IN VIVO DIVERSE SPECTRUM OF NEOPLASMS INDUCED IN 30-DAY-OLD RATS AND *IN VITRO* NEOPLASTIC TRANSFORMATION OF RAT MAMMARY EPITHELIAL CELLS FOLLOWING A SINGLE PULSE OF N-ETHYl-N-NITROSOUREA

By

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ABSTRACT

IN VIVO DIVERSE SPECTRUM OF NEOPLASMS INDUCED IN 30-DAY-OLD RATS AND *IN VITRO* NEOPLASTIC TRANSFORMATION OF RAT MAMMARY EPITHELIAL CELLS FOLLOWING A SINGLE PULSE OF N-ETHYL-N-NITROSOUREA

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The specific aim of these studies were to determine the *in vitro* N-Ethyl-N-Nitrosourea (ENU)-induced spectrum of neoplasms and the *in vitro* ENU-induced rat mammary epithelial cell neoplastic transformation.

I. *In Vivo* Project. Male and female 30-day-old Sprague-Dawley (CD) and Berlin Druckrey (BD-IV) rats were inoculated intraperitoneally with a single dose of 45, 90 and 180 mg/kg of ENU. Previous experiments demonstrated that a single high dose of ENU (180 mg/kg i.p.) administered to 30-day-old female CD rats was capable of producing mammary tumors (MTs) in 100% of the animals with a 95% malignancy rate. The high rate of malignancy, local invasion and metastases to the lung were the particular features of this model. The first two parts of the *in vivo* project were a continuation of further characterization of this animal model for human breast cancer. To study the spectrum of neoplasms and the incidence of MTs in the absence of ovarian hormones stimulalation, CD male rats have been inoculated with similar doses of ENU as female CD rats. ENU induced mammary

tumors in 46% of the 30-day-old CD male rats with 3.3% lung metastases. This study also revealed that ENU induced a wide spectrum of neoplasms in histogenetically unrelated tissues.

Besides mammary and neurogenic tumors, ovarian tumors (OT) of an unusual testicular-like type (arrhenoblastoma) have been detected in 18% of the CD and 47% of BD-IV rats inoculated with high doses of ENU transplacentally and postnatally as compared to 2% (CD) and 3% (BD-IV) controls. Diethylnitrosamine (DEN) administered intraveneously postnatally did not produce ovarian tumors different from control levels. Some of these OT (6%) produced metatastases throughout the peritoneum by implantation. Hormonal assays indicated that some tumors produced testosterone, estradiol or estrone.

II. In Vitro Project. In the first part, characteristics of cultured normal mammary epithelial cells (NE) derived from Lewis and CD rats and ENU-induced adenocarcinoma cells (MA) derived from CD and Sprague-Dawley Fisher (CDF) rats have been described and compared. The NE and MA cells of ductular origin have been shown to grow in culture as a cell multilayer with cobblestone-like appearance, domes and squames formation and piled-up colonies (MA). The rate of proliferation and squames detachment in confluent cultures of MA cells have been increased up to 20% by the presence of epidermal growth factor (EGF) in the medium. Rhodanile blue staining, scanning and

transmission electron microscopy have shown partially keratinized shed cells.

The NE cells have not grown in soft agar or formed tumors when inoculated into appropriate hosts. The opposite has been true in each case for the MA cells. Karyotypes of NE and MA cells have revealed a hypodiploid modal number of chromosomes.

The second part has been designated to investigate the earliest alterative changes of _in vitro_ NE cells exposed to a single ENU pulse (25, 50, 100 and 500 ug/ml). Depending on doses, after ENU exposure, the cytometric hypodiploid histograms of NE cells have shown a second peak of cells with DNA content in tetraploid or octaploid range. The ENU-exposed NE cells (NET) have regained their resting hypodiploid pattern after 75 to 120 hours. DNA synthesis and cell count histograms have shown that NET cells significantly increased DNA synthesis ($p < 0.05$) above the control and, depending on doses, blocked mitosis up to 75 to 120 hours. The chromosomal study has shown a dose and time effect relationship. Most of the chromosomal aberrations, isochromatid breaks and chromatid exchanges have been observed at 6 and 24 hours post-exposure. Trisomy of chromosome #10 has been expressed with constancy (30%) on NET cells. The sequence of phenotypic alterations (termed "stage I-V") has been monitored by daily observation. The NET cells, after a period of approximately 30 days post-ENU exposure, have shown a

marked proliferation of morphologically altered cells (piled-up colonies, stage III) which subsequently acquired the capacity to form colonies in soft agar (stage IV) and finally became tumorigenic (stage V) when the cells have been transferred into an isologous host. The histology of these tumors resembled highly anaplastic carcinomas.

The ENU-induced reproducible sequence of *in vitro* neoplastic transformation constitutes a valuable model for the study of human breast cancer in particular and carcinomas in general.

"I always make special notes about evidence that contradicts me; supportive evidence I can remember without trying!"

Charles Darwin

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This PhD. dissertation represents a major achievement of my life and an open door to the scientific community. At the end of this hard but proficient chapter of my life I realize how important it is for a scientist to enjoy the freedom of creativity in a democratic society. My thoughts of profound recognition and appreciation are going to my mother who brought me to this "land of opportunity", to Mrs. Mary Foster who introduced me to Dr. Koestner, who kindly offered me the opportunity of starting and finishing my graduate studies. For 6 years Dr. Adalbert Koestner was my mentor and academic advisor. With kindness and patience he guided my scientific career up to this point. I greatly appreciate and thank him for the great opportunity he offered me and all the help in finishing my program. I wish also to express my appreciation to the following people: Dr. Clifford Welsch, member of my graduate committee, for his professional scientific advice and critiques in reviewing my manuscripts, Dr. Stuart Sleight and Keiji Marushige for their encouragement and guidance.

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TABLE OF CONTENTS

TABLE OF CONTENTS--continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES--continued

LIST OF FIGURES--continued

LIST OF FIGURES--continued

LIST OF FIGURES--continued

LIST OF FIGURES--continued

CHAPTER 1

A Review of N-Ethyl-N-Nitrosourea: Mechanism of Action as Chemical Carcinogen

The specific aim of these studies was to determine the *in vivo* N-ethyl-N-nitrosourea (ENU)-induced spectrum of neoplasia and *in vitro* ENU-induced rat mammary epithelial cell neoplastic transformation.

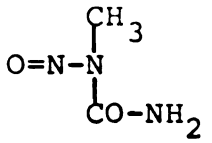
ENU has been extensively used as a transplacental neurocarcinogen capable of inducing neurogenic tumors in nearly 100% of rats exposed prenatally under optimal conditions. Susceptibility increases with advancing gestation culminating at parturition time and declining postnatally. After 30 days of age it becomes difficult, if not impossible, to induce neurogenic tumors in rats with a single dose of ENU.[1-4] Although neuroectodermal cells and epithelial cells of the mammary gland are the primary target cells for the oncogenic effects of ENU, high doses of ENU administered to young rats induced a wide spectrum of neoplasms.[5-8] This indicates that ENU is capable of transforming a variety of cell types of different organs. In addition to the high doses, the age of the rats may play an important role in explaining these results. The ENU-induced spectrum of neoplasms in multiple histogenetically unrelated tissues in the experiment could be related to the rate of DNA synthesis and cellular replication which is essential for the process of neoplastic transformation.

For a better understanding of ENU mechanism of action as chemical carcinogen a short review of the literature should be beneficial.

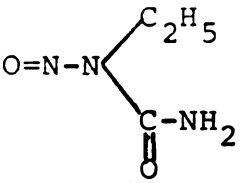
Chemical Carcinogen

Two acyl-alkyl nitrosamides, methyl- and ethylnitrosourea (MNU and ENU) have been shown to be potent resorptive carcino-

gen.[1,4,10]



Methylnitrosourea



Ethylnitrosourea

Nitrosamines and nitrosamides can be distinguished according to the type of substitution of alkyl and acyl residues for R_1 and R_2. Acylalkylnitrosamides are significantly less stable chemically than alkylnitrosamines. Decomposition of ENU and MNU occurs spontaneously at slightly alkaline pH and does not depend on enzymatic activation.[9] Subcutaneous injection produces mostly local sarcomas, while systemic application leads to tumor induction, determined by the chemical structure of the compound.[1,9]

Historical Perspectives. Until the late 1960's it was generally believed that alkylation of the N-7 of G (guanine) of nucleic acids was the biologically important event. The foundation for this assumption was based on studies with such alkylating agents as sulfur mustard, dimethyl sulfate and methyl methansulfonate. These classes of directly acting agents that react primarily at the N-7 of G are, however, not particularly carcinogenic.[11] The particular and unusual reactivity of N-nitroso alkyl compounds was first elucidated in 1968 when Singer et al[12] working with tobacco mosaic virus and its RNA demonstrated that N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), recognized at that time as a powerful mutagen and carcinogen, was demonstrated to cause methylation of DNA, but

to have markedly different site affinities and conformation dependence than the classical methylating agents.[13] The interest in N-nitroso compounds was further stimulated when in 1970 Lawley and Thatcher[14] found O^6-MeG, first detected by Loveless,[15] to be present in MNNG-treated nucleic acids but not in dimethyl sulfate-treated nucleic acids. The difference between MNNG and dimethyl sulfate was also evident in their biologic effects, the latter being a very weak carcinogen.[11]

Further works by Goth and Rajewsky[16] and Lawley and Warren[17] established that a carcinogenic N-nitroso ethylating agent, ENU, reacted in vivo to a relatively greater extent at the O^6 of G than did MNU, as reported by Kleihues and colleagues.[18,19]

An attractive hypothesis was then proposed which said that the decisive factor in carcinogenesis was not the absolute extent of modification of DNA but rather the relative amount of O^6-alkyl G formed. This hypothesis was supported by Gerchman and Ludlum's that this base could cause mispairing and thus be a possible candidate for inducing genetic changes.[20-22] Accumulation and persistence of O^6-MeG or EtG in some target tissues has been correlated with carcinogenicity[23,24] but this association has not been consistently confirmed.[25,26]

Chemical Reactions of Ethylnitrosourea and Other N-nitroso Compounds. Figure 1-1 summarizes the action of direct acting alkylating agents on RNA and DNA.

Simple Alkylating Agents Acting on Nucleic Acids

1) Reactivity:
   methyl > ethyl > higher homologues

2) Preferential Reactivity:
   alkyl sulfate → N
   N-nitroso ⟶ O
   alkylalkane sulfonate ⟶ N > O

3) Possible Sites of Reactions:
   All rings, nitrogens and oxygens
   (except sugar attachment sites)
   phosphodiester
   2'-O of ribose

Figure 1-1. General conclusions on the reactivity of direct acting alkylating agents toward both RNA and DNA. N indicates nitrogens and O represents oxygens.[20]

Studies of ethylation of nucleic acids with ENU revealed that approximately 80% of the total ethyl groups were on oxygens.[27,28] The predominant products were ethylphosphotriesters (50-60%). All other oxygens were also modified. In addition to O^6-EtG, the products included O^2-EtC, O^2-EtT (or U), and O^4-EtT (or U). In the case of of RNA, the 2'-O or ribose was also ethylated.[29,30]

In their now classic experiments, Goth and Rajewsky administered (^{14}C-ethyl) ENU to 10-day-old BD-IX rats under conditions that ultimately led to virtually all the animals developing tumors of the brain but no other tissue.[16] They then isolated DNA from the brain and other tissues at various times after the single treatment with the carcinogen. They reported that O^6-EtG was not removed rapidly from the DNA of the brain (target organ) whereas it was greatly reduced in other organs such as the liver. From this observation they evolved the concept that it was not the extent of alkylation

of the O^6 of G that was specifically important in carcinogenesis, but rather the fact that O^6-EtG was not removed enzymatically from a given cell population.[21]

Mutagenic Reactions of Alkylating Agents.[11] Mutagenesis is the result of a change in transcription of a nucleic acid that can be a naturally occurring event and thus has probably contributed to biologic evolution. Mutation can occur without chemical modifiation when the polymerase that directs transcription has, for any reason, a high error rate, and the resulting mistakes are not corrected by a proofreading enzyme. Inasmuch as genetic change normally occurs at a very slow rate, it may be assumed that transcription is generally faithful.

When a chemical modification is introduced, several types of effects are possible. 1) Transcription may be unaffected, as seems to be the case when N-7 alkyl G or N^6-alkyl A is present.[31,32] 2) Transcription may be stopped by the modified base not being recognized at all, which is likely to result when substituents are introduced on sites involved in base pairing or when the substituent is bulky and steric factors play a role.[33] 3) Modifications can be mutagenic if the tautomeric equilibrium is changed by the modification and the modified base thus has the base-pairing properties of another base.[34,35] 4) A potential mutagenic modification can also be either lethal or have no effect, depending on whether the modification occurs at a site essential or non-essential for protein or nucleic acid structure or function.

The major derivative formed upon ENU reaction with nucleic

acids is ethylphosphotriesters. No evidence has thus far been obtained which suggests that this is a mutagenic action.[11]

All the in vitro experiments on mutations resulting from alkylation of nucleosides can be summarized as follows: No predictions of base pairing can be made on the basis of chemical structure. The observed mispairing is not specific, but neither is it totally nonspecific. The derivatives that have been shown to mispair are substituted on the N-3 of C and U, the O^2 and O^4 of U (or T), the O^2 of C, and the O^6 of G.[11,21]

Repair of Alkyl Derivatives: Kinetics, Possible Mechanisms and Biologic Consequences. Before postulating repair and its mechanisms, one must know and consider the chemical stability of the alkyl products in DNA under physiologic conditions. It has long been known that 3- and 7- alkyl purine nucleosides are easily depurinated from DNA due to the great lability of their glycosyl bonds.

Figure 1-2 summarizes some of the data and conclusions regarding the stability of alkyl derivatives of DNA.

Cells differ in their ability to remove modified nucleosides,[36] and there have been attempts to correlate excision ability with enzyme activities. It has been postulated that enzymes exist that recognize conformational changes resulting from modifications of nucleic acids.[11] Another enzyme activity has been found in cell extracts that seems to excise O^6-alkyl G,[37,38] but not 7-alkyl G, and the lack of enzymatic excision of O^6-alkyl G has been correlated with the tumorigenic potential of mammalian cells.[39] With regard to bacteria, E. coli cells usually have limited capacity for removing O^6-

Stability of Alkyl Derivatives in DNA

1. In Vitro pH 7, 37C
   - a) O^6 alkyl G, O^2 alkyl T, O^2 alkyl C, O^4 alkyl T — no dealkylation; glycosyl bond breakage detected in 10 days
   - b) N-3 alkyl A — glycosyl bond cleavage $t_{1/2}$=26 hours
   - c) N-7 alkyl G — glycosyl bond cleavage $t_{1/2}$ = 155 hours
   - d) Stability of glycosyl bond much greater in polynucleotides than in mononucleotides ($t_{1/2}$=N-7 alkyl G in monomer = 6 hours).

2. In Vivo pH 7, 37C
   - a) All alkyl bases excised (dealkylated); phosphotriesters stable.
   - b) Loss of alkyl derivatives occurs most rapidly in first day; it varies greatly, depends on period observed.
   - c) Complete excision (or dealkylation) not observed for O-alkyl derivatives after 5 days.
   - d) There is a strong possibility that multiple enzymes are responsible for loss of alkyl bases.

Figure 1-2. General conclusions regarding the stability of alkyl derivatives in DNA, under physiologic conditions. Alkyl refers to methyl or ethyl substituents. $t_{1/2}$ = half-life.

methylguanine from DNA, but an inducible repair function is expressed after the exposure of cells to low concentrations of alkylating agents. This repair pathway, termed the adaptive response, allows rapid and error-free repair of O^6-methyl-guanine residues. Olsson and Lindahl[40] has established that the methyl group is transferred to a cysteine residue on the enzyme protein and that guanine is thus generated in the DNA substrate. Rapid assay of the O^6-methylguanine DNA transmethylase using antibodies specific for O^6-methylguanine to precipitate this base was described.[41] The mammalian O^6-methylguanine-DNA transmethylase was also able to remove (at a slower rate) ethyl groups from O^6-ethylguanine in DNA forming S-ethyl-cysteine, but did not attack any of the other methylated bases present in DNA methylated with MNU.[41] The mammalian O^6-methylguanine methyltransferase no longer seems active in some human tumor cell lines, in particular in lines from cells transformed with DNA tumor viruses, whereas normal diploid fibroblasts and other control cells express this repair function.[42]

The N-nitroso alkylating agents, particularly ENU, have an affinity for modifying all oxygens in nucleic acids. It may well be that the formation and fate of O^6-alkyl G and other O-alkyl derivatives formed by the potent carcinogenic N-nitroso compounds are fortuitously good indicators of additional reactions contributing to cancer.

Activation of Oncogenes by N-Nitroso Compounds. Detection and isolation of transforming genes (oncogenes) from human tumors have provided the means to investigate, at the mole-

cular level, events involved in the development of human neoplasia. A broad spectrum of human cancer contain such transforming genes, a group of at least three structurally different genes, H-ras, K-ras, and N-ras, which code for highly related proteins designated p 21. Sukumar et al[22] demonstrated that a single point mutation was responsible for the malignant activation of the H-ras-1 locus in MNU-induced mammary carcinomas in Buf/N female rats.

Molecular characterization of one of the genes revealed that the twelfth codon was GAA instead of GGA of the normal allele, encoding glutamic acid instead of glycine.[22] These results indicate that chemical carcinogenesis represents an adequate model to study the role of transforming ras genes in human neoplasia.

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CHAPTER 2

Diverse spectrum of tumors in male Sprague-Dawley rats following single high doses of N-ethyl-N-nitrosourea

SUMMARY

In this study, 30-day-old male Sprague-Dawley rats, were inoculated intraperitoneally with a single dose of 45, 90 and 180 mg/kg of N-ethyl-N-nitrosourea (ENU). A wide spectrum of neoplasms occurred. The most common tumors were those of the mammary gland and of the nervous system. Although the incidence of mammary tumors was highest in the two high-dose groups (90 and 180 mg/kg ENU), the incidence of neurogenic tumors was highest in the 45 mg/kg dose group. Mammary tumor development led to early death and precluded development of tumors of the nervous system, which require a longer latency period. A variety of neoplasms of other organs have been associated particularly with high doses of ENU, including ameloblastic tumors, carcinomas of the thyroid, prostate, kidney, pancreas, intestine and lung, hemilymphatic tumors and sarcomas. It is concluded that large doses of ENU are capable of expanding the tumor spectrum of young male rats beyond the target organs generally affected with lower doses, as described in earlier reports.

INTRODUCTION

N-ethyl-N-nitrosourea (ENU) has extensively used as a transplacental neurocarcinogen capable of inducing neurogenic tumors in nearly 100% of rats exposed prenatally under optimal conditions. Susceptibility increases with advancing gestation culminating at parturition time and declining postnatally. After 30 days of age it becomes difficult, if not impossible, to induce neurogenic tumors in rats with a single

dose of ENU.[1-4]

In a recent study we used 30-day-old female Sprague-Dawley (CD) rats in an attempt to correlate molecular damage with tumor incidence in selected organs following single inoculation of high doses of ENU.[5] A surprisingly high incidence of mammary tumors (MTs) (up to 100% in the 90 and 180 mg/kg dose groups) resulted in early death and precluded the development of tumors of the nervous system (a prime target organ), which require a longer latency period than MTs.[6]

These findings prompted us to repeat the experiment using 30-day-old male rats. In this report we describe the spectrum of neoplasms induced with high single doses of ENU in susceptible young male CD rats.

MATERIALS AND METHODS

Carcinogen

ENU crystals[a] were freshly dissolved in phosphate/citrate -buffered saline at pH 4.2 (1 part buffer to 14 parts saline) before administration. The crystalline carcinogen was stored in a dessicator at -20 C.

Animals

Four groups of 30 rats (three experimental groups and one control group) were used (Table 2-1). All rats were 30-day -old male specific-pathogen-free (SPF) cesarean-delivered (CD) rats.[b] They were housed in plastic cages (two rats per cage)

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a Synthesized by Dr. Ming Chang, Veterinary Pathobiology Department, Ohio State University, Columbus, Ohio.
b Charles River Laboratory, Portage, Michigan.

in temperature-controlled rooms (24 C) provided with 10 hours electric light per day. They were fed autoclaved Purina Lab Chow 5010 C and water ad libitum. Rats in the three experimental groups were inoculated intraperitoneally with freshly prepared ENU solutions in single high doses of 45, 90 and 180 mg/kg, respectively. The control group was inoculated with an equal volume of phosphate/citrate-buffered saline solution at pH 4.2. Rats were observed daily and weighed weekly after treatment. Most rats died or were sacrificed because of tumors of the nervous system, hemilymphatic system, or mammary glands or because of cachexia. A complete necropsy was performed shortly after spontaneous death or euthanasia of moribund animals. The histologic sampling procedures were equal among all groups. Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4-6 u and stained with hematoxylin and eosin (H&E). Special stains, such as Masson's trichrome, periodic acid-Schiff (PAS), and Movat were used when indicated. Tumors of all organs were studied. Mammary neoplasms were classified according to the suggested classification of mammary tumors in rats by the World Health Organization.[7,8] Morphologic criteria for malignancy were pleomorphism, layering of secretory epithelium, infiltration of surrounding tissue, high mitotic activity, anaplasia and metastases.

Tissues for electron microscopy were fixed in 4% glutaraldehyde, postfixed in osmium tetroxide, and, after processing, embedded in Epon, cut with an ultramicrotome (1 u sections), and stained with 1% toluidine blue. Blocks were selected for electron microscopic examination, and ultrathin sections were

contrasted with uranyl acetate and lead citrate. Survival times were analyzed by one-way analysis of variance. Significant differences between means were determined by the LSD ($P = 0.05$).

RESULTS

Incidence and Survival Time

The incidence of tumors in all ENU treatment groups (80 -86%) was substantially higher than in the control group (7%) (Table 2-1). There was, however, no difference in either the tumor incidence or the number of tumors per rat among the three ENU dose groups. A significant difference existed in survival time between the control group and treated groups and also among the treated groups. The mean survival time decreased with increasing doses of ENU (Table 2-1). Another difference among the three dose groups was the incidence of tumors of the mammary gland and nervous system (Table 2-2). The rats affected with MTs per dose group were 7 (23%) in the 45 mg/kg group, 14 (46%) in the 90 mg/kg group, and 12 (40%) in the 180 mg/kg group. While 19% of the total number of MTs occurred in the 45 mg/kg group, 40% occurred in the 90 mg/kg group and 41% occurred in the 180 mg/kg group. The two highest ENU doses resulted not only in a higher incidence of mammary neoplasias but also in an increased incidence of carcinomatous and sarcomatous types of MTs. There was a direct correlation between dose of ENU and the percentage of malignant MTs (10%, 21% and 27% malginancy per dose group). The overall mean MT induction time was 140 days, with a mean of 105 days in the highest ENU dose group (180 mg/kg). The mean survival time of the rats bearing malignant MTs was 280 days ($\pm$5) days. The opposite was true for neurogenic tumors; 46% of the total number of neurogenic tumors developed in the low-dose group (45 mg/kg), 26% developed in the 90 mg/kg group, and 28% developed in the

Table 2-1. Number of tumors and survival time in male Sprague-Dawley rats treated with ENU intraperitoneally at 30 days of age.

Dose of ENU	No. of rats with tumors (30 rats per group; percentage in parentheses)	Mean number of tumors per rat	Mean survival time (days) (mean + SEM)
0	2(7)	0.1	491 + 4
45 mg/kg	24(80)	1.7	425 + 16
90 mg/kg	26(86)	1.2	367 + 19
180 mg/kg	24(80)	1.6	294 + 14

180 mg/kg group. The number of rats per group affected by neurogenic tumors was 20 (41%) in the 45 mg/kg group, 14 (28%) in the 90 mg/kg group, and 15 (31%) in the 180 mg/kg group. The mean survival time of rats bearing neurogenic tumors per dose group was 447 days for 45 mg/kg group, 386 days for 90 mg/kg group, and 350 days for 180 mg/kg group.

Histologic Classification

The histologic classification and the number of various types of neoplasms are listed in Table 2-2.

Neurogenic Tumors. Tumors of the nervous system are similar to those described in detail in previous publications.[1-4] In addition to the neurogenic tumors mentioned (Table 2-2), 10% and 15% of the rats from the high-dose groups developed alterative changes such as focal malacia and gliosis.

Mammary Tumors. Benign MTs (42% of all MTs) were either fibromas or fibroadenomas consisting of fibrotic encapsulated masses with various degrees of intercalated glandular structures (fibroadenomas). Malignant MTs were either fibrosarcomas, adenocarcinomas, or carcinosarcomas. Fibrosarcomas consisted of anaplastic fusiform fibroblastic cells with whorl formation and a high rate of mitosis (5-6 per high power field). One fibrosarcoma had metastasized to the axillary lymph nodes, mediastinum and lung. Adenocarcinomas (Figure 2-1) consisted of irregular ductular and acinar structures intercalated with a dysplastic stroma often heavily infiltrated with mast cells. The multilayered cells exhibited an increased nuclear-cytoplasmic ratio, pleomorphism and a high rate of mitosis. Marked necrosis and cystic ductular structures filled with eosinophil-

Table 2-2. Incidence and classification of tumors induced with ENU in male Sprague-Dawley rats inoculated at 30 days of age.

	Number of tumors per dose/group (30 rats per group)			
Neoplasias	0	45 mg/kg	90 mg/kg	180 mg/kg
Central Nervous System	-	18	12	11
Oligodendrogliomas	-	12	8	9
Astrocytomas	-	2	-	2
Mixed gliomas	-	2	4	-
Ependimomas	-	2	-	-
Peripheral Nervous System	-	7	2	4
Anaplastic neurinomas	-	7	2	4
Mammary gland tumors	-	10	21	21
Fibromas	-	5	7	7
Fibroadenomas	-	-	3	2
Fibrosarcomas	-	1	-	2
Adenocarcinomas	-	4	9	11
Carcinosarcomas	-	-	2	-
Thyroid gland	2	8	2	2
Follicular carcinomas	-	2	1	-
Follicular adenomas	-	3	1	-
C-cell adenomas	2	3	-	2
Pituitary gland	-	1	1	-
Chromophobe adenoma	-	1	1	-
Bone	-	-	-	1
Osteosarcoma	-	-	-	1
Odontogenic tumor	-	-	-	2
Ameloblastic Odontoma	-	-	-	2
Pancreas	-	2	-	2
Islet cell carcinoma	-	1	-	2
Exocrine adenocarcinoma	-	1	-	-
Prostate gland	-	-	1	2
Adenocarcinoma	-	-	1	2
Skin	-	2	-	1
Hemangiopericytoma	-	2	-	1
Liver	-	1	1	-
Hepatocellular				

Table 2-2 (cont'd)

Neoplasias	Number of tumors per dose/group (30 rats per group)			
	0	45 mg/kg	90 mg/kg	180 mg/kg
carcinomas	-	1	1	-
Hemilymphatic System	-	2	3	4
Lymphosarcoma	-	2	1	2
Myeloid leukemia	-	-	2	2
Spleen	-	-	-	1
Hemangiosarcoma	-	-	-	1
Large Intestine	-	1	-	-
Carcinoma	-	1	-	-
Kidney	-	1	-	2
Renal carcinoma	-	1	-	2
Adrenal gland	-	1	-	-
Pheochromocytoma	-	1	-	-
Lung	-	-	2	1
Adenomas	-	-	1	-
Squamous cell carcinomas	-	-	1	-
Peritoneum	-	-	-	1
Mesothelioma	-	-	-	1
Totals	2	54	46	53

Figure 2-1. Mammary tumor adenocarcinoma showing ducts with multilayered neoplastic epithelial cells (H&E, x 120)

Figure 2-2. Mammary tumor adenocarcinoma metastases to the lung exhibiting vascular neoplastic emboli (arrows). (H&E, x 120)

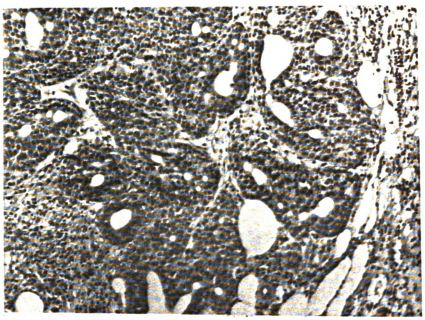

Figure 2-1

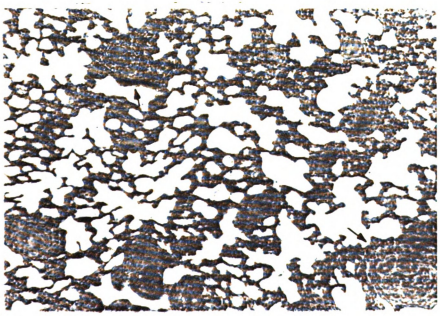

Figure 2-2

ic material were often associated with this type of tumor. Two of the rats with adenocarcinomas (one each in the 45 and 180 mg/kg ENU dose groups) had lung metastases characterized by multiple epithelial emboli and tumoral masses invading the lung parenchyma (Figure 2-2). The carcinosarcomas were characterized by malignancy of both epithelial and mesenchymal components. In addition, 8% of the rats from the treated groups free of mammary neoplasms developed mammary gland hyperplasia.

Electron micrographs of carcinoma cells exhibited the characteristic pattern of mammary epithelial cells. The cells consisted of a central nucleus with a large nucleolus. The free margin had numerous microvilli and desmosomal junctions. Free ribosomes and tubules of rough-surfaced endoplasmic reticulum (RER) were widely distributed throughout all parts of the cell. Some of the tubules formed cisternae. Numerous electron-dense secretory granules and a moderate number of lipid bodies occurred intracytoplasmically and within intercellular spaces.

Ameloblastic Odontomas. These tumors were detected in two rats (180 mg/kg ENU dose) with a survival time of 280 days. The tumors contained many developing teeth of various sizes and shapes resembling normal or atypical tooth germs, with enamel and cementum in varying quantities and, in addition, epithelial and mesenchymal odontogenic tissues with scattered island of bone and osteoid (Figure 2-3).

Hepatocellular Carcinomas. These tumors were encountered in 2 rats (45 and 90 mg/kg ENU dose groups) with a survival

Figure 2-3. Ameloblastic odontoma showing irregular structures consisting of mesenchymal odontogenic tissue (O), dentin (D) and cementum (C). (H&E x 120)

Figure 2-4. Hepatocellular carcinoma, trabecular pattern. (H&E, x 48)

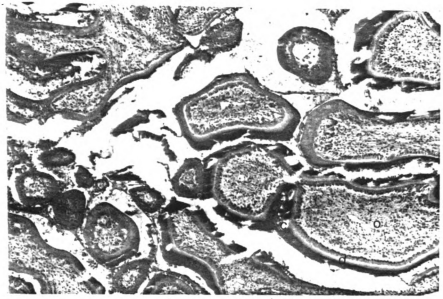

Figure 2-3

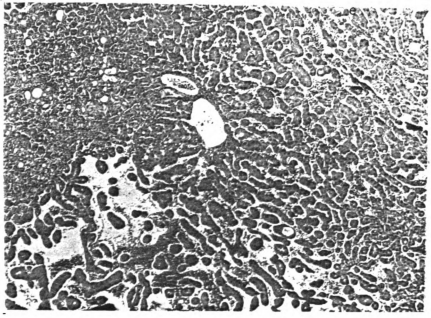

Figure 2-4

time of 415 and 320 days, respectively. The tumors had a trabecular pattern with moderate necrosis, telengiectasia and fibrosis (Figure 2-4). In addition, multiple nodules and vascular emboli consisting of well-differentiated hepatoblasts were detected in the lung. In other liver-tumor-free rats, hepatic changes consisting of focal cellular alterations, nodular hyperplasia, spongiosis hepatis, focal necrosis, biliary hyperplasia and cholangiofibrosis were observed in 20%, 23% and 30% of the treated group rats, compared with foci of cellular alteration in only 5% of the control group rats.

Thyroid Tumors. These tumors occurred in all dose groups and controls in equal number except for the 45 mg/kg group, where four times the number of the other groups occurred. The mean survival time of rats with thyroid tumors was 450 (±15) days. Only 3 of the 14 tumors (all in experimental groups) had characteristics of malignancy. They were diagnosed as follicular carcinomas (2 in the 45 mg/kg and 1 in the 90 mg/kg ENU dose groups) and were characterized by a papillary pattern with invasion of the capsule and adjacent tissue. No metastases to other organs was detected.

Tumors of the Pancreas. These were detected in 4 ENU-inoculated rats. The mean survival time of rats bearing these tumors was 320 (±10) days. Three were islet cell tumors recognized macroscopically as solitary nodules 0.5-1 cm in diameter. The tumors (one in the 45 mg/kg group and two in the 180 mg/kg group) were islet cell carcinomas characterized by the lack of a capsule, a well-defined neuroendocrine pattern, cellular anaplasia, and local invasion. Invasion of the sur-

Figure 2-5. Pancreas. Islet cell carcinoma. Aggregates of exocrine acini incorporated into the neoplasia are clearly apparent. (H&E, x 120)

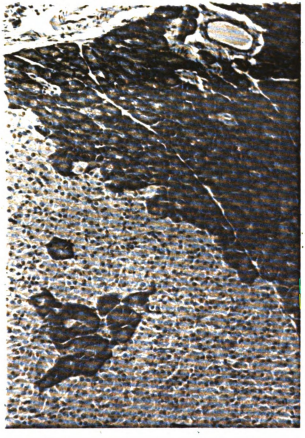

Figure 2-5

rounding exocrine pancreas, with aggregates of exocrine acini incorporated into the neoplasia, was clearly apparent (Figure 2-5). Hyperplasia of the islet cell of the pancreas was recognized in 10% of the additional rats from the high-dose groups. One tumor was an adenocarcinoma of the exocrine pancreas, with a high degree of anaplasia and many mitoses. The exocrine pancreatic adenocarcinoma had metastasized to the mesenteric lymph nodes and liver.

Carcinoma of the Large Intestine. This type of tumor was seen in one rat, with metastases to the adjacent mesenteric lymph node. The animal was terminated at 510 days of age. An osteosarcoma arising from sacral vertebrae was diagnosed in 1 rat at the age of 330 days. This tumor metastasized to the lung, right atrium and adrenal gland.

Adenocarcinomas of the Prostate Gland. These tumors were detected in 3 rats. The mean survival time of rats bearing this type of tumor was 260 ($\pm$2) days. These tumors were anaplastic and had metastasized to lymph nodes, liver and lung and invaded adjacent tissue. Mucosal papillary hyperplasia of the urinary bladder and prostate gland hyperplasia with squamous metaplasia were observed in 10% of the additional rats from the 2 high-dose groups. None of those changes were observed in the control group.

Hemangiosarcoma of the spleen. This type of tumor was detected in one rat with a survival time of 320 days which had metastasized to the liver and kidney.

Hemangiopericytoma. 3 ENU-exposed rats developed this type of tumor in the subcutis of the submaxillary region with

a mean survival time of 450 (±2) days.

Mesothelioma. This tumor was detected in 1 rat with a survival time of 406 days. The tumor had spread throughout the serosal surface of the abdominal cavity.

Renal Carcinomas. These tumors developed in 3 exposed rats, with a survival time of 260 (±2) days. One of the tumors (45 mg/kg group) was a solid carcinoma developing in the cortex and invading the adjacent renal parenchyma. The other two tumors (180 mg/kg group) were solid clear-cell carcinomas occupying large areas of the corticomedulla at one pole of the kidney. They consisted of large polyhedral cells with small hyperchromatic nuclei and clear cytoplasm. Foci of small aggregates of polyhedral cells with eosinophilic granular cytoplasm were interspersed throughout. Local invasion of the surrounding renal tissue was apparent.

Primary Tumors of the Lung. These were seen in three instances: two were adenomas and one was a squamous cell carcinoma characterized by the presence of numerous keratin pearls, invasion, necrosis and inflammation. The survival time for the rat bearing a squamous cell carcinoma was 253 days.

Neoplasms of the Hemilymphatic System. These were seen in 9 rats: five lymphosarcomas and four myeloid leukemias. All nine hemilymphatic neoplasms occurred in ENU-exposed rats and affected liver, spleen, kidneys and lymph nodes. All the hemilymphatic neoplasms occurred in rats under 1 year of age (the mean survival time was 245 ± 5 days).

DISCUSSION

The results indicate that single postnatal ENU exposure of 30-day-old male rats has the potential of inducing a wide spectrum of neoplasms in multiple, histogenetically unrelated tissues.

The most striking and unusual finding was the high incidence of MTs in male rats, not previously described as a consequence of transplacental or postnatal ENU exposure. In a previous experiment using female rats, 30% of the ENU-induced MTs were ovarian-hormone-independent.[5] Although the hormone responsiveness of the ENU-induced MTs in the male rats of the present study is not known, it is reasonable to assume that they are largely ovarian-hormone-independent, because they were transformed in the absence of substantial quantities of these hormones. In vitro experiments provided evidence that N-methyl-N-nitrosourea (MNU) induced rat neoplastic mammary epithelial cells were able to grow in culture medium free of ovarian hormone.[9]

The inverse correlation between the doses of ENU and neurogenic tumor incidence can be explained by the shorter survival time in the high-dose groups precluding development of neurogenic tumors, which have a longer latency period than MTs. In the high-dose-treated groups we saw a high incidence of alterative changes of central nervous system, such as glial nodules associated with focal malacia, which can be considered early neoplastic proliferation. Fourteen rats from the 90 and 180 mg/kg dose groups in our experiments were terminated be-

cause of malignant MTs at 9 months of age (280 days), which is lower than the mean survival time of rats bearing neurogenic tumors in the high-dose groups (386 and 350 days, respectively). Early neoplastic proliferations of the central nervous system were recognized in 8 of these rats. Given a longer survival time, it is probable that true tumors could have developed from these changes.

Of special interest are the ameloblastic odontomas, a tumor type not previously reported in CD rats associated with ENU exposure. This tumor occurs in man and has been reported with relatively high frequency in some areas of Africa. Malignant behavior has been recognized, and lung metastases have been described occasionally.[10] In dogs, this tumor has been encountered with low frequency, behaving as a slow-growing benign neoplasm.[11] Ameloblastic odontomas apparently arise from the primitive odontogenic epithelium, resembing the dental lamina.

Tumors of the liver have not been reported in experiments using ENU as a carcinogen. The liver has been generally considered to be a non-target organ in ENU carcinogenesis. However, we observed hepatocellular carcinomas in 2 ENU-exposed rats. The spontaneous incidence of liver tumors in male CD rats was reported to be 1% at 24 months of age,[14] a survival time which is considerably higher than that of rats from the ENU-treated groups.

Some of the tumors listed in Table 2-2 are reported to occur spontaneously in the rat.[12-14] Tumors of the exocrine pancreas, bone and odontogenic apparatus were not encountered

spontaneously in historical controls of CD male rats at 24 months of age.[13,14] Only 2 adenomas of the thyroid gland occurred in the control group. The reason no other tumors were noticed is the age of the animals (491 days) when the experiment was terminated. Spontaneous tumors in rats usually occur later in life, peaking at 2 years and beyond.[12, 13,14]

The mean survival time for the ENU-exposed rats was approximately 1 year. Most of the malignant tumors occurred in the high-dose groups, in which none of the rats survived the first year of life. It is a fair assumption that these tumors are treatment-related. The number of animals used in the experiment and the low incidence of most tumors preclude statistical analysis of these neoplasms other than mammary and neurogenic tumors, where the high incidence is convincing.

It is expected that a systemically acting carcinogenic agent might increase the incidence of naturally occurring tumors and shift their appearance to a younger age. Also, exposure to a carcinogen may render spontaneously occurring benign tumors more malignant and anaplastic. Neoplasias affected in that manner were considered treatment-related. In support of the assumption that malignancies are treatment-related is the presence of proliferative changes (pre-neoplastic alterations) in organs and tissues of treated animals without tumors. Such proliferative changes occurred in the brain, liver, pancreas, mammary gland, and urogenital system. None of these changes occurred in the control group.

Although neuroectodermal cells and epithelial cells of the mammary gland are the primary target cells for the oncogenic effect of ENU, high doses of ENU administered to young rats induce a wide spectrum of neoplasms. This indicates that ENU is capable of transforming a variety of cell types of different organs. In addition to the high doses, the age of the rats may play an important role in explaining these results. During the first weeks of life, the cells of many organs have a high turnover rate, including those of the male mammary gland.

The ENU-induced spectrum of neoplasms in multiple histogenetically unrelated tissues in our experiment could be related to the rate of DNA synthesis and cellular replication, which greatly augment the process of neoplastic transformation.[15,16,17]

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CHAPTER 3

Testicular-like tumor in the ovary of rats induced by chemical carcinogens

SUMMARY

Ovarian tumors which simulated testicular-like structures were detected in 18% of the Sprague-Dawley (CD) and 47% of BD-IV (Berlin Druckrey IV) rats inoculated with high doses of N-ethyl-N-nitrosourea (ENU) transplacentally and postnatally as compared to 2% (CD) and 3% (BD-IV) in controls. Diethylnitrosamine (DEN) administered intravenously postnatally did not produce tumors different from control levels. The carcinogens increased the incidence of ovarian tumors (OT) with a testis-like tubular pattern. Some of these OT (6%) produced metastases throughout the mesentery by implantation. Malignant mammary tumors (MT) associated with OT were recognized in 54% of exposed rats. Hormonal assays indicated that some of the tumors produced testosterone, estradiol or estrone.

INTRODUCTION

Since Pick (1905)[23] first described a tumor with testicular-like structure in the human female and called it "adenoma testiculare ovarii", the origin of and appropriate nomenclature for this type of tumor has been a controversial issue. Investigators have described this tumor in the literature under different names such as: arrhenoblastoma,[1,13,15,17,19,20,22] androblastoma,[10,21,32,33,34,35] Sertoli-like tumor,[29,33,35] granulosa cell tumor or "folliculoma lipidique",[3] and sex cord-stromal tumor.[11,12,27,28] These tumors characteristically develop in young women during the third decade of life, or younger.[1] Pathologists have distinguished three histological patterns of these tumors: differentiated tubular type,

undifferentiated pattern and intermediate pattern.[1,38] Javert and Finn (1951) estimated that 22% of these neoplasms are malignant. Generally, tumors of undifferentiated pattern, rather than those of predominantly tubular type, metastasize. Metastases appear late during the course of the disease and may be found in the liver, kidney, lung and paraaortic nodes.[14]

The histogenesis of these tumors remains debatable. Curtis[4] suggested that this tumor represents a one-sided development of teratoma. The term arrhenoblastoma was first proposed by Meyer[19] and was used by him in a morphological sense. Meyer suggested that arrhenoblastomas arise from certain indifferent, ambivalent, but male directed cells which he presumed had persisted in the rete ovarii since the intermediate phase of gonadogenesis and which were capable of proliferating to produce tubules.[19] Subsequently, the term arrhenoblastoma was adopted for a group of virilizing ovarian neoplasms. The manifestations of masculinization vary in degree and not all arrhenoblastomas are recognizably virilizing tumors. Indeed, the androgenic activity of an arrhenoblastoma is not always related to its content of Leydig-like (interstitial) cells. In certain cases of tubular testicular adenoma, tubular androblastoma, or Sertoli testicular cell tumor, the hormonal effects may be either estrogenic or androgenic.[1]

Mackinley (1957) suggested that arrhenoblastomas and their mixed variants are granulosa-cell tumors in varying degrees of differentiation.[1] Teilum[32,35] termed the tumors homologous androblastomas based upon the morphological identity of certain gonadal tumors in the male and female which led to

the concept of using the tumor classification for identical testicular and ovarian tumors. The term "androblastoma" (from "andros" meaning man) is a designation for neoplasms derived from the mesenchymal core of both testis and ovary and, in either sex, showing a tendency to reflect the specific testicular architecture irrespective of the qualitative hormone-producing properties.[33]

Similar testicular-like tumors have been described in animal ovaries, in mice,[25,26] rats,[7,12] agoutis,[36] shrews,[39] mares,[15,20] Indian Deshi hens,[9] and cats.[12] Tubular adenomas of the ovary were observed spontaneously in old Wistar rats usually 900 to 1,000 days of age.[17] In mice, mares, and Indian Deshi hens, the tumors were associated with virilizing changes.[9,15,25] In rats and agoutis the tumors were not associated with hormone production.[7,16]

In our experiments, the rat strains Berlin Druckrey (BD-IV) and Sprague-Dawley (CD) were chosen for chronic carcinogenic experiments using N-ethyl-N-nitrosourea (ENU) and diethylnitrosamine (DEN) as chemical carcinogens to study the incidence and pathology of tumors of the nervous system, mammary gland, liver and kidney, well known target organs for these chemicals.[6,18,30,31] Besides the tumors of known target organs (described previously), 21% of the experimental animals developed an unusual type of ovarian tumor with testicular-like structures (arrhenoblastomas). Although the ovary was not previously known to be a target organ for ENU or for DEN, our experiments proved that ENU especially was able to produce a relatively high yield of ovarian tumors with testi-

cular-like structure. The purpose of the present study was to define the frequency of ENU and DEN-induced ovarian neoplasias in different rat strains, to characterize the morphology, metastasis behavior and hormonal characteristics of these relatively rare and controversial testicular-like tumors, and to investigate the possible relationship between these neoplasms and other types of tumors, especially mammary tumors.

MATERIALS AND METHODS

Carcinogen

ENU crystals[a] were dissolved shortly before administration in phosphate/citrate-buffered saline at pH 4.2 (1 part buffer to 14 parts saline). The crystalline carcinogen was stored in a dessicator at -20 C.

DEN[b] was freshly prepared in saline solution at pH 6.6.

Animals

All animals used (240 in experimental groups and 120 in control groups) were female specific-pathogen-free (SPF) Sprague-Dawley (CD)[c] and BD-IV[d] rats (Table 3-1). Eight experimental groups of 30 rats each were utilized: three groups of CD rats were inoculated IP at 30 days of age with freshly prepared ENU solution in doses of 180, 90 and 45 mg/kg; one group of rats were offspring of CD mothers inoculated intra-

a Synthesized by Dr. Ming Chang, Veterinary Pathobiology Department, Ohio State University, Columbus, Ohio.
b Fisher Company, Livonia, Michigan.
c Charles River Laboratory, Portage, Michigan.
d Harlan Industries, Indianapolis, Indiana.

venously (TPIV) with ENU on the 20th day of gestation; two groups of BD-IV rats were inoculated IP at 30 days of age with freshly prepared ENU solution at doses of 90 and 45 mg/kg; two groups of CD rats were inoculated intravenously at 30 days of age with freshly prepared DEN solution at doses of 160 and 80 mg/kg. Control animals (60 CD and 60 BD-IV rats) were inoculated IP at 30 days of age with saline solution. Animals were housed in plastic cages (two rats per cage) in a temperature-controlled room (24 C) with 10 hours of electric light per day. They were fed autoclaved Purina Lab Chow 5010 C and water ad libitum. The rats were observed daily and weighed weekly after treatment until death. Most of the rats were sacrificed or died because of overgrowing mammary gland, nervous system, liver or kidney tumors, or because of cachexia.

Morphological Studies

A complete necropsy was performed shortly after spontaneous death or euthanasia of moribund rats. The histological sampling procedures were identical in all groups. The tissues were fixed in 10% buffered formalin or in Bouin's solution and prepared for light microscopic examination. They were sectioned at 4-6 u and stained with hemotoxylin and eosin (H&E), periodic acid-Schiff (PAS) or osmium. The tissues for electron microscopy were fixed in glutaraldehyde and osmic acid and, after proper processing, were embedded in Epon, cut at 1 u with an ultramicrotome and stained with 1% toluidine blue. Blocks were selected from thick sections for electron microscopic examination following sectioning and contrasting

with uranyl acetate and lead citrate.

Hormonal Assays

Blood samples were collected from rats prior to euthanasia under anesthesia using ketamine hydrochloride; serum was separated by centrifugation and immediately frozen at -20 C. Hormonal assays for testosterone, estradiol and estrone were performed on serum samples collected from 8 experimental rats and 8 control rats of the same ages and strains. In addition, two samples of intracystic fluid were also examined for hormonal content. Hormonal assays on serum samples were performed by simple radioimmuno-assay techniques.[24] The values of hormonal assays were estimated by standard error of mean.

RESULTS

Tumor Induction

A high percentage of rats in this experiment developed neoplasms of the target organs for the specific carcinogens (eg. mammary gland (100% in high dose CD rats; 45% in high dose BD-IV), central nervous system (80% in high dose CD rats; 50% in high dose BD-IV rats), and kidney (10% in high dose CD rats;0% in BD-IV rats)). The incidence and pathology of tumors in other organs have been reported previously.[30,31] In addition, 21% of the rats developed tumors of the ovary. Ovarian tumors developed in 50 rats from the experimental groups (ENU - 48 tumors; DEN - 2 tumors) that received chemical carcinogens and in 3 rats from the control groups (Table 3-1). Considering the ENU exposed rats separately, the CD rats had an 18% and the BD-IV a 47% incidence of ovarian tumors of testicular type. The ovarian tumors in the BD-IV rats exceeded in incidence all other tumors encountered in this strain. In the control groups, two rats (BD-IV) developed granulosa cell tumors of the ovary and one (CD) rat developed a testicular (Sertoli's cell)-like tumor. The survival time ranged from 223 to 590 days in experimental rats with a mean survival time of 426 days for experimental groups and 850 days for control rats.

Clinical Findings

The majority of CD rats inoculated with ENU postnatally developed mammary tumors (MT).[32] In 76% of the rats which developed OT, MT were also present. In some of the rats, zonal alopecia (inguinal and abdominal regions) were observed.

Table 3-1. Incidence of ovarian tumors in rats inoculated with chemical carcinogens

Carcinogen	Strain of Rat	Number of Rats	Number of Rats with Ovarian Tumors	Percent
ENU 45 mg/kg IP	CD*	30	7	23
ENU 90 mg/kg IP	CD	30	5	17
ENU 180 mg/kg IP	CD	30	7	23
ENU 20 mg/kg TPIV	CD	30	3	10
Total		120	22	18
ENU 45 mg/kg IP	BD-IV**	30	11	37
ENU 90 mg/kg IP	BD-IV	30	15	50
Total		60	26	47
DEN 80 mg/kg IV	CD	30	1	3
DEN 160 mg/kg IV	CD	30	1	3
Total		60	2	3
Total Experiment Groups		240	50	21
Control Group	CD	60	1	2
	BD-IV	60	2***	3
Total-Control Groups		120	3	1

* CD - Sprague-Dawley

** BD-IV - Berlin Druckrey

*** Granulosa Cell Tumor

Tumors larger than 1 cm were detected by abdominal palpation. The rats which developed OT larger than 2-3 cm usually experienced metrorrhagia. No other signs of virilization or feminization were observed clinically.

Macroscopic Appearance

The malignant MT associated with OT were recognized in 54% of experimental rats, the rest of the MT were classified as fibroadenomas or cystadenomas. The OT were unilateral, ranging from 0.2 to 5 cm in diameter (Figure 3-1). They were usually solid, encapsulated, lobulated tumoral masses, white-yellowish in color (Figure 3-2). Some contained multiple cysts filled with serosanguinous or hemorrhagic fluid. Cystic endometrial hyperplasia and polyps were detected in 27% of the rats bearing OT. Metastases of OT were detected as multiple miliary nodules disseminated throughout the mesenterium in 6% of the rats affected by OT. Two rats died because of ruptures of large (4-5 cm in diameter) hemorrhagic OT. The OT weighed from 2 to 40 grams. The opposite ovary appeared smaller than normal. In one case an OT was associated with a vaginal leiomyosarcoma.

Microscopic Appearance

Tubular-like structures were present in all OT examined histologically. The histologic appearance of the tumors varied considerably, consisting of cysts, cords, undifferentiated mesenchymal cells, or of solid cellular masses arranged in tubular structures of varying sizes resembling seminiferous tubules and lined by Sertoli-like cells (Figure 3-3). A thin fibrous capsule surrounded the mass. The neoplastic cells

Figure 3-1. Gross morphology of a testicular-like tumor in the ovary of a rat. The tumor metastasized by implantation throughout the mesenterium. Serum testosterone was 2250 pg/ml.

Figure 3-2. Sagittal section of the above tumor showing an encapsulated, solid lobulated mass.

FIGURE 3-1

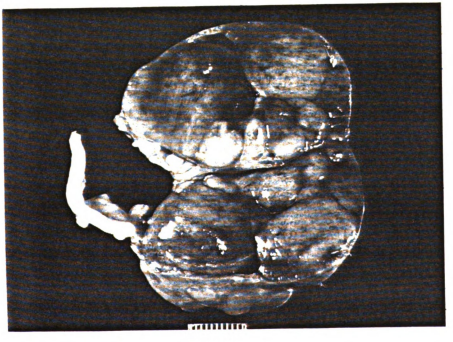

FIGURE 3-2

Figure 3-3. Testicular-like tumor. Sertoli cells arranged in tubules and pseudotubular structures. Bar = 50 um.

Figure 3-4. Photomicrograph illustrating tubular arrangement of spindle-shaped Sertoli cells. Bar = 150 um.

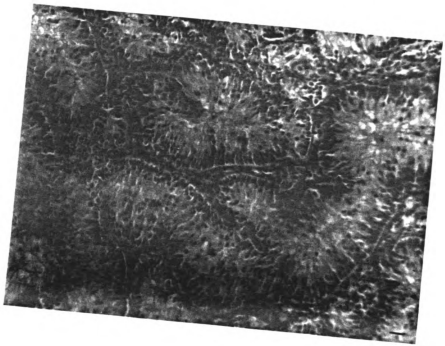

FIGURE 3-3

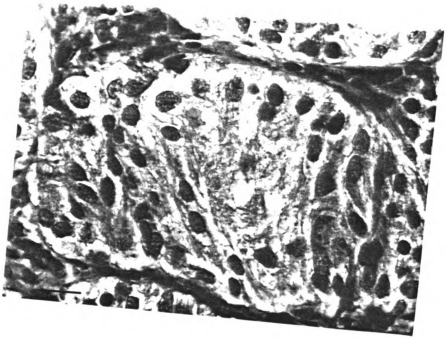

FIGURE 3-4

were characterized by large spindle-shaped or polyhedral cells with basally-located nuclei and indistinct light eosinophilic foamy cytoplasm containing many indistinct vacuoles and projecting out into a tubular lumen; usually the centers of such lumens were filled with similar appearing cells (Figure 3-4). Numerous intractyoplasmic lipid droplets were identified with osmium stain. Cords of cells were recognized especially in malignant tumors. The cords consisted of the same palisading cell type lining a thin fibrovascular stroma. Cell borders were indistinct due to the compact arrangement of tumor cells. The nuclei were oval and hyperchromatic. The tumor cells exhibited the heterochromatic Barr-body of the female nucleus. Fifty percent of the tumors were observed originating from the hilus region and ovarian parenchyma (Figure 3-5), 26% were seen arising from the ovarian capsule (surface epithelium), and 24% of the tumors were seen developing intracystically from the stromal wall (Table 3-2). In some of the smaller OT, atretic follicles and normal or degenerating corpora lutea were seen in addition to the tubular structures. Granulosa cell tumors observed in control BD-IV rats exhibited a characteristic pattern with Call-Exner bodies. They were devoid of tubular structures. One testicular-like tumor (1.5 cm in diameter) in a control rat (CD, 870 days old) was indistinguishable from induced OT. The metastases which occurred in 6% of the OT in experimental groups appeared as miliary nodules seeding the mesentery and reproducing the same tubular-like structure lined by Sertoli-like cells. The metastases spread by implantation from malignant OT through ruptured tumor capsules. The opposite ovaries were usually

Figure 3-5. Tubular structures with Sertoli cells arising in the hilus region of the rat ovary (arrow). Bar = 50 um.

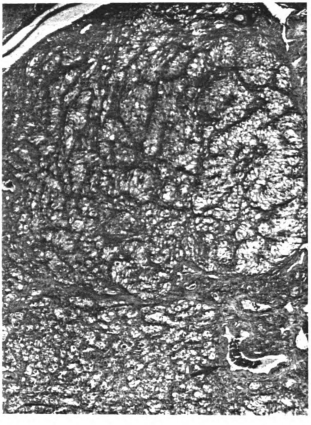

Figure 3-5

Table 3-2. Classification of ovarian tumors and other related alterative changes induced by chemical carcinogens

		Number	Percent
I.	Ovary: Testicular-like tumors	50	21
II.	Origin		
	1. Hilus and ovarian parenchyma	25	50
	2. Capsule	13	26
	3. Intracystic walls	12	24
III.	Metastases		
	1. Mesentery	3	6
IV.	Uterine, ovarian and mammary changes		
	1. Endometrial cystic hyperplasia	11	27
	2. Leiomyosarcoma	1	2
	3. Ovarian atrophy of germinal epithelium (opposite ovary)	25	50
	4. Mammary tumors	38	76
	Adenocarcinomas	27	54
	Fibroadenomas	8	16
	Cystadenomas	3	6

atrophic.

Ultrastructural Evaluation (EM).

As a rule, the tumor cell sheets were separated from the stromal connective tissue by a basal membrane (Figure 3-6,7). The cells were closely apposed to each other; occasionally intercellular spaces occurred. The cells were elongated, with their longitudinal axis often oriented at right angles to the basal membrane. Between adjacent cells, occasional desmosome -like contacts were formed. The nuclear shape varied and was either elongated or ovoid; most nuclei were moderately indented (Figure 3-6). The cells had moderate numbers of organelles; however, there was an increase in smooth endoplasmic reticulum and in mitochondria with tubular cristae (Figure 3 -6). The Golgi cmplexes were inconspicuous. Lipid droplets were seen in the cytoplasm of all tumors. Ribosomes and polysomes occurrred regularly (Figure 3-8). Occasional annulate lamellae and small dispersed profiles of rough endoplasmic reticulum were identified. The two granulosa cell tumors found in the control BD-IV rats exhibited distinct ultrastructural pattern characterized by chromatin rich nuclei and numerous large lipid granules in the cytoplasm. No ultrastructural difference was observed between induced testicular-like tumors and the spontaneous one found in the control (CD) group.

The mean standard error of testosterone levels in the experimental group was 37.5% higher than in the control group (Figure 3-9). The serum testosterone content was in one case of malignant metastasizing OT especially high (2250 pg/ml).

Figure 3-6. Electron micrograph of tubules composed of indented nuclei of Sertoli cells with cytoplasm containing mitochondria (M), free ribosomes (R) and secretory granules (S). Uranyl acetate-lead citrate. (x 6000).

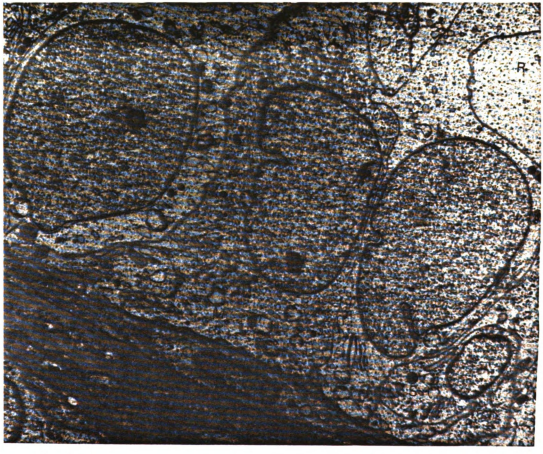

Figure 3-6

Figure 3-7. Electron micrograph of a ductular structure illustrating Sertoli cells lined by a thin basement membrane (B). The cytoplasm contains vesicles (V) and secretory granules (S). Uranyl acetate-lead citrate. (x 6000).

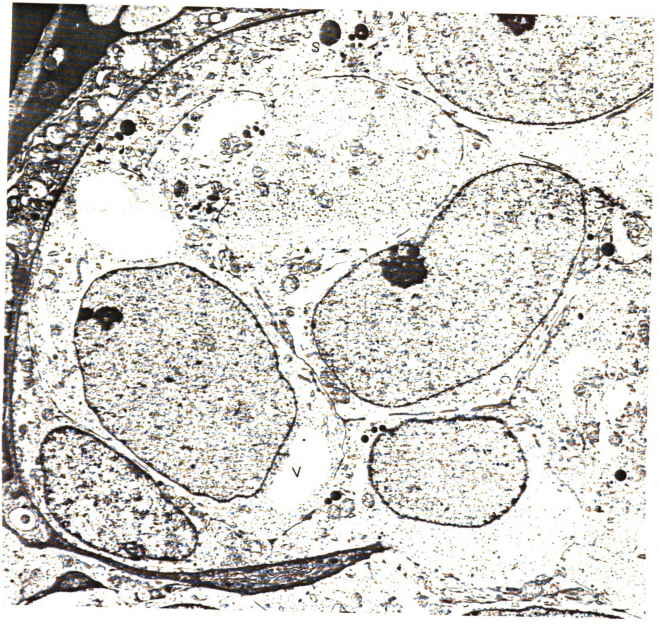

Figure 3-7

Figure 3-8. Higher magnification electron micrograph of Sertoli cells lined on a thin basement membrane (B). The nuclei contain homogeneous disperse chromatin. The cytoplasm contain large vesicles (V), rough endoplasmic profiles (RE) and mitochondria (M). The cells are attached to each other by desmosomes (arrows). (x 10000).

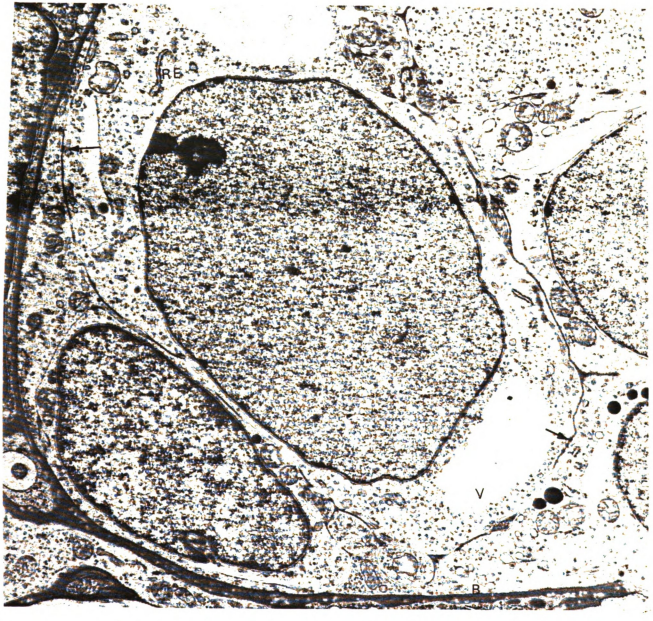

Figure 3-8

Figure 3-9. Serum testosterone in rats with testicular (Sertoli's cell)-like tumors of the ovary related to tumor diameter. The mean value in 8 rats with ovarian tumors (636 ± 109 pg/ml) was elevated 46% above controls (437 ± 27 pg/ml) (mean = solid line; standard error = shaded area).

Figure 3-10. Serum estrone in rats with testicular (Sertoli's cell)-like tumors of the ovary related to tumor diameter. The mean concentration in 8 rats with ovarian tumors (174 ± 11 pg/ml) was elevated above serum estrone levels in control rats (156 ± 9 pg/ml) but the differences were not significant.

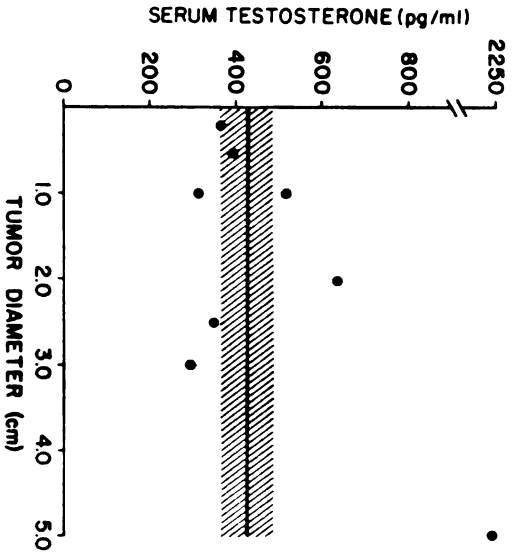

Figure 3-9

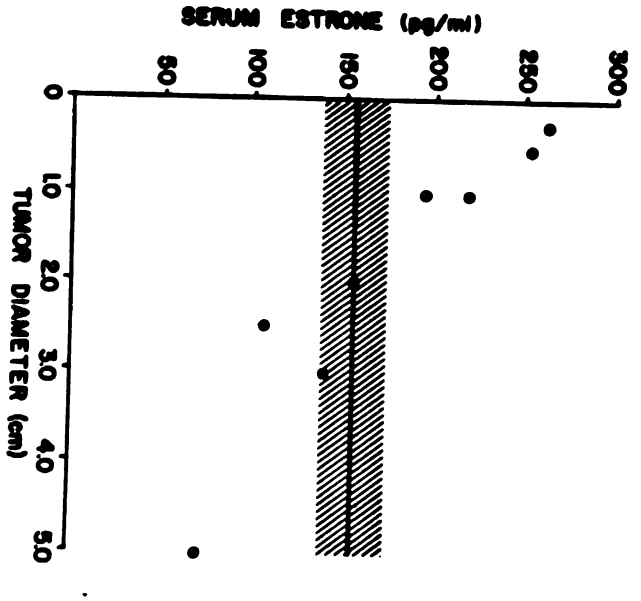

Figure 3-10

Figure 3-11. Serum estradiol in rats with testicular (Sertoli's cells)-like tumors of the ovary related to tumor diameter. The mean value in 8 rats with ovarian tumors (108 $\pm$ 6 pg/ml) was elevated significantly above serum estradiol levels in control rats (90 $\pm$ 4 pg/ml) (mean = solid line; standard error = shaded area).

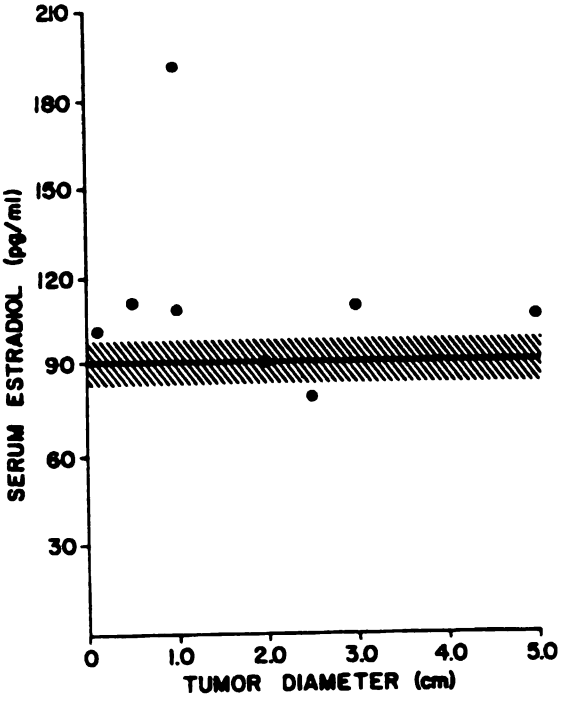
210
180
150
120
90
60
30
SERUM ESTRADIOL (pg/ml)
0
1.0
2.0
3.0
4.0
5.0
TUMOR DIAMETER (cm)

Figure 3-11

The testosterone content of the intracystic tumoral fluid in the other two malignant OT was also elevated (1027 and 5702 pg/ml). The estrone level in malignant OT was 50% (Figure 3-10) and the estradiol level was 75% above the mean control (Figure 3-11). The latter two hormones were not elevated in the cystic fluid.

DISCUSSION

The carcinogens (ENU and DEN) used in our experiments increased the OT incidence and shifted their occurrence to a younger age (mean survival time of 426 days). The tumor type was uniformally of a testis-like tubular pattern with a high rate of malignancy. The BD-IV rat is considered a strain which is relatively resistant to chemical carcinogens.[6] Indeed, in comparison with CD rats, BD-IV rats developed a lower incidence of nervous system and mammary gland tumors.[30,31] Surprisingly the opposite was true for ovarian tumors, BD-IV rats had a significantly higher ovarian tumor rate than CD rats (Table 3-1). With the highest dose of ENU used (90 mg/kg IP), 50% of the female BD-IV rats developed testicular-like tumors in the ovaries. The OT in the BD-IV rats exceeded in incidence all other tumors encountered in this strain.

A good correlation between the incidence of OT and MT was established. Of 22 CD female rats bearing OT, 18 had also mammary adenocarcinomas. In BD-IV rats exposed to ENU, 10 out of 26 rats had OT and MT concurrently. Nine of the MT were adenocarcinomas. Four rats bearing OT and MT (for which hormonal assays were done) expressed a higher than normal serum

estradiol. None of the OT-bearing rats with increased serum testosterone developed MT. Hormonal assays were not performed in all OT bearing rats. However, the hormonal increase in 8 rats bearing OT combined with cystic endometrial hyperplasia (38% of rats bearing OT) and malignant MT is a good indication that some of the OT were functional. The association of OT with malignant MT in BD-IV rats (9 out of 10) which, contrary to CD rats, are not prone to develop MT, could be related to increased ovarian hormone production in rats bearing OT, which probably stimulated the progression of malignant MT.

Histologically and ultrastructurally the cells populating the seminiferous-like structures are of epithelial origin. They are large cells with ovoid and grooved nuclei, indistinct foamy cytoplasm and secretory lipid droplets, similar to the Sertoli tumor cells described in dogs.[2] The clinical, histological and hormonal findings illustrate that some of the OT are functional. Increased serum-testosterone levels in some and increased estrone and estradiol levels in other rats (Figure 3-9,10,11) contrast the opinions of other authors who claim that these types of tumors are nonfunctional in rats.[3, 7]

The induction of OT in 30-day-old female CD and BD-IV rats with chemical carcinogens is possible because the ovary remains an embryonic organ for a period of time after the female reaches puberty. At the 30th day of age and thereafter, growth and development of ovarian components continue, especially toward the formation of new follicles. The granulosa and Sertoli cells have the same embryological anlage, derived

from mesenchymal blastema which forms the sex cords (granulosa cells in females and Sertoli cells in males).[40] A high rate of DNA synthesis and cellular replication occur in the ovaries, constituting an ideal target for alkylating carcinogens. For unknown reasons the neoplastic transformation differentiates mostly towards testicular-like tumors resembling Sertoli cells. As a consequence of this differentiation the hormonal behavior varies since Sertoli cells may produce estrogens as well as testosterone.[1,3,5,7] Alterations in enzyme pathways in neoplastic tissue should explain why some of these tumors result in high levels of testosterone and others do not.[5,8,20,37,40]

Controversy continues regarding the histogenesis of these tumors, called arrhenoblastomas, androblastomas, Sertoli-Leydig tumors, tubular adenomas or sex cord stromal tumors by different authors. Since this is a relatively rare tumor in man and there is not a good biological model, it has been difficult to clarify the pathogenesis. Indeed, some of these tumors arise from the hilus region, probably as Meyer suggested,[19] from the male-directed cells that have persisted in the rete ovaries after gonadogenesis. However, a good proportion of the tumors also arise from the ovarian capsule or from the stromal wall, which opens the alternative possibility that they develop despite their frequent testicular morphology, directly from ovarian stroma. There is no reason to believe that only rudimentary testicular cords are capable of forming tubular structures, inasmuch as the ovarian mesenchyme is also capable of differentiating into tubular structures, sex cords, Sertoli cells and Leydig cells.[1,21,33,40] Clarifica-

tion of the origin and pathogenesis of this controversial type of human ovarian neoplasm may likely result from more careful scrutiny of spontaneous and induced ovarian neoplasms of animals and a comparison of the morphology and physiology of human and animal ovaries.

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CHAPTER 4

Characteristics of normal rat mammary epithelial cells and N-ethyl-N-nitrosourea -induced mammary adenocarcinoma cells grown in culture

SUMMARY

The characteristics of normal mammary epithelial cells derived from Lewis and Sprague-Dawley rats and ENU-induced adenocarcinoma cells derived from Sprague-Dawley and Fisher rats and grown in monolayer cultures were compared. After collagenase treatment the rat mammary epithelial cell aggregates were placed in a hormone-supplemented medium. The normal mammary epithelial cells (NE) attached to the surface of the culture dish within 50 hours, whereas the mammary adenocarcinoma cells (MA) attached within 24 hours and grew as a cell multilayers. After the colonies of NE and MA cells became confluent, the culture entered a steady state in which the cells from the upper layer were shed into the medium. The rate of proliferation and squame detachment in confluent cultures was increased by the presence of epidermal growth factor. Rhodanile blue staining and transmission electron micrscopy showed that the shed cells were partially keratinized. In addition, the normal cells were unable to grow in soft agar or to form tumors when inoculated into appropriate hosts. The opposite was true in each case for the mammary adenocarcinoma cells. Karyotypes of normal and neoplastic mammary rat epithelial cells revealed a hypodiploid modal number of chromsomes.

INTRODUCTION

The administration of relatively high doses of certain polycyclic aromatic hydrocarbons, e.g., DMBA, MCA or alkylated nitrosoureas e.g., MNU, ENU to female rats invariably results in a high incidence of mammary carcinomas.[1-4] The carcinogen-

ic efficiency of these chemicals and the morphological and physiological similarities of the induced carcinomas to human breast cancer provides the basis for the attractiveness of this system as an important model to examine mechanisms of mammary gland carcinogenesis. To date, chemical carcinogenesis of the rat mammary gland has been examined primarily in vivo; few laboratories have attempted to examine this neoplastic process in vitro. A prerequisite for a thorough analysis of rat mammary gland carcinogenesis in vitro is the availability of cell culture techniques capable of maintaining and propagating normal and neoplastic rat mammary epithelial cells. During the past two year, we have developed techniques to excise and isolate normal rat mammary epithelial cells and mammary carcinoma cells derived from ENU-induced rat mammary carcinomas. In this commmunication, we describe these techniques as well as characteristics of these cells maintained in cell cultures.

MATERIALS AND METHODS

Source and Isolation of Cells. Normal mammary tissue (NE) was obtained from the whole fat pad (inguinal and axillary) of normal female Sprague-Dawley[a] rats (SNLR/CD1) and normal Lewis[a] rats (SNLR/L1), at fifty days of age. Under ketamine hydrochloride anesthesia,[b] the whole fat pad containing the mammary gland was removed and transferred to a small Erlenmeyer flask

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a Charles River Laboratories, Portage, Michigan.
b Vetalar, Parke Davis, Morris Planes, New Jersey.

containing Eagle's Minimum Essential Medium (MEM) with Earle's Salts and 2 mM L-glutamine.[c] Fragments of the whole mammary gland were transferred to a 60 mm Petri dish and under a dissecting microscope remnants of connective tissue, fat and lymph nodes were removed using a small forceps with fine tips and an iris scissors. After dissection, the tissue contained mostly mammary epithelium and fat tissue. The fragments were transferred to a concave glass plate and minced with crossed sterile scalpels. The finely minced tissue was then placed in a 100 ml Erlenmeyer flask containing 50 ml MEM, 0.1% collagenase,[d] 4,000 I.U. penicillin[c] and 2,000 mg streptomycin[c] and was incubated at 37 C in a gyratory water bath for 40 minutes. The cell suspension was then pipetted vigorously to further disperse cells and then was filtered through one layer of 74 um opening nylon mesh.[d] The filtrate was centrifuged at 100 x g to remove cell debris, and the resulting pellet was resuspended in 4 ml of Dulbecco's Modified Eagles Medium (DMEM)[c] supplemented with 25% horse serum (HS)[c] and a hormone combination consisting of 20 ug /ml each of hydrocortisone, insulin, and prolactin,[e] and 10 ng /ml epithelial growth factor (EGF),[f] and 1% antibiotics (penicillin and streptomycin[c]). Initially, the cells and cell clusters were plated at a high density into 60 mm tissue culture dishes and incubated in a humidified 10% CO_2 incubator at 37 C. Six hours after the cellular components started to settle down they were examined under an inverted microscope. Free cells,

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c Gibco, Grand Island, New York.
d Worthington Biochemical Corp., Freehold, New York.
e Sigma Chemical Co., St. Louis, Missouri.
f Collaborative Research, Waltham, Massachusetts.

cell aggregates and ductular structures, some closely resembling terminal end buds, were photographed using a Nikon F_5 UFX Automatic Camera[g] and Panatomic X film.[h] At 8 to 10 hour intervals, the suspended cells in each dish were removed by decanting to another dish. Fresh growth medium was added to these dishes. This process was repeated 5 times. The initial dishes usually contained mesenchymal cells, but the cells plated out in subsequent dishes were mostly epithelial cells. Most of the epithelial colonies developed from cell aggregates and duct-like structures which attached to the bottom of the dishes, between 20-50 hours post-seeding. Every 2 days half the medium was removed and fresh growth medium was added to every dish. Ten days later multiple epithelial colonies of various sizes surrounded by mesenchymal cells were recognized in several dishes, but mostly in dishes that were seeded at later time periods. Using a marker objective mounted on the inverted Nikon microscope the epithelial-like colonies were identified and the mesenchymal cells were removed using a rubber policeman and differential trypsinization (gradually increasing trypsin concentrations from 0.01 to 0.05% with 0.2% EDTA). The procedures were repeated several times until only epithelial-like colonies remained. Confluency in culture dishes was obtained in 2 months.

Induction of Mammary Tumors. Mammary adenocarcinomas (MA) were induced in female Sprague-Dawley[a] and Fisher[a] rats by a single i.p. injection of ENU as previously described.[4-6] Between 60

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g Nikon, Tokyo, Japan.
h Eastman Kodak Co., Rochester, New York.

days and 80 days post-inoculation, the rats developed multiple mammary tumors. The rats were euthanized using CO_2, and mammary tumors were excised surgically under aseptic conditions, cleared of surrounding connective tissue, and cut into 2 mm^3 segments for cell culture preparation. The remainder of the tumor was fixed in buffered formalin, sectioned and stained with hematoxylin and eosin (H&E) for histopathological examination. Tissue dissociation and isolation of neoplastic epithelial cells was achieved in the same manner as described above for normal mammary epithelium. Confluency of the adenocarcinoma cells in culture dishes was obtained in 2 weeks.

Routine Tissue Culture Techniques. Once growth was established, the cells were transferred to T75 flasks and maintained as described above. At later passages (P_{10}) the concentration of serum was lowered from 25 to 10%. For subculture of normal and neoplastic mammary epithelial cells, growth medium was aspirated and the cells washed once with Hanks' Buffered Saline Solution (HBSS)[c] and cells dissociated with 0.25% trypsin and 0.02% EDTA.[c] Treatment with trypsin was for 3 to 5 minutes at 37 C. The treatment was repeated twice. Epithelial cells (NE and MA) strongly attached to the dishes for the first passages and therefore were difficult to remove. The cells were never totally removed from the flask in which they achieved confluency for the first time; these cells were maintained for future subcultures in case of cell loss. The medium was changed every other day. The cells were counted using a hemocytometer. For the first passages, the cell aggregates were difficult to disrupt during preparation of the cell suspension; the cells were

lysed in 0.1 M citric acid containing 0.1% crystal violet at 37 C for 1 hour and the nuclei were counted. The cells were frozen in liquid nitrogen (N_2) in DMEM + 2 mM glutamine supplemented with 20% HS and 10% DMSO as a cryoprotectant.

To determine seeding efficiency, the cells were seeded at 4.2×10^5/60 mm Petri dish and scored after 4 hours when the maximum number of cells had attached but before mitosis started.[7] The formula for seeding efficiency is:

$$\frac{\text{No. of Cells Recovered}}{\text{No. of Cells Seeded}} \times 100 = \text{Seeding Efficiency}$$

Plating efficiency was calculated by seeding, at a low density, 600 and 1000 cells/60 mm Petri dish. The cells grew as discrete colonies. The colonies were counted after 8 days and the results were expressed as the plating efficiency. The formula for plating efficiency is:

$$\frac{\text{No. of Colonies Formed}}{\text{No. of Cells Seeded}} \times 100 = \text{Plating Efficiency}$$

The population doubling time was calculated expressing the cell number as a function of $\log_2$ by the multiplication of the common logarithm of the cell count by the factor 3.33.[8] The formula used for the calculation of population doubling time is:

$$\text{Number of population doublings} = \log_{10}(N/N_0) \times 3.3$$

Where: N = number of cells in the growth vessel at the end of a period of growth.

N_0 = number of cells planted in the growth vessel.

The squames accounted for the calculation of population doubling time.

Cloning and Anchorage Independent Growth. The mammary adenocarcinoma epithelial cells were cloned using dilution cloning technique.[9] The ability of MA rat mammary epithelial cells to form colonies in agar was determined as described by MacPherson[10] with minor modifications. The basal layer, which contained 5 ml of 2% Sea Plaque Agarose[i] supplemented with DMEM, 25% HS, and 0.2% sodium bicarbonate was formed in 60 mm plastic Petri dishes. Formation of the basal layer was followed by the addition of a second layer (1.5 ml) 0f 0.33% Sea Plaque Agarose supplemented as above, containing 7.5×10^4 dilutions of cells/plate. Plates were incubated at 37 C in an atmosphere of 10% CO_2. After 5 to 6 weeks, colonies greater than 40 cells were counted using an inverted phase microscope.

Oncogenicity. Suspensions of cells diluted to 1 and 2×10^6 cells in 0.05 ml of culture medium were inoculated subcutaneously into the interscapular region of newborn Sprague-Dawley and Fisher rats and nude mice were examined three times weekly for progressively growing tumors at the site of inoculation. Six to eight weeks later, the animals were sacrificed and all palpable nodules and tumors were surgically excised and prepared for routine tissue cultures and histological examination.

Chromosome Analysis. Chromosome analysis was performed according to methods described in the literature[11,12] with minor modifications. Briefly, cells grown in T75 flasks in a logarithmic phase of growth were treated with 0.04 ug Colcemid/ml[c] for 1

hour before being harvested with trypsin solution. The cell suspension was then centrifuged at 1000 x g for 10 minutes. The supernatant was removed and washed twice with HBSS. After the last wash the supernatant was removed and, without disturbing the cell pellet, a hypotonic solution (distilled water) was added, the pellet resuspended, and the tube was incubated at 37 C for 15 minutes. After hypotonic treatment, the cells were fixed in methanol:glacial acetic acid (3:1) for 15 minutes and slides were prepared by dropping the cell suspension with a narrowed tip Pasteur pipet onto the slide; the preparation was subsequently rapidly air dried. G-banding was performed by a modification of the method of Yunis[12] using a trypsinization time of about 15 seconds.

Electron Microscopy. The cells grown in Petri dishes or T75 flasks were harvested by trypsinization or by using a fine, sharp spatula. To obtain perpendicular sections through the cell multilayers, the cells were fixed directly in a Petri dish and then processed. The entire film of cells was removed and fixed in 0.1% glutaraldehyde for 3 hours. Then the cells were refixed in 3% glutaraldehyde carefully to avoid disrupting the pellet. The pellet was fixed at room temperature for 2 to 5 hours. This second fixation hardened the pellet so that it could be handled more easily for dehydration and embedding. The pellet was then washed in 0.1M phosphate buffer, post-fixed in 1% osmium tetroxide for 1 hour, washed again with phosphate buffer and three times in 30% alcohol for 10 minutes each time. Each block was stained with uranyl acetate and lead citrate. The sections were examined on a Phillips 201 Electron

Microscope.

For scanning electron microscopy (SEM), cultures were fixed in Petri dishes with glutaraldehyde and processed as designated above, except that the uranyl acetate was omitted. After dehydration with an ethanol series, fragments of the dish were cut out under alcohol and critical point dried in liquid CO_2. The specimens were coated with gold-palladium and examined in a Cambridge S4-10 Scanning Electron Microscope.

Scoring of Detergent-Insoluble Cells and Cornified Cell Envelopes. This procedure was performed according to the technique used by Green.[13] The squames shed into the medium were harvested and centrifuged. The sedimented squames were resuspended in a small volume of buffer and counted in a hemocytometer chamber. The rate of squame production was calculated for the interval during which the accumulation took place every 3 days and cumulated at 20 days when the cells were subcultured. The cells shed were suspended in 12 ml of 1% sodium dodecylsulfate (SDS); after incubation of 5 minutes, the suspension was centrifuged at 2000 rpm for 10 minutes, the supernatant was removed and the pellet was resuspended in 0.20 ml of buffer. A small aliquot was removed and the cells counted in a hemocytometer chamber using Nikon phase optics. To count cornified envelopes, ß-mercaptoethanol was added to the detergent-treated suspension to 1%, and after 5 minutes an aliquot was counted in the hemocytometer. The cell ghosts were then incubated at 90 C for 10 minutes, centrifuged at 2000 rpm for 10 minutes and prepared for EM as described above.

Rhodanile Blue Staining for Keratin. Cultures grown on Lab-

Tek tissue culture chamber slides[i] and slides made from squame suspensions were fixed in 10% buffered formalin, stained with 1% Rhodanile Blue (a 1:1 mixture of 2% Rhodamine B and 2% Nile Blue A in water)[j] for 3 minutes and then destained in running tap water for about 30 seconds to one minute, until the excess red dye was eliminated from non-keratinocyte areas, leaving them light blue, and the keratinocyte colonies bright red.[13,14]

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i Miles Laboratories, Naperville, Illinois.
j Matheson Co., Inc., Norwood, Ohio.

RESULTS

A. Primary Cultures of Normal Rat Mammary Gland

Two normal primary epithelial cell lines (SNLR/L1 and SNLR/CD1) from young virgin female Sprague-Dawley and Lewis rats were obtained.

1. Growth Characteristics. Examination under an inverted microscope revealed that the normal mammary epithelial cells grew from aggregates of ductular structures in DMEM with HS and hormone supplements (Figure 4-1). They attached to the surface of the culture dish within 20-50 hours and grew out very slowly as a cell multilayer. They grew well on plastic substrate but poorly on glass. Three distinct morphological cell types were observed after 5 days in culture. The first cell type was a cuboidal shaped cell, possibly of ductular or alveolar origin (Figure 4-2). The second type was a spindle-shaped, fibroblast-like cell usually located between islands of cuboidal cells or even beneath the cuboidal epithelial colonies. The third type was a larger, ovoid-shaped lipocyte with numerous large intracytoplasmic lipid droplets. The lipocytes and fibroblast-like cells were gradually eliminated during the first 2-5 weeks of culturing. The epithelial colonies continued to expand, gradually coalescing and finally forming a confluent cell multilayer. The cells reached confluency in a 60 mm Petri dish in about 2 months. The final cell population was of epithelial type (NE): consisting of cuboidal-shaped cells forming a typical "cobblestone" appearance with dome formation (Figure 4-3). The NE cells had a strong cell to cell adherence and were resistant to trypsinization. The cell growth

Figure 4-1. Inverted microscopic appearance of NE free cells, cell aggregates and ductular structures, some closely resembling terminal end buds (arrow), six hours post-seeding after collagenase treatment. (x 60).

Figure 4-2. NE cells colonies appearance, free of mesenchymal components, 4 weeks post-seeding, after repeated mechanical and differential trypsinization procedures. (x 60).

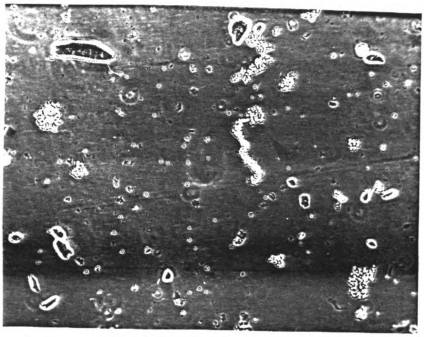

Figure 4-1

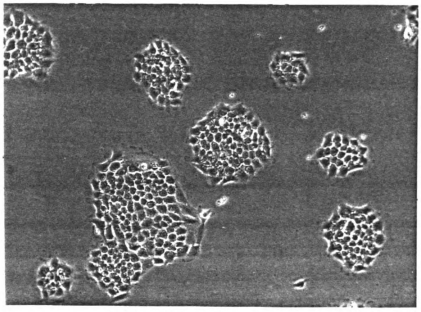

Figure 4-2

Figure 4-3. Appearance of confluent NE cell culture with cobblestone-like pattern and domes formation 2 months post-seeding. (x 120).

Figure 4-4. Scanning electron microscopy of NE cells expanding large pseudopod-like membranes at the periphery and centrally forming stratified cell layers of squamous keratinized cells. (x 200).

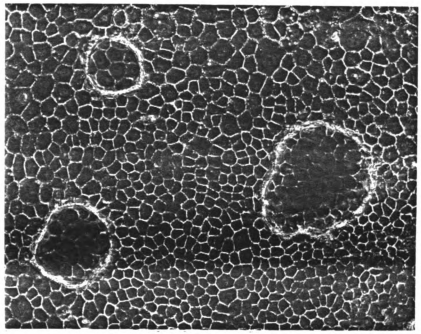

Figure 4-3

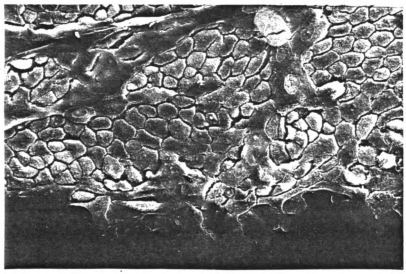

Figure 4-4

characteristics illustrated that the population doubling time (PDT) decreased with increased passages (Table 4-1). For NE cells, PDT decreased from 95 (Lewis rat) and 100 hours (Sprague-Dawley rat) initially to 33 and 22 hours, respectively, after multiple passages. The seeding efficiency and plating efficiency increased with the passage numbers for NE Lewis rat cells. The seeding efficiency for NE Sprague-Dawley rat cells decreased slightly with passage numbers and the plating efficiency increased with the passage numbers. The cells would not grow readily at low cell inocula and grew very slowly until a critical cell number (10^5/ml) was reached. The NE cells (Sprague-Dawley rat) which were maintained in culture up to 80 passages, and became transformed, greatly changed their phenotype. The cells no longer formed tight colonies, became more adherent-independent, and piled-up, forming spheroids which floated freely in the medium.

Scanning Electron Microscopy (SEM). Scanning electron microscopy confirmed these observations. The NE cells grew in a monolayer at the periphery, and had expanding large pseudopod-like membranes with centrally forming stratified cell layers showing varying degrees of differentiation toward squamous keratinized cells (Figure 4-4). The cell monolayer was recognized by the cuboidal-shape and the many microvillous projections on the surface (Figure 4-5). The second layer, more differentiated cells, were flattened with polygonal outlines and fewer, shortened microvilli.

Transmission Electron Microscopy (TEM). Ultrastructural examination revealed three structural markers that may be used to

Table 4-1. Comparative characteristics of normal and neoplastic rat mammary epithelial cells

Cell Line	Passage	Population Doubling Time (Hrs.)	Seeding Efficiency (4 hrs. post-seeding)	Squame Production	Plating Efficiency (8 days post-seeding)	Saturation Density (no. cells x 10^5 sq. mm.)	Modal Chromosome Number (per 50 metaphases	Tumorigenicity Rats/Mice
			%	%	%			
SMLR/CD 1* (neoplastic)	5	80					38(32-77)+	1/5
	9	70					38(28-70)	
	15	25	26				36(32-60)	3/5
	33	22						
	36	21	96	6	6	6	38(32-80)	4/5 3/5
SMLR/CDF 1** (neoplastic)	3	82						
	5	80						
	15	74	37	25	5	2	46(36-88)	4/5 3/5
	30	60						
SMLR/L 1*** (normal)	6	95						
	10	86	20		0.2			
	16	60						
	20	25	73	2	4	3	40(32-46)	0/5 0/5
SMLR/CD 1* (normal)	6	108						
	20	90			0.1	4	38(22-80)	
	30	60		2				
	40	40	64					
	60	25	50		4	4	36(32-88)	5/10 0/5
	80	22	44					

* Derived from a Sprague-Dawley female rat
** Derived from a Fisher female rat
*** Derived from a Lewis female rat
+ Range in parenthesis

identify mammary epithelial cells: desmosomes, tonofibrils, and intracytoplasmic vacuoles.[15] Ultrathin sections, cut perpendicular to the surface of the outgrowth, formed a stratified squamous epithelium (Figure 4-6). The basal layer exhibited multiple well developed villous projections. The cells from the upper layers showed either short villous projections or an absence of villi. Cells were connected by numerous desmosomes with attached tonofilaments (Figure 4-7). Similar tonofilaments were also present in the cytoplasm and especially as a perinuclear rim. The number of mitochondria, smooth endoplasmic reticulum, rough endoplasmic reticulum, free ribosomes and Golgi apparatus was variable.

Squame Production in Confluent (NE) Cell Cultures. Squame production was observed in NE cells when EGF was present in the medium. The cells from stratified epithelium (2-3 layers) gradually lost their nuclei, became more flattened, partially keratinized, contained a large number of phagosomes and an increased density of microfilaments resembling the filaments observed in normal human mammary epithelial cells.[15] These were the squames or keratinized cells which shed into the medium, depending on how long they were kept at confluency. About 2% of the total cells were shed into the medium during a 20-day post-seeding period.

Chromosome Analysis. The NE cells maintained a near diploid chromosome constancy (modal 38) with a range between 32 to 80 (Table 4-1). The chromosome pattern was characteristic of the rat species.

Figure 4-5. Scanning electron microscopy of NE cells from the basal layers showing a cobblestone-like pattern with numerous microvilli projections on the surface. (x 2600).

Figure 4-6. Ultrathin section, cut perpendicular to the surface of the outgrowth revealed that the NE cells formed a stratified squamous epithelium. The second cell layer showed increased intracytoplasmic perinuclear tonofilaments. (x 700).

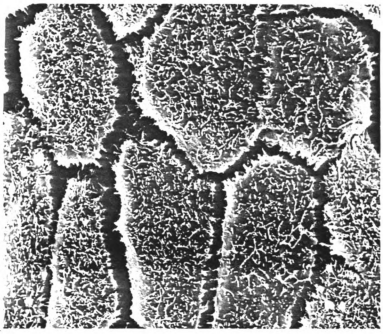

Figure 4-5

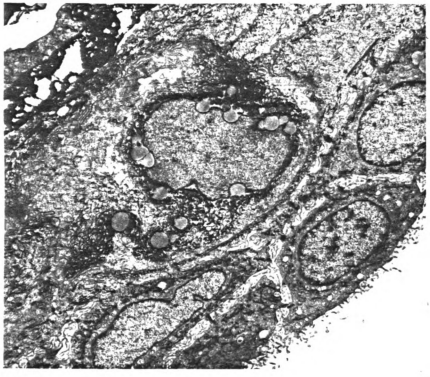

Figure 4-6

B. Primary Cultures of ENU-Induced Rat Mammary Gland Adenocarcinoma Cells

Two primary neoplastic epithelial cell cultures were obtained from adenocarcinomas induced in Sprague-Dawley and Fisher rats by inoculation with ENU (MA cells).

Growth Characteristics. After collagenase treatment, the MA cells grew in culture from cell aggregates and continued to grow as a cell multilayer and also showed a faster rate of growth than NE cells for the first passages. The MA cells formed domes and piled-up colonies, forming spheroids of cells floating into the medium after approximately 5 passages (Figure 4-8). Within the epithelial colonies, cells were mainly cuboidal, possessing large nuclei with prominent nucleoli and striking perinuclear granulation, closely adherent to one another and resistant to trypsinization procedure.

The PDT for MA cells decreased from 80 and 82 to 40 and 22 hours, and the seeding efficiency increased with passages. The plating efficiency and saturation density was slightly higher than that of the NE cells (Table 4-1).

Scanning Electron Microscopy (SEM). The MA cells formed cell multilayers and multifocal spheroids. The MA cells continued to divide at confluency, settled on top of the formed layer, forming spheroids (Figure 4-9).

Transmission Electron Microscopy (TEM). Ultrastructural examination of MA cells revealed the same markers as were observed with NE cells. The MA cells formed a stratified squamous epithelium up to 5 layers thick. An increased accumulation of filaments and electron dense bodies in the cytoplasm of shed cells with

gradual degeneration and disappearance of nuclei, ribosomes, mitochondria and alteration of desmosomes has been observed. Occasionally, blister-like structures (domes) were formed between the upper stratified squamous epithelium (Figure 4-10). Round, dense intracytoplasmic bodies, probably representing lipid or phagosomes, were constantly present; they were large and more numerous in stratified cells. Some of these electron dense bodies formed large intracytoplasmic aggregates in the stratified cells which caused the superficial layer to have a dome-like appearance (Figure 4-11).

Squame Production in MA Cell Cultures. The process of squames formation in cultures containing EGF was increased in MA (especially in STLR/CDF1) cells than in NE cells (Table 4-1). Addition of 10 ng/ml of EGF to the tissue culture medium substantially increased the rate of cell replication and squame formation compared with tissue culture medium without EGF (Figure 4-12). The percentage of squames harvested during 20 days period ranged from 6 to 25% for MA cells. About 70% of the squames harvested consisted of cells with pycnotic nuclei or without nuclei. Treatment of squames with 1% sodium dodecylsulfate (detergent) and 1% β-mercaptoethanol (reducing agent) showed cell ghosts with thick cornified envelopes. The well preserved marginal band was clearly visible on electron photomicrographs (Figure 4-13). Rhodanile blue staining indicated that the shed cells were partially keratinized.

Chromosome Analysis. The MA cells derived from Sprague-Dawley rat were in a hypodiploid range with a modal number of 38 and a range of 22 to 80. The MA cells derived from a Fisher rat

Figure 4-7. Transmission electron microscopy appearance of NE cells showing numerous surface microvillous projections (M), desmosomes with attached tonofilaments (D), and intracytoplasmic vacuoles (V). (x 22000).

Figure 4-8. Inverted microscope appearance of MA cells forming a large piled-up colony. (x 60).

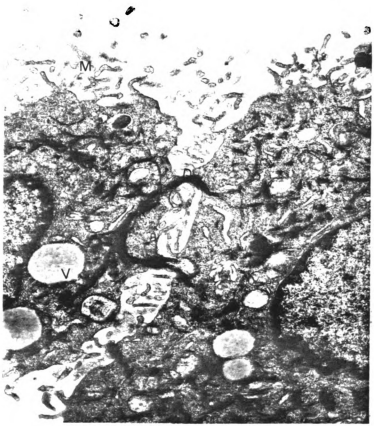

Figure 4-7

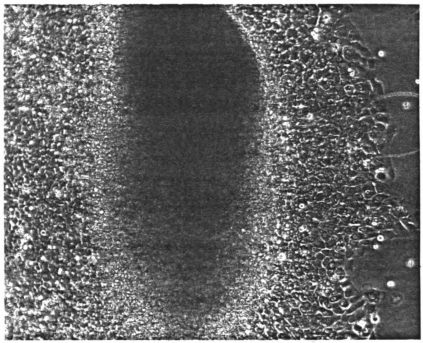

Figure 4-8

Figure 4-9. Scanning electron microscopy appearance of MA cells showing squamous differentiation and mitosis on the top of differentiated layer from which piled-up colonies will form. (x 200).

Figure 4-10. Transmission electron microscopy appearance of MA cells with blister-like structure (dome) formed between the upper stratified squamous epithelium. (x 7000).

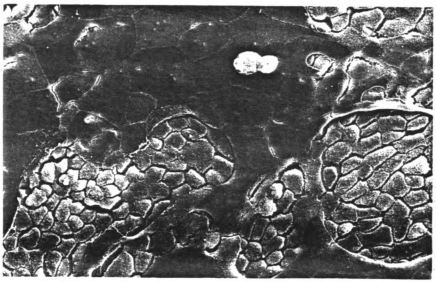

Figure 4-9

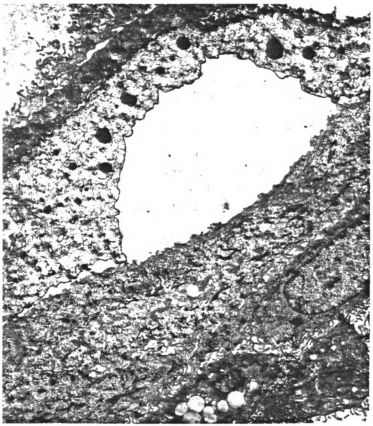

Figure 4-10

Figure 4-11. Transmission electron microscopy appearance of intracytoplasmic electron dense body aggregates in in the stratified cells which occasionally caused a "dome-like" appearance of the tissue culture. (x 7000).

Figure 4-13. Photomicrograph of squames showing marginal bands (post-treatment with detergent and reducing agents). (x 7000).

Figure 4-12

Figure 4-13

Figure 4-12. Graphic representation of the EGF effect on MA cell cultures. The squames formation was significantly increased on tissue cultures containing EGF in the medium.

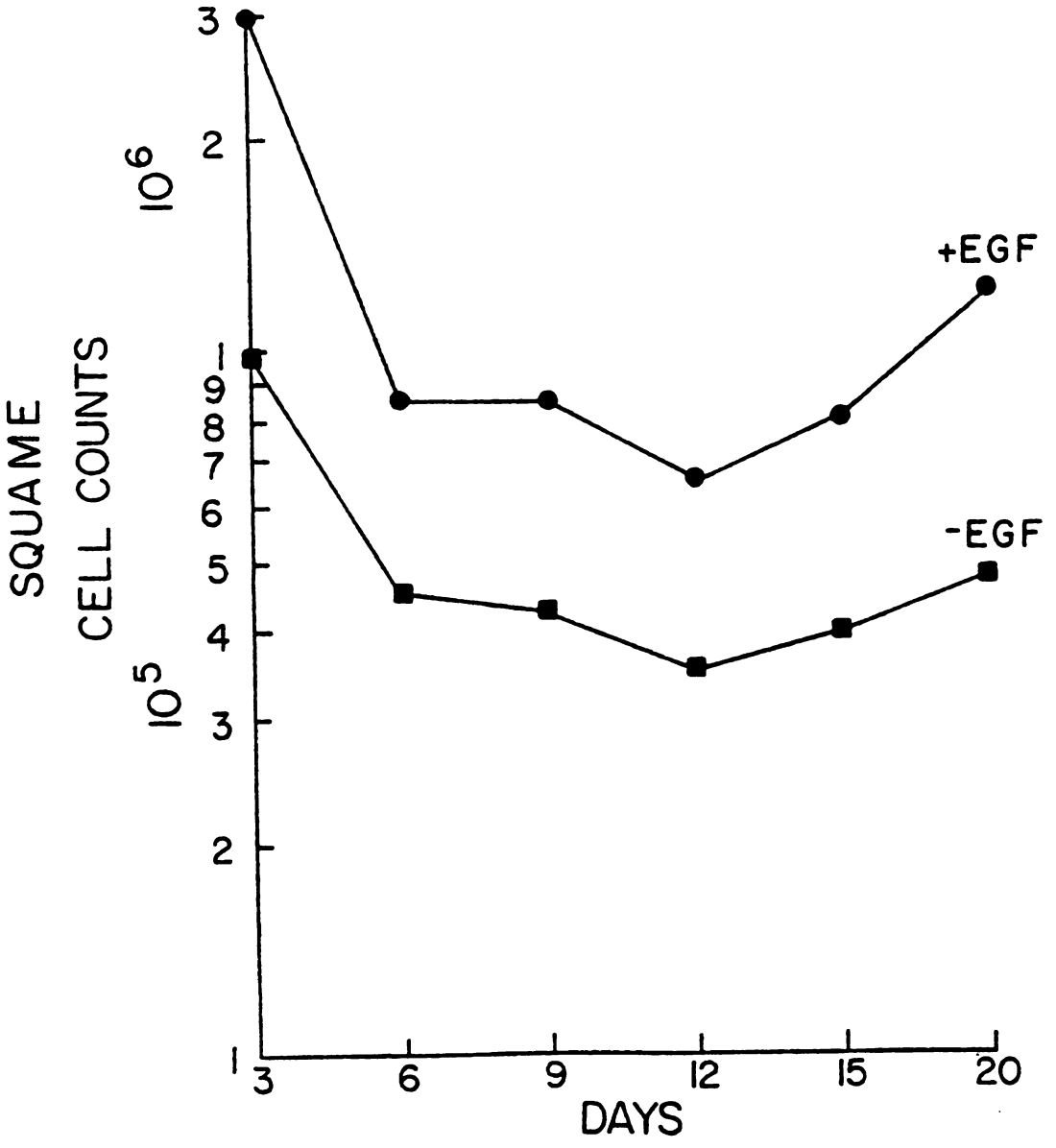
SQUAME
CELL COUNTS
10^6
10^5
3
2
1
9
8
7
6
5
4
3
2
1
+EGF
-EGF
3
6
9
12
15
20
DAYS

Figure 4-12

were in a near diploid number with a modal number of 46 and a range of 36 to 88 (Table 4-1).

Soft Agar Assay. MA cells grew readily in soft agar culture at later passages (after passage 10). Cell passages under 10 grew slowly and formed smaller and fewer colonies (0.05%). MA cells formed colonies in soft agar which were counted after a period of 5 weeks. Colonies containing 40 or more cells were scored, and ranged in size from 1.2 to 200 um. Two of the multicellular colonies were removed with a Pasteur pipet and successfully replated in monolayer culture. NE cells did not form colonies in soft agar.

Oncogenic Potential. Oncogenic potential differed with the passage number of MA cells (Table 4-1). Earlier passages of MA cells (up to 10 passages) inoculated into isologous newborn rats and nude mice gave poor tumor yield but tumor yield increased up to 60-80% at passages beyond 15. The histological appearance of these tumors was carcinoma and adenocarcinoma. One of the NE cell cultures (Sprague-Dawley rat) at passage 80 gave an incidence of 50% tumors which indicates that they became spontaneously transformed in vitro. The other NE primary cells (Lewis rat) up to passage 20 didn't show any indication of spontaneous transformation. The inoculum was negative, even in concentrations of 6×10^6 per newborn rat.

DISCUSSION

The results of the present study indicate successful demonstration of in vitro growth of NE cells and for the first time MA cells derived from ENU-induced rat mammary adenocarcinoma. The cells grew in DMEM containing 25% HS, supplemented with hormones and EGF. Both the NE cells and the MA cells grew as a cell multilayer rather than monolayer, forming up to 5 layers of stratified squamous epithelial cells. EGF increased proliferation and squame formation and induced keratinization in cultured MA cells, an observation heretofore not reported in rat MA cells. The colonies usually extended laterally by cell proliferation and movement, while differentiation began in the center and expanded on the whole surface at confluency, where upper cells formed cornified envelopes which were insoluble in detergents and reducing agents. EGF has been reported to increase the rate of cell replication in human breast cancer cells in vitro[16-19] and of mice mammary gland explants[21,22] and to induce differentiation with squame and keratin formation of cultured human epidermal cells.[13,23,24] At confluency, an increased number of cells were in terminal differentiation phase and when these cells were subcultured they no longer attached to the dish substrate. Those cells accounted for the greatly increased percentage of squame production after 2-3 days when the medium was changed for the first time and the squame quantitated. Then the curve of squame production continued to decrease slightly to the 12th day postculture and raised again toward the 20th day when the cells were subcultured (Figure 4-12).

This phenomenon in addition to strong adherence to the substrate and to each other makes it difficult to evaluate growth parameters with accuracy. As mentioned earlier, terminal differentiation occurs and this can lead to loss of up to 25% (MA) of the cell population in 20 days in confluent cultures. The light microscopic and ultrastructural characteristics of the cultured mammary epithelial cells (NE and MA) resemble those observed in the epidermis in vivo[13,14,23,24] but distinct differences in the morphology of cornified squames indicate an incomplete maturation process. The squames formed in culture had only loosely packed filaments rather than a typical dense keratohylin matrix.

Cultures of normal or neoplastic cells grew as a cell multilayer with formation of domes and piling up (MA) similar to features described in human and rodent epithelial cells.[15,25,26,28] Overlapping and multilayering in culture vessels reveals that the cultures are not conventional monolayers. Multilayering occurs in the initial outgrowth from some explanted epithelial tissues [13,14,23,24] but has not commonly been reported in primary cultures of dissociated normal epithelium. This seems to be a characteristic of the growing pattern of some epithelial cells rather than a consequence of excessive crowding.

Dome Formation. This is a characteristic of many epithelial cell monolayers.[23,26,28,29,30] Some authors have suggested that domes represent in vitro analogues of mammary secretory acini[29] and that their presence is due to local variations in transcellular transport mechanisms.[28] As mentioned above, the cells, during earlier passages, form numerous droplets of unidentified

structures (possible phagosomes) which sometimes accumulated in the stratified cells and could have mediated dome formation. Also, domes were recognized in the stratified cells which were ready to shed, cells which probably lost their transcellular capabilities. Why domes form when and where they do is still an insufficiently explained phenomenon.

Growth characteristics provide insufficient information to distinguish between normal and malignant epithelial cells. However, the ability of cells to grow in soft agar and to form colonies appears to be a reliable indicator of malignancy of ENU-induced MA cells. Consistent with this view is our observation that the NE cells failed to grow in soft agar whereas MA cells grew in the agar.

Interestingly, the NE cells (SNLR/CD1) when transplanted to isologous hosts or nude mice formed palpable anaplastic carcinomas (P_{80}). It appears that at least a portion of the NE cells are very sensitive to spontaneous neoplastic transformation in vitro. MA cells from the earlier passages (up to 10) usually do not produce tumors when they are inoculated into isologous newborn rats. It seems that neoplastic cells originating from ENU-induced adenocarcinomas required multiple passages in vitro until they can induce a high yield of tumors in the appropriate hosts.

The chromosomal pattern was typical of rodentia species. The NE cells (or even those NE cells that spontaneously transformed in vitro) maintained a near diploid modal chromosome number (38 to 40 chromosomes). The MA cells maintained primarily hypodiploid modal number (38 chromosomes). Quantitaion of DNA

by cytofluorometry (see Chapter 5) revealed that rat NE cells in culture had a reduced amount of DNA compared to peripheral blood lymphocytes and fresh, dissociated mammary epithelial cells. Karyotypic analysis showed that these cells were also hypodiploid. Thus in the process of becoming established in cell cultures, rat NE cells lost DNA and chromosomes. These changes may be essential for stable, in vitro growth.

The origin of mammary epithelial cells involved in carcinogenesis continues to be a debatable topic. Information regarding the region of the ductal tree from which the epithelium was obtained may be important with respect to an understanding of carcinogenesis. The ductal system starts at the skin level as large ducts in the nipple branching downward into smaller ductules and, during pregnancy, presumably developing terminal alveoli.[31] In ultrastructural studies of terminal ductules, investigators have not been able to agree on the number of cell types comprising the epithelium.[15,26,32] However, a cell has been consistently recognized containing bundles of cytoplasmic filaments resembling epidermal tonofilaments.[15,27,28,31,32] These cells were also connected by desmosomes. The cells observed in our experiments had similar characteristics. It is possible that they derived from filament-containing cells of the duct.

It appears from the available data that rodent mammary tissue cultures, even with the inconsistency of frequent in vitro spontaneous neoplastic transformation, remain a valuable source of information for the understanding of the complexity of malignant mammary neoplasia.

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CHAPTER 5

N-ethyl-N-nitrosourea-induced in vitro neoplastic transformation of rat mammary epithelial cells

SUMMARY

Normal mammary epithelial cells (NE), originating from Sprague-Dawley (CD) and Lewis female rats, were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 25% horse serum (HS) and hormone supplements. At passage 3, after most of the cells were selectively separated as epithelial cell monolayer, the cells were treated with N-ethyl-N-nitrosourea (ENU) in various doses (25, 50, 100 ug ENU/ml) to study the process of in vitro mammary epithelial neoplastic transformation. The immediate effect of ENU on NE cells was monitored for cellular DNA content measured by flow cytometry, DNA synthesis by (^{3}H) thymidine incorporation assay and chromosomal aberrations using Giemsa banding techniques.

The NE cytofluorometric histograms showed a hypodiploid population. Depending on doses, after ENU exposure, the NE showed a second peak of cells with DNA content in tetraploid or octaploid range. The ENU-exposed NE cells (NET) regained their hypodiploid pattern after 75 to 120 hours. DNA synthesis histograms showed that NET cells (25, 50 and 100 ug ENU/ml) increased DNA synthesis above the control and, depending on doses, blocked mitosis up to 75 to 120 hours. The chromosomal study showed a dose and time effect relationship. Most of the chromosomal aberrations, isochromatid breaks, were at 6 hours post-exposure. Post-ENU exposure, the cell counts and cytology of NET cells showed an increased number of cells and multinucleation compared with NE.

The sequence of phenotypic alteration (termed "stage I-V") was monitored by daily observation. The NET, after a period

of approximately 30 days post-exposure, showed a marked proliferation of morphologically altered cells (piled-up colonies) which subsequently acquired the capacity to form colonies in soft agar and finally became tumorigenic (stage V) when the cells were transferred into an isologous host. The histology of these tumors resembled highly anaplastic carcinomas.

INTRODUCTION

Ethylnitrosourea (ENU)-induced carcinogenesis *in vitro* has been investigated by direct application of carcinogen to fibroblasts and neurogenic cells.[1-3] ENU is a potent alkylating agent, and in contrast to some other carcinogens it is thought to act directly, not requiring enzymatic activation.[2,4] Since the half-life *in vivo* is 30 minutes,[2,5] it is considered to act relatively rapidly both *in vivo* and *in vitro*. Possible targets in the cell are DNA, various species of RNA, and a variety of proteins.[4,6,7]

The interval between primary carcinogen-cell interaction and the onset of tumor growth represents a largely unidentified phase in the process of carcinogenesis, both at the molecular and cellular levels. In this phase many characteristic phenotypic and constitutional alterations occur in the presumptive cancer cells. For a better understanding of this phase of neoplastic transformation *in vivo* experiments would be beneficial. However, this approach is hampered by the complex cellular composition of intact tissues, together with the fact that only a minor fraction of their constituent cells undergo the alterations ultimately resulting in the expression of a

"malignant" phenotype. The problem is further aggravated when dealing wiht epithelial systems because of the difficulty to separate the epithelial cells from the mesenchymal component and grow them as continuous cell lines *in vitro*. Culturing of mammary epithelial cells (NE) *in vitro*, therefore, constitutes one of the most difficult problems in the study of *in vitro* neoplastic transformation.

In recent years a number of papers dealing with *in vitro* neoplastic epithelial transformation, using mostly tissue explants were reported.[8-13] There have been only a few attempts of *in vitro* neoplastic transformation of mammary gland epithelium. Dao used rat organ mammary culture exposed to 7,12-dimethylbenz(a)anthracene as a chemical carcinogen[14] while Cohen used dissociated mammary cells exposed to nitrosomethylurea.[15]

The multistage process of carcinogenesis is well recognized. Cellular changes associated with the initiation and promotion of neoplasia are studied most easily *in vitro*, where the environmental factors can be controlled and cellular responses monitored. Such studies have been difficult to investigate with epithelial cells *in vitro* since there was no satisfactory model system.

Recently, a culture system has been described that satisfies the criteria required for an *in vitro* model of carcinogen-induced mammary gland epithelial cell neoplasia. The cells grown *in vitro* have been identified by morphologic, scanning and transmission electron microscopic criteria as epithelial in origin.[16] The cultured cells, of ductular or

alveolar origin, have been derived from target cell types of a tissue known to be susceptible to ENU-induced neoplasia *in vivo*. In this system, cells were treated in primary culture with ENU soon after isolation from the animal. The NE cells survived *in vitro* for extended periods of time (months rather than days) which is important for analysis of long term changes following exposure to carcinogens. One should be aware, however, of the frequent "spontaneous" neoplastic transformation in *in vitro* rat systems.

In the present study, we investigated the earliest ENU-induced structural and morphological changes in cultured rat mammary epithelial cells. DNA content, rate of DNA synthesis, chromosomal aberrations and cell counts were analyzed at various times up to 120 hours post-ENU exposure. The multistep phenotypic alterations of rat mammary epithelial cells were recorded and morphologically altered cells were implanted into compatible hosts for *in vivo* carcinoma development.

MATERIALS AND METHODS

Source and Isolation of Cells

Epithelial cells for primary normal mammary gland (NE) tissue culture were obtained from the whole mammary gland fat pad (inguinal and axillary) of normal female, specific-pathogen-free (SPF) Sprague-Dawley (CD) and Lewis rats[a] at 50 days of age by previously described techniques.[16] Briefly, after

collagenase (0.1%) treatment, the cell suspension was filtered through one layer of 74 micron opening nylon mesh[b] and seeded in a 60 mm Petri dish containing DMEM[c] supplemented with 25% HS[c] and a hormone combination consisting of 20 ug/ml each of hydrocortisone, prolactin and insulin,[d] and 10 ng/ml of epithelial growth factor (EGF)[e], and 1% antibiotics (penicillin and streptomycin).[c]

The cells were incubated in a humidified 10% CO_2 atmosphere at 37C. At about 8 to 10 hour intervals, the suspended cells in each dish were removed from the incubator and decanted to another dish. This process was repeated about 5 times. For further separation of epithelial cells, differential trypsinization and mechanical separation was applied to the dishes containing epithelial colonies. In about 2 months, dishes with pure epithelial colonies were obtained.

Carcinogen Pulse

At passage 3 (P_3), the tissue culture dishes were treated with 25, 50, 100 and 500 ug/ml of the carcinogen and mutagen[17] acylalkylnitrosamide N-ethyl-N-nitrosourea (ENU). ENU crystals were dissolved shortly before administration in phosphate/citrate-buffered saline at pH 4.2 (1 part buffer to 14 parts saline) and added to the dishes containing medium without serum. The duration of treatment was for a 2 hour period,

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a Charles River Laboratory, Portage, Michigan.
b Cistron Corporation, Elmsford, New York.
c GIBCO, Grand Island, New York.
d Sigma Chemical Company, St. Louis, Missouri.
e Collaborative Research, Waltham, Massachusetts.

after which the supernatant was removed, the cells washed twice with Hank's Buffered Saline Solution (HBSS)[c] and refed with complete medium.

Characterization of NE Treated Cell Lines (NET)

The morphologic appearance of neoplastic cell lines was monitored by phase-contrast microscopy[f] and recorded photographically. Tumors obtained after reimplantation of NET cultured cells were characterized by their histologic appearance.

Flow Cytometric Deterimination of Cellular DNA Content

NE cells P_3, 1 x 10^6 were diluted in hypotonic propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate).[18] Peripheral blood leukocytes (PBL) and a suspension of freshly disaggregated rat mammary tissue cells were treated in an identical manner to determine where the 2N peak occurred in the DNA histograms. Samples with greater than 1% cell clumps were discarded. The instrument used was the Coulter Cell Sorter Model TPS-Large Laser-Two Color System.[g] The argon-ion laser emitted light at 488 nm and the output power was set at 400 nm. Longpass filters used were 515 and 630 nm. Amplification was set so that the 2N modal peak of normal PBL and disaggregated rat mammary tissue peaks occurred in channel number 6. The coefficients of variation for the PBL and mammary cells were 12.1% and 14.2% respectively. Data were collected for each sample such that 10,000 cells were counted in the peak channel. Samples were collected at 24, 48, 72 and 96 hours

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f Nikon Diaphot-TMD, Nippon Kogaku K.K., Tokyo, Japan.

g Coulter Electronics, Hialeah, Florida.

after exposure to 0, 50, 100 and 500 ug ENU/ml of culture medium.

DNA Synthesis

Primary NE at P_3 and P_4 were seeded in 60 mm Petri dishes at concentrations of 7 x 10^5/ml. When the cells reached approximately 50-60% confluency, the treatment with ENU was applied as described above in doses of 0, 25, 50 and 100 ug ENU/ml of culture medium. Three dishes from each dose level were pulse labeled with (^{3}H) thymidine (2uCi/ml) at 24 hour intervals up to 120 hours. The cells were incubated at 37C for 1 hour, trypsinized and processed for the (^{3}H) thymidine incorporation assay. The collected cells were rinsed several times with HBSS, then incubated with 1 ml of 5% trichloracetic acid (TCA) at 37C for 1 hour and centrifuged at 4430 g for 15 minutes at 37C. TCA-insoluble material was dissolved in 0.2 ml of o.2 N NaOH for 15 minutes at 37C. Finally, 100 microliter aliquots (TCA-soluble and TCA-insoluble in NaOH) were transferred to scintillation vials and counted in 9.00 ml of an aqueous counting scintillation fluid.

Cell Counts

Three dishes from each dose level were counted using trypan blue dye exclusion technique and hemocytometer at 24 hour intervals up to 120 hours.

Chromosome Analysis

Chromosome analysis was performed according to established methods[19,20] with minor modifications. Briefly, cells in a logarithmic phase of growth were treated with Colcemid 0.04 ug/ml[c] for 1 hour then harvested with 0.25% trypsin[c] solution.

The cell suspension was then centrifuged at 114 g for 10 minutes and the supernatant was removed and, without disturbing the cell pellet, distilled water was added and the pellet resuspended, and incubated at 37C for 15 minutes. Following hypotonic treatment, the cells were fixed in methanol:glacial acetic acid (3:1) for 15 minutes and slides were prepared by dropping the cell suspension with a narrowed Pasteur pipet from a height of 3-4 feet above the slide and rapidly air dried. G-banding was performed by a modification of the method of Yunis[20] with a trypsinization time of about 15 seconds.

ENU-induced chromosomal aberrations were scored according to World Health Organization (WHO)[21] and others.[3,23]

Colony Formation and Cloning in Semisolid Agar Medium

The ability of rat mammary epithelial cells to form colonies in agar was determined as described by MacPherson[25] with minor modifications. The basal layers contained 5ml of 2% Sea Plaque Agarose[h] supplemented with DMEM, 25% HS and 0.2% sodium bicarbonate in 60 mm plastic Petri dishes. A second layer (1.5 ml) of 0.33% Sea Plaque Agarose supplemented as above, containing 7.5×10^4 dilutions of cells/plate was added. Plates were incubated at 37C in a 10% CO_2 atmosphere. Five to six weeks after plating, colonies containing greater than 40 cells were counted using an inverted phase microscope.[f] Cloned sublines of established "parental" tumorigenic cell lines were obtained by aspiration of single colonies from the cloning medium with a Pasteur pipet followed by trypsinization for 1-2 minutes then transferred to a 60 mm Petri dish for further culture.

Oncogenicity

Suspensions of cells (stage V NET and control NE) diluted to 1 to 2 x 10^6 cells in 0.05 ml of culture medium were inoculated subcutaneously into the interscapular region of 10 newborn CD and Lewis rats and 10 athymic nude mice. The rats were examined three times weekly for progressively growing tumors at the site of inoculation. Six to eight weeks later, the animals were sacrificed and all palpable nodules and tumors were surgically excised and prepared for routine tissue culture, electron microscopy and histological examination as described above.

Statistics

Quantitative changes were assessed for significance by the two way analysis of variance followed by the Duncan's Multiple Range Test post-test. Changes were considered significant if $p \leqslant 0.05$.

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h Marine Colloids Division, FMC Corporation, Rockland, Maine.

RESULTS

Untreated NE in Culture (Controls)

The NE cells were obtained from CD female rats and maintained in culture as was described in Materials and Methods. After approximately two months, a pure epithelial cell culture consisting of cuboidal cells arranged in a "cobblestone-like" pattern and showing dome formation at confluency was obtained (Figure 5-1). The morphological and electron microscopic characterization of these cells were previously reported.[16] The NE cells had a slow rate of growth for the first 3 passages. Although the growth rate increased in later passages it remained slower than that of the NET cells. The population doubling time (PDT) decreased with increased passages, and the plating efficiency remained low when compared to NET cells. The NE cells did not form colonies in soft agar and did not produce tumors when implanted into appropriate hosts, with the exception of one cell line which continued to grow, forming piled-up colonies at P_{60}, grew in soft agar and induced tumors (P_{80}) in 50% of newborn CD rats upon inoculation. Five other primary epithelial cells were maintained in culture for about 2-4 passages when they degenerated and died. Another NE culture, derived from a Lewis rat, continued to grow and at P_{35} showed no indication of transformation (4 months in culture).

ENU-Treated Cells (NET) and Development of Neoplastic Mammary Gland Cell Lines

In contrast to the control cultures, NET cells, from 2 cell lines exposed to ENU *in vitro*, underwent a characteristic and reproducible sequence of phenotypic alterations preceeding

Figure 5-1. Inverted microscopic appearance of confluent normal mammary epithelial cells culture with cobblestone-like pattern and dome formation. (x 120).

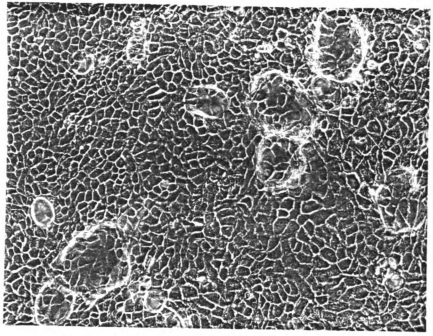


Figure 5-1

the expression of neoplastic properties (Figure 5-2). The duration of the individual phases of this process, termed "stage I-V",[1,8] was similar in both cultures. There were variations in time sequences relative to the dose of ENU. For instance the recovery period following exposure to 500 ug ENU/ml was longer when compared to doses of 25, 50 and 100 ug ENU/ml; at doses of 500 ug/ml 50% of the cells died out and small epithelial-like colonies were observed to develop among the larger sensecent cells after a latency period of 12 days.

Stage I

During the early phase of observation (up to 6 days), primary NET cells showed slight differences from the corresponding controls. An increased number of multinucleated cells (binucleated and trinucleated) in NET cells (50 and 100 ug ENU /ml) was observed after 72 hours post-treatment in contrast to controls (Figure 5-3). Depending on dose, the NET cells showed a slight to severe altered morphology, consisting of large, polyhedral or heterogeneous cells with loose cell to cell adherence (senescent cells). The monolayer failed to maintain the tight "cobblestone-like" pattern. The multinucleated and senscent cells died out after a short period of time (approxmately 5-10 days).

Stage II

After a few days (7-15 days), isolated colonies of small cuboidal epithelial cells developed and maintained this morphology throughout stage II (Figure 5-4). These cells had a faster rate of growth than NE cells although multilayer growth and formation was similar to the homogeneous cuboidal appearance of

Figure 5-2. Diagrammatic representation of the *in vitro*-*in vivo* system for pulse carcinogenesis by ENU in rat mammary epithelial cells.

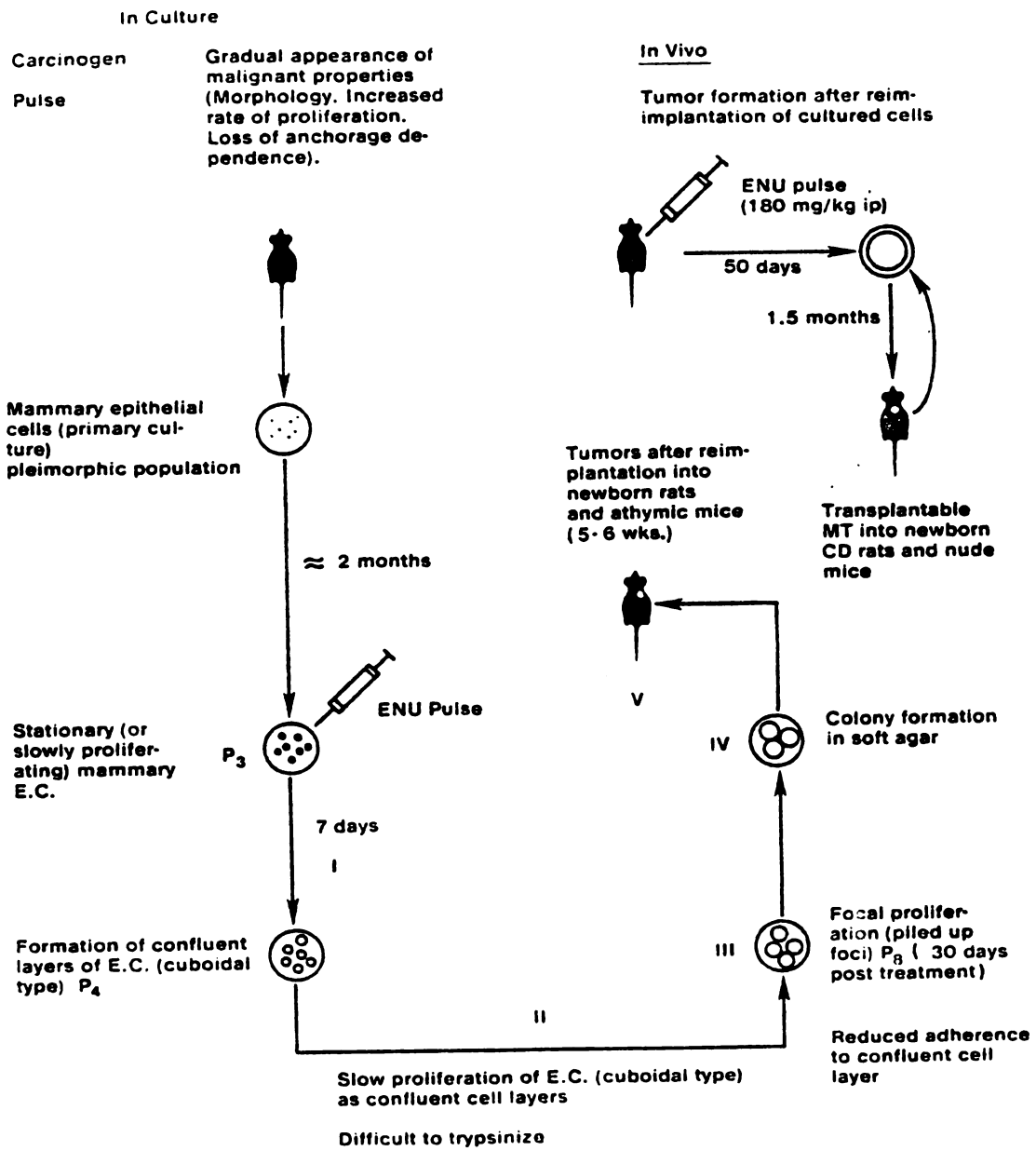

In Culture
Carcinogen
Pulse
Gradual appearance of malignant properties (Morphology. Increased rate of proliferation. Loss of anchorage dependence).
In Vivo
Tumor formation after reimplantation of cultured cells
ENU pulse (180 mg/kg ip)
50 days
1.5 months
Mammary epithelial cells (primary culture) pleimorphic population
≈ 2 months
Tumors after reimplantation into newborn rats and athymic mice (5-6 wks.)
Transplantable MT into newborn CD rats and nude mice
ENU Pulse
Stationary (or slowly proliferating) mammary E.C.
P3
V
IV
Colony formation in soft agar
7 days
I
Formation of confluent layers of E.C. (cuboidal type) P4
III
Focal proliferation (piled up foci) P8 (30 days post treatment)
II
Reduced adherence to confluent cell layer
Slow proliferation of E.C. (cuboidal type) as confluent cell layers
Difficult to trypsinize

Figure 5-2

Figure 5-3. Stage I NET cells (ENU 100 ug/ml) 72 hours post-treatment showing increased numbers of senescent and multinucleated cells. (x 200).

Figure 5-4. Stage II NET cells (ENU 100 ug/ml) 7 days post-exposure. Epithelial colony (center) surrounded by sensecent cells. (x 200).

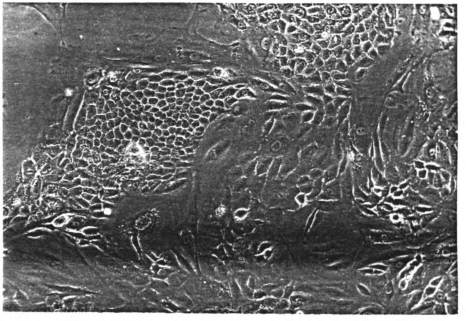


Figure 5-3

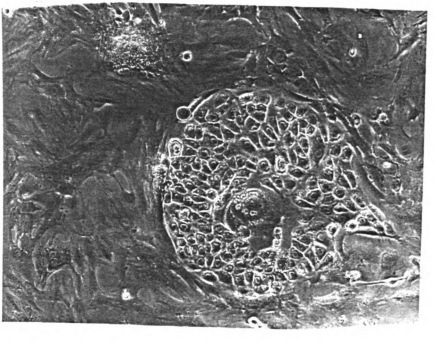


Figure 5-4

NE cells. This phase extended from 7 to 30 days post ENU-exposure.

Stage III

A subsequent phase of moderate proliferation resulted in the formation of piled-up foci (spheroids) of epithelial cells, 30 days post-exposure to ENU (Figure 5-5). These "piled-up" foci persisted and slowly grew larger and were easily removed when flushed gently with medium. These cells maintained their previous morphologic appearance, grew as cell multilayers with a tendency to form piled-up colonies. The piled-up colonies were sensitive to trypsin during this period. The overall PDT was decreased and the maximum cell number per unit was increased in this phase. Implants of stage III NET cells into newborn rats failed to grow.

Soft Agar Assay (Stage IV)

Spheroids from NET cells were collected by flushing and were recultured for 2 more passages in liquid medium and then seeded into soft agar. The NET cells formed colonies in soft agar cultures and were scored after a period of 5-6 weeks. The average initial cloning efficiency was 0.2% (P_6) and then increased to 4% (P_8). The colonies diameters ranged in size from 1.2 to 200 um (Figure 5-6). Two of the multicellular colonies were removed with a Pasteur pipet, trypsinized and replated in liquid media. The cells grew as a cell multilayer and retained the ability to pile-up.

Tumorigenicity (Stage V)

The tumorigenicity of the cells was assessed by subcutaneous inoculation of cell suspensions at 1 to 2 x 10^6 cells

Figure 5-5. Stage III NET cells (ENU 100 ug/ml) 30 days post-exposure. A focus of active cellular proliferation precluding development of piled-up colony. (x 48).

Figure 5-6. Soft agar colony of NET cells 5 weeks post-seeding. (x 48).

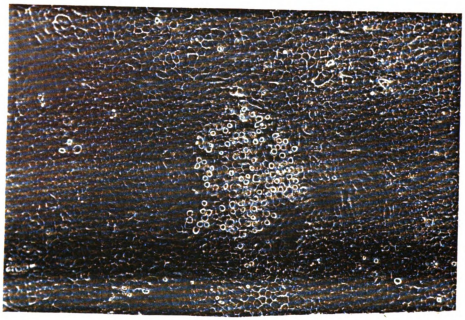


Figure 5-5

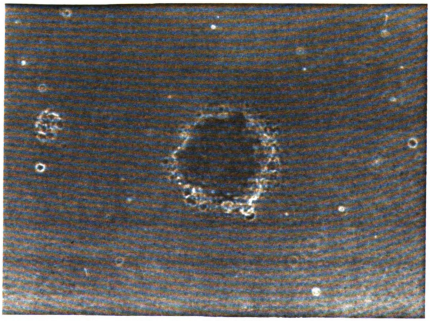


Figure 5-6

into newborn isologous rats. After 6 weeks post-inoculation, tumors developed at the site of inoculum in 8/10 Lewis and 9/10 CD inoculated rats and 5/10 nude mice. The gross, histologic and electron microscopic examination of the tumors recovered from the implanted rats revealed a carcinomatous pattern (Figure 5-7,8). The cell lines obtained from the reimplanted NET cells not only phenotypically resembled the original cells, but they also maintained the characteristic of piling-up. The time to move from stage I to stage V took an average of 70-90 days or 10 passages.

Characteristics of the Immediate Changes in NE Post ENU-Treatment

Cytometric measurements of cellular DNA content. Figure 5-9 shows the DNA histograms following the addition of ENU to the cell cultures. The ordinate represents the frequency (number of cells per channel) while the abscissa represents the channel number (from 1 to127). Columns represent ENU concentration; from left to right are 0, 50, 100 and 500 ug ENU/ml. Rows represent 24, 48, 72 and 96 hours following exposure to ENU. In the untreated cells the modal population was channel number 4 which was hypodiploid compared with the normal cells that had the 2N peak in channel 6. Although a distinct peak was not present, a shoulder was evident in the area of channel 8 representing tetraploid cells. On days 2 and 3 the untreated cells showed a distinct second peak with modal channels of 18 and 20, respectively, which was slightly greater than an octaploid population of cells. At 24 hours following the addition of 50 ug ENU/ml to the cultures the hypodiploid peak was still

Figure 5-7. Gross appearance of tumors 5 weeks after the implantation of 2×10^6 NET cells subcutaneously into nude mice.

Figure 5-8. Histological appearance of a solid carcinoma harvested from a nude mouse transplanted with 2×10^6 NET cells. (H&E x 200).

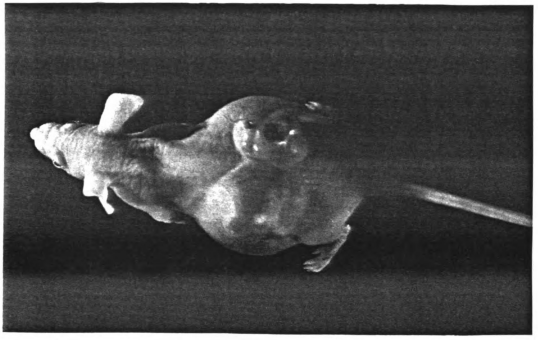


Figure 5-7

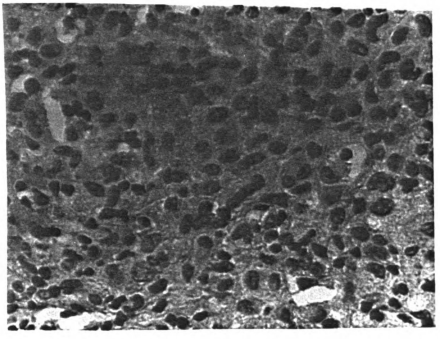
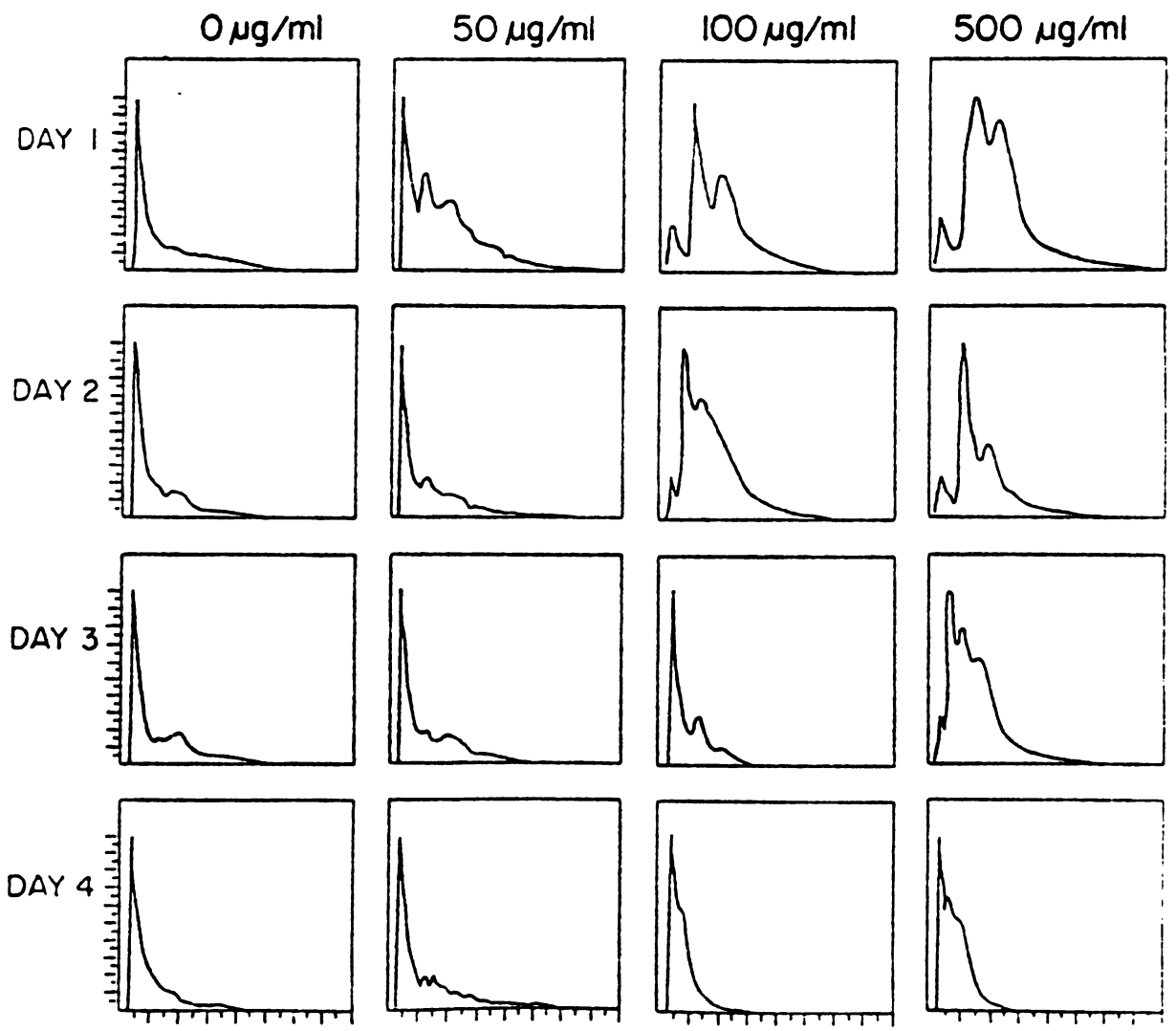


Figure 5-8

Figure 5-9. Cytometric DNA histogram following the addition of ENU to the cell cultures.



Cytofluorometric DNA histograms of normal rat mammary gland cells exposed to various doses of ENU.

Figure 5-9

present but there were also other peaks that appeared. The modal channel numbers for these peaks were 11 and 20, respectively. On days 2 through 4 there was a recovery towards the distributions seen with the untreated cells. Similar changes occurred where 100 and 500 ug ENU/ml were used. With these higher doses the modal channel numbers for the peaks beyond the tetraploid region were similar to those modal channel numbers seen at the 50 ug ENU/ml dose.

In Figure 5-10 the ordinate shows the percent of resting cells. This was the number of cells that appeared in channels 1 through 11 divided by the number of cells counted, expressed as a percentage. This figure was used to demonstrate the rate at which the treated cells returned to their resting or hypodiploid state. Over the 4 days of the experiment, about 60% of the untreated cells were in this hypodiploid region. There was a dose dependent decrease with time in the fraction of hypodiploid cells and the rate of recovery to the hypodiploid state was inversely related to dose ($p < 0.05$).

DNA Synthesis

The diagrammatic representation of (^{3}H) thymidine incorporation assay of the normal mammary gland epithelial cells treated with ENU is illustrated in Figure 5-11. Peak rates of thymidine incorporation occurred at 96 hours following treatment with ENU and in control cultures. Cells treated with ENU however, had rates of incorporation greater than control cells (p 0.05).

The histogram of cell counts processed (Figure 5-12) in parallel with incorporation assay (Figure 5-11) illustrated

Figure 5-10. Diagrammatic representation of the DNA content illustrating the percent of resting cells exposed to various doses of ENU during a 4 day period. This figure was used to demonstrate the rate at which the treated cells returned to their resting or hypodiploid state.

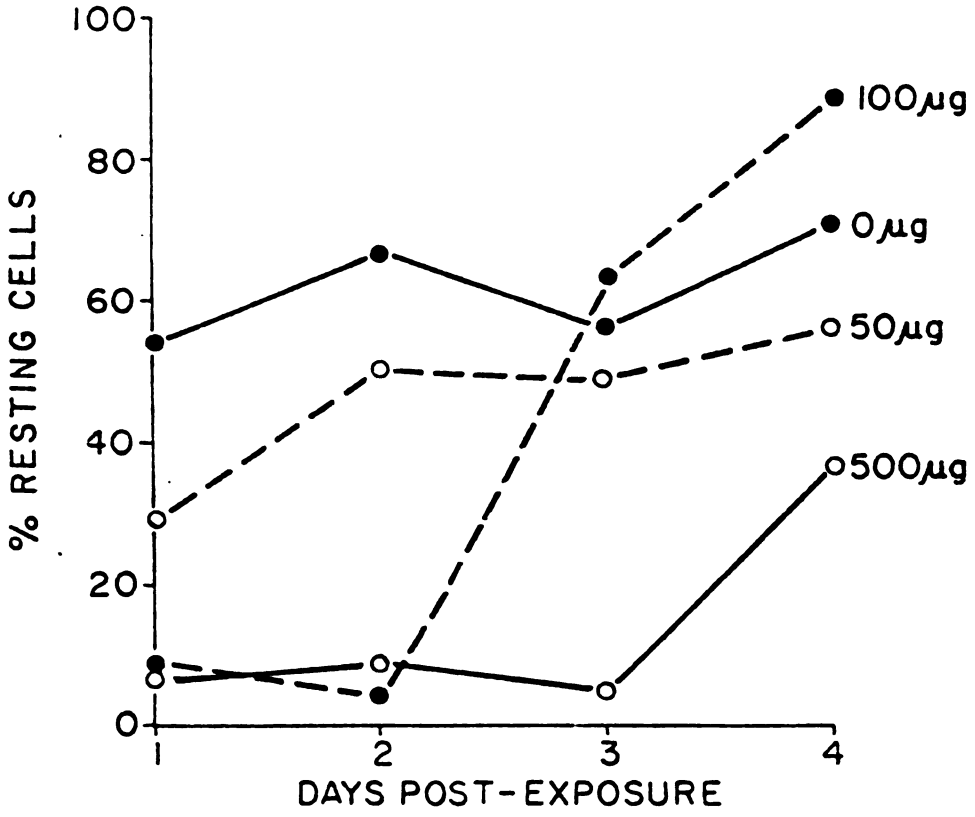

100
80
60
40
20
0
% RESTING CELLS
1
2
3
4
DAYS POST-EXPOSURE
100µg
0µg
50µg
500µg

Figure 5-10

Figure 5-11. Diagrammatic representation of (^{3}H) thymidine incorporation assay of the NE cells exposed to various doses of ENU.

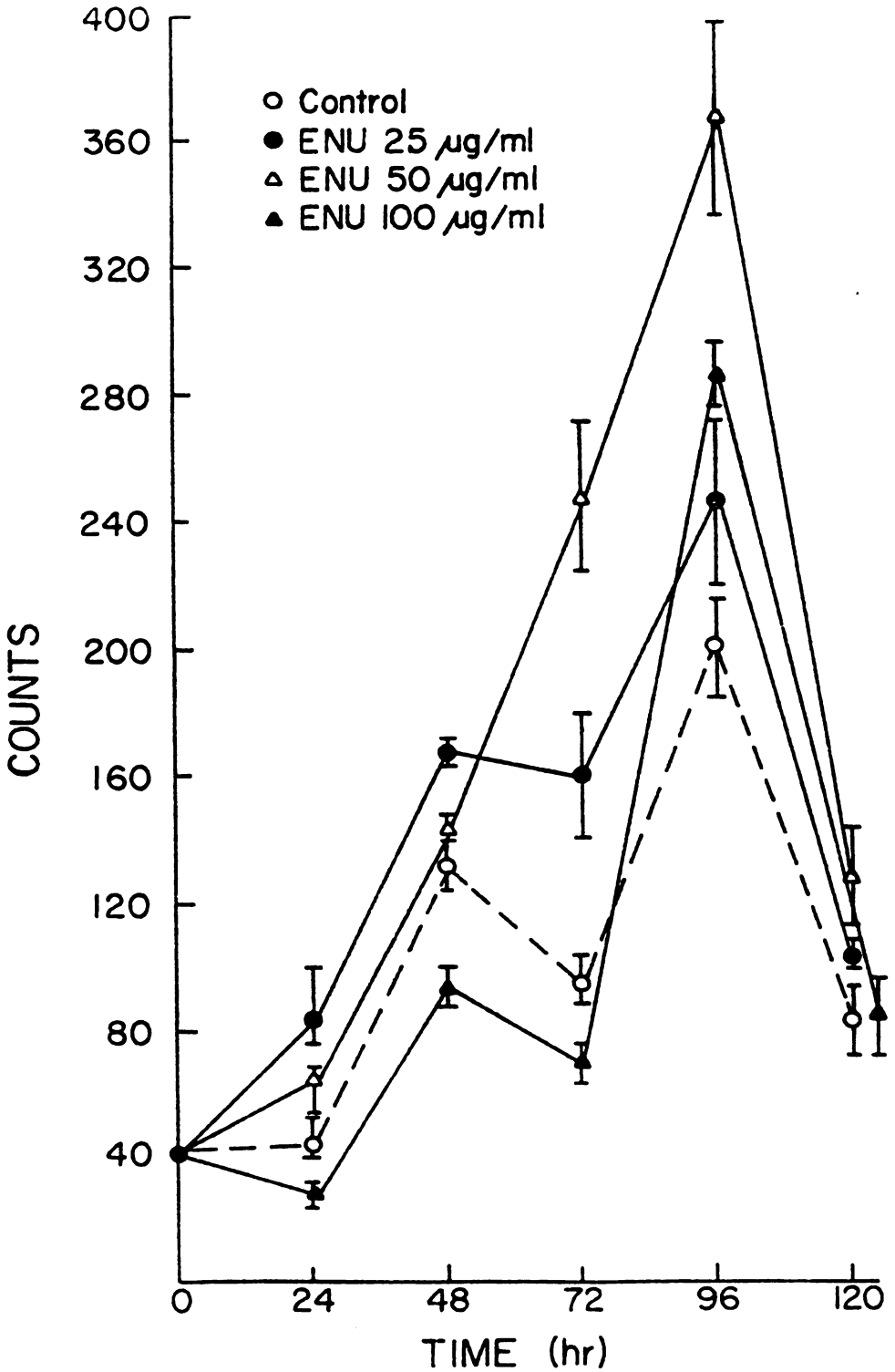

400
360
320
280
240
200
160
120
80
40
COUNTS
Control
ENU 25 µg/ml
ENU 50 µg/ml
ENU 100 µg/ml
0
24
48
72
96
120
TIME (hr)

Figure 5-11

Figure 5-12. Diagrammatic representation of the NE cell counts exposed to various doses of ENU.

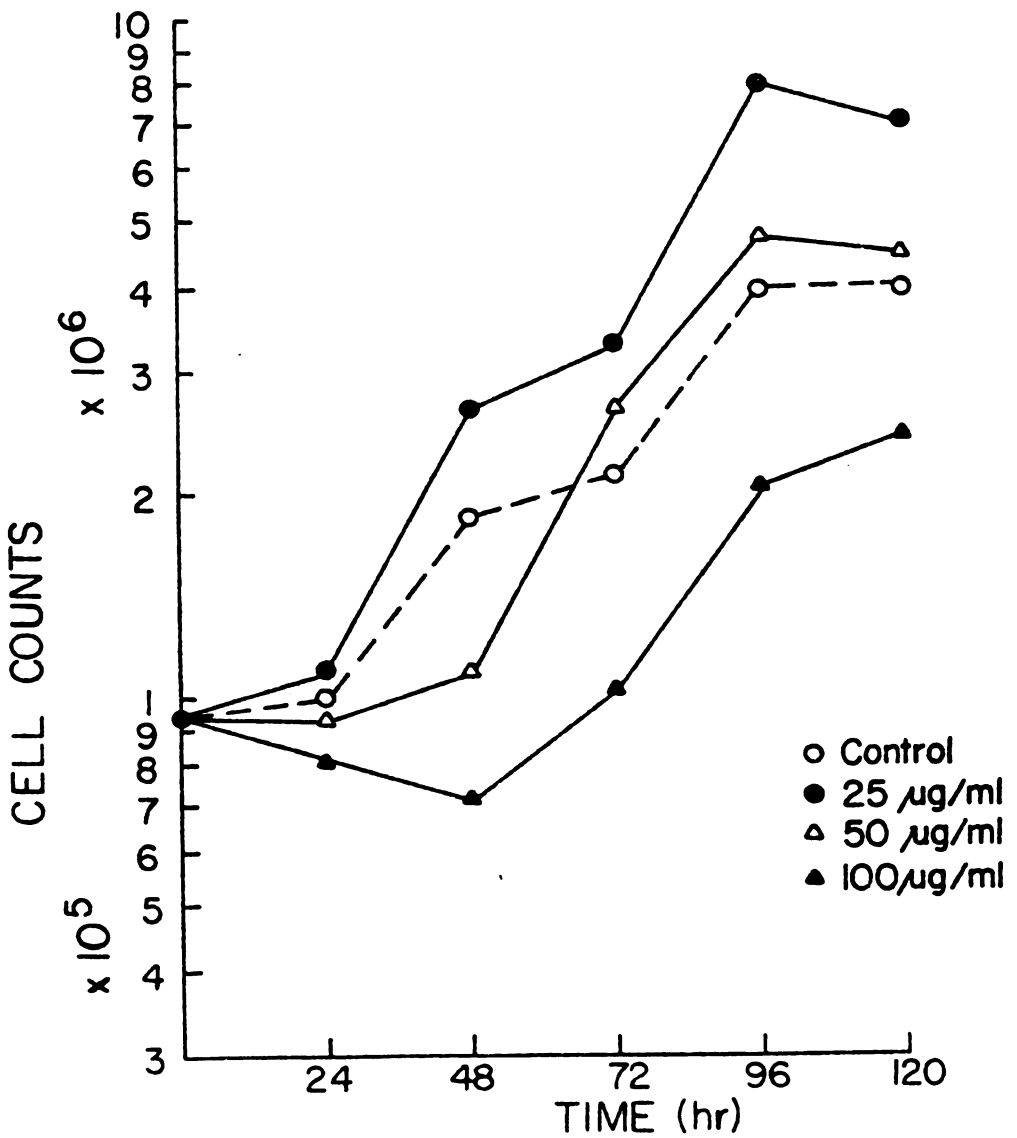

CELL COUNTS
x 10^6
x 10^5
10
9
8
7
6
5
4
3
2
1
9
8
7
6
5
4
3
24
48
72
96
120
TIME (hr)
Control
25 µg/ml
50 µg/ml
100µg/ml

Figure 5-12

that the number of cells, treated with 25 and 50 ug ENU/ml, were slightly increased above the control throughout the test period (24 to 120 hours). There was a significant difference at 96 hours with 50 ug ENU/ml ($p < 0.05$).

The cytologic examination of the cells exposed to 50 and 100 ug ENU/ml showed multinucleated cells which increased progressively from 24 hours up to 120 hours.

Cytogenetic Findings

The NE cells showed a near normal diploid chromsome number with a modal number of 38 ranging between 36-60. The findings from chromosome studies after ENU exposure of NE cell cultures are illustrated in Table 5-1. In each experiment 2 or more doses of ENU were tested. A dose-related effect at 25, 50, 100 and 200 ug ENU/ml was demonstrated. At 500 ug ENU/ml up to 72 hours none or few mitoses were identified and only 50 metaphases were recorded at 72 to 96 hours. A dose as low as 25 ug ENU/ml produced chromosome damage in 16% of the cells. In cultures exposed to ENU, 24 hours before harvest, abnormal metaphases were almost half the number of those treated at 6 hours. At 96 hours post-treatment, abnormal cells decreased to near control values.

The predominant type of aberrations observed in this experiment, regardless of dose and time, were the isochromatid and chromatid breaks (Table 5-1). Most of the exchanges were seen when cells were exposed to ENU, 24 hours before harvest. Small DNA-containing particles called double minutes (dm) were observed in the metaphases of cells exposed to ENU and increased with higher doses and after 72 hours and 96 hours post-exposure.

Table 5-1. Effect of ENU *In Vitro* on chromosomes of rat mammary cultures (P_4 and P_5).

Dose ENU ug/ml	No. Experiments	Percent Cells with Aberrations	Types of Aberrations/100 cells: Single Chromatid Breaks	Iso-chromatid Breaks	Exchanges	Multiple Breaks	D.M. (metaphases with D.M.)
Control	2	9(8-10)	4	5			
ENU added 6 hours before harvest							
25	2	15(14-16)	6	9			
50	2	25(20-30)	5	10	5	3	2
100	2	45(40-50)	20	10	5	5	5
200	2	50(48-52)	30	15		5	5
ENU added 24 hours before harvest							
25	2	10(9-11)	10				
50	2	12(10-14)	5	3	2		2
100	2	25(15-26)	10	2	10		3
200	2	27(17-29)	12	4	10		1
500	2	MI					
ENU added 72 hours before harvest							
100	2	15(10-18)	5	5	1		4
200	2	20(15-22)	5	5	5		5
500	2	20*(15-25)	8	10	2		8
ENU added 96 hours before harvest							
100	2	10(6-14)	4	2			4
200	2	12(6-16)	6	2	2		2
500	2	18(8-10)	8	2	2		6

MI - Mitotic Inhibition
* - Counted only 50 metaphases
D.M. - Double Minutes

The number of "dm" per cell was between 2 and 5. Fewer exchanges (Figure 5-13) and multiple breaks were mostly related with the higher doses of ENU.

Giemsa banding studies of NET cell line karyotypes consistenly revealed the presence of chromosomal markers. Trisomy of chromosome #10 was found with higher frequency (30%) (Figure 5-14). Trisomy of chromosome #19 appeared as an incidental finding on the same metaphase (Figure 5-14).

Karyotyping of NET cells recovered from tumors after reimplantation into appropriate hosts, revealed many abnormalities, such as translocation, dm, and loss of sex chromosomes. The chromosome numbers were increased in reimplanted NET cells showing a modal number of 48 with a range of 36 to 96 chromosomes per metaphase.

Figure 5-13. NET cell metaphase, 72 hours post-ENU exposure, revealing isochromatid exchanges (arrow). (x 1200).

Figure 5-13

Figure 5-14. NET cell metaphase karyotype (stage IV) showing trisomy (1) of chromosome #10, trisomy (2) of chromosome #19 and double minutes (3). (x 1200).

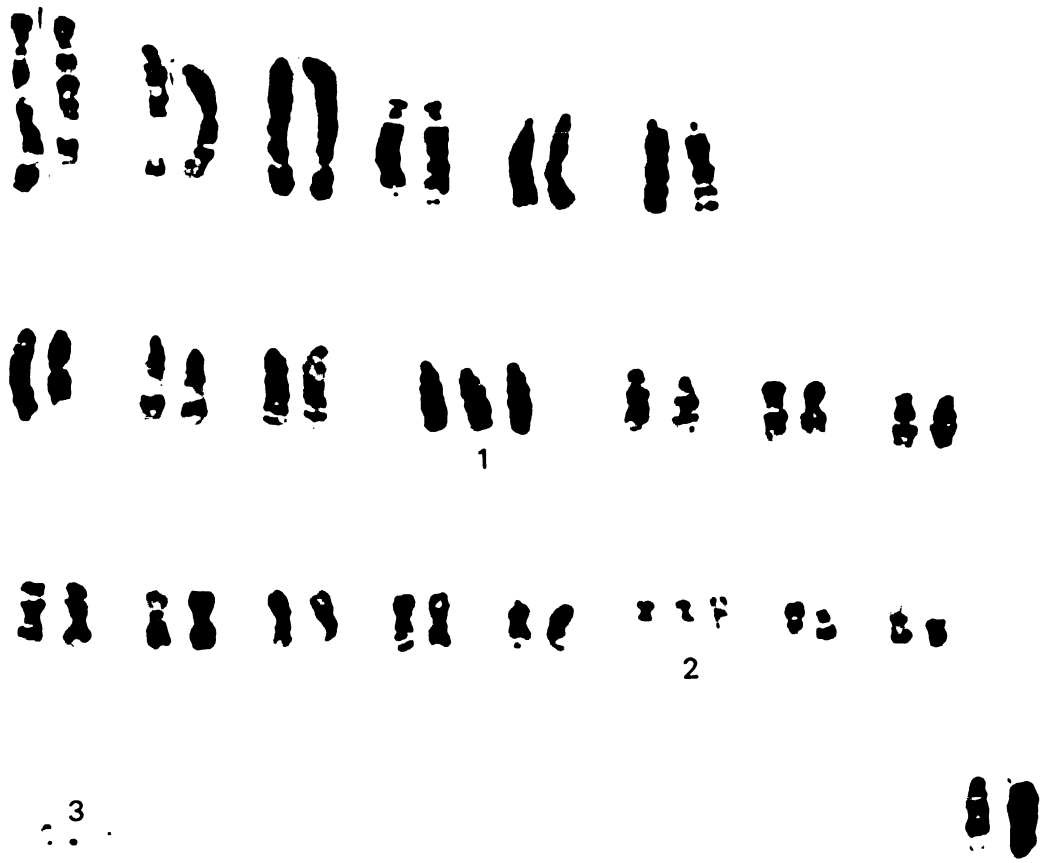


Figure 5-14

DISCUSSION

As it was demonstrated in vivo,[26,27] the present study indicates that mammary gland tissue constitutes a target organ for the mutagenic and direct acting carcinogen effect of ENU. In the in vitro system, the average interval between ENU exposure and the first observation of tumorigenicity (morphologically transformed mammary epithelial cells) was approximately 70-90 days. This was similar to the mean induction time of in vivo mammary tumor development induced with high doses of ENU in CD female rats.[26,27]

Quantitation of DNA by cytofluorometry revealed that rat mammary cells in culture had a reduced amount of DNA compared with PBL and fresh, dissociated mammary tissue. Karyotypic analysis showed that these cells were also hypodiploid. Thus in the process of becoming established in cell culture, rat mammary epithelial cells lose DNA and chromosomes. These changes may be necessary for stable, in vitro growth.

The DNA frequency distributions of untreated mammary epithelial cells in culture had the appearance of a randomly dividing cell population. The shoulder on the hypodiploid peak represented the tetraploid population. This population was not more apparent due to the relatively low concentration of tetraploid cells and the low amplification setting used for the cytofluorometer. A high amplification setting probably would have made the tetraploid peak more apparent. Also present in the untreated cells were some cells with a near-octaploid amount of DNA. These cells probably represented multinucleated cells that were seen on phase contrast microscopy

and stained preparations. Cell clumps did not interfere with the study since we discarded any sample with greater than 1% cell clumps. Since the untreated and treated NE cells had identical peak modal channel numbers, it is not likely that ENU altered the binding of propidium iodide for DNA.

The effect of ENU on DNA frequency distributions of rat mammary epithelial cells was to increase, in a bimodal fashion, the number of cells with greater than the resting, hypodiploid amount of DNA. The modal channels for the 2 additional peaks were around 11 and 20. These peaks may represent tetraploid and octaploid cells, respectively. Subjective microscopic evaluation of the cell cultures showed that treated-cultures had more multinucleated cells than controls.

The results of the experiment studying incorporation of thymidine showed that ENU-treated cells incorporated thymidine at a greater rate than control cells. This serves as indirect evidence that ENU stimulated DNA synthesis. These data coupled with the results showing accumulation of near tetraploid and near octaploid cells following treatment with ENU suggested that ENU stimulated DNA synthesis and blocked cell division. These effects of ENU were only temporary; the rate of thymidine incorporation decreased along with control cells and the DNA distributions returned toward pre-exposure distributions by the end of the experiment.

The explanation of the observations described above most likely resides in the fact that alkylating agents, although dependent on proliferation, are not cell-cycle specific, and the drugs may act at any stage of the cycle. However, the toxicity

is usually expressed when the cell enters the S phase and progression through the cycle is blocked at the G_2 (premitotic) phase.[28] Cells appear more sensitive in late G_1 or S then in G_2, mitosis, or early G_1. Polynucleotides are more susceptible to alkylation in the unpaired state than in helical form. During replication of DNA, positions of the molecule are unpaired. The cells accumulated behind the block at G_2 may have a double complement of DNA while continuing to synthesize other cellular components, such as protein and RNA. This may result in unbalanced growth, with the formation of enlarged or giant cells that may continue to synthesize DNA, making as much as four or five times the normal complement.[29] These mechanisms of action of ENU may account for the results in the present study. ENU may block those tetraploid cells from dividing and stimulate them to synthesize more DNA, bringing them up to a near octaploid state prior to dissipation of the effect.

Chromosome analysis of <u>in vitro</u> rat NE exposed to ENU indicated both dose and time effect. The greatest number of cells showing chromosomal damage occurred when cells were treated 6 hours before harvest (Table 5-1). Types of breaks were principally chromatid and isochromatid breaks suggesting that one mechanism of action of ENU could be on the chromsome structure.

Trisomy of chromsome 10 was the most consistent marker associated with <u>in vitro</u> ENU-induced neoplastic transformation of rat mammary epithelial cells, though trisomy of 19 chromosome was also found. Excess chromosome 10 is presumed to arise by mitotic nondysjunction and rearrangements, but its significance in the neoplastic process is not known. Comparison of chromo-

somal findings in these early stages with those of gross tumors would be important. However, good metaphases are difficult to obtain directly from solid tumors.

The presence of "dm" in metaphases was first considered to be a specific finding in neurogenic tumors,[30,31] but recently these were recognized in many other tumor types especially in breast cancer.[33] Their origin and significance remain debatable. Recent observations have demonstrated that there is a close interrelationship between "dm" and so-called homogeneously staining regions (HSR).[33] Thus, it has been shown that "dm" can be transformed into HSR and vice versa,[30, 31] but they also can originate from other regions.[34] The importance of these "dm" in carcinogenesis is not known.

ENU-treatment of NE cells showed a dose-effect relationship expressed by various phenotypic and numeric and structural chromsomal aberrations. However, the significance of these changes is unknown and may have only indirect relevance to carcinogenicity, for example as an indicator of cell damage.

Phenotypic Changes

In regard to phenotypic changes, a common feature of stage III was a tendency toward squamous differentiation and irregularity of nuclear size. The cells at this stage inoculated into isologous hosts did not induce tumors. Neoplastic cell lines transformed *in vitro* invariably derived from stage III foci. This stage probably is equivalent with the *in vivo* proliferative nodules (wholemount preparation) observed at the terminal end buds (TEB) of rat mammary gland post ENU-treatment.

[26,35,36] Evidence suggests that malignant mammary tumors may develop from these proliferative nodules. However there is no direct evidence that other types of carcinomas arise from islands of altered squamous cells *in vivo*.[8]

More work is needed to determine if the described *in vitro* epithelial cell neoplastic transformation could be related with activation of the "ras" oncogene as it was demonstrated with nitroso-methylurea.[37]

The ENU-induced reproducible sequence of *in vitro* neoplastic transformation constitutes a valuable model for the study of earliest neoplastic alterations of human breast cancer in particular and carcinomas in general.

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VITA

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