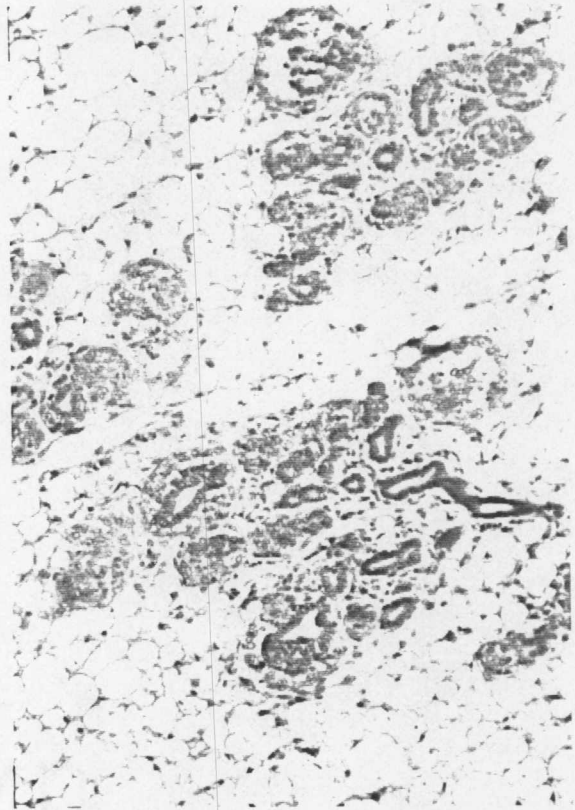


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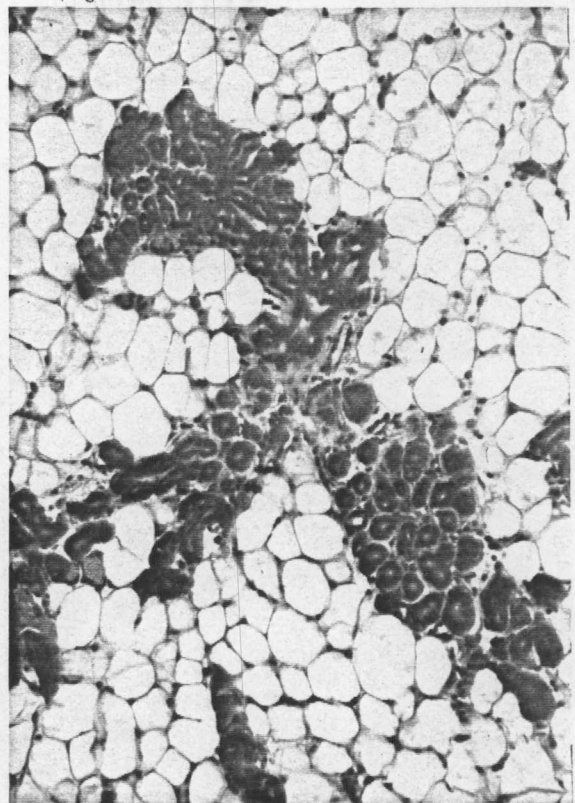


Figure A-24

VITA

Stephen Paul Ethier was born in Chicopee, Massachusetts, on October 26, 1955. He received his education in Chicopee and graduated from Chicopee High School in 1973. The following September he enrolled at St. Anselms College in Manchester, New Hampshire, where he majored in Biology. In the spring of 1977 he graduated from St. Anselms College and later that year began a Masters program in Radiological Health at the University of Michigan. Upon completion of the Masters degree he entered the Ph.D. program in Radiation Biology at The University of Tennessee, Knoxville, which he received in December of 1982.

The author is currently a post-doctoral investigator at the Michigan Cancer Foundation in Detroit, Michigan.

In the summer of 1979 the author was married to Beth Bowling of Mt. Clemens, Michigan. During the summer of 1982 the author played short stop for the Competitive Inhibitors which during that season compiled the best record in the history of Bio-Med School softball.