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The effect of transplacental exposure to diethylstilbestrol on mammary gland development in the peripubertal female ACI rat

Vassilacopoulou, Dido, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE EFFECT OF TRANSPLACENTAL EXPOSURE TO DIETHYLSTILBESTROL ON MAMMARY GLAND DEVELOPMENT IN THE PERIPUBERTAL FEMALE ACI RAT

by

DIDO VASSILACOPOULOU

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THE EFFECT OF TRANSPLACENTAL EXPOSURE TO DIETHYLSTILBESTROL ON MAMMARY GLAND DEVELOPMENT IN THE PERIPUBERTAL FEMALE ACI RAT

by

Dido Vassilacopoulou

Adviser: Professor Elizabeth S. Boylan

Female ACI rats were exposed to diethylstilbestrol (DES) in utero, to evaluate effects on: 1) mammary gland development 2) mammary gland sensitivity to natural and synthetic estrogens, and 3) mammary gland sensitivity to endogenous epidermal growth factor (EGF). Additionally, a study was undertaken to examine certain aspects of morphological and physiological differences observed between rats displaying intact or unilateral agenesis of the genital tract. Pregnant rats were injected with vehicle (sesame oil) or DES (total dose, 8.0 μg) on days 15 and 18 of gestation. DES-exposed and control offspring were ovariectomized at 34 days of age and sacrificed at day 53 to ascertain the morphology of the mammary glands in peripubertal rats. A separate group of DES-exposed and control animals was implanted with Elvax pellets containing estradiol 17B (E2) or DES at 5 or 11 ng adjacent to the third mammary gland pair. Furthermore, an additional group of rats was subjected to bilateral sialoadenectomy at

the day of ovariectomy to remove the major source of endogenous EGF. Mammary glands of DES-exposed animals exhibited atypical mammary gland morphology, tending to hypo- or hyper-differentiation, and seemed to be refractory to stimulation by DES or E2, when compared to controls. Sialoadenectomy had no apparent effect on mammary gland morphology in either the DES-exposed or vehicle-exposed groups.

Uterine and mammary morphological differences were observed between rats with intact genital tracts and unilateral agenesis of the reproductive tract. The timing of vaginal opening was found to be accelerated in rats with unilateral agenesis. These pronounced differences resulted in exclusion of all rats with unilateral agenesis from the tabulation of data on the effects of prenatal exposure to DES which were described above.

DEDICATION

To my mother Evangelia my sister Despina and my brother Tasso whose love, support and constant encouragement made the completion of this project possible.

To my nephews George and Ari whose arrival in our family made being away from home alot more difficult.

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INTRODUCTION

The objectives of this dissertation were to investigate the effects of prenatal exposure to the synthetic estrogen diethylstilbestrol (DES) on mammary gland development of the peripubertal ACI rat, and on the differences in response of the mammary epithelium of DES-exposed rats to stimulatory molecules. Therefore, this INTRODUCTION includes sections on the embryology and postnatal development of the mammary gland, followed by sections on the control of mammary development by hormones and growth factors, and finally sections on the chemistry, metabolism and effects of perinatal exposure to DES.

EMBRYOLOGY OF THE MAMMARY GLAND

Mammary glands are organs that are derived from the ventral skin. In most mammals, mammary rudiments do not form as separate individual buds from the ectoderm. In contrast they appear as a formation of a 'mammary band', an ectodermal thickening running along each side on the lateral-ventral side of the body wall (Kratochwil,1975). This band breaks up soon to give rise to the individual mammary gland rudiments.

The rodent mammary epithelium first appears at 10-11 days in the rat embryo, with enlargement of a single layered ectoderm forming the mammary streak. This streak is a

bilateral line of cells that extends from the anterior limb bud to the posterior limb bud (Myers, 1917; Turner and Gomez, 1933;). On the 12th day of gestation, the mammary bud appears. Propper (1968) has concluded that the formation of the mammary band is the result of inductive stimuli that originate from the underlying mesenchyme. By the 14th day the mammary bud changes to a bulb-shaped structure with a narrow neck. At the same time the gland's sexual phenotype is determined (Sakakura, 1987).

Kratochwil and his collaborators have indicated that in males, mammary mesenchyme is the target tissue for androgens (Kratochwil, 1971; Kratochwil and Schwartz, 1976; Durnberger and Kratochwil, 1980). Androgen receptors on the mammary mesenchyme are induced by mammary epithelium (Heuberger et al., 1982). If androgen is present, the mammary mesenchyme responds by condensing around the epithelium causing its destruction (Sakakura, 1987).

The female mammary gland exhibits no growth during the 11th to the 16th day of gestation. This interval is called the resting phase. Late in the 16th day the mammary bud undergoes rapid proliferation forming the mammary sprout (Sakakura, 1987). At day 17, the sprout extends downward penetrating the fat pad precursor tissue which appeared on day 14. At this stage, the first evidence of branching is observed. During the following few days until birth, the mammary epithelium grows until the resulting mammary gland has

formed 15-20 branching ducts (Sakakura, 1987). To explore the functional differentiation occurring concurrently with morphological changes, Ceriani (1970) carried out studies *in vitro* which demonstrated that ductal epithelial cells in explanted 16 day fetal rat mammary rudiments have the ability to synthesize casein when cultured in the presence of insulin, glucocorticoid, and prolactin. Only authentic mammary cells had the capability to respond to these particular hormones.

Although the fetal mammary gland seems somewhat functionally differentiated in the 16 day old embryo, the mammary anlagen is apparently not irreversibly committed to mammary morphogenesis at this stage. Experiments by Kratochwil (1969) have shown that the fetal mammary tissue responds to salivary mesenchyme by exhibiting dichotomous pattern of branching, which is consistent with salivary glands, as opposed to monopodial branching pattern which is exhibited by the typical mammary gland. From these experiments and the experiments performed *in vitro* by Ceriani (1970), it seems logical to propose that the mammary epithelial cells are committed to their specific secretory function before birth, but the commitment is still capable of being modified if other signals are present.

The persistence of a capacity to respond to inductive signals in the postnatal mammary epithelium has been demonstrated through experiments in the adult mouse mammary gland (Sakakura et al., 1979). These investigators have

demonstrated that the 14 day old mammary or salivary mesenchyme directs organo-specific development in the adult mouse gland that is dependent on the source of mesenchyme used. The same investigators have shown that, in C3H mice carrying the mammary tumor virus, mammary tumors appeared earlier and more frequently in glands that had received fetal mammary mesenchyme when compared with those that did not receive mesenchyme implantation (Sakakura et al., 1979).

Sakakura et al., (1982) have also identified two distinct types of mesenchyme associated with the mammary epithelium. One is the dense mammary mesenchyme, which is important in mammary gland morphogenesis. This type of mesenchyme is first recognized morphologically in the 13 day old embryo, as two to three layers of fibroblasts surrounding the mammary epithelium. The other mesenchymal tissue type is the mammary fat pad precursor, which then converts into the fat pad. This type of tissue first appears on the 14th day of gestation. Preadipocytes containing fatty substances are observed on the 16th-17th day of gestation. It has been demonstrated that fat pad precursor tissue is very important to embryonic mammary gland morphogenesis (Sakakura, 1982) but the nature of the mechanisms involved is not yet understood. Shortly after birth the entire area occupied by the fat pad precursor tissue has been changed to white fat tissue. The fat pad is very essential for the gland's postnatal morphogenesis.

Nipple development occurs between day 18 and 20 of

gestation in rodents. A circular invagination of the epidermis occurs around the mammary cord, and at the same time, the epidermis at the bottom of the mammary pit is lifted to give rise to the anlage of the nipple (Sakakura, 1987).

In the human, the milk streak is first recognized during the 4th week of gestation in the 2.5 mm embryo (Russo and Russo, 1987). The parenchymal cells start to invade the underlying stroma between the 7th and 8th week of gestation. Between the 10th and 12th week of gestation epithelial buds first appear. Additional branching is observed around the 15th week. There seems to be some disagreement in the literature on the exact time that this additional development which marks the branching stage of embryonic mammary gland development occurs (Russo and Russo, 1987).

The last intrauterine developmental events of the human mammary gland are still under question. Russo and Russo (1987) report that during the last weeks of intrauterine life the development of very primitive lobular structures has been observed. These investigators suggest that the secretory activity observed in newborns is not confined to the primitive alveolar structures but rather is a generalized response induced of the entire gland to the maternal hormonal levels.

POSTNATAL MAMMARY GLAND DEVELOPMENT

Shortly after birth, the mouse mammary duct exhibits some growth. This event is caused by the presence of circulating

maternal hormones. With the elimination of these hormones the terminal end buds (TEB) that were present at birth disappear (Daniel and Silberstein, 1987). The TEB's reappear again during the 3rd week of life. Terminal end buds are the 'driving force' behind ductal morphogenesis. They produce a supply of differentiated ductal and myoepithelial cells for elongation of the subtending ducts (Daniel and Silberstein, 1987). Histological analysis of end buds has revealed that, on the luminal side, they are lined with cells that are continuous with, and also give rise to the luminal cells of the subtending duct. These cells contain microvilli on the luminal side and junctional complexes on the lateral side (Daniel and Silberstein, 1987). The basal surface is lined by cap cells, which lack differentiated features. It has been suggested that cap cells may represent a pluripotent stem cell population that has the capacity to differentiate into both mammary ductal and mammary myoepithelial cell types (Daniel and Silberstein, 1987).

In the rat, the growth of the mammary gland is identical in both male and female animals up to the third week of life (Russo and Russo, 1978). During the first week of life the mammary gland is composed of a main duct that branches into 3-5 secondary ducts. During the following two weeks, the secondary ducts branch dichotomously to give rise to 6 generations of ducts (Russo and Russo, 1978). At 21 days of age TEB density per mm has reached its maximum. At the same

time alveolar bud (AB) number starts to increase. This increase is also characterized by a decrease in TEB density (Russo and Russo, 1978). The number of ABs continues to increase till the 62nd day of postnatal life. After this age the number of ABs remains constant in virgin female rats (Russo and Russo, 1978).

The onset of the estrous cycle marks the start of an additional event that affects the gland's morphology. Some ABs start to differentiate into smaller units, called the alveoli. Each AB develops into a cluster of 10-12 alveoli, forming one lobule (Russo and Russo, 1978). At 84 days of age the mammary gland demonstrates a steady population of TEB, AB, and lobules in the virgin animal. Involution occurs after 14 months of age. This stage is characterized by the reduction of the TEB number, and size reduction in the ABs and lobules (Russo and Russo, 1978).

During pregnancy, TEBs rapidly differentiate to ABs and then into lobules. At this time only a few terminal ducts are seen in the mammary gland. The alveolar epithelium assumes a secretory function which is continued throughout the lactation period (Russo and Russo, 1978). Post- pregnancy involution involves a reduction in size and number of the lobular acinar structures. The functional differentiation of the gland induced by pregnancy, can be detected morphologically even after involution. The number of lobules seen in regressed glands following pregnancy is greater than the number of

lobular structures seen virgin animals following regression (Russo and Russo, 1978).

In humans, the breast tissue undergoes a complete series of postnatal changes as well. These changes can be divided into two phases: the developmental phase (through puberty) and the differentiation phase (during pregnancy)(Russo and Russo, 1987). Glandular morphology undergoes developmental changes up to the time of lobular formation. In lobular formation development and functional differentiation take place at the same time. Lobular development is extended by repeated pregnancies (Russo and Russo, 1987).

In the absence of child bearing, the size and number of lobules is reduced. Mammary involution in humans starts in premenopausal women as a function of declining ovarian function (Vorherr, 1974; Russo and Russo, 1987). The menopausal stage of mammary involution consists of a drastic reduction in epithelial structures and fat deposition (Vorherr, 1974; Russo and Russo, 1987).

HORMONAL CONTROL OF MAMMARY GLAND DEVELOPMENT

The growth and development of the mammary gland is under multiple hormonal controls. The types of hormones that play an important role in mammary gland morphogenesis are discussed in this section.

In general, ductal growth and maintenance of mammary gland are controlled by ovarian steroids. Estrogen has two

prominent effects on the mammary gland: (1) it stimulates ductal growth and (2) it increases progesterone receptor (PR) concentrations (Haslam, 1987).

Current hypotheses on how estrogen exerts proliferative responses in mammary cells include the following: (1) estrogen acts as a direct mitogen on mammary epithelium, (2) it acts as an indirect stimulator by eliciting paracrine, autocrine, and /or endocrine production of estrogen-induced growth factors. G. Shyamala and A. Ferenzy (1984) have reported the possibility that estrogen effects are initiated in the mammary stroma since, after estrogen stimulation, increased stromal DNA synthesis is observed 24 hours before epithelial DNA synthesis. Recently, S.Z. Haslam (1988) has indicated that estrogen can have a local, non-systemically mediated effect on mammary cell proliferation. In addition, the investigator indicated that estrogen can also promote mammary cell proliferation as the result of a systemically mediated estrogen effect which could possibly involve the production of an estrogen-dependent growth factor. In these experiments, Elvax 40P pellets containing estradiol 17B were placed directly into the mammary gland to elicit a localized effect, or subcutaneously to produce a systemic effect. The effect of estrogen on epithelial cell proliferation was measured by its effect on mammary gland morphology from whole mount examinations and on DNA synthesis histoautoradiography. However, in this study the age and developmental stage of the

gland were crucial in determining the mode of mitogenic action (local or systemic) that estrogen was exerting. Using the same methodology and steroid autoradiography Daniel et. al., (1987) have indicated that no estrogen receptors could be detected in the cap cell layer of terminal end buds, the zone of most active DNA synthesis. On the other hand, estrogen receptors could be detected in the luminal cells of the end buds, in ductal epithelium and in stromal cells adjacent to ducts. They concluded that estrogen may be acting locally on both epithelial and nonepithelial target cells, and possibly in conjunction with extramammary factors, directly stimulating mammary ductal growth.

Apart from any directly mitogenic function estradiol 17B stimulates the secretion of other hormones both in vitro and in vivo. Specifically, it stimulates the secretion of thyroid-stimulating hormone and prolactin by pituitary cells in culture (Topper and Freeman, 1980). In addition, it has been shown recently that mammary tumor cells secrete growth factors in culture, and that the secretion of such growth factors is possibly regulated by estrogen (Salomon et al., 1984, Dickson et al., 1985, Haslam, 1987).

Progesterone appears to be required for alveolar formation (Topper and Freeman, 1980). It is possible that the proliferative effects of progesterone are independent of estrogen since it was observed that in ovariectomized adult virgin mice, progesterone had mitogenic effects on the

epithelial cells. The effects of progesterone though were enhanced by the presence of estrogen (Haslam, 1987). Progesterone appears to be responsible for alveolar development in the mouse, while it does not seem to be required for mammary ductal elongation (Freeman and Topper, 1978, Haslam, 1987). Progesterone also exhibits antilactogenic activity. It has been suggested that this quality of the hormone is mediated by cyclic AMP (Topper and Freeman, 1980).

Ovarian steroids are responsible for ductal growth and branching, but lobulo-alveolar development and extensive growth of alveolar epithelium during pregnancy require prolactin (Topper and Freeman, 1980). Prolactin is a hormone of the anterior pituitary gland. Secretion of prolactin is subject to diurnal variation (Vonderhaar, 1987). Regulation of prolactin secretion is achieved by several pathways; prolactin-inhibiting factors (PIF) and prolactin-releasing factors have been identified as regulatory molecules along with dopamine (Nicoll et al., 1970, Valverde et al., 1972, Lu and Meites., 1973, Frantz, 1977, Vonderhaar, 1987). The exact mechanism that is involved in the uptake and intracellular processing of prolactin that result in the hormone's action in its target tissues is not yet clearly understood. It is possible that prolactin exerts its growth-promoting effects via direct interaction with the mammary tissue, but it is yet unclear if it is the immediate mitogen (Topper and Freeman,

1980). Studies on the effect of prolactin on mammary gland development have demonstrated that, in hypophysectomized, ovariectomized and adrenalectomized rats, the only combination of hormones that produce lobulo-alveolar development was estrogen, progesterone and prolactin. No lobulo-alveolar development was observed when prolactin was not administered in these triply operated animals. Glandular development was also impaired when prolactin was administered alone, in the absence of ovarian steroids (Lyons, 1958, Lyons et al., 1958). So it appears that these three hormones are required for complete morphogenesis of the mammary gland. In vitro studies have shown that prolactin plays a very important role in the maintenance of the lobulo-alveolar morphological state of the gland. The four hormone combination that can achieve this morphologically developed state in vitro, is insulin, aldosterone, hydrocortisone and prolactin (Vonderhaar, 1987). If prolactin is removed from this combination after the lobulo-alveolar state has been achieved, in vitro, alveolar structures regress, leaving only a ductal parenchyma (Banerjee et al., 1983, Vonderhaar, 1987).

Prolactin plays the key role in the functional differentiation of the gland. It plays a major role in lactogenesis and the maintenance of lactation. It is also responsible for the regulation of the synthesis of milk-specific fatty acids and the uptake of fatty acids by the mammary epithelial cells (Vonderhaar, 1987).

Growth hormone (GH) in combination with estradiol promotes ductal growth in adolescent rodents (Topper and Freeman, 1980). The role that this hormone plays in the functional differentiation of the mammary gland is not well understood. However, it seems to satisfy the anterior pituitary requirement for lobulo-alveolar growth in triply operated mice (animals from which the ovaries, pituitary gland and adrenal glands have been removed); this does not stand true for the triply operated rat (Toppper and Freeman, 1980). G.B Silberstein and C.W Daniel (1987) have reported that bovine and mouse growth hormone placed in slow-release implants stimulated local end bud formation in ovariectomized, unprimed mice. Contralateral and ipsilateral glands were left unaffected, suggesting that the hormone did not act systemically, but locally.

Glucocorticoids seem to be required for maximal ductal growth in addition to enhancing the formation of lobules during pregnancy (Topper and Freeman, 1980). Glucocorticoids appears to have lactogenic functions as well. Along with insulin they seem to be responsible for the accumulation of rough endoplasmic reticulum (RER) in mammary secretory cells from midpregnant and virgin mouse mammary glands (Oka and Topper, 1971).

Placental Lactogen (choriomammotropin) in the rat is secreted as Placental Lactogen I and II. These two hormones differ considerably in size and do not cross react, and both

hormones seem to be important for mammary development during pregnancy (G. Thordarson and F. Talamantes, 1987). Very limited information is available on the mechanism of action of Placental Lactogen. It is not known if specific Placental Lactogen receptors exist in the mammary gland or whether Placental Lactogen acts on this tissue via the receptors of other lactogenic hormones as prolactin or growth hormone (G. Thordarson and F. Talamantes, 1987).

Thyroid hormones do not seem to be absolutely necessary for ductal growth, but seem to be important for the synthesis and secretion of α-lactalbumin (Topper and Freeman, 1980, G. Thordarson and F. Talamantes, 1987). In addition, thyroxin (T3) appears to be important for the stimulation of lactose synthetase activity and casein synthesis in mammary explants from pregnant mice by estradiol 17B (E2) (Topper and Freeman, 1980).

GROWTH FACTORS AND MAMMARY GLAND DEVELOPMENT

In addition to endocrine hormones, growth factors have been found to have proliferative effects on the mammary gland. Epidermal growth factor (EGF) is single chain polypeptide consisting of 53 amino acid residues, and was originally isolated from the submaxillary glands of mice (G. Carpender and S. Cohen, 1979). It has been shown that mouse mammary epithelial cells possess functional receptors for EGF (Y.

Takehani and T. Oka, 1983). Coleman et al., (1988) using slow release plastic implants capable of delivering EGF locally to the mammary gland have shown that EGF is capable of having a multitude of physiological effects on the mammary glands of ovariectomized mice. The local effects included formation of new end buds, the restoration of normal end bud histomorpholgy, increase of ductal diameter and reinitiation of epithelial cell DNA synthesis. Local EGF effects did not include any lobulo-alveolar growth. Induction of stromal EGF receptors by mammary epithelium was also suggested in this study. Using the same methodology S. Coleman and G. Silberstein (1990) have shown that the implanted EGF caused a very pronounced inhibition of ductal growth in virgin hormonally intact C57 mice. This inhibition was found to be fully reversible and was preceded by the disappearance of EGF receptors in the cap cell layer of the end bud epithelium and the stromal cells located adjacent to the epithelium.
The authors concluded that EGF plays an important growth regulatory role in mouse mammary ductal morphogenesis.

EGF has been seen to have an inhibitory effect on milk protein production in cultured mouse mammary epithelial cells at different stages of pregnancy. On the other hand EGF stimulated cell proliferation in a primary mouse mammary epithelial cell culture at different stages of pregnancy (Y. Takehani,and T. Oka, 1983). It was concluded from this study that EGF participates in the regulatory mechanisms of the

development and functional differentiation of the mouse mammary gland during pregnancy. Furthermore, impaired lactation performance was observed in sialoadenectomized mice. The capacity of sialoadenectomized mothers to nurse pups was much less than the capacity of intact mothers. This diminished capacity was manifested in high offspring mortality within 5 days of birth. The mammary glands of sialoadenectomized mothers were much smaller and were producing less milk than the glands of intact mothers. Interestingly when sialoadenectomized mothers were injected with EGF their lactating capacity increased to the level of the intact mothers (S. Okamoto and T. Oka, 1984). In view of the finding that EGF seems to inhibit precocious differentiation of the mammary gland during pregnancy the authors give, among others, the following explanation for their findings: pregestational sialoadenectomy caused inhibition in the growth of the mammary gland, which, in turn results in a decreased production of milk during lactation which then results in high offspring mortality. The number of high affinity receptors for EGF increases steadily and reaches a maximum during midpregnancy as the gland undergoes rapid proliferation. During lactation when the glands reach functional differentiation, high affinity receptors for EGF decline (Edery, 1985).

These observations, taken together, lead to the conclusion that EGF is a potent proliferation stimulator and

functional differentiation inhibitor on the mammary gland.

Other growth regulatory peptides are seen to influence the growth of the gland. These include transforming growth factors and insulin-like growth factors.

The presence of transforming growth factors (TGFs) in human breast cancer cells indicates that these peptides might play an important role in the regulation of mammary growth (Salomon et al., 1984, Knabbe et al., 1987). TGFs can be subdivided into at least two groups based on their functional interactions with EGF (Frolik et al., 1983). One group, TGF-α, is able to compete with EGF for its receptor (Todaro et al., 1980) while the other, TGF-β, does not. Transforming growth factor-β can inhibit proliferation of epithelial cells of many origins. This inhibition has also been seen in mammary epithelial cells after mammary glands have been treated with TGF-β containing Elvax pellets (Silberstein et al., 1987). In 4 days, TGF-β had reduced end bud number by approximately 75%. However this inhibition was fully reversible, and after the removal of the implant, structural reorganization of terminal ducts into end buds and resumption of ductal elongation was observed. The authors of this study suggested that TGF-β is either acting to induce a negative growth regulator or is itself such a molecule.

Insulin-like growth factors (IGFs) are molecules that share a 47% homology with insulin and have been found to be potent mitogens for human mammary tumor cells. Distinct cell

membrane receptors for IGF I and II are present in human breast cancer cells. Somatomedin, the third member of the IGF family, is seen to be a strong mitogen for cultured human tumor cells (Dembinski and Shiu, 1987). It has been shown that IGF I can substitute for insulin, in organ culture, in the functional differentiation of the lactating mouse mammary gland (Dembinski and Shiu, 1987, Prosser and Topper, 1986).

Finally, studies on the effects of cholera toxin (CT) implants on the mouse mammary glands have shown that elevated levels of intracellular cAMP around the CT-containing Elvax pellet, promoted rapid growth and normal ductal morphogenesis in the gland (Silberstein et al., 1984). In addition, Sheffield et al.,(1985) demonstrated that CT can also act systemically to induce alveolar and lobulo-alveolar development in mice. These mammogenic effects of CT were not dependent on concurrent administration of estradiol and progesterone in hormonally intact mice. Ovariectomized and hypophysectomized animals did require treatment with estradiol and progesterone in addition to deoxycortisone acetate and T4 in order to manifest the mammogenic effects of CT.

DES HISTORY

Diethylstilbestrol (DES) was first synthesized in 1938 by Sir E. Charles Dodds, in the Courtlaud Laboratory of the University of London (Dodds, 1938). DES is a very potent nonsteroidal compound that can be administered orally and can

act as effectively as the most potent estrogen. In the 1940's DES was widely used to prevent miscarriages and to help avoid pregnancy complications (Herbst 1981). It was estimated that most of the pregnant women that received DES were treated in the late 1940's and early 1950's. The drug was officially banned in the U.S. in 1971 by the F.D.A. for use during pregnancy, after its use during that time was associated with the appearance of clear cell adenocarcinoma of the vagina, in seven young women in one Boston hospital (Herbst et al.,1971; Greenwald at al., 1971). This type of cancer, up to that time, was seen almost exclusively in patients over the age of fifty (Herbst et al., 1971). It was suggested that the risk for developing clear cell adenocarcinoma of the vagina following DES-exposure was small, approximately 1 or fewer per 1000 (Herbst 1984). Furthermore, it was suggested that these cancers appeared more frequently after the age of 14 years, reaching a peak at 19, after which the development of such tumors declined (Herbst 1984). A central registry for such cases was established in late 1971 (Herbst et al., 1972).

The initial rationale for using DES during pregnancy came from two Harvard physicians, Dr. George Van Siclen Smith and Dr. Olive Watkins Smith who found that DES appeared to stimulate the pituitary directly to produce progesterone despite of the hormone levels already present in the organism, thus bypassing the body's natural balancing mechanisms. According to these investigators, DES was to be used for its

ability to stimulate the body's own production of progesterone which of course is essential for the maintenance of pregnancy (*Orenberg* 1981).

Non-neoplastic disorders of the genital tract of DES-exposed daughters include cervical ectropion and vaginal adenosis (Herbst 1981). Cervical ectropion is defined as the presence of glandular (columnar) epithelium in the ectocervix. Vaginal adenosis is defined as the presence of glandular (columnar) epithelium in the vagina (Herbst 1981). The normal lining of the vagina is stratified squamous epithelium with no glandular structures. Almost all clear cell adenocarcinomas of the vagina and the cervix have been shown to be accompanied by adenosis and ectropion respectively (Herbst 1981). It seems then logical to propose that these non-neoplastic disorders of the genital tract provide some foundation for the appearance of this type of cancer.

DES STRUCTURE AND METABOLISM

Diethylstilbestrol (4, 4' dihydroxy-α-β-diethylstilbene) or DES is a non-steroidal compound, first synthesized by Sir Charles Dodds in 1938 (Dodds et al., 1938). It shares all the biological effects of the steroidal estrogens but belongs to the stilbene type estrogen structural class along with hexestrol and dienestrol (McLachlan et al., 1984). It has been shown that most of the molecular structures of estradiol are superimposable with the structure of DES (Duax and Weeks,

1980). The high estrogenicity of DES has been attributed to the spacing of the two free hydroxyl groups in addition to the trans position of the ethyl moieties (Korach et al., 1985). DES is related to estradiol in terms of its capability to interact with the estrogen receptor and effects on estrogen target tissues (McLachlan et al., 1984). DES has the capability to bind to the estrogen receptor with an affinity similar to that exhibited by the natural estrogen estradiol 17B (Korach et al., 1985).

Following ingestion, DES undergoes extensive oxidative metabolism in the organism leading to a wide variety of oxidative metabolites in most laboratory animals and also in humans (Metzler, 1981a; Metzler, 1981b). DES oxidative metabolites include DES 4'-4''semiquinone and DES-4'-4''quinone; the later tautomerizes to Z,Z-dienestrol (Z,Z-DIES), a metabolite found in all of the species studied (Metzler, 1982). This oxidation of DES is mediated by peroxidases and appears to be of particular interest for the mechanism of DES carcinogenicity (Metzler, 1984). Oxidative metabolites of DES have been seen both in rat and hamster fetuses (Miller et al., 1982; Gottschilch and Metzler, 1984). Studies on the oxidative metabolism of DES in organ culture have revealed that the primary target for its carcinogenicity, the fetal genital tract, has the capability to metabolize this compound (Maydl et al., 1983). The intermediates of the oxidative pathway are capable of binding covalently to nucleic

acids and protein (Metzler, 1984). In addition, these intermediates are likely to be responsible for mutations in S. cerevisiae. Furthermore, the peroxidative pathway seems to be required for the transformation of Syrian hamster embryo cells by DES (McLachlan et al., 1982).

Aside from oxidative metabolism, conjugation to glucuronic acid is a major pathway in DES metabolism in the rat (Fisher et al., 1976). DES is primarily metabolized by the liver in rodents and humans (Metzler and McLachlan, 1978). The major metabolite of this pathway is diethylstilbestrol monoglucuronide (DESG). This glucuronic acid conjugate enters the intestine via the bile in the rat, were it is hydrolyzed to DES in the distal small intestine prior to absorption and distribution to the organism (Fisher et al., 1976). The conjugates are excreted in the urine (Metzler, 1981). Limited data from human subjects suggest that the metabolism of DES in the human in similar to that observed in laboratory animals (Fisher et al., 1976).

The mechanism of DES transplacental carcinogenicity is still not known. It is possible that effects of DES exposure are the results of both the estrogenicity of this compound and of its metabolic activation to a chemical carcinogen.

DES EFFECTS IN RODENT MODELS

The teratogenic effects of DES in the genital tract of rodents have been well documented. Pregnant CD-1 mice treated

with DES on days 9-16 of gestation, a time which corresponds to major organogenesis in the genital tract of the mouse, resulted in vaginal adenosis in 15% of the 35 day old female offspring (Newbold and McLachlan, 1982). Vaginal adenocarcinoma was seen in 2 out of 91 animals treated prenatally with DES (Newbold et al., 1981). Other cervicovaginal abnormalities observed after prenatal exposure to DES included cervical enlargement, squamous metaplasia in the endocervical canal, excess keratinization of the ectocervix and vagina and basal cell metaplasia in the upper vagina (Newbold and McLachlan, 1982).

Treatment with moderate doses DES on days 15 to 19 of gestation resulted in the induction of persistent urogenital sinus (lack of differentiation into separate vagina and urethra) and hypertrophy of the portio vaginalis in ICR/Jcl mice (Nomura and Kanzaki, 1977). Lung and ovarian tumors were induced in ICR/Jcl mice exposed prenatally to small amounts of DES (Nomura and Masuda, 1980). Long term effects on the adult female CD-1 mouse genital tract following transplacental exposure to DES included the following morphological abnormalities: excess keratinization, cervical enlargement, uterine squamous metaplasia and cystic endometrial hyperplasia (McLachlan et al., 1980). Uterine squamous metaplasia, cervical and vaginal adenocarcinomas were seen in old CD-1 mice following prenatal exposure to DES (Walker, 1983). Long term effects of prenatal exposure to DES were also observed in

the non-exposed female offspring of DES-exposed CD-1 mice. Uterine adenocarcinomas and ovarian cystadenomas were seen in these F-2 generation animals and their appearance was attributed to DES and aging (Walker, 1981).

DES exposure also resulted in ovarian inflammation and oviductal malformations in addition to salpingitis and microscopic alterations of the oviduct (Newbold et al., 1982). Abnormal ovary development including gross enlargement and cystic morphology was also observed in the Sprague Dawley rat following prenatal exposure to DES (Boylan, 1978).

Increased frequency of atypical uterine epithelia, cystically dilated uterine glands and thickened vaginal epithelium was observed in ACI rats exposed to DES <u>in utero</u> (Rothschild et al., 1988). Persistent estrous along with ovarian and endometrial tumors were seen in prenatally exposed albino rats (Napalkov and Anisimov, 1979). Impaired fertility was observed in the rat following prenatal exposure to 120 μg DES during the third week of gestation (Boylan, 1978); exposure to lower doses of DES (1.2 μg) did not affect the fertility of these animals (Boylan, 1978). Reproductive performance of mouse female offspring following prenatal DES-exposure was also impaired (Walker, 1983; McLachlan et al., 1975).

In male mice, prenatal exposure to DES resulted in the induction of undescended testes and their hypogenesis (Nomura and Kanzaki, 1977), reduced reproductive ability, epididymal

cysts, and retention of Muellerian duct vestiges (McLachlan et al., 1975). Undescended hypoplastic testis and the retention of Muellerian structures was also seen in the rat (Vorherr, 1979). Early studies have indicated that male rats exposed to high doses of DES lacked prostates and exhibited inhibited development of seminal vesicles, epididymides and vasa deferentia (Greene et al., 1939). Rete testis carcinoma, an extremely rare type of cancer was observed in male CD-1 mice following transplacental exposure to DES (Newbold et al., 1985).

Neonatal treatment with DES resulted in the abnormal presence of columnar epithelium in the vaginal fornices. The columnar cells appeared as single layers in the area of fornical lining epithelium or as glandlike or cystic structures in the subepithelial stroma (Plapinger and Bern, 1979). Adult NMRI mice treated neonatally with DES developed adenosis of the cervicovaginal epithelium and also showed signs of a beginning malignant transformation in the adenosis regions (Forsberg and Kalland, 1981). Persistent vaginal cornification that was observed following neonatal treatment with DES was found to be an ovary independent event (Takasugi et al., 1970), suggesting a disturbance in the hypothalamic gonadotropin secretion (Bern and Talamantes, 1981). Neonatal DES exposure resulted in a high incidence of vaginal adenosis in CD-1 mice (Newbold and McLachlan 1982). In view of the fact that this lesion does not appear to be a common

abnormality following prenatal treatment with DES it was concluded that the time of DES exposure may be critical in the appearance of these lesions (Newbold and McLachlan, 1982). Ovarian effects of neonatal treatment with DES in BALB/cCrgl mice include increased incident of polyovular follicles per ovary and increased frequency of mice with polyovular follicles (Iguchi et al., 1986). Thus, the timing of exposure to DES dictates some of the teratologic carcinogenic consequences of DES.

The mammary gland is the target of many hormones, and furthermore it is one of the few organs which exists as rudimentary ducts until it undergoes full development and differentiation in pregnancy. The advancing end bud is the target for many carcinogens and thus, the mammary gland is an obvious site for action by perinatal natural and synthetic steroid treatment. Evidence from rodent models has confirmed that neonatal hormone treatment can affect mammary morphology and physiology and can result in increased incidence of mammary adenocarcinoma (Bern and Talamantes, 1981).

Prenatal exposure to DES resulted in the development of prominent nipples in both male and female rat fetuses at term (Boylan, 1978). Furthermore, incorporation of 3H-uridine was altered in the primary duct epithelium in the 5-day old pups (Bergman and Boylan 1982). The growth and development of the mammary anlage were inhibited following prenatal exposure to high doses of DES (12-42 mg/rat) during days 12-21 of

gestation (Greene et al., 1939). Transplacental administration of DES has been observed to promote the incidence of palpable tumors in rats treated postnatally with 7,12-dimethylbenz[a]anthracene (DMBA) (Boylan and Calhoon, 1979,1983). In addition, studies on DES-exposed hamster progeny treated postnatally with DMBA have indicated an enhancing effect of the prenatal exposure on tumor latency and multiplicity (Rustia and Shubick, 1979). The DES-exposed progeny also had significantly higher incidence of malignant tumors such as mammary carcinomas and carcinomas of the forestomach (Rustia and Shubick, 1979). Long term effects on mammary gland morphology were observed in 14 month old Sprague-Dawley rats. Lobular hyperplasia was observed in rats exposed to 1.2 μg DES in week 3 of gestation and both ductal and lobular hyperplasia were observed in rats exposed to 120 ug during the same period (Rothschild et al., 1988). Prenatal exposure to DES alone was not able to increase the incidence of mammary tumor development in 14 month old Sprague-Dawley rats (Boylan and Calhoon, 1979). Transplacental exposure to DES alone was effective in adult female offspring of the ACI rat strain in producing mammary tumors (Rothschild et al., 1987). The combination of both the prenatal and postnatal treatment with DES resulted in increased tumor multiplicity and decreased tumor latency (Rothschild et al., 1987).

Thus, exposure to DES during the perinatal period can produce teratogenic and carcinogenetic effects on the

developing mammary glands and reproductive tracts of male and female rodents. Still very limited information exists on the effects of perinatal DES exposure on the multitude of growth factors and hormones that influence the development of these target organs. Further understanding of these of these interactions could possibly be the key in understanding the mechanism of action by which DES exerts its effects on these tissues.

ACI RAT MODEL

The ACI rat strain first originated at Columbia University in 1926. The strain resulted from a cross between August line 1561 and the Copenhagen line 2331 (which possesses an Irish marker). The name of this strain originated from the initials of that cross; ACI or A*C (Curtis et al., 1931). This strain is characterized by the occurrence of unilateral renal agenesis and hydronephrosis in 30% and 26% of male and female animals respectively; this event is always accompanied by the absence of a uterine horn or vas deferens in the ipsilateral side (Fujikura 1970). The mechanism by which these defects are inherited appears to be polygenic and it is not compatible with simple Mendelian transmission (Crammer and Gill, 1975). This strain is of particular interest because the female animals of this strain have an "essentially 0 incidence of mammary tumors" during their lifetime (Segaloff and Maxfield 1971). This characteristic along with the

finding that they show increased mammary gland sensitivity to long term systemic estrogen treatment (Curtis et al., 1931; Dunning and Curtis 1946; Dunning et al., 1947), make this strain a very useful tool for the study of mammary cancer.

The ACI rat strain has been shown to have a high mammary tumor response to DES treatment (Stone et al., 1979). Comparison studies between the ACI and Sprague Dawley rat strain on the effectiveness of postnatal DES treatment have revealed that only the ACI rats responded with a high incidence of mammary adenocarcinomas and increased pituitary weights. Pituitary prolactin-cell adenomas were only seen in ACI rats along with elevated plasma prolactin levels . On the other hand, elevated uterine weights and the induction of pyometritis was only seen in Sprague-Dawley rats (Stone et al., 1979).

Different carcinogens were found to enhance the ability of DES to induce mammary tumors in this strain. The chemical carcinogen, DMBA, and X-irradiation were found to act synergistically in the production of mammary adenocarcinomas in the ACI rat (Shellabarger et al., 1980, 1978). Synergistic effects between DES administration and radiation were also reported by Segaloff and Maxfield (1971). An increased incidence of mammary carcinomas was observed when animals were irradiated while under the influence of DES. All of these findings taken together indicate that the ACI rat model is an important tool for broadening our understanding of the effects

of prenatal DES exposure on the development and carcinogenesis of the mammary gland.

THESIS OBJECTIVE

Work from our laboratory has previously shown that transplacental exposure to DES alone is capable of producing mammary tumors in the adult female offspring. From these earlier data it was unclear whether prenatal DES had a direct initiating action on the fetal mammary gland, or whether prenatal DES exposure had altered the functioning of the hypothalamic-pituitary axis in such a way as to enhance mammary tumorigenesis. In order to investigate further the possibility that DES had a direct action, and in recognition of the difficulties of determining morphological differences in fetal cells, the state of the peripubertal mammary gland was studied, in anticipation that the gland would express its altered character. This was done by studying the following: 1) the morphological developmental pattern of the peripubertal ovariectomized ACI gland, 2) the effects of local delivery to the mammary gland of natural and synthetic estrogens, and 3) the effects of endogenous EGF on DES-exposed and vehicle-exposed mammary gland morphology.

Chapter 1

THE EFFECT OF PRENATAL EXPOSURE TO DIETHYLSTILBESTROL ON MAMMARY GLAND MORPHOLOGY IN THE PERIPUBERTAL FEMALE ACI RAT.

A portion of this Chapter published previously in Proc. AACR., 30: 215, 1990.

ABSTRACT

We have previously shown that prenatal exposure to diethylstilbestrol (DES) results in an increased sensitivity to carcinogens in both ACI and Sprague Dawley rats, and in the development of mammary tumors in a small percent of young female ACI rats. In this study, the effect of prenatal exposure to DES on mammary gland morphology in the peripubertal ACI rat was investigated. Pregnant rats were injected with vehicle (sesame oil) or DES (total dose, 8.0 μg) on days 15 and 18 of gestation. The DES-exposed group contained 47 rats in 28 litters and the vehicle-exposed group contained 51 rats in 34 litters. DES-exposed and control offspring were ovariectomized at 34 days of age and sacrificed 19 days later. At selected intervals between d34 and d53 the vagina was inspected to determine the time of vaginal opening. At sacrifice, the 3rd mammary gland pair was removed, fixed and stained for histological analysis. The pituitary glands and the entire reproductive tract were removed for subsequent analysis. The nature of the response elicited by prenatal exposure was judged by examining whole mount preparations of the DES-exposed and vehicle-exposed glands. The degree of lobuloalveolar development was evaluated on a subjective scale ranging from I (least differentiated) to V (most differentiated). The majority of the glands in the DES and vehicle-exposed groups (71.5% and 98% respectively) were classified as II or III on the scale of differentiation. Only

mammary glands from the DES-exposed group exhibited the extreme differentiation grades I and V.

PRENATAL EXPOSURE	GRADE OF DIFFERENTIATION				
	I	II	III	IV	V
DES	24.5%	46%	25.5%	0%	4%
Sesame Oil	0%	44%	54%	2%	0%

The time of vaginal opening was accelerated in the DES-exposed group suggesting an ovary-independent DES effect. Comparison of reproductive organ and pituitary weights between DES-exposed and control groups did not show any significant differences. These results, namely the presence of morphological extremes in the DES-exposed group, along with our previous findings, suggest a possible DES effect directly on the mammary gland during the intrauterine period. This effect is manifested later on during the peripubertal period as morphological alteration in the mammary gland's developmental pattern in approximately 30% of the DES-exposed population.

INTRODUCTION

Diethylstilbestrol (DES) is a synthetic estrogen that shares many biological effects with the steroidal estrogens (McLachlan et al, 1984). It is a small planar molecule that has the ability to cross the placenta (Shah and McLachlan 1976).

The carcinogenetic effect of prenatal exposure to DES on reproductive tract tissue has been well demonstrated in rodents (McLachlan et al, 1980; Vorherr et al, 1979; Rustia and Shubick, 1976). These carcinogenetic effects of the drug have also been seen in other organ systems, e.g. renal carcinomas in Syrian hamsters and liver tumors in male European hamsters (Reznic-Shuller, 1979). In addition, data from numerous rodent models suggested that exposure to DES or sex steroids during the perinatal period could alter the normal pattern of mammary gland development (Bern and Talamantes, 1981; Boylan, 1978; Greene et al, 1939, 1940).

Work from our laboratory using the ACI rat strain has indicated that prenatal exposure to DES alone is effective in producing mammary tumors in the adult female offspring (Rothschild et al, 1987). The 5 tumor bearing animals in that study were obtained from 4 different litters, suggesting that the occurrence of these tumors was not an isolated effect on one litter, but probably a direct effect of prenatal DES exposure. The other mammary glands in these tumor bearing

animals were found to exhibit moderate ductal and lobulo-alveolar proliferation with some evidence of secretion. On the other hand, mammary glands from DES-exposed animals that did not develop mammary tumors, were found to be largely undifferentiated showing a lower degree of proliferation than most of the mammary glands in the vehicle-exposed group.

The specific aim of this study was to investigate the possible action of transplacental DES on the mammary gland by studying morphological deviations from the gland's normal developmental pattern in the peripubertal ACI rat. Rats were ovariectomized at day 34 to eliminate endogenous ovarian hormones which could have affected mammary gland morphology, and the degree of development of the mammary gland was ascertained at day 53.

MATERIALS AND METHODS

Rats of the ACI strain were obtained from the Harlan-Sprague Dawley, Inc. (Indianapolis, IN). Animals were housed in a temperature and light-controlled animal facility and supplied with water and Purina rodent chow ad libitum. Virgin female animals were mated at approximately 3 months of age. One or two females were placed with one male rat in the late afternoon, and successful mating was ascertained by the presence of sperm in the vaginal smear the following morning. This time was considered as day 0 of pregnancy. Pregnancy was confirmed by weighing the animals every 2 days to establish appropriate weight gain. Pregnant rats were assigned randomly to one of 2 treatment groups, DES or vehicle.

On days 15 and 18 of gestation, pregnant females in the experimental group were injected with 4.0 μg DES in 0.3 ml of sesame oil for a total dose of 8.0 μg of DES per animal. Control animals were injected on the same days with 0.3 ml of the vehicle (sesame oil) only. With an average body weight of 160g, this dose of DES approximated 50 μg/kg. Pregnant females were allowed to deliver and raise their pups to weaning.

At 34 days of age, the female offspring were anesthetized with a mixture of Ketamine and Xylazene (200 mg/20 mg; 0.06 ml mixture/ 100 g rat), administered i.m, and were subjected to bilateral dorsal ovariectomy. At selected intervals between

d34 and d53, the vagina was inspected to determine approximate time of vaginal opening.

DES-exposed and vehicle-exposed animals were asphyxiated with carbon dioxide at 53 days of age, 19 days following ovariectomy. The third pair of mammary glands was removed by gentle separation of the mammary tissue from the skin with scalpels for preparation of whole mounts.

The entire genital tract and pituitary gland were also removed and fixed in 10% neutral buffered formalin. The gross morphology of the genital tract was recorded as intact or unilateral agenesis, according to the presence or absence of bilateral urogenital structures. Both the weight of the fixed genital tract and the wet weight of the pituitary were recorded.

HISTOLOGY

Each mammary gland was spread and compressed as much as possible between glass slides to insure a flat surface. The tissue was then fixed in 70% ethanol and stained with alum carmine. Following staining, the tissue was dehydrated through an ethanol series and finally stored in 100% ethanol.

Each whole mount was then photographed using a Wild stereomicroscope. Tissues were categorized according to the degree of ductal branching and lobulo-alveolar development on a subjective scale from I-V using the following classifications:

Class I= low degree of differentiation, some terminal end buds

present, very few alveoli;

Class II= approximately 50% of the gland possessing alveolar buds;

Class III= more than 50% of the gland with alveoli;

Class IV= moderate lobuloalveolar expansion; alveolar buds tending to obscure ducts.

Class V= extensive lobuloalveolar development; reminiscent of mid-pregnant state.

After initial inspection, the accuracy of mammary gland classification, was confirmed by two additional observers. There was a high degree (>95%)of agreement on mammary gland classification for classes I, IV, and V. There was somewhat lesser agreement (75-80%) between classes II and III. of mammary glands into classes II or III. Cases in which there was disagreement were referred to a third observer for final decision.

Analysis

There were 51 DES-exposed rats in 27 litters and 64 vehicle-exposed rats in 34 litters available for analysis. Animals exhibiting unilateral agenesis of the genital tract were excluded from mammary gland evaluation as a result of prominent differences in time of vaginal opening and uterine weight (see Chapter 4). Also the fact that there was an unequal proportion of rats with unilateral agenesis in the two groups (4 in DES-exposed and 13 in vehicle-exposed)

contributed to the decision to exclude these from consideration. Thus, the group sizes were: 47 DES-exposed rats and 51 vehicle-exposed rats.

The mammary gland morphology data are graphically represented as histograms with a curve fitted on the histograms for better visual representation of data distribution. Statistical analysis of the data included parametric and non-parametric tests. T-tests and the non-parametric equivalent Kruskal-Wallis one way analysis of variance were performed on the mammary gland data to test for statistical significance. T-test analysis looks for differences in the location of the mean and assumes normal distribution. In order to check the t-test results the Kruskal-Wallis was performed. This test works with ranked data (in our case, the five classes of mammary proliferation represented these ranks) and looks to see if the data intermesh. In addition, the Kolmogorov-Smirnov two sample test was applied to further analyze the data. This is a non-parametric test that tests differences between two distributions. This test is sensitive to differences in the characteristics of distributions of the two samples such as location, dispersion and skewness.

The uterine and pituitary data were expressed as mean$\pm$SD and subjected to analysis of variance to test for statistical significance.

RESULTS

Whole mount preparations from the third right and left glands were prepared in order to evaluate the nature of the response elicited by prenatal exposure to DES in the ovariectomized peripubertal rat. The degree of lobulo-alveolar development was evaluated on a subjective scale ranging from I (least differentiated) to V (most differentiated). Examples of each category are shown in Figure 1; distinguishing features are given in the figure legend.

Table 1 presents the distribution of morphologies in the two exposure groups. The majority of the DES-exposed glands (67%) were categorized as low to moderate alveolar expansion, that is, classes II or III. No glands were found to exhibit class IV differentiation score, while 4% of the DES-exposed glands exhibited a very proliferated morphology, and were classified as class V. Interestingly, 24.5% of the DES-exposed glands exhibited a very rudimentary glandular morphology and were classified as class I.

Almost all of the vehicle-exposed mammary glands (98%) were categorized as low to moderate alveolar development, that is, classes II or III. Only 2% of the vehicle-exposed glands demonstrated a moderate level of lobuloalveolar development, or class IV differentiation score. No class I or V glands were observed in this exposure group. The above data are

Figure 1. Whole mounts of mammary glands:

a. Class I: Low degree of differentiation, some terminal end buds (arrow), few alveoli present.

b. Class II: Approximately 50% of the gland possessing alveolar buds (arrow).

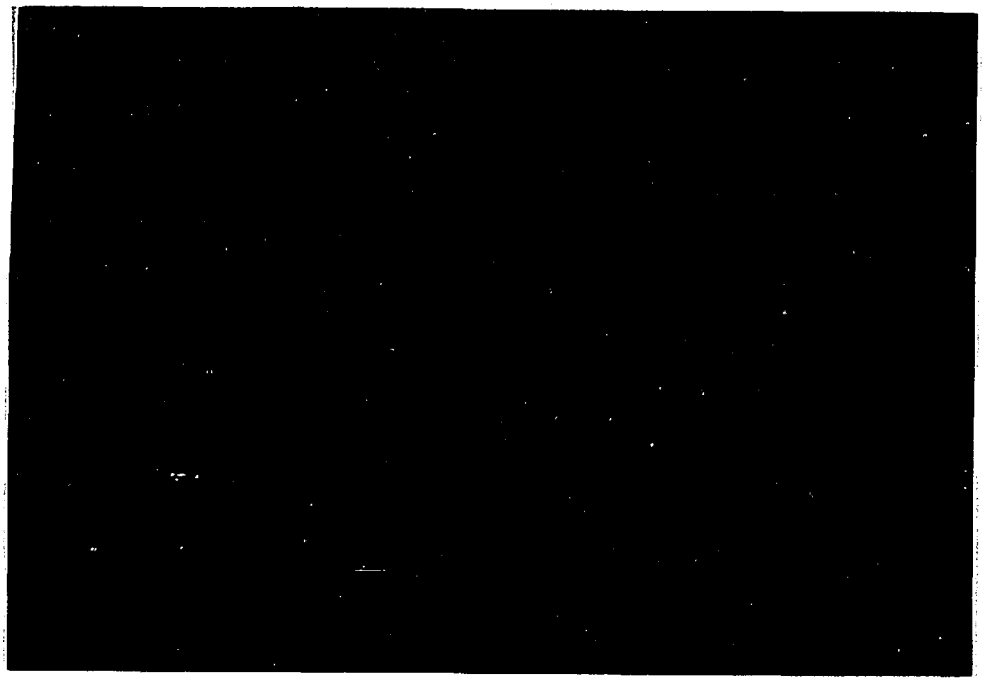


Figure 1: Whole mounts of mammary glands:

c. Class III: More than 50% of the gland with alveoli.

d. Class IV: Moderate lobulo-alveolar expansion. (arrow=lobule).

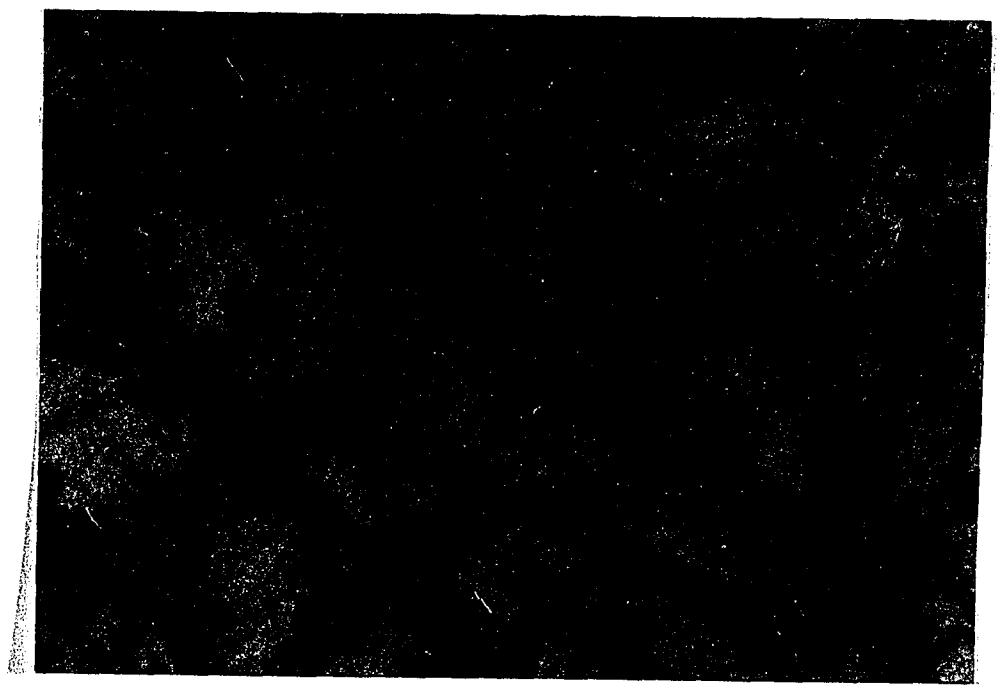


Figure 1. Whole mounts of mammary glands:

e. Class V: Extensive lobulo-alveolar development.

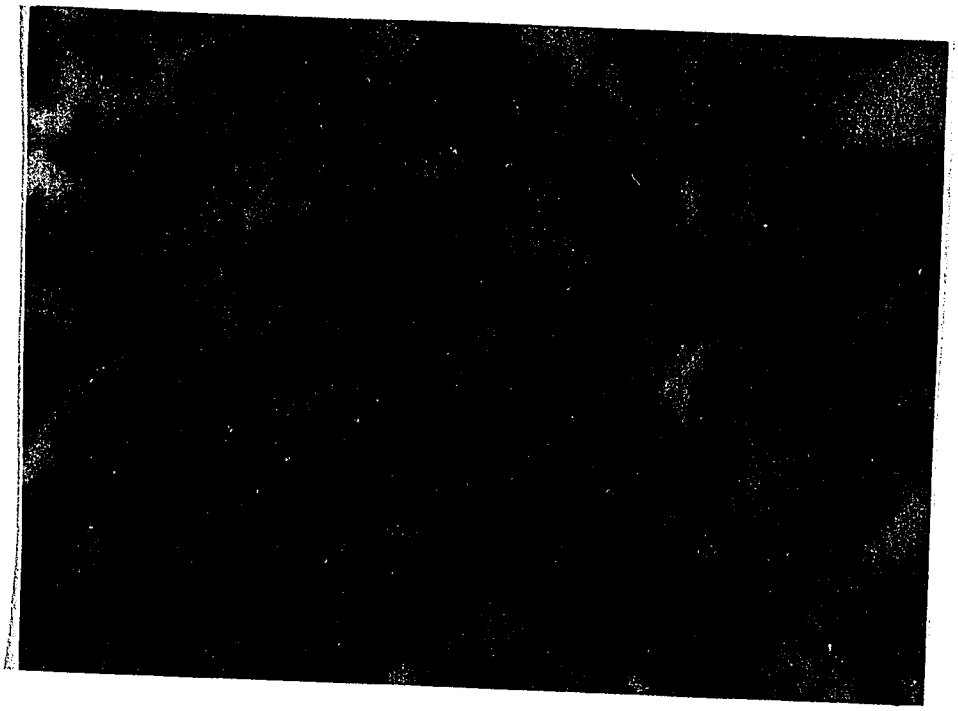


Table 1.

Class of Differentiation of Mammary Glands (MG) from Rats Exposed Prenatally to DES or Vehicle

Prenatal Exposure	[1]Class	Left MG n=47	Right MG n=47	Total n=94
DES*	I	12(26%)	11(23%)	23(24.5%)
	II	22(47%)	21(45%)	43(46%)
	III	11(23%)	13(28%)	24(25.5%)
	IV	0(0%)	0(0%)	0(0%)
	V	2(4%)	2(4%)	4(4%)
		n=51	n=51	n=102
SO*	I	0(0%)	0(0%)	0(0%)
	II	24(47%)	21(41%)	45(44%)
	III	26(51%)	29(57%)	55(54%)
	IV	1(2%)	1(2%)	2(2%)
	V	0(0%)	0(0%)	0(0%)

1. See methods and Figure 1 for an explanation of the various classes.
* This table only includes data from animals with intact reproductive tracts.

schematically represented in Figures 2 and 3 were the actual incidence of mammary gland scores is indicated in the histograms (right axis) and the computer-generated distribution curve is superimposed (left axis). Data were subjected to T-test, Kruskal-Wallis one way analysis of variance and Kolmogorov-Smirnov statistical analyses. The distribution of morphologies was found to differ significantly between the two exposure groups ($P \leq 0.01$).

In order to investigate the possibility of asymmetry in the degree of mammary gland development, the scores of each mammary gland pair were compared, and the gland differential (+1, -1, or 0) was calculated for each animal. No gland pair differed in score more than 1 grade; 12 pairs were discordant in the DES-exposed group; in the vehicle-exposed group there was 9 discordant pairs. The overall mean in gland differential in each group was +0.07, clearly demonstrating very high degree of symmetry in gland differentiation in both exposure groups. This can be visually confirmed by inspection of Figures 2 and 3, comparing the distributions between right and left, and by comparing the distributions in Table 1.

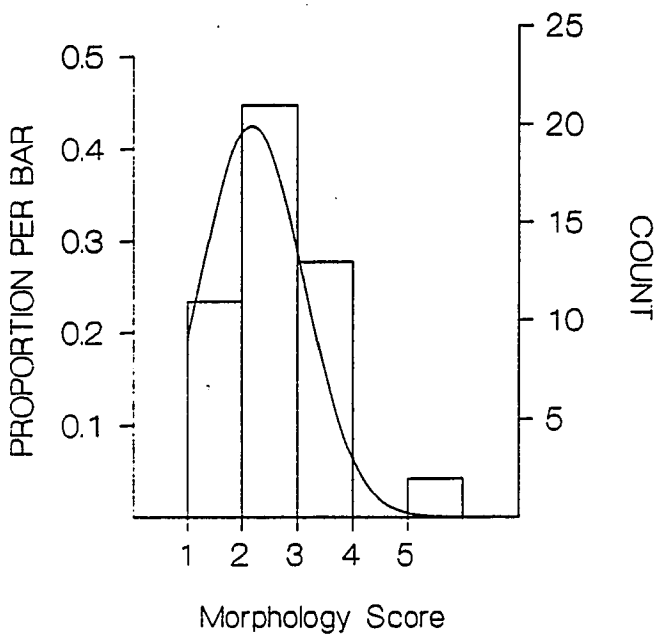
Effect of Prenatal DES Exposure on Pituitary and Uterine Weights.

The entire reproductive tract and the pituitary were removed at the time of necropsy; organ weights and gross morphological appearance were ascertained. These data are

Figure 2. Distribution of mammary gland differentiation scores in DES-exposed female offspring. Top: Right glands. Bottom: Left glands.

DES Exposed Control Group

Right Morphology



Left Morphology

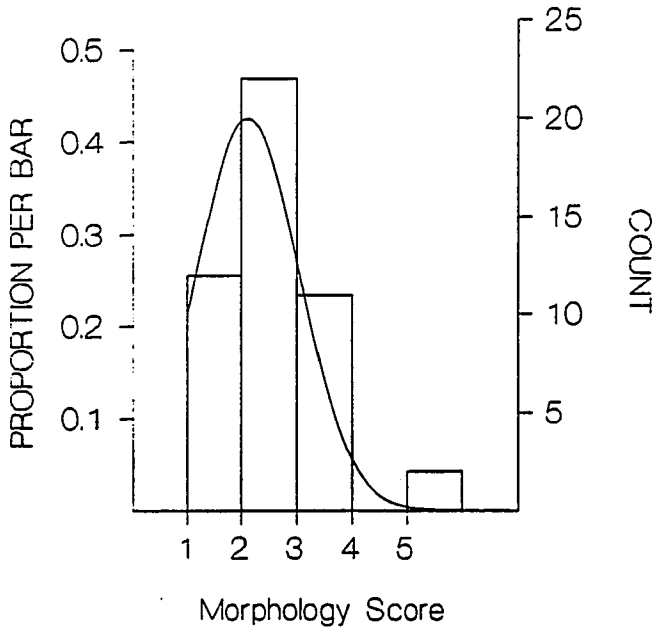
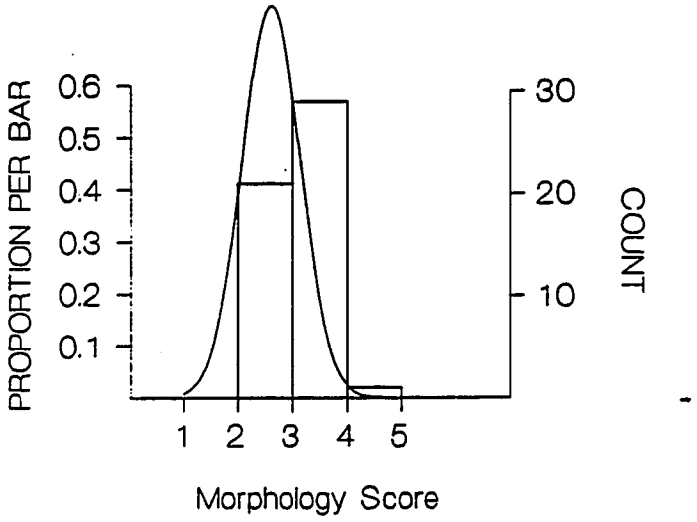


Figure 3. Distribution of mammary gland differentiation scores in vehicle-exposed female offspring. Top: Right glands. Bottom: Left glands.

Vehicle Exposed Control Group

Right Morphology



Left Morphology

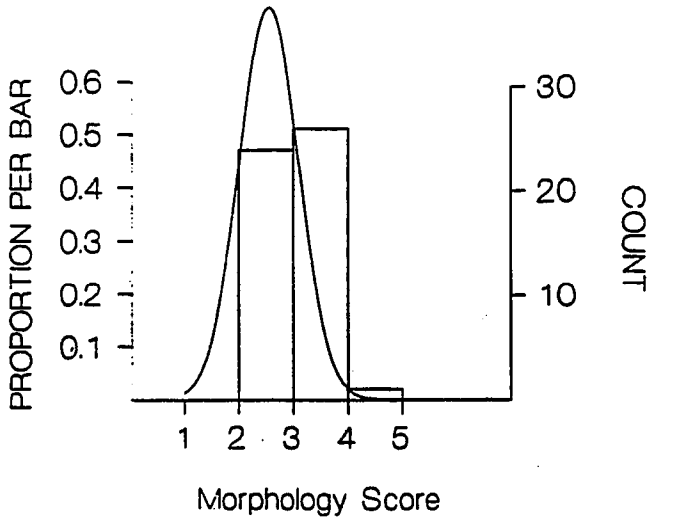


Table 2.

Mean Pituitary and Uterine Weights from DES and Vehicle-exposed Rats

Prenatal Exposure	[1]Genital Morphol.	[2]Mean Pituitary Weight (mg±SD)	[2]Mean Uterine Weight (mg±SD)
DES	INT	6.8±0.6 (n=47)	118±60 (n=22)*
	UA	6.5±0.7 (n=4)	238±36* (n=4)
SO	INT	6.9±0.7 (n=49)	126±58 (n=43)*
	UA	7.7±0.7 (n=13)	221±57* (n=13)

1. Refers to the status of the morphology of the genital tract: UA=unilateral agenesis, INT=intact.
2. Some pituitary glands and reproductive tracts from DES and vehicle-exposed animals were lost in the process of extraction or tissue preparation.

* The weights of the unilateral agenesis reproductive tracts in both exposure groups were statistically different from intact reproductive tract weights, $P \leq 0.01$

summarized on Table 2.

No significant differences were found for the weights of the pituitary glands between the prenatal exposure groups (P=0.08), or between rats with intact genital tracts or unilateral agenesis (P=0.08).

In addition, no significant differences were observed for the weights of the intact (P=0.83) or unilateral agenesis reproductive tracts (P=0.70) between the prenatal exposure groups. On the other hand, the weights of the intact reproductive tracts in DES-exposed or vehicle-exposed animals, were found to be highly significantly different ($P \geq 0.01$) from the unilateral agenesis reproductive tract weights in both prenatal exposure groups.

Effect of Prenatal DES Exposure on Time of Vaginal Opening

The effects of prenatal exposure to DES or vehicle on time of vaginal opening are summarized in Table 3. Data from 41 DES-exposed animals and 50 vehicle-exposed animals with intact reproductive tracts are shown here. In addition, the same table includes data from 4 DES-exposed and 13 vehicle-exposed rats with unilateral agenesis of the reproductive tract.

The majority of the vehicle-exposed rats had closed vaginas at the time of sacrifice at day 53. Only 18% of the animals in this exposure group with intact genital tracts had open vaginas at the time of sacrifice. On the other hand, 69%

Table 3.

Time of Vaginal Opening in Rats Exposed Prenatally to DES or Vehicle.

Prenatal Exposure	[1]Genital Morphology	Age at Which Vagina Found Open ≤34	≤49	≤53
DES	INT n=41	1(2%)	4(10%)	7(17%)
	UA n=4	0(0%)	3(75%)	3(75%)
SO	INT n=50	0(0%)	0(0%)	9(18%)
	UA n=13	0(0%)	0(0%)	9(69%)

1. Refers to the status of the morphology of the genital tract: UA=unilateral agenesis, INT=intact. Differences in population numbers are due to the fact that time of vaginal opening inspections commenced after the onset of the experiment.

of the animals with unilateral agenesis in this exposure group had open vaginas at the time of sacrifice.

DES-exposed animals appeared to have an earlier time of vaginal opening than the vehicle-exposed rats with intact or unilateral agenesis reproductive tract morphologies. Ten percent of the DES-exposed animals with intact genital tracts were seen to have vaginal opening between days 35 and 49, and 2% of the total number of animals in this exposure group even had vaginal opening before day 34. Furthermore, 75% of the DES-exposed rats with unilateral agenesis had open vaginas before day 49.

DISCUSSION

Our data demonstrate that prenatal exposure to DES alone is capable of inducing atypical mammary gland morphology in the peripubertal ovariectomized ACI rat. The DES-exposed offspring exhibited a whole range of differentiation scores ranging from very undifferentiated morphology to extensive lobulo-alveolar development. The two extreme morphological patterns (I and V) found in the DES-exposed group were absent in the control, sesame-oil exposed glands.

Our previous work (Rothschild et al, 1987) has shown that transplacental exposure to 8.0 μg of DES alone was capable of inducing mammary tumorigenesis in adult female ACI rats in a small but statistically significant number of animals. The mammary glands of the tumor bearing animals demonstrated lobulo-alveolar expansion equivalent to that judged as grade IV here. However, the majority of the glands from intact, 10 month old rats exposed to 0.8 or to 8.0 μg DES in this earlier study exhibited a largely undifferentiated morphology equivalent to class I and II. Similar morphological events are observed in our study on younger ovariectomized animals. One fourth of the DES-exposed glands in the prepubertal rat exhibited a largely undifferentiated morphology and were classified as grade I. Inhibitory effects of prenatal exposure to DES have also been observed in mice (Nagasawa et al, 1980), where a single injection of 5 μg DES on d12 of

pregnancy resulted in long term inhibition of gland development. In addition, neonatal treatment of Balb/c females with estradiol 17B resulted in an initial inhibition in the degree of ductal branching (Tomooka and Bern, 1982).

A small percentage of the DES-exposed glands in this experiment had extensive lobulo-alveolar development even after the rats were subjected to ovariectomy, suggesting an ovary-independent effect of DES. This atypical glandular developmental pattern found in the ovariectomized peripubertal animals confirm and extend the previous findings on adult ovary-intact rats from our laboratory and suggest a possible direct effect of the exposure in utero to DES on the embryonic mammary tissue.

Previous attempts to show significant differences in mammary gland morphology between intact DES-exposed and vehicle-exposed Sprague-Dawley rats at 2 months of age were not successful (Boylan and Calhoon 1983). Although the DES-exposed rats developed many more palpable mammary tumors after a postnatal challenge with 10mg of DMBA than the control vehicle-exposed animals, no morphological differences in the gland's development were found prior to DMBA administration. By contrast, the results of the present study indicate that morphological deviations from normal development are evident in 53d old ACI rats following exposure in utero to DES.

Our data show that prenatal exposure to DES alone did not result in altered pituitary or uterine weights either in

animals with intact reproductive tracts or in animals with unilateral agenesis. These data are consistent with another study which showed that uterine weights of DES and control prepubertal CD-1 mice were similar (Maier et al, 1985). The same investigators showed that prenatal DES exposure in the mouse alters subsequent uterine response to estrogen causing a significantly decreased growth response to short term estrogen exposure.

Previous efforts from our laboratory to describe hormonal imbalances in DES-exposed 2 month old Sprague Dawley rats (Boylan et al, 1983) have shown that serum estrogen and progesterone levels were normal and that serum prolactin levels were depressed compared to controls. We have also indicated that prenatal exposure to 0.8 μg and 8.0 μg DES alone resulted in increased pituitary weights in a dose dependent way in intact adult animals.

Various mechanisms have been proposed to explain the teratogenic and carcinogenetic effects attributed to intrauterine exposure to DES. DES has been shown to exert its teratogenic effects in the 19 day old rat fetus, at least in part, through its estrogenic activity (Henry et al, 1984). Direct fetal injections of DES into 19 day rat fetuses caused a dose related incident of cleft phallus, hypospadias, and incomplete coiling of oviducts. Direct fetal injection of estradiol elicited similar urogenital malformations, but estradiol was about 100 fold less potent than DES. It was

concluded from this study that, DES can act directly in the fetus without requiring maternal mediation. Since estradiol produced similar urogenital abnormalities as DES, it was concluded that prenatal exposure to estrogens is teratogenic, and that DES acted on the embryo through its estrogenic activity. While these data support the contention that DES is acting via the estrogen receptor, it is possible that both DES and maternal estrogens share some other pathway resulting in malformation and cancer.

The rat mammary epithelium is determined as mammary tissue by the 17th day of embryonic development (reviewed by Sakakura, 1987). Estrogen receptors are first present in the 16 day old mouse embryo and can be found only in the mesenchymal cells surrounding the epithelial rudiment (Narbaitz et al, 1980). These findings suggest that DES could act through its estrogenicity on the estrogen receptors present in these mesenchymal cells. It is possible then that these "affected" mesenchyme cells could direct the abnormal epithelial development of the DES-exposed gland.

Alternatively, DES could be directly genotoxic on the mammary gland epithelium. There are many reports which provide evidence supporting a direct genotoxic method of action (Buenaventura et al, 1984; Hill and Wolf, 1982; Mehta and von Borstel, 1981; Rudiger et al, 1979). One example is the capability of DES to increase sister chromatid exchange (SCE) both in bone marrow cells of SD rats, and in cultured FR

3T3 rat fibroblasts (Closer and Cerni, 1984). Furthermore, Liehr et al. (1985) have reported the presence of covalently modified DNA nucleotides in the kidneys of male Syrian hamsters following chronic DES treatment.

On the other hand, DES has been found to cause aneuploidy and neoplastic transformation in Syrian hamster embryo cells (Tsutsui et al, 1983). The same doses that cause aneuploidy in these cells have been found to cause arrest or abnormal mitotic spindles. Thus, by the disruption of microtubules and the production of aneuploidy it is possible that neoplastic transformation may result (Tucker and Barrett 1986). Furthermore, DES has been found to induce neoplastic transformation in Syrian hamster embryo cells without measurable mutations at two loci (Barret et al, 1981). Thus, the question as to the mechanism of DES action remains unresolved.

Our study points to the possibility that DES could be acting as an initiator of carcinogenesis during the intrauterine period. This initiation is manifested morphologically as atypical glandular development as early as the peripubertal period. This glandular defect is expressed well before maturity when the hormonal state of the animal promotes mammary growth and differentiation.

Chapter 2

MAMMARY GLAND RESPONSIVENESS TO NATURAL AND SYNTHETIC ESTROGENS FOLLOWING PRENATAL EXPOSURE TO DIETHYLSTILBESTROL IN THE FEMALE ACI RAT.

A portion of this Chapter published previously in Fed. Proc. A692, 1991.

ABSTRACT

We have previously shown that prenatal exposure to diethylstilbestrol (DES) results in atypical mammary gland development in peripubertal female ACI rats, and promotes mammary tumorigenesis in adult animals. The purpose of this study was to investigate differences in the response to local stimulation by estrogenic compounds in the mammary glands of ACI rats exposed in utero to DES or vehicle. Pregnant rats were given injections of DES (8.0 μg) or vehicle (sesame oil) on days 15 and 18 of gestation. The DES-exposed group contained 63 rats in 57 litters and the vehicle-exposed group contained 62 rats in 59 litters. DES and vehicle-exposed offspring were subjected to bilateral dorsal ovariectomy on d34. Local administration of the estrogenic compounds was achieved by surgical implantation of DES or estradiol 17B (E2)-containing Elvax 40P pellets, two weeks following ovariectomy, at d49. These pellets were implanted adjacent to the third mammary gland pair, the right pellet with E2 or DES at 5 or 11 ng, the left pellet of Elvax only. Animals were sacrificed 4 days following implantation at d53. At sacrifice, the 3rd mammary gland pair was removed fixed and stained for histological analysis. Stained mammary gland whole mounts were classified according to the degree of glandular proliferation on a subjective scale ranging from I to V (from least to most differentiated). Vehicle-exposed

mammary glands responded strongly and consistently to both doses of E2 and DES, with increased ductal and lobulo-alveolar differentiation. The DES-exposed glands were significantly less responsive to stimulation by 11 ng DES pellets. Similar trends to reduced proliferative response were seen in DES-exposed groups implanted with 5 ng DES and 11 ng E2 pellets. DES and vehicle-exposed mammary glands had equivalent positive responses to 5 ng E2 implants. The tendency of the DES-exposed mammary glands to be refractory to stimulation by E2 and DES may be related, at least in part, to the relative hypo-differentiation of some glands.

INTRODUCTION

The growth and development of the mammary gland is under multiple hormonal controls. In general, ductal growth and maintenance of mammary gland are controlled by the 2 major classes of ovarian steroids: estrogens, and progestogens.

Estrogen has two prominent effects on the gland: (1) it stimulates ductal growth and (2) it increases progesterone receptor concentrations. There is evidence that estrogen effects are initiated in the mammary stroma, since after estrogen stimulation, increased stromal DNA synthesis is observed 24 hours before epithelial DNA synthesis (Shyamala and Ferenzy, 1984).

Although the effects of estrogen on mammary gland growth and progesterone receptor concentration were known, the mechanism(s) of action of estrogens <u>in vivo</u> remained to be elucidated. It was unclear whether estrogens exerted mitogenic effects on the mammary cells (stroma or epithelium) or whether estrogens exerted their proliferation-promoting effects via other growth-stimulatory molecules produced elsewhere in the body and delivered to the mammary gland via the systemic circulation. In order to address this question, Haslam (1988) undertook to determine if the mitogenic effects of estrogen <u>in vivo</u> on the mouse mammary gland were the result of a localized or of a systemically mediated mode of action. These experiments indicated that estrogen can act both locally

and systemically to produce mitogenic effects on the mouse mammary gland. The investigator used Elvax 40P pellets containing estradiol 17B and placed them directly into the mammary gland to elicit a localized effect, or subcutaneously, in the intrascapular region, to produce a systemic effect. The effect of estrogen on epithelial cell proliferation was measured by its effect on mammary gland morphology from whole mount examinations and on DNA synthesis using DNA histoautoradiography. However, in this study the age and developmental stage of the gland were crucial in determining the mode of mitogenic action (local or systemic) that estrogen was exerting. In the immature mammary gland estrogen acted locally to stimulate mammary epithelial cell DNA synthesis, while mammary epithelial DNA synthesis, in the adult animals, was elicited only when high estrogen doses, enough to produce a systemic response, were administered. Other investigators, using the same Elvax 40P methodology have indicated that estradiol 17B was capable of inducing direct local stimulation on the mammary glands of castrated virgin C57/BL/crl mice (Daniel et al, 1987).

Progesterone appears to be required for alveolar formation (Freeman and Topper, 1978). Progesterone was recently found to play an important role in stimulating epithelial DNA synthesis in the adult mouse mammary gland (Haslam, 1988). It was postulated by the investigators that progesterone is exerting its effects via estrogen-dependent

progesterone receptor.

In light of these results, we sought to determine the effects of local stimulation by natural or synthetic estrogens on the DES-exposed mammary glands of the peripubertal ACI rat. We have shown that prenatal exposure to DES alone is effective in producing mammary tumors in the adult female offspring of ACI rats (Rothschild et al, 1987). This strain has been shown to have a very low incidence and late age of onset of spontaneous mammary tumors (Segaloff and Maxfield, 1971). These findings, along with the fact that adult female ACI rats respond to DES treatment with a high incidence of mammary tumors (Stone et al, 1979), made this strain an interesting model for the purposes of that and subsequent studies.

Recently, we have shown that prenatal exposure to DES alone is capable of altering the normal morphological developmental pattern of the mammary gland in the peripubertal ovariectomized ACI rat (Chapter 1). It is possible that prenatal exposure to DES caused a permanent alteration of the fetal mammary cells and/or their stromal environment. This action resulted postnatally in atypical morphology in the peripubertal mammary gland. The nature of the DES action on the mammary gland was not revealed by these studies.

The specific aim of this study was to investigate the nature of the action of prenatal exposure to DES on the peripubertal mammary gland of ovariectomized rats by prenatal exposure to DES. Having found that exposure to DES <u>in utero</u>

alters the morphological development of the mammary gland in the young ACI rat, we wanted to study the sensitivity of the DES-exposed gland to estrogenic compounds, in the absence of ovarian steroids that could potentially affect the response of the DES-exposed glands to these molecules. The effects of stimulatory molecules such as DES or estradiol 17B, delivered locally, were studied in order to establish whether differences existed in the responsiveness of the DES-exposed mammary gland, and to possibly relate these differences to the altered morphological pattern observed in the peripubertal animals following prenatal exposure to DES.

MATERIALS AND METHODS

Treatment of Pregnant Rats

Details concerning the origin, mating and treatment of adult ACI rats may be found in the Materials and Methods section of Chapter 1. The total number of DES exposed offspring was 72 in 64 litters; there were 71 vehicle-exposed offspring in 67 litters. Animals exhibiting unilateral agenesis of the genital tract were excluded from mammary gland evaluation because of prominent differences in time of vaginal opening and uterine weight (see Chapter 4). Thus the effective group sizes were: 63 DES-exposed and 62 vehicle-exposed. The female offspring from each litter were assigned randomly to the different postnatal treatment groups.

Treatment of Female Offspring

At 34 days of age, the female offspring were subjected to bilateral dorsal ovariectomy following anesthesia with a mixture of Ketamine and Xylazene (200 mg/20 mg; 0.06 ml mixture/ 100 gr rat) administered i.m. Two weeks following ovariectomy offspring from both exposure groups were implanted with Elvax 40P pellets containing either DES or estradiol 17B at doses of 5 or 11 ng. The third right mammary gland was used as the target tissue, while the contralateral gland was implanted with a control pellet. We have previously determined that there are no inherent differences in

right/left in mammary morphology in this strain (Chapter 1).

Following anesthesia the third mammary gland was exposed after a ventral mid-line incision and pinned back on the dissecting board. After the nipple was located, the experimental implant was placed next to the advancing duct, by opening a small pocket in the underlying fat pad, and by pushing the implant into the pocket with a pair of very fine forceps. The same procedure was used for implantation of the control pellet on the contralateral side. Following implantation, wound clips were used to close the skin, which was observed to heal quickly and without complications. Animals were kept under a warm lamp until the effects of the anesthetic wore off, and the rats were observed to walk about in the cage.

At selected intervals between d34 and sacrifice, the vagina was inspected to determine the approximate time of vaginal opening.

Elvax preparation procedure

Experiments in this paper are based on the procedures of Silberstein and Daniel (1987). These investigators showed that Elvax pellets are very useful in studying the nonsystemic effects of biologically active molecules in developing mammary tissues. Elvax 40P, an ethylene vinyl-acetate copolymer is an implant material that causes no inflammatory response, and is capable of gradual local response of a great variety of

molecules *in vivo*.

Implants containing DES or estradiol were prepared as described by Rhine et al., (1980). Ethylene vinyl acetate copolymer (40% vinyl-acetate by weight) was washed in several changes of 95% ethanol for 1 week prior to being used. Elvax was then dissolved in methylene chloride to give a 20% solution (w/v). The substance to be suspended in the polymer was added to the Elvax solution and the mixture was vortexed for 10 seconds, in order to ensure proper mixing. The mixture was then transferred to an already cooled glass mold. After the mixture was poured, it remained on dry ice for 10 minutes. The frozen slab was transferred to a cold plate and was stored in the freezer for 2 days at -20 C. The slab was then dried for 2 additional days at room temperature in a desiccator under a mild vacuum (600 torr). After the pellet had dried it was cut into small pieces (about 2x1x1.5 mm). The final weight of the implants was about 2 mg; each pellet contained 5 or 11 ng of DES or estradiol. Control pellets were prepared separately according to the same methodology, containing only Elvax.

Necropsy-Histology

DES-exposed and vehicle-exposed animals were asphyxiated with carbon dioxide 4 days following implantation at d53 of age. Necropsy and histology procedures were carried out as described in Chapter 1.

Analysis

For statistical methods see chapter 1 (Materials and Methods).

RESULTS

Effect of Local Estradiol-17b (E2) Stimulation on the Morphology of DES-exposed and Vehicle-exposed Mammary Glands.

Low Dose (5ng). The effects of the E2-containing Elvax pellets on the morphology of the DES-exposed peripubertal glands, are summarized in Figure 1. As in Chapter 1, both the actual score frequencies (histograms) and the computer-generated curve fitting the distribution of gland scores (curve) are presented. Data from 13 rats in 11 different litters are shown here. The right mammary glands responded to the local stimulation of 5 ng E2 by exhibiting a class III differentiation score in the majority of the cases (62%). No class I score was observed in any of the pellet bearing glands. On the other hand, 8% of the contralateral glands demonstrated this lowest grade of differentiation. In addition, a class III differentiation score was observed in only 38% of the left, control pellet bearing glands suggesting a stimulatory effect of the estradiol on the right glands.

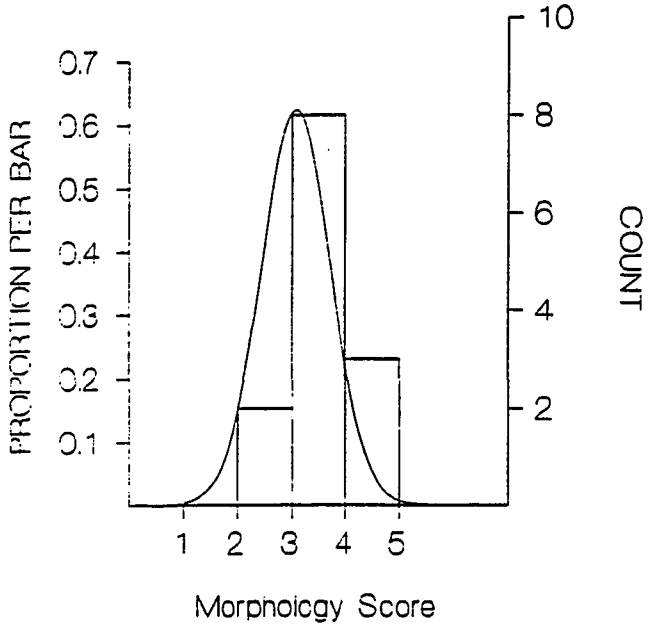
Similar local stimulatory events were observed in the vehicle-exposed glands. These results are summarized in Figure 2. Data from 15 rats in 15 different litters are shown here. A class III score was observed in 73% of the cases on the experimental side, while only 47% of the contralateral glands demonstrated this differentiation score. The majority of the left glands (53%) exhibited a class II grade in this

Figure 1. Effect of E2 (5 ng)-containing Elvax pellets on the morphology of DES-exposed mammary glands. Right side (top panel)=E2 pellet. Left side (bottom panel)=control pellet.

DES Exposed Exptl Group (Post E2-5)

Right

Morphology



Left

Morphology

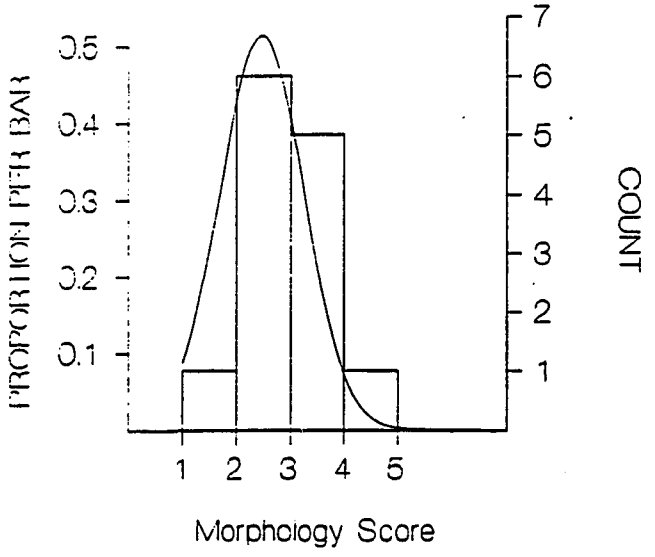
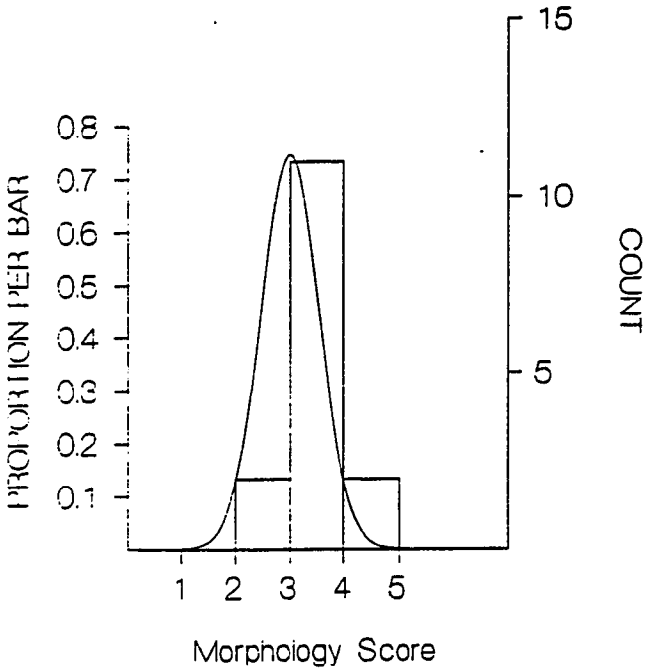


Figure 2. Effect of the E2 (5 ng)-containing Elvax pellets on the morphology of vehicle-exposed mammary glands. Right side (top panel)=E2 pellet. Left side (bottom panel)=control pellet.

Vehicle Exposed Exptl Group (Post E2-5)

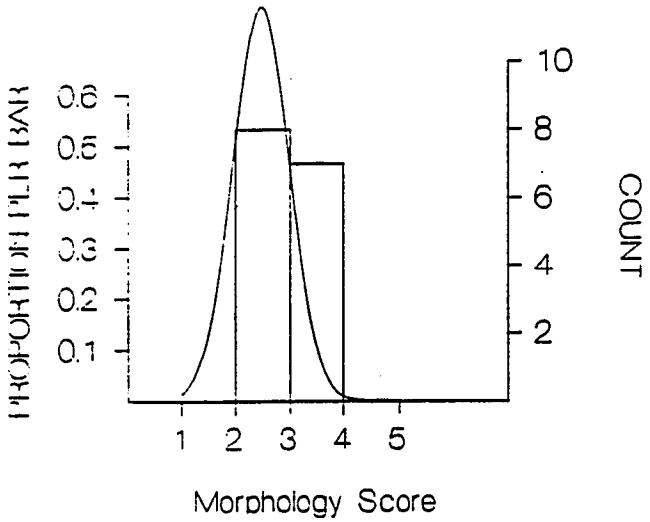
Right

Morphology



Left

Morphology



case. No class I differentiation scores were observed in the vehicle-exposed group. This observation is consistent with our previous findings that indicate that the class I differentiation score is unique to the DES-exposed animals.

Although these results suggest a mild local stimulatory effect of the estradiol-containing pellet on the morphology of the ACI mammary gland, no significant differences were observed between the prenatal exposure groups.

High Dose (11ng). The effects of high dose estradiol-containing pellet are summarized in Figure 3. Data from 15 rats in 15 different litters are summarized here. The DES-exposed mammary glands responded to the local stimulation with class II and Class III differentiation scores in 40% and 47% of the cases respectively. Only 13% of the estradiol stimulated glands exhibited a class IV differentiation scores. The contralateral glands demonstrated in majority (40%) a class III differentiation score, while 20% of these control glands had class II morphology and 20% demonstrated class I developmental score. The lowest grade of morphological development was once again observed only in the contralateral side.

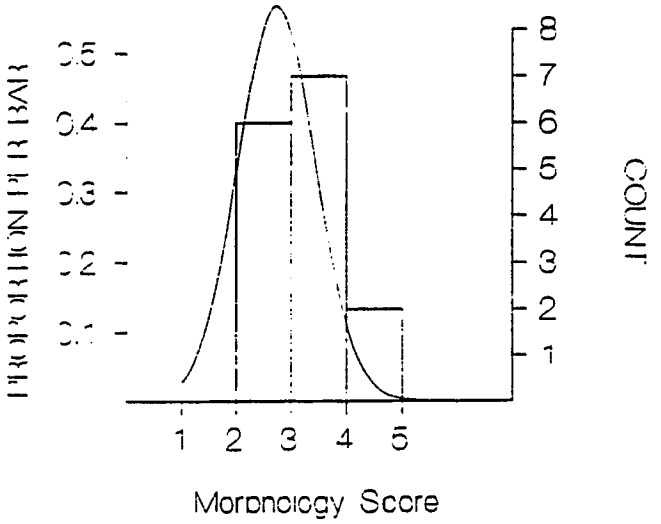
The vehicle-exposed mammary glands response to the local estradiol stimulation is summarized in Figure 4. Data from 15 rats in 15 different litters are summarized here. It was observed that most of the experimental glands (60%) responded with a class III score. In addition, only a small percentage

Figure 3. Effect of the E2 (11 ng)-containing Elvax pellets on the morphology of DES-exposed mammary glands. Right side (top panel)=E2 pellet. Left side (bottom panel)=control pellet.

DES Exposed Expti Group (Post E2-11)

Right

Morphology



Left

Morphology

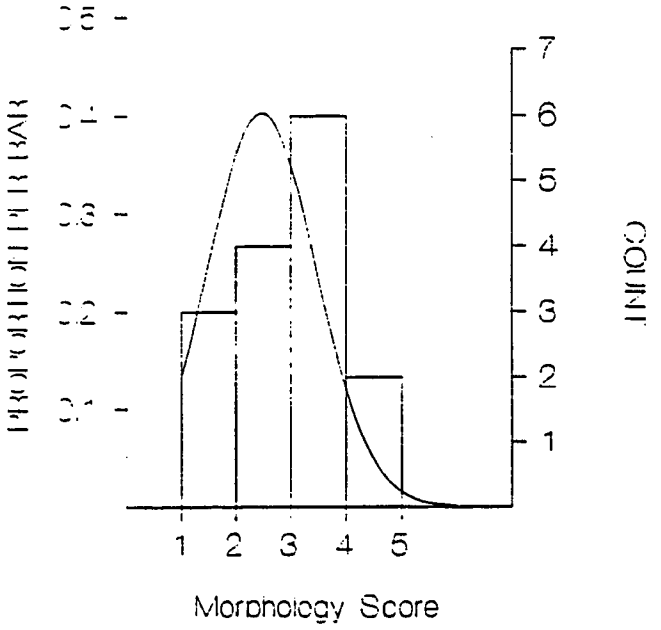
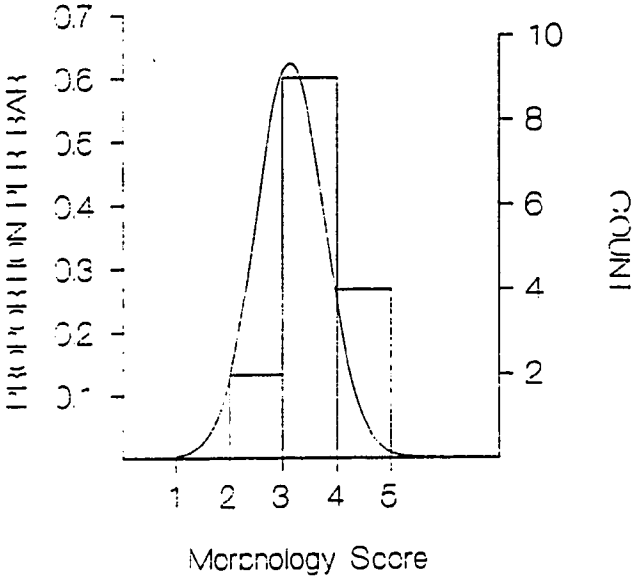


Figure 4. Effect of E2 (11 ng)-containing Elvax pellets on the morphology of vehicle-exposed mammary glands. Right side (top panel)=E2 pellet. Left side (bottom panel)=control pellet.

Vehicle Exposed Exptl Group (Post E2-11)

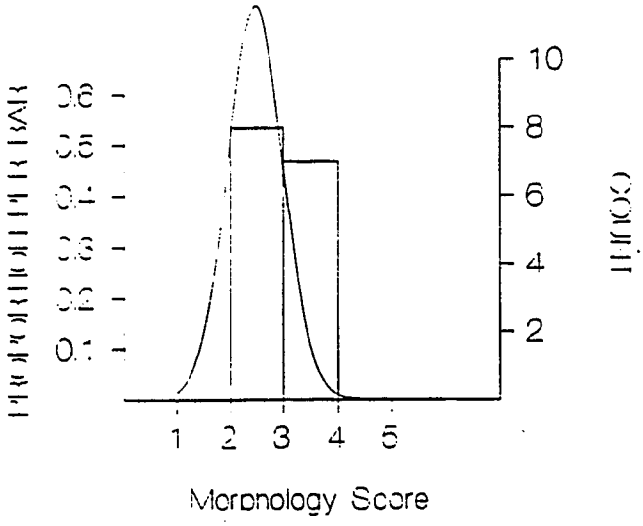
Right

Morphology



Left

Morphology



of the vehicle-exposed, estrogen-stimulated mammary glands demonstrated a class II differentiation score. The contralateral glands showed a great increase in the occurrence of class II morphology and a decrease in the occurrence of class III morphology when compared to the experimental side. These findings suggest a more pronounced local stimulatory effect of the 11 ng E2 implant on mammary gland morphology.

When the effect of the 11 ng E2 containing implants are seen together as a function of the prenatal exposure treatment a difference on the level of glandular stimulation is observed. A stronger stimulatory effect of the E2 pellet on the vehicle-exposed glands than on the DES-exposed group is implied by these data. Statistical analysis of these findings suggest a possible difference between the two prenatal exposure groups (P=0.109)

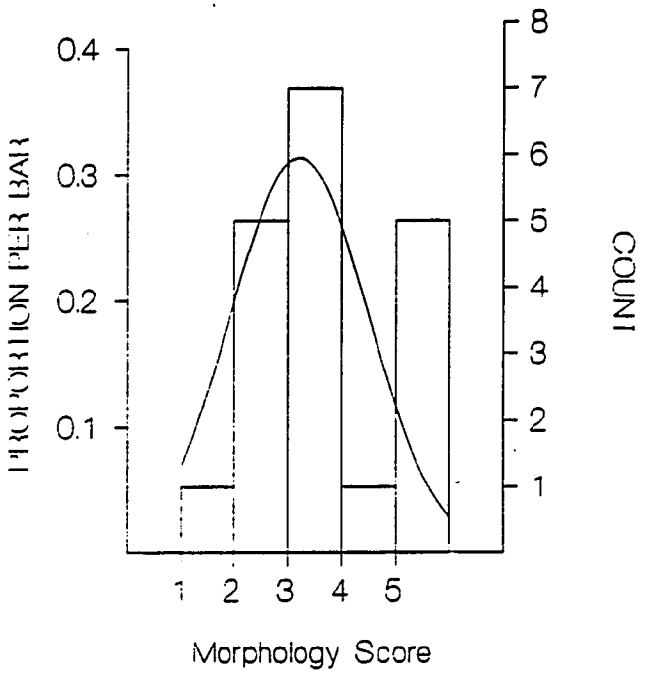
Effect of Local Diethylstilbestrol Stimulation on the Morphology of DES-exposed and Vehicle-exposed Mammary Glands.

Low Dose (5ng). The local stimulatory effects of the low-dose DES-containing pellet are summarized in Figure 5. Data from 19 DES-exposed animals in 18 different litters are shown here. The pellet-bearing glands responded to the local stimulant with a class III morphology in 37% of the glands examined. Class II morphology was seen in approximately 26% of the glands. Both class I and V differentiation scores were present in this group of animals in 5% and 26% of the cases

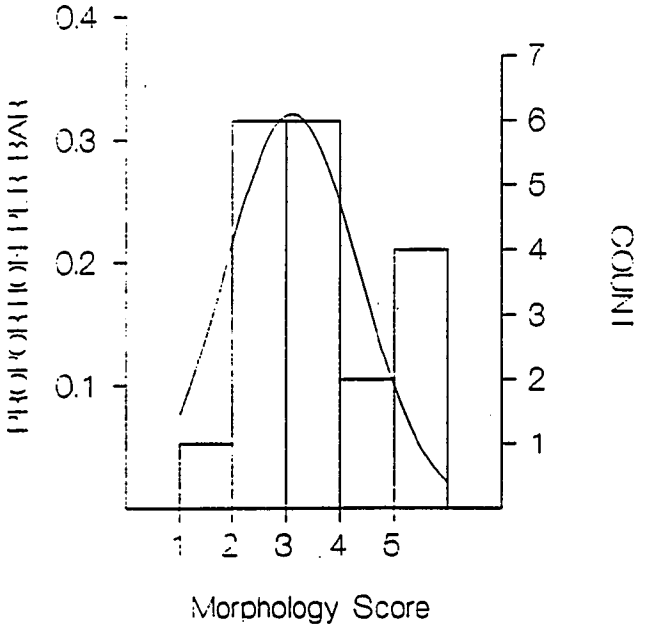
Figure 5. Effect of the DES (5 ng)-containing Elvax pellets on the morphology of DES-exposed mammary glands. Right side (top panel)=DES pellet. Left side (bottom panel)=control pellet.

DES Exposed Exptl Group (Post DES-5)

Right Morphology



Left Morphology



respectively. The contralateral glands showed a slightly decreased incidence of class III and a small increase in class II scores. Again, class I and V differentiation grades were present in the contralateral side in the approximately the same percentages as in the experimental side. Thus the local 5 ng DES pellet caused essentially no stimulation in these glands.

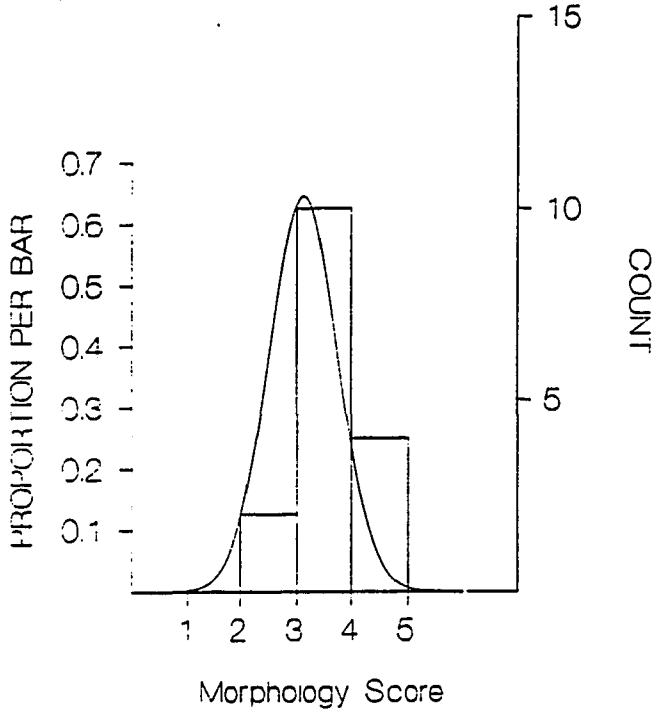
The effects of the low dose DES pellet on the vehicle-exposed glands are summarized in Figure 6. Data from 16 animals in 15 different litters are shown here. The experimental glands responded to the local stimulant with a class III morphology in 62.5% of the cases. Class IV differentiation score was observed in 25% of the glands, and only 12.5% of these glands showed a class II grade. The morphological profile of the contralateral glands was quite different. An increased incidence of class II grade was observed, 50% as opposed to 12.5% in the experimental side. The occurrences of class III and IV morphologies were significantly reduced when compared to the experimental side. These results demonstrate a stimulatory effect of local DES on the vehicle-exposed glands. The pattern of stimulation seen in the vehicle-exposed glands is different from that observed in the DES-exposed animals, as the latter tended to show less morphological differences between the experimental and control sides. Despite these observations, statistical analysis of these data did not indicate any significant differences in the

Figure 6. Effect of the DES (5 ng)-containing Elvax pellets on the morphology of vehicle-exposed mammary glands. Right side (top panel)=DES pellet. Left side (bottom panel)=control pellet.

Vehicle Exposed Exptl Group (Post DES-5)

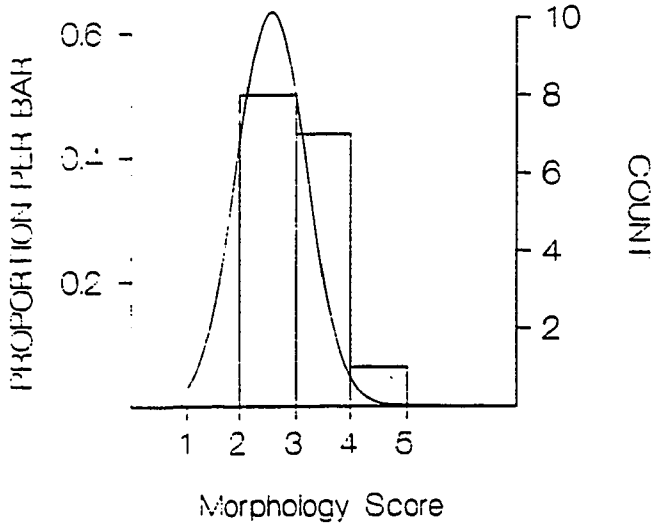
Right

Morphology



Left

Morphology



pattern of gland response to this type of stimulation as a function of the prenatal treatment.

High Dose (11ng) The effects of the high dose DES pellet are summarized in Figure 7. Data from 16 DES-exposed rats from 13 different litters are shown here. The majority of the DES-exposed glands (81.25%) responded to the local stimulation with class III differentiation score, while class II and IV represented only 12.5% and 6.25% of the glands respectively. The control side's morphological pattern was very different from the one observed in the experimental glands. Here, many more class II glands were seen, (37.5%) as opposed to 12.5% in the experimental side. In addition, a significant drop in the class III gland, when compared to the experimental glands, was observed. Class I glands were only seen in the control side, in 12.5% of the control glands. These results suggest an overall local stimulatory effect of the pellet on the DES-exposed glands.

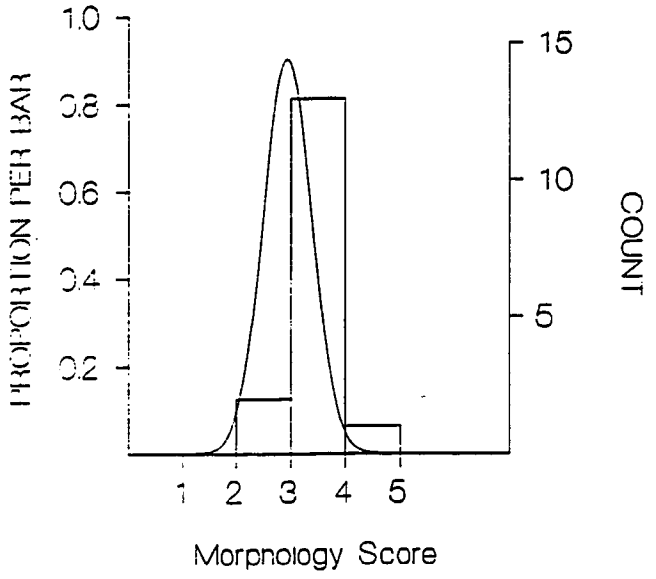
The effect of the high dose DES containing pellet on vehicle-exposed glands are summarized in Figure 8. Data from 16 rats in 14 different litters are shown here. The majority of the experimental glands (62.5%) responded to the DES-containing pellets with class IV developmental score, while the rest demonstrated class III morphology. No classes I, II or V were observed in this group of animals. By contrast, the majority of the contralateral glands (75%) demonstrated class II morphology, while class III and IV glands were seen in

Figure 7. Effect of DES (11 ng)-containing Elvax pellets on the morphology of DES-exposed mammary glands. Right side (top panel)=DES pellet. Left side (bottom panel)=control pellet.

DES Exposed Exptl Group (Post DES-11)

Right

Morphology



Left

Morphology

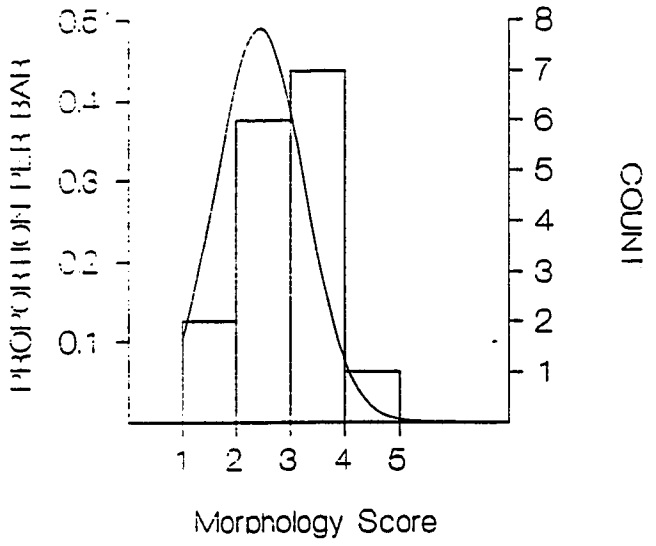
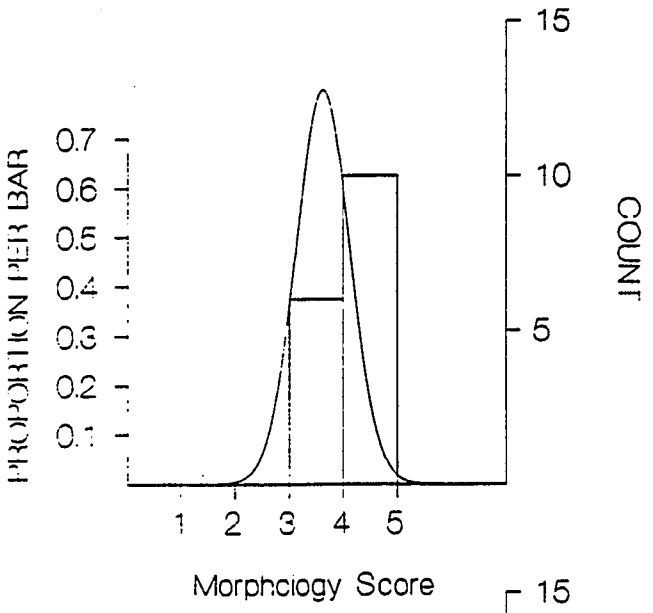


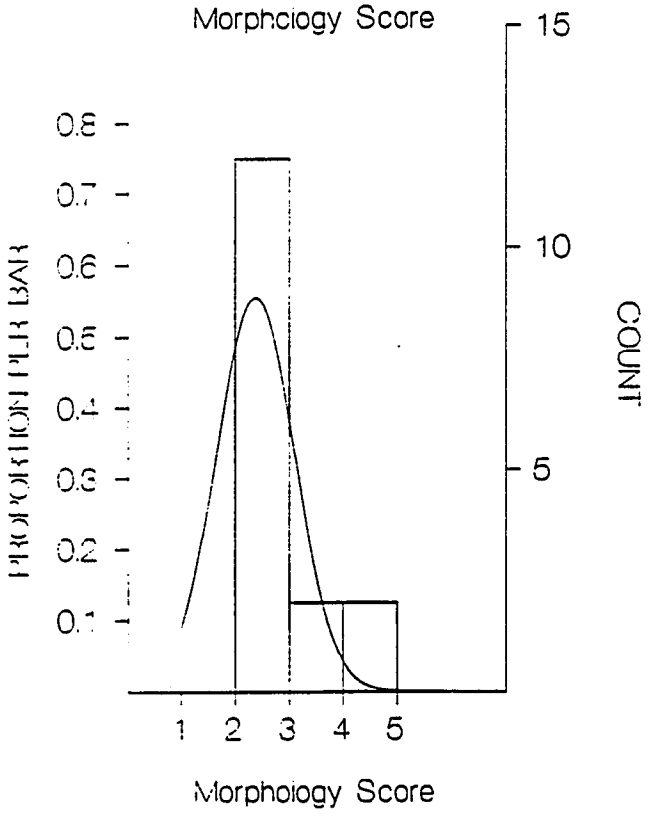
Figure 8. Effect of the DES (11 ng)-containing Elvax pellets on the morphology of vehicle-exposed mammary glands. Right side (top panel)=DES pellet. Left side (bottom panel)=control pellet.

Vehicle Exposed Exptl Group (Post DES-11)

Right Morphology



Left Morphology



12.5% of the glands examined each. These findings strongly suggest a stimulatory effect of the DES-containing pellets on the mammary glands of these vehicle-exposed rats.

Comparisons on the degree of the response elicited by the implants on both the DES-exposed and the vehicle-exposed mammary gland morphology demonstrated a difference on the degree of response elicited. The majority of the DES-exposed glands responded to the local stimulant with class III degree of differentiation. On the other hand, the vehicle-exposed glands demonstrated in the most cases moderate lobulo-alveolar expansion or class IV differentiation score. When the effects of these DES containing implants were subjected to statistical analysis, significant differences between the two exposure groups were observed ($P \leq 0.001$).

Effects of Prenatal Exposure to DES or Vehicle and Postnatal Treatment to DES or Estradiol on Uterine and Pituitary Weights.

The data from the two prenatal exposure groups and all the postnatal treatment groups are summarized in Tables 1 and 2. DES exposure plus postnatal treatment did not seem to affect uterine and pituitary weights.

Effects of Prenatal Exposure to DES or Vehicle and Postnatal Treatment to DES or Estradiol-17b on Time of Vaginal Opening.

The data from the prenatal exposure groups and all the

Table 1.

Mean Uterine Weights from DES and Vehicle-exposed Rats.

Prenatal Exposure	Postnatal Treatment	[1]Genital Morphol.	Mean Uterine Weight (mg±SD)
DES	DES (5ng)	INT (n=12)	102±24
		*UA (n=2)	177±30
	DES (11ng)	INT (n=7)	101±36
		UA (n=4)	185±71
	E2 (5ng)	INT (n=10)	122±32
		UA (n=2)	196±24
	E2 (11ng)	INT (n=9)	148±64
		UA (n=2)	211±70
SO	DES (5ng)	INT (n=13)	121±44
		UA (n=1)	146
	DES (11ng)	INT (n=14)	121±44
		UA (n=2)	133±18
	E2 (5ng)	INT (n=13)	132±46
		UA (n=3)	140±74
	E2 (11ng)	INT (n=13)	94±31
		UA (n=2)	162±8

1. refers to the status of the morphology of the genital tract: UA=unilateral agenesis, INT=intact. Some reproductive tracts were lost in the process of tissue preparation.

* The weights of the unilateral agenesis reproductive tracts were statistically different from intact reproductive tract weights, $P \leq 0.01$.

Table 2.

Mean Pituitary Weights from DES and Vehicle-exposed Rats.

Prenatal Exposure	Postnatal Treatment	[1]Genital Morphol.	Mean Pituitary Weight (mg±SD)
DES	DES (5ng)	INT (n=17)	6.6±1.8
		UA (n=1)	6.5
	DES (11ng)	INT (n=10)	6.5±0.8
		UA (n=4)	6.2±0.6
	E2 (5ng)	INT (n=12)	6.4±1.7
		UA (n=1)	6.6
	E2 (11ng)	INT (n=10)	7.0±1.6
		UA (n=2)	7.1±1.4
SO	DES (5ng)	INT (n=12)	7.0±0.9
		UA (n=1)	6.0
	DES (11ng)	INT (n=15)	6.6±0.6
		UA (n=2)	6.1±0.7
	E2 (5ng)	INT (n=13)	6.7±0.8
		UA (n=3)	5.4±1.6
	E2 (11ng)	INT (n=16)	6.9±0.6
		UA (n=3)	6.6±0.7

1. refers to the status of the morphology of the genital tract: UA=unilateral agenesis, INT=intact. Some pitutary glands were lost in the process of extraction.

Table 3.

Time of Vaginal Opening in Rats Exposed Prenatally to DES Postnatally Implanted With E2 or DES pellets.

Prenatal Exposure	[1]Postnatal Treatment	[2]Genital Morphol	Age at Which Vagina Found Open ≤49	≤53
DES	DES(5 ng) n=20 lit=18	INT n=19	3(16%)	4(21%)
		UA n=1	1(100%)	1(100%)
	DES(11ng) n=16 lit=14	INT n=12	1(8%)	3(25%)
		UA n=4	2(50%)	3(75%)
	E2(5ng) n=16 lit=14	INT n=13	0(0%)	0(0%)
		UA n=3	3(100%)	3(100%)
	E2(11ng) n=17 lit=14	INT n=15	1(7%)	2(14%)
		UA=2	2(100%)	2(100%)

1. refers to the type of postnatal treatment; DES or E2 (5 and 11 ng)= implantation with Elvax pellets containing 5 or 11 ng of DES or E2. Differences in population numbers are due to the fact that time of vaginal opening inspections commenced after the onset of the experiment.
2. refers to the status of the morphology of the genital tract: INT=intact, UA=unilateral agenesis.

Table 4.

Time of Vaginal Opening in Rats Exposed Prenatally to Vehicle and Postnatally Implanted with E2 or DES pellets.

Prenatal[1] Exposure	[2]Postnatal Treatment	[3]Genital Morphol.	Age at Which Vagina Found Open ≤49	≤53
SO	DES(5ng) n=15 lit=14	INT n=14	0(0%)	3(21%)
		UA n=1	1(50%)	1(50%)
	DES(11ng) n=18 lit=16	INT n=16	0(0%)	4(25%)
		UA n=2	1(50%)	1(50%)
	E2(5ng) n=16 lit=15	INT n=13	0(0%)	1(8%)
		UA n=3	2(67%)	3(100%)
	E2(11ng) n=19 lit=17	INT n=16	0(0%)	1(6%)
		UA n=3	1(33%)	3(100%)

1. SO=sesame oil.
2. refers to the type of postnatal treatment; DES or E2 (5 and 11 ng)= implantation with Elvax pellets containing 5 or 11 ng of DES or E2. Differences in population numbers are due to the fact that time of vaginal opening inspections commenced after the onset of the experiment.
3. refers to the status of the morphology of the genital tract: INT=intact, UA=unilateral agenesis.

postnatal treatment groups are summarized in Tables 3 and 4. Data from 69 DES-exposed and 68 vehicle-exposed animals are shown here. The majority of the animals in both exposure groups and all the postnatal treatments had closed vaginas at the time of sacrifice. Animals exhibiting unilateral agenesis were found in every exposure and postnatal treatment group. No vehicle-exposed animal with intact morphology, was found to have vaginal opening before the day of pellet implantation, d49. On the other hand, a small percentage of the DES-exposed intact animals did have open vaginas at that time. These findings are consistent with our previous findings (see Chapter 1) and suggest an earlier time of vaginal opening in the DES exposure group. No significant differences in the time of vaginal opening were observed as a function of the type of pellet implanted.

DISCUSSION

Our data demonstrate that the mammary glands of prenatally DES-exposed rats tend to be refractory in their response to local stimulation by E2 or DES, when compared to the response exhibited by vehicle-exposed mammary glands to the same type of local stimulation. While significant differences were seen only when a 11 ng DES pellet was used, there was an indication that the DES-exposed glands were less responsive to 5 ng DES and 11 ng E2. As the degree of stimulation by these pellets was not as pronounced in the vehicle-exposed glands as when the 11 ng DES pellet was used, a refractory response was more difficult to detect.

Exposure to estrogens during the perinatal period has been seen to affect the development of the mammary gland in rodents. Prenatal exposure to estrogens has been observed to have teratogenic effects on nipple development in the rat (Greene et al, 1939a; Greene et al, 1939b; Greene et al, 1940). Work from our laboratory has also shown that prenatal exposure to DES resulted in nipple teratogenesis in neonatal rats (Boylan, 1978). Prenatal exposure to estradiol dipropionate has resulted in transient inhibition of mammary gland branching in neonatal mice (Jean, 1969). Neonatal treatment with estradiol 17B has been seen to cause mammary gland regression in the rat (Nagasawa et al, 1974). In mice, neonatal treatment with estradiol 17B or DES resulted in

reduced ductal branching (Tomooka and Bern, 1982). In addition, prenatal exposure to DES resulted in long term inhibition of mammary gland development in mice (Nagasawa et al, 1980). Inhibition of nipple and/or duct development has been also seen in mice following prenatal exposure to estradiol benzoate in mice (Hoshino, 1979).

It is possible that DES acts on the embryonic mammary gland via its estrogenic ability. It has been shown that DES can act directly to the rat fetus to produce teratogenic effects similar to the ones produced by estradiol (Henry et al, 1984). Direct effects of estrogen on the fetal mouse mammary gland have been reported (Raynaud and Raynaud, 1954). Severe inhibition of mammary gland development and abnormal nipple development were seen when estrogens were injected directly into the mouse fetus. It is possible that DES is having similar effects on the development of the ACI rat mammary gland.

The mammary stroma seems to be involved in the epithelial response to hormones. Contact between stroma and epithelium appears to be critical for epithelial DNA synthesis response to estradiol (McGrath, 1983). Mesenchymal cells appear to be a likely site for DES action during the intrauterine period. Estrogen receptors can be found only on the mesenchymal cells surrounding the embryonic mammary gland (Narbaitz et al, 1980). It is possible that <u>in utero</u> DES treatment affects the normal development of these important mesenchymal cells.

These affected stromal cells could in turn direct the altered sensitivity patterns to local stimulation seen in the DES-exposed mammary glands.

The results of the present study suggest that the morphological hypo-differentiation observed in the DES-exposed glands of peripubertal ovariectomized ACI rats can be extended to the gland's response to local stimulation by natural or synthetic estrogens.

Chapter 3

THE EFFECT OF SIALOADENECTOMY ON MAMMARY GLAND MORPHOLOGY IN OVARIECTOMIZED ACI RATS FOLLOWING PRENATAL EXPOSURE TO DIETHYLSTILBESTOL (DES).

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ABSTRACT

We have previously shown that prenatal exposure to DES causes atypical mammary gland development in prepubertal female ACI rats. This study was undertaken to determine the role of endogenous epidermal growth factor (EGF) in the development of the DES-exposed and control (vehicle-exposed) ACI rat mammary gland by removing the major source of circulating EGF, the submandibular glands. Pregnant ACI rats received vehicle or DES (8.0 μg total dose) on days 15 and 18 of gestation. The DES-exposed group and vehicle-exposed groups each contained 51 pups. Approximately half the animals in each exposure group were subjected to bilateral sialoadenectomy as well as bilateral ovariectomy at day 34 and sacrificed 19 days later. The other half was subjected to bilateral ovariectomy and sham sialoadenectomy. The degree of lobulo-alveolar development was evaluated by examining stained wholemounts of the third mammary gland pair. Each gland was assigned a score on a scale of I (= least differentiated) to V(= most differentiated).

Results for grades I-V respectively in the DES-exposed and postnatally sialoadenectomized group were: 15%; 37.5%; 37.5%; 10%; 0%; in the DES-exposed and postnatally sham operated group: 0%; 65%; 13%; 17%; 5%; in the vehicle-exposed and postnatally sialoadenectomized group: 0%; 59%; 37%; 4%; 0%; in the vehicle-exposed and postnatally sham-operated

group: 0%; 65%; 35%; 0%; 0%;.

Our data demonstrate that sialoadenectomy did not alter the pattern of glandular morphology expected in DES-exposed and vehicle-exposed ovariectomized female ACI rats. These results suggest that EGF is not playing any major trophic (or inhibitory) role in the ovariectomized peripubertal rat, and that removal of the major source of endogenous EGF has no differential effect on the DES-exposed mammary gland.

INTRODUCTION

The growth and development of the rodent mammary gland has been found to be regulated by a multitude of hormones and growth factors. One of these regulatory molecules is epidermal growth factor (EGF). EGF is a single polypeptide chain, that consists of 53 amino acid residues. The main site of EGF production in mice are the submandibular glands (G. Carpender and S. Cohen, 1979). EGF has been shown to stimulate lobulo-alveolar growth in the mammary gland of non-ovariectomized mice following estrogen-progesterone priming (Vonderhaar, 1987). Furthermore, local application on the mammary gland of EGF-containing Elvax pellets has been shown to be effective in re-initiating ductal growth and morphogenesis in growth arrested glands of ovariectomized mice (Coleman et al, 1988). In addition, the effects of the EGF-containing pellet were shown to be confined in the area around the implant and were dose and time dependent. Among the effects of EGF observed by these investigators were formation of new end buds and reinitiation of epithelial DNA synthesis. When the same methodology was applied in hormonally-intact animals, local EGF application was found to inhibit normal ductal growth (Coleman and Daniel, 1990). The effects of EGF implants in this study were found to be restricted in the area around the implant and were fully reversible. Reduced levels of DNA synthesis and size reduction of the EGF-treated end

buds characterized this EGF directed inhibition. In light of these findings, it was suggested by these investigators that EGF plays an important role in mouse mammary morphogenesis, in that it can act as either a mitogen or a growth inhibitor depending on the growth state of the gland (Coleman and Daniel, 1990).

Functional EGF receptors have been identified in normal mammary epithelial cells in culture (Takehani and Oka, 1983). Studies on the characterization and quantification of the EGF receptor in mouse mammary tissue have shown that there is a constant decrease in high affinity receptor level with increasing age (Edery et al, 1985). This finding was true also for the beginning stages of gestation and lactation. During midpregnancy, a time of rapid mammary proliferation, high affinity receptor levels reach maximal levels, but during midlactation high affinity EGF receptor levels become minimal. It was concluded by these studies that the high affinity receptor levels of EGF that exist in the mouse mammary gland are modulated differently according to the physiological state of the gland.

EGF was found to inhibit casein production while it promotes cell growth in mammary epithelial cells in culture derived from mice at different stages of pregnancy (Takehani and Oka, 1982). Hormone-dependent differentiation of mammary epithelial cells is inhibited by the presence of EGF in culture (Takehani and Oka, 1983). Aside from its role during

functional differentiation, EGF has been found to be a growth and morphological development regulator in organ culture (Turkington, 1969; Tonelli and Sorof, 1980).

Studies on the effects of submandibular gland EGF on mammary tumorigenesis have shown that this growth factor plays an important role in this process (Kurachi et al. 1985). Sialoadenectomy (removal of the submandibular salivary glands) of young CH3/Hen mice (14-22 weeks old) reduced the mammary tumor incidence occurring later in life (Kurachi et al, 1985). Furthermore, the same study indicated that, the small number of sialoadenectomized animals that did develop mammary tumors had increased tumor latency and decreased rate of tumor growth. Studies of the effect of endogenous EGF on the success rate of transplantation of a spontaneous mammary tumor into nude mice have indicated that EGF is a crucial factor in this process (Tsutsumi et al, 1987). Sialoadenectomy diminished the success rate of that tumor into recipient nude female mice by about 38%. Replacement EGF therapy in sialoadenectomized mice enhanced the success rate of tumor implantation. Recently, it was shown that EGF may be an important factor in mouse mammary carcinogenesis (Inui et al, 1989). These investigators have shown that sialoadenectomy reduced the incidence of precancerous and cancerous mammary lesions and lowered the success rate of mammary cancer transplantation in two strains of mice.

In view, of the potentially important role of endogenous

EGF in mammary development and, more importantly, in carcinogenesis, we sought to investigate the possible role of this growth factor in the processes involved in the development of the normal or DES-exposed ACI rat mammary gland.

Work from our laboratory has shown that prenatal exposure to DES alone results in mammary tumorigenesis in the adult, hormonally intact female offspring of the ACI rat strain (Rothschild et al,1987). Furthermore, we have recently shown that prenatal exposure to DES alone is effective in producing morphological alterations in the developmental pattern of the mammary gland in the prepubertal, ovariectomized ACI rat female offspring (Chapter 1).

The specific aim of this study was to investigate the effect of endogenous EGF in the control of growth and differentiation in the normal and DES-exposed rat mammary gland. This project was undertaken in order to broaden our understanding of the potentially altered physiological state of the rat mammary gland following exposure to DES <u>in utero</u>.

MATERIALS AND METHODS

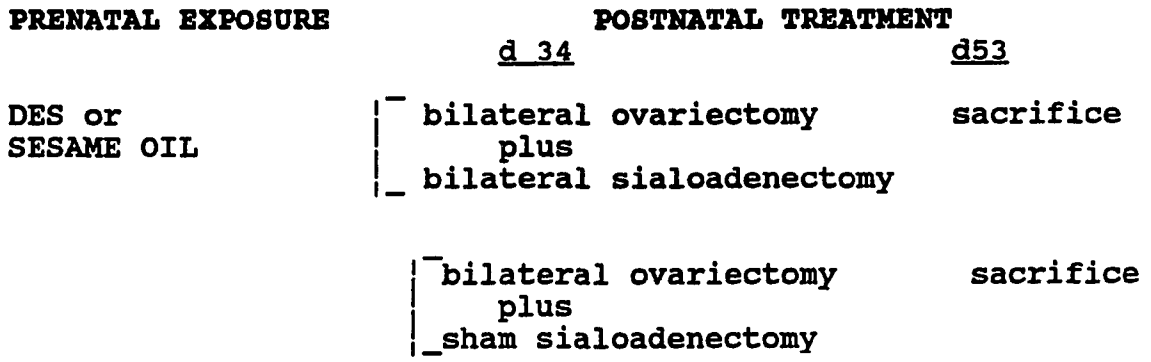
Treatment of Pregnant Females

On days 15 and 18 of gestation, pregnant females in the experimental and control groups were injected with DES or sesame oil as per the methods explained in Chapters 1 and 2. Pregnant females were allowed to deliver naturally and raise their pups to weaning. The total number of DES-exposed offspring was 51 in 28 litters, and 51 vehicle-exposed offspring in 37 litters. The female offspring from each litter were assigned randomly to the different postnatal treatment groups.

Treatment of Female Offspring.

At 34 days of age, both the DES-exposed and vehicle-exposed female offspring were subjected to bilateral dorsal ovariectomy following anesthesia with a mixture of Ketamine and Xylazene (200 mg/20 mg; 0.06 ml mixture / 100gr rat) administered i.m. At the same time animals of both exposure groups were subjected to sialoadenectomy or were sham-operated. A schematic representation of the experimental protocol is given in Fig.1. Sialoadenectomies were performed by first making a small (1 1/2-2 cm) midline incision in the ventral cervical area. The submandibular glands were easily located and carefully ligated with surgical clamps before being excised. Upon removal of the submandibular glands, minimal or no bleeding was observed. Following excision,

Figure 1.


PRENATAL EXPOSURE
POSTNATAL TREATMENT
d 34
d53
DES or
SESAME OIL
bilateral ovariectomy
plus
bilateral sialoadenectomy
sacrifice
bilateral ovariectomy
plus
sham sialoadenectomy
sacrifice

wound clips were used to close the skin. Sham-operated animals were subjected to the same midline incision, but the submandibular glands were left unaffected. The same wound clips were used in these animals as well. Animals were kept under a warm lamp until the effects of the anesthetic wore off, and the rats were observed to walk about in the cage. At selected intervals between d34 and sacrifice, the vagina was inspected to determine the approximate time of vaginal opening.

Necropsy-Histology

DES-exposed and vehicle-exposed animals were asphyxiated with carbon dioxide at 53 days of age, 19 days following ovariectomy and sialoadenectomy or sham sialoadenectomy. At necropsy the entire genital tract was removed and fixed in 10% neutral buffered formalin. The ACI rat strain is characterized by the occurrence of unilateral renal and uterine agenesis in approximately 26% of the female animals (Fujikura, 1970). The genital morphology exhibited by each animal and the weight of the fixed genital tract were recorded.

Mammary glands were spread stained, photographed, and evaluated as per the methods explained in Chapters 1 and 2.

Analysis

For statistical methods see chapters 1 and 2 (Materials

and Methods). As for chapters 1 and 2, rats displaying unilateral agenesis were excluded from the analysis of mammary gland morphology.

RESULTS

Prenatally DES-exposed + Postnatally Sialoadenectomized Rats

The effects of sialoadenectomy on the morphology of the DES-exposed peripubertal glands are shown on Table 1 and are presented graphically in Figure 2. With rats displaying unilateral agenesis removed, data from the remaining 20 rats in 15 different litters are summarized here. The majority of the glands in this group (85%) exhibited low degree of alveolar development (Class II) to moderate degree of lobulo-alveolar expansion (Class IV). Fifteen percent exhibited the lowest degree of developmental score (Class I). There were no class V glands in this group.

Prenatally DES-exposed plus Postnatally Sham-operated Rats.

The effects of postnatal sham operations on the morphology of the DES-exposed peripubertal glands are shown in Table 1 and Figure 2. Data from 23 rats in 13 different litters are shown here. The majority of these glands (95%) were seen to exhibit developmental scores ranging from Class II to Class IV. A small percentage of these animals was observed to exhibit Class V glandular morphology.

While both distributions were somewhat atypical for DES-exposed glands in that the prenatally DES-exposed and postnatally sialoadenectomized group lacked class I, no statistical differences were observed between postnatal

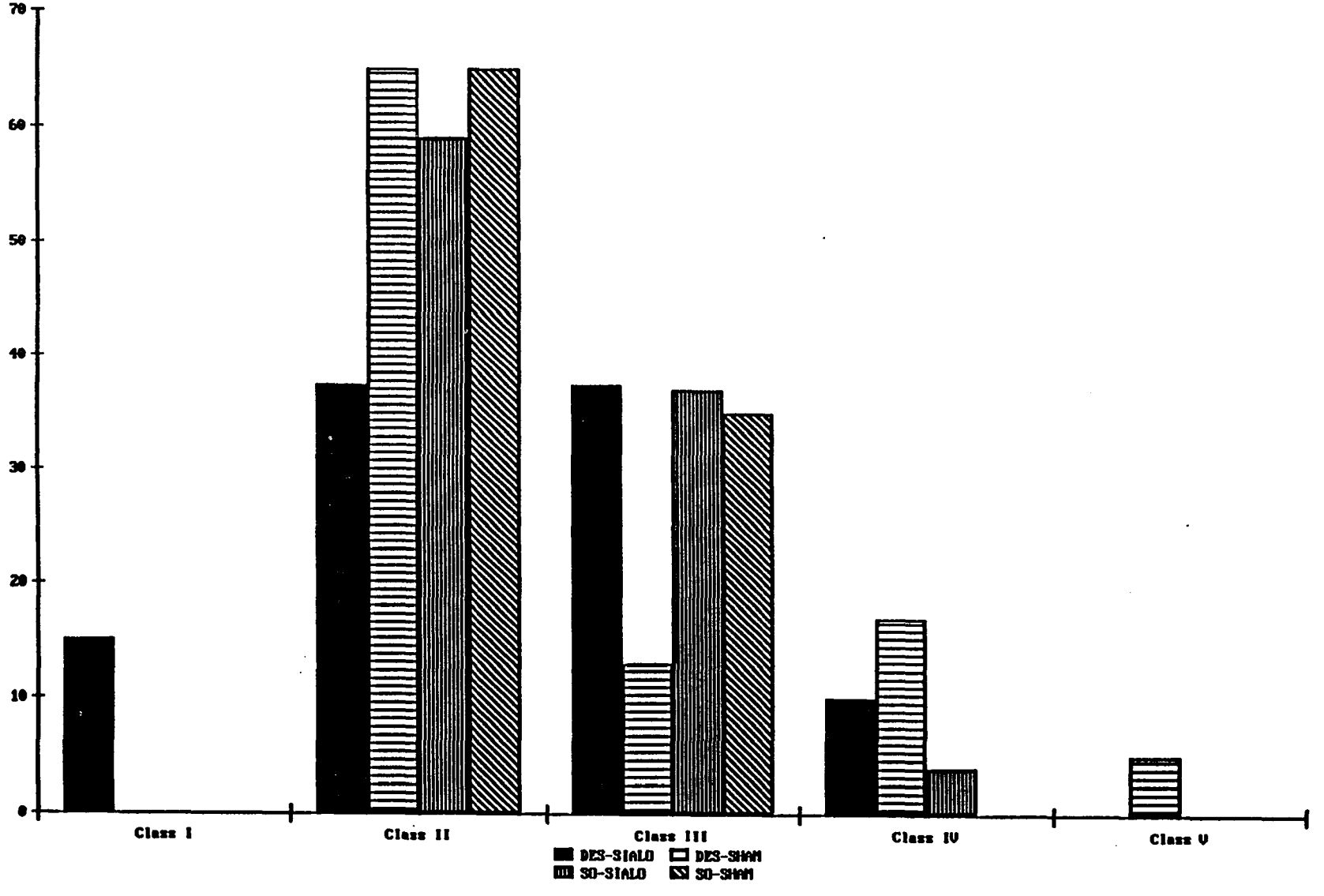
Table 1.

Mammary Gland Developmental Score Comparisons (n=# of rats).

Prenatal Exposure	[1]Postnatal Treatm.	Class	No and Percent of Glands (R+L)
DES n=20	sialo	I	6 (15%)
		II	15 (37.5%)
		III	15 (37.5%)
		IV	4 (10%)
		V	0 (0%)
DES n=23	sham	I	0 (0%)
		II	30 (65%)
		III	6 (13%)
		IV	8 (17%)
		V	2 (5%)
SO n=23	sialo	I	0 (0%)
		II	27 (59%)
		III	17 (37%)
		IV	2 (4%)
		V	0 (0%)
SO n=20	sham	I	0 (0%)
		II	26 (65%)
		III	14 (35%)
		IV	0 (0%)
		V	0 (0%)

1. refers to the type of postnatal treatment: sialo=sialoadenectomy, sham=sham-operated animals.

Figure 2. Mammary gland developmental scores in animals with intact reproductive tracts in prenatally DES or vehicle-exposed and postnatally sialoadenectomized or sham-operated female ACI rats.


70
60
50
40
30
20
10
0
Class I
Class II
Class III
Class IV
Class V
DES-SIALO
DES-SHAM
SO-SIALO
SO-SHAM

treatment groups in the DES-exposed animals. It is likely that the absence of the extreme spectrum of morphologies is a function of the group sizes which were reduced when the rats exhibiting unilateral agenesis were removed from the analysis.

Prenatally Vehicle-exposed plus Postnatally Sialoadenectomized Rats.

The effect of postnatal sialoadenectomy on the morphology of the vehicle-exposed peripubertal glands are shown in Table 1 and Figure 2. Data from 23 rats in 19 different litters are shown here.

All of the animals in this exposure and postnatal treatment group exhibited Class II to IV proliferation scores. Ninety six percent were evaluated as Classes II and III, and only 4% of these glands demonstrated Class IV differentiation score. No class I or V morphologies were seen in these rats.

Prenatally Vehicle-exposed plus Postnatally Sham-operated Rats.

The effects of postnatal sham operations on the morphology of the vehicle-exposed peripubertal glands, are shown in Table 1 and Figure 2. Data from 20 animals in 18 different litters are shown here. All of the mammary glands in this prenatal exposure and postnatal treatment group had differentiation scores ranging from Class II to Class III. Again, no Class I or Class V, differentiation scores were

observed in this group. As with the DES-exposed glands, no statistical differences were observed between postnatal treatment groups in the vehicle- exposed animals.

Time of Vaginal Opening

The effects of prenatal exposure to DES or vehicle and postnatal treatment on the time of vaginal opening in female ACI rats exhibiting intact or unilateral agenesis genital morphologies are summarized in Table 2. It is clear that, whether DES-exposed or not, whether sialoadenectomized or not, rats with unilateral agenesis had an earlier time of vaginal opening.

Uterine Weights

The effects of prenatal exposure to DES or vehicle and postnatal sialoadenectomy or sham operations on uterine weights in ACI rats with intact or unilateral agenesis genital morphologies are summarized in Table 3. Both prenatal exposures and postnatal treatments did not seem to affect the fixed weights of the genital tracts. Uterine weights appeared similar in all four groups. Collectively animals exhibiting unilateral agenesis of the genital tract tended to have heavier uterine weights than rats with intact genital morphologies ($P \leq 0.01$), although the weights for the 2 DES-exposed groups (sialoadenectomized and sham-operated) displaying unilateral agenesis were unusually low.

Table 2.

Time of Vaginal Opening in Rats Exposed Prenatally to DES or Vehicle and Postnatally Sialoadenectomized or Sham-operated.

Prenatal Exposure	[1]Postnatal Treatm.	[2]Genital Morphol	Age When Vagina Found Open ≤49	≤53
DES	sialo n=26	INT n=20	0(0%)	1(5%)
		UA n=6	3(50%)	3(50%)
	sham n=25	INT n=23	3(13%)	5(22%)
		UA n=2	1(50%)	1(50%)
SO	sialo n=27	INT n=23	0(0%)	1(4%)
		UA n=4	0(0%)	3(75%)
	sham n=24	INT n=20	0(0%)	1(5%)
		UA n=4	0(0%)	2(50%)

1. refers to the type of postnatal treatment: sialo=sialoadenectomy, sham=sham-operated animals.
2. refers to the status of the morphology of the genital tract: UA=unilateral agenesis, INT=intact.

Table 3.

Uterine Weights of Rats Exposed Prenatally to DES or Vehicle and Postnatally Sialoadenectomized or Sham-operated.

Prenatal Exposure	[1]Postnatal Treatm.	[2]Genital Morphol.	[3]Uterine Weights (mg±SD)	Range
DES	Sialo	INT n=20	144±18.07	117-180
		UA n=6	138±38.81*	100-187
	Sham	INT n=23	140±37.96	88-208
		UA n=2	162±82.02*	104-220
SO	Sialo	INT n=23	179±48.75	52-250
		UA n=4	250±66.13*	186-326
	Sham	INT n=20	143±48.73	59-207
		UA n=4	288±102.53*	216-361

SO=sesame oil

1. refers to the type of postnatal treatment; sialo= sialoadenectomy, sham= sham-operated animals.
2. refers to the status of the morphology of the genital tract:INT= intact genital morphology, UA= unilateral agenesis.
3. numbers represent litter means, not individual measurements.

* The weights of the unilateral agenesis reproductive tracts in both exposure and postnatal treatment groups, were statistically different from intact reproductive tract weights, $P \leq 0.01$.

DISCUSSION

Our data demonstrate that sialoadenectomy appears to have no effect on the glandular morphology pattern seen in DES-exposed and vehicle-exposed ovariectomized female ACI rats. The DES-exposed and vehicle-exposed offspring demonstrated the expected range of mammary gland differentiation scores given the reduced sample sizes necessitated by the elimination of rats with unilateral agenesis. Furthermore, sialoadenectomy or sham operations did not seem to affect the time of vaginal opening or uterine weights in both prenatally DES-exposed and vehicle-exposed ACI rats.

EGF has been implicated in the mechanisms of mammary carcinogenesis. Sialoadenectomy has been found to reduce the incidence of precancerous and cancerous lesions and to lower the success rate of mammary cancer transplantation in two strains of mice (Inui et al, 1989). In addition, sialoadenectomy has been shown to markedly reduce the levels of plasma EGF from 0.28 ± 0.13 ng/ml to less than 0.1 ng/ml in female nude mice (Tsutsumi et al, 1987). Furthermore, sialoadenectomy performed in CH3/HeN mice resulted in a fewer number of mammary tumors in these animals with a longer period of latency and a slower growth rate (Kurachi et al, 1985). In contrast, it has been shown that sialoadenectomy did not alter the incidence of mammary cancers induced by the chemical carcinogen 7,12-dimethylbenz[a]antharacene in the rat (Ravdin

et al, 1987). In other studies, EGF has been seen to play a role in skin tumor induction by another chemical carcinogen, methylcholanthracene, in mice (Rose et al, 1976; reviewed by Stoscheck and King, 1986).

Two hypotheses could account for the lack of effect of sialoadenectomy in this experiment: 1) it is possible that other sources of EGF in the ACI rat tissues, could still provide sufficient amounts of the growth factor that compensate for the removal of the primary EGF source, or 2) EGF has no active role in the stage of mammary gland development studied here. The qualities exhibited by EGF on the complex mechanisms involved in normal rat mammary gland development and mammary carcinogenesis are not well understood and require further investigation.

Chapter 4

DISTINGUISHING MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF ACI RATS WITH UNILATERAL AGENESIS OF THE UROGENITAL SYSTEM.

ABSTRACT

A study was undertaken to examine certain aspects of morphological and physiological differences observed between ACI rats with intact or unilateral agenesis of the reproductive tract. It was found that animals exhibiting unilateral agenesis of the genital tract possessed considerably thicker uterine horns, without any additional gross morphological differences between the two morphology categories. Similarly, the fixed weight of the unilateral agenesis genital tracts was found to be greater than the weight observed in intact genital tracts. Upon microscopic examination of uterine structure from animals in both genital morphology and exposure groups, no histological changes (other than size) were found in any of the groups examined. Surface area comparisons of cross-sections of intact and unilateral agenesis uteri revealed a considerable surface area difference between these two genital morphology states. These events were found to be independent of the type of prenatal exposure. By contrast, the wet weight of the pituitary glands in both genital morphology groups was found to be very similar. The type of prenatal exposure did not seem to affect these results.

When the effect of genital unilateral agenesis on mammary morphology was examined it was found that, in both exposure groups, the majority of mammary glands in these animals tended

to have higher degrees of glandular proliferation than the mammary glands of rats with intact genital tracts. Time of vaginal opening was also found to be affected by genital morphology. DES-exposed rats with genital unilateral agenesis were found to have an earlier time of vaginal opening than their genitally intact counterparts. The same findings were true for the vehicle-exposed animals, although vaginal opening in unilateral agenesis DES-exposed animals seemed to occur earlier than in unilateral agenesis vehicle-exposed rats, suggesting independent, additive effects.

These data indicate uterine and mammary morphological differences between animals with intact and unilateral agenesis of the genital tract which are independent of the prenatal exposure group. Use of the ACI rat model, therefore, must take into account these significant differences exhibited by rats with this genital condition.

INTRODUCTION

The ACI rat strain originated at Columbia University in 1926 (Curtis et al. 1939) as a cross between the August line 1561 and Copenhagen line 2331; the latter possessed an Irish marker, and thus the name ACI or A*C was given to the offspring of that cross. This strain is characterized by unilateral renal agenesis and hydronephrosis in 30% of the males and 26% of the females (Fujikura, 1970). Renal agenesis was always associated with absence or hypoplasia of a uterine horn or vas deferens and epididymis on the ipsilateral side. Absence of the corresponding ovary and uterus was reported to be common in female animals with renal agenesis by some investigators (Morgan, 1953). Others have reported that, although the uterine horns were absent in female animals with renal agenesis, ovaries were always present (Fujikura, 1970). These abnormalities have been thought to be the result of an insult to developing embryological structures, including the ureteric bud and the mesonephric and Mullerian duct systems (Cramer and Gill, 1975). The transmission of these defects do not follow a simple Mendelian pattern of inheritance and appear to be polygenic (Morgan, 1953; Cramer and Gill, 1975).

One of the more striking characteristics of this strain is that adult females have an almost zero incidence of spontaneous mammary tumors in their lifetime (Segaloff and Maxfield, 1971). However, these rats are susceptible to the

induction of mammary cancer by chronic, systemic diethylstilbestrol (DES) administration (Dunning et al, 1947, 1952). Reference to other experiments relating to mammary carcinogenesis in this system may be found in the Introduction of this thesis.

Work from our laboratory has shown that transplacental exposure to DES alone results in the development of mammary tumors in adult female rats of the ACI strain (Rothschild et al, 1987). The combination of prenatal exposure to DES plus postnatal DES treatment resulted in significantly greater tumor multiplicity and decreased tumor latency in these animals. Furthermore, prenatal DES exposure alone resulted in increased frequency of atypical uterine epithelia, cystically dilated uterine glands and thickened vaginal epithelia in female ACI rats. Prenatal exposure plus postnatal DES treatment resulted in increased incidence of squamous metaplasia of the luminal epithelium and in cystically dilated uterine glands (Rothschild et al, 1988). We have further shown that, prenatal exposure to DES is capable of eliciting atypical mammary gland morphology in the peripubertal, ovariectomized ACI rat (Chapter 1). Thus the ACI rat strain, is a very useful tool in the study on the effects of prenatal DES exposure and/or DES postnatal treatment in mammary development and tumorigenesis.

In the course of studies in the effects of DES, the question arose as to whether animals with unilateral agenesis

(UA) responded differentially to DES. Surprisingly there is little information in the literature on the morphology of the mammary gland and the reproductive tract in this strain, nor has attention been paid to possible unique features of ACI rats with UA.

The purpose of this study was to investigate aspects of morphological and physiological differences between intact (INT) and UA genital morphologies in an effort to distinguish between effects of prenatal exposure to DES or vehicle and effects of these genital tract morphologies. Data presented here were collected from experiments presented in chapters 1-3 in order to describe characteristics unique to ACI rats with UA on as large a pool of animals as possible.

MATERIALS AND METHODS

Animals referred to in this chapter reflect a combination of those from chapters 1-3 grouped, as appropriate, according to prenatal exposure. Here contrasts are made between subsets of each exposure group on the basis of the morphology of the urogenital tract: INT or UA.

Treatment of Pregnant Rats and Female Offspring

Pregnant rats and their female offspring were treated as per the methods described in chapters 1-3. At day 34, rats were ovariectomized. In cases where an ovary (and uterine horn) was not immediately apparent through the dorsal incision, a careful exploration of the abdominal fat pad was undertaken. Those rats with no visible ovary or uterine horn on one side were tentatively identified as UA, awaiting confirmation at necropsy.

Necropsy-Histology

DES-exposed and vehicle-exposed animals were asphyxiated with carbon dioxide 19 days following ovariectomy, at 53 days of age. Mammary glands were removed by gentle separation of the mammary tissue from the skin, using surgical scalpels. The nature of the response elicited in the mammary gland following *in utero* exposure to DES or vehicle was judged by examining whole mount preparations of the third mammary gland

pair.

At necropsy the entire genital tract was removed and fixed in 10% neutral buffered formalin. It must be emphasized that careful inspection of the abdominal fat pad at necropsy revealed no evidence of a remaining ovary. The genital morphology (ie. INT or UA) exhibited by each rat and the weight of the fixed genital tract were recorded. At the same time, the pituitary gland was also removed, and its wet weight was recorded.

Both types of genital morphologies were photographed using a Wild stereomicroscope. Uterine horns from animals exhibiting INT or UA were transected to obtain a representative sample of each. All tissues were dehydrated through a series of Cellosolve and Toluene, embedded in paraffin, sectioned at 10 um, and stained with hematoxylin and eosin. Following staining, 2 diameters of the uterine sections from both genital morphologies were measured, using a Nikon Optiphot microscope and the surface area computed. Photomicrographs were obtained using the same microscope. Mammary glands were fixed, stained, evaluated and photographed as per the methods described in chapters 1-3.

Statistics

Data were expressed as the mean$\pm$SD and were analyzed for statistical significance using analysis of variance.

RESULTS

Macroscopic Differences between Intact and Unilateral Agenesis Uteri.

It should be noted that all rats studied here had been ovariectomized at d34. Therefore comparisons are restricted to the remaining portion of the reproductive tracts. No differences in ovarian morphology were noted at the time of ovariectomy, between animals with unilateral agenesis (UA) or intact (INT) reproductive tracts. The gross morphological differences between animals exhibiting UA of the urogenital tract morphology and animals exhibiting intact genital morphology are shown in Figure 1. Animals with intact genital tracts possessed long and slender uterine horns; cervices and vaginas were typical of peripubertal rats. Rats exhibiting unilateral agenesis, while lacking a uterine horn on one side, appeared to have considerably thicker uterine horns on the other when compared to those found in intact rats. Cervical and vaginal morphologies in UA animals were seen to be comparable to those of intact rats. By inspection, no differences genital morphology were observed between animals exhibiting unilateral agenesis as a function of the type of prenatal exposure. The same was true for animals with intact genital morphologies. No other gross morphological differences were seen to exist between animals in both morphology categories.

Figure 1. Reproductive tracts displaying intact and unilateral agenesis morphologies.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Weight comparisons between INT and UA genital tracts in DES-exposed and vehicle-exposed ovariectomized ACI rats are summarized in Table 1. DES-exposed and vehicle-exposed animals with intact genital tracts were seen to have very similar weights of the fixed genital tract, although there was wide variation of weights in each group. Similarly, DES-exposed and vehicle-exposed animals with UA were also found to have comparable weights of the fixed genital tract. These data demonstrate that there is a significant difference in the weight of the fixed genital tract between animals exhibiting unilateral agenesis or intact genital morphologies ($P \leq 0.01$), and that these differences are attributed to the status of the animal's genital morphology rather than the prenatal exposure group.

Microscopic Differences between Intact and Unilateral Agenesis Uteri.

In order to examine possible microscopic differences in the uterine structure between animals exhibiting INT or UA genital morphologies from both exposure groups, representative cross-sections were prepared of the uteri from the following offspring: 11 DES-exposed offspring with INT genital morphologies, 8 DES-exposed offspring with UA genital morphologies, 8 vehicle-exposed offspring with INT genital morphologies, and 7 vehicle-exposed offspring with UA genital morphologies; each animal was from a separate litter.

Table 1.

Weight Comparisons between Intact and Unilateral Agenesis Genital Tracts in DES-exposed and Vehicle-exposed ACI Rats.

Prenatal Exposure	[1]Genital Morphology	Uterine Weight (mg±SD)	Range
DES	INT (n=60)	118.25±49.92	44-331
	UA (n=13)	201.92±50.90*	85-273
SO	INT (n=96)	121.45±46.02	34-263
	UA (n=21)	192.23±64.04*	59-367

1. refers to the status of the morphology of the genital tract: INT=intact genital morphology, UA=unilateral agenesis.

* The weights of the unilateral agenesis reproductive tracts, in both exposure groups, were statistically different from pooled intact reproductive tract weights, $P \leq 0.01$.

Sections of uterine tissue from DES-exposed intact offspring appeared indistinguishable from the vehicle-exposed intact controls. Uterine luminal epithelia appeared normal with a single layer of cuboidal cells, and uterine glands appeared normal without any evidence of dilation. When the cross-sectional surface area was calculated, DES-exposed intact and vehicle-exposed intact uteri had comparable surface area measurements, (Table 2). The same was true for animals with UA in both exposure groups. However, UA animals from both exposure groups, had significantly enlarged uterine surface area measurements, when compared to INT animals ($P \leq 0.01$).

Surprisingly, microscopic morphology of uterine tissue from DES-exposed offspring with UA appeared identical to the uterine epithelial morphology exhibited by vehicle-exposed offspring with UA. The great difference in size was attributable to a proportionally larger amount of stroma and myometrium in UA when compared to INT organs (Figures 2 and 3).

These data demonstrate clearly that it is the morphological state of the genital tract, rather than the prenatal exposure group that account for the differences seen in the surface area measurements in these animals.

Table 2.

Cross-sectional Area Comparisons between Intact and Unilateral Agenesis Uteri in DES-exposed and Vehicle-exposed ACI Rats.

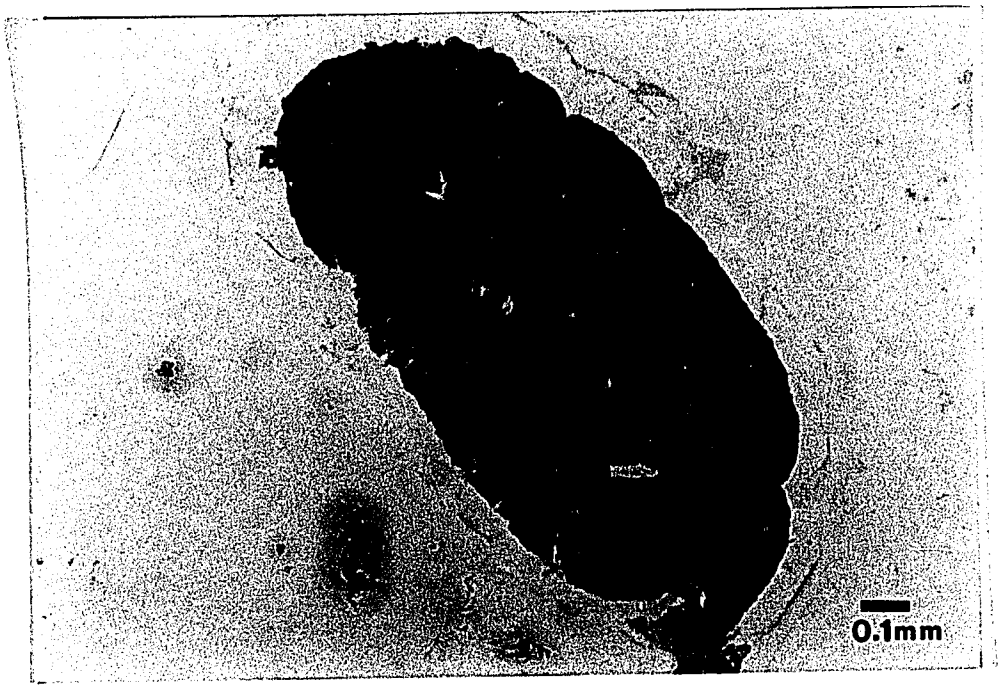
Prenatal Exposure	[1]Genital Morphology	Surface Area (mm^2)	Range
DES	INT (n=11)	0.45±0.32	0.19-1.41
	UA (n=8)	2.1±1.41*	0.39-3.92
SO	INT (n=8)	0.46±0.49	0.03-1.41
	UA (n=7)	2.20±1.11*	0.47-3.76

1. refers to the status of the morphology of the genital tract: INT= intact genital morpholgy, UA=unilateral agenesis.

* The surface area of the unilateral agenesis uteri, in both exposure groups were statistically different from the surface area of the intact uteri, $P \leq 0.01$.

Figure 2. Cross section of uterine horn from vehicle-exposed ACI rat with intact reproductive tract.

Figure 3. Cross section of uterine horn from vehicle-exposed ACI rat with unilateral agenesis.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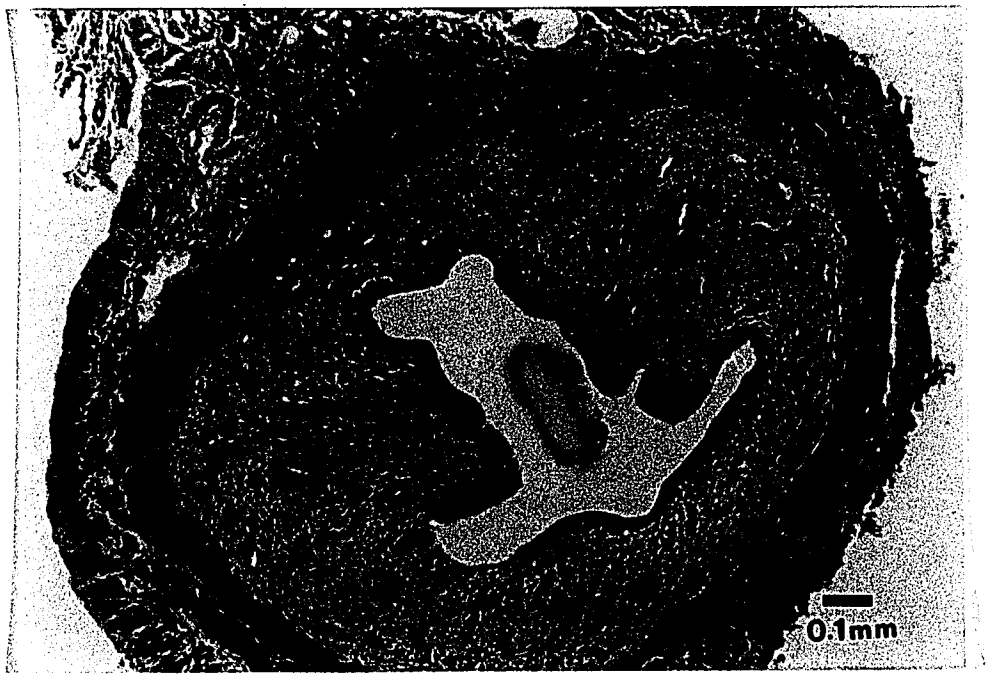
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Time of Vaginal Opening Differences between Intact and Unilateral Agenesis Genital Morphologies in Prenatally DES-exposed or Vehicle-exposed Ovariectomized Peripubertal ACI Rats.

Time of vaginal opening data in both exposure and genital morphology groups are summarized in Table 3. DES-exposed animals with unilateral agenesis genital morphologies tended to have an earlier time of vaginal opening when compared with DES-exposed animals with intact genital tracts. Half of the unilateral agenesis animals in this exposure group had open vaginas at day 49 of life, while only 11% of the genitally intact animals had open vaginas at that time. Furthermore, only 17% of the rats with intact genital morphologies had open vaginas at the time of sacrifice, day 53, while 67% of the unilateral agenesis animals had open vaginal morphologies at day 53.

Only 20% of the vehicle-exposed, intact animals, had open vaginas at the time of sacrifice. By contrast, most of the unilateral agenesis animals in this exposure group (67%) had open vaginal morphology at day 53.

Mammary Gland Morphology Differences between Intact and Unilateral Agenesis Genital Morphologies in Prenatally DES-exposed and Vehicle-exposed Ovariectomized Peripubertal ACI Rats.

Mammary gland developmental score comparisons between

Table 3.

Comparisons of Time of Vaginal Opening between DES-exposed or Vehicle-exposed Female ACI Rats, Exhibiting Intact or Unilateral Agenesis Genital Morphologies.

[1]Genital Morphology	Prenatal Exposure	Age When Vagina Found Open ≤d34	≤d49	≤53
INT n=124	DES n=64	1(2%)	7(11%)	11(17%)
	SO n=60	0(0%)	0(0%)	12(20%)
UA n=23	DES n=6	0(0%)	3(50%)	4(67%)
	SO n=17	0(0%)	0(0%)	11(65%)

1. refers to the status of the morphology of the genital tract: INT= intact, UA= unilateral agenesis.

rats in both genital morphology and exposure groups are shown in Figure 4 and summarized in Table 4. Data from 158 DES-exposed rats in 83 litters and 188 vehicle-exposed rats in 93 litters are shown here. Inspection of the distribution of scores in the DES exposed groups reveals a pronounced shift toward higher scores in UA rats. This is most clearly demonstrated in Class V where 50% of UA rats exhibit this highest degree of development as opposed to 6% in the INT group, and in Class I where no UA scores were found.

As expected, vehicle-exposed glands from animals with INT genital morphologies demonstrated developmental scores of Class II-Class IV. Only 3% of the mammary glands in intact animals exhibited moderate lobulo-alveolar development or Class IV developmental score. On the other hand, 42% of the glands in this exposure group with UA, were classified to have Class IV degree of proliferation. Furthermore, 8% of the mammary glands from animals with UA in this exposure group had Class V proliferation score. This type of developmental score was not seen in any of the 152 vehicle-exposed animals with INT genital morphology. The lowest degree of development seen consistently in this exposure group is Class II. Animals with INT genital morphologies had Class II proliferation scores in 51% of the glands examined, but only 11% of the glands in rats with UA agenesis exhibited this degree of proliferation score. Again, a clear shift to higher mammary gland differentiation was associated with the UA morphology.

Figure 4. Mammary gland developmental score comparisons between DES and vehicle-exposed ACI rats with intact and unilateral agenesis reproductive tracts.

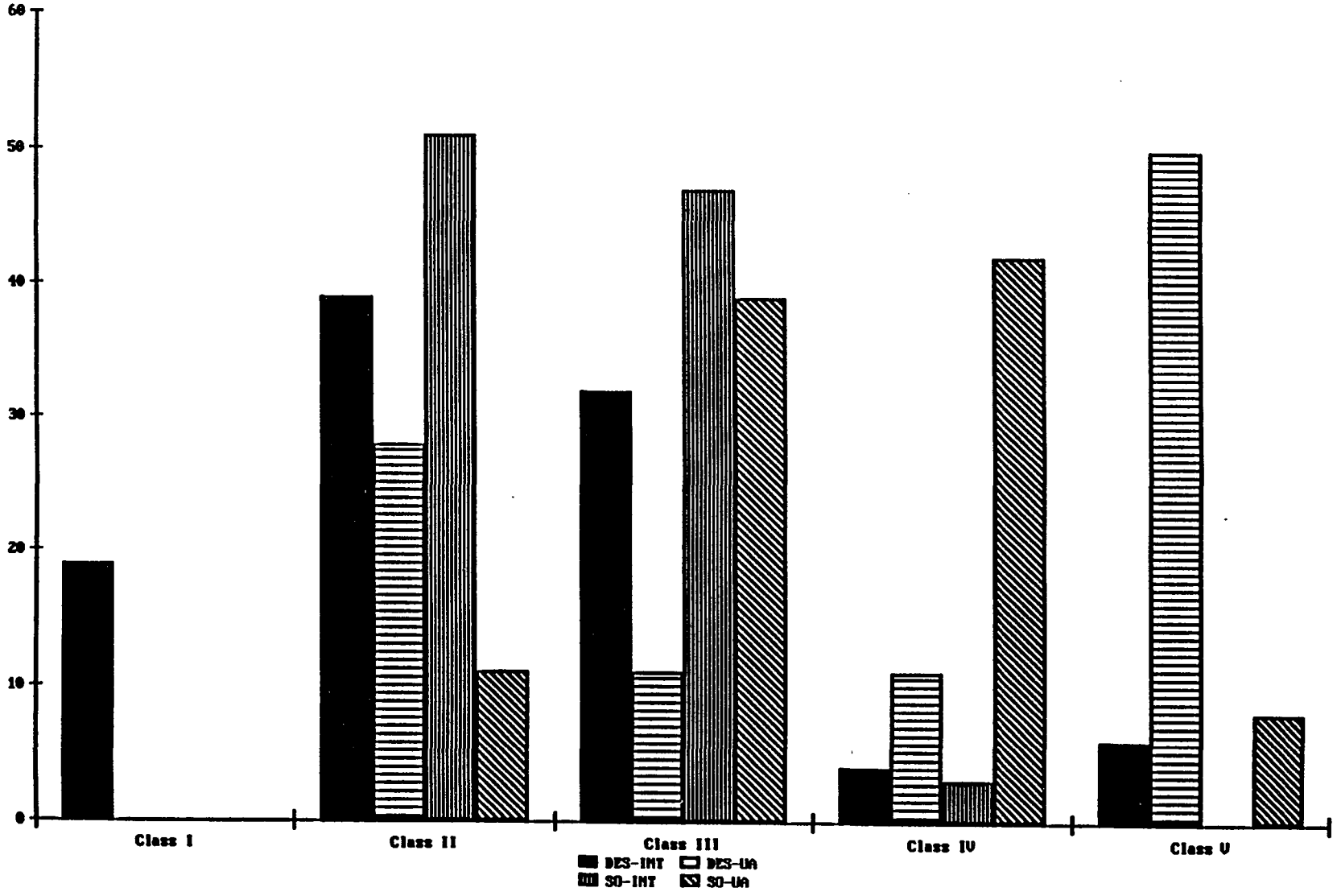
60
50
40
30
20
10
0
Class I
Class II
Class III
Class IV
Class V
DES-INT
DES-UA
SO-INT
SO-UA

Table 4.

Comparisons of Mammary Gland Development Scores between Intact and Unilateral Agenesis Genital Morphologies in Prenatally DES-exposed and Vehicle-exposed Peripubertal ACI Rats.

Prenatal Exposure	[1]Genital Morphology	[2]MG Morphology	
DES n=158 Lit=83	INT n=140	I	27(19%)
		II	54(39%)
		III	45(32%)
		IV	6(4%)
		V	8(6%)
	UA n=18	I	0(0%)
		II	5(28%)
		III	2(11%)
		IV	2(11%)
		V	9(50%)
SO n=188 Lit=93	INT n=152	I	0(0%)
		II	77(51%)
		III	72(47%)
		IV	3(3%)
		V	0(0%)
	UA n=36	I	0(0%)
		II	4(11%)
		III	14(39%)
		IV	15(42%)
		V	3(8%)

SO=sesame oil

1. Refers to the status of the morphology of the genital tract: INT=intact, UA=unilateral agenesis.
2. Refers to the developmental score of the gland; for detailed description, see "Materials and Methods".

Pituitary Wet Weight Differences between Intact and Unilateral Agenesis Genital Morphologies in Prenatally DES-exposed and Vehicle-exposed Ovariectomized Peripubertal ACI rats.

The data on pituitary wet weight comparisons between both exposure and genital morphologies groups are summarized in Chapter 1, Table 2. No significant differences were observed, in the wet weight of the pituitary glands between animals with intact and unilateral agenesis genital morphologies in either of the prenatal exposure groups.

DISCUSSION

The results of this study suggest that there are morphological and physiological differences between ovariectomized peripubertal female ACI rats with intact or unilateral agenesis reproductive tracts. The renal system structural defects observed in this strain have been characterized (Fujikura, 1970; Morgan, 1953). These defects have been attributed to the failure of the mesenchyme of the urogenital ridge to support development of these tubular structures (Cramer and Gill, 1975). To the best of our knowledge this is the first study that reports additional physiological differences between female animals with intact or unilateral agenesis reproductive tracts.

One of the most striking gross morphological differences between ovariectomized animals in the two genital morphology categories was the finding that the remaining uterine horn in the animals exhibiting unilateral agenesis was substantially enlarged in diameter, and heavier when compared to the uterine horns observed in the genitally intact animals. This finding was independent of the type of prenatal exposure. It is possible that some kind of hormonal compensatory mechanism is operating in these animals.

Interestingly, a previous study from our laboratory (Rothschild et al, 1988) has shown that the uteri in intact non-ovariectomized adult ACI rats were considerably heavier

than the uteri of animals with unilateral agenesis in keeping with the presence of essentially only one half of a reproductive tract in UA animals. Our findings in the ovariectomized peripubertal ACI animals show the opposite. To date, no studies have been performed investigating differences in the hormonal milieu between ovariectomized female ACI rats exhibiting intact or urogenital agenesis genital morphologies.

Although macroscopic differences were quite evident between the two types of genital morphologies in these rats, we were not able to identify any microscopic deviations from normality in the structure of the uteri in animals with unilateral agenesis.

Additional physiological differences between animals in both genital morphology groups included differences in mammary gland development score as well as differences in time of vaginal opening. Mammary glands from animals exhibiting unilateral agenesis tended to have higher degrees of proliferation when compared with the developmental scores of mammary glands from animals with intact genital morphologies. Interestingly, none of the DES-exposed animals with unilateral agenesis exhibited Class I differentiation score, a finding observed in 24% of the DES-exposed animals with intact genital morphologies. Furthermore, a small number of prenatally vehicle-exposed glands exhibited Class V differentiation score, an event not seen in identically treated vehicle-

exposed animals with intact genital morphologies.

Time of vaginal opening in animals of both exposure groups was also affected by genital morphology. DES-exposed animals had an earlier time of vaginal opening irrespective of the type of genital morphology. The time of vaginal opening in DES-exposed and vehicle-exposed rats with unilateral agenesis seemed to be earlier than that observed in animals of both exposure groups with intact genital morphologies. Pituitary weights between animals of both prenatal groups and genital morphologies did not exhibit any major differences.

The presence of the morphological differences observed in the INT or UA genital tracts of ACI rats suggests an alteration in the hormonal state of the animal due to the inherited genital malformations. It is possible that compensatory over-production of ovarian steroids occurs somewhere in ACI rats exhibiting unilateral agenesis. The origin of this implicated estrogen source is unknown. This estrogen/progesterone overproduction may, in turn, be responsible for the additional physiological events observed in these rats. In the case of DES-exposed ACI rats with unilateral agenesis, the added effect of prenatal exposure and altered hormonal state could result in an earlier time of vaginal opening than in the DES-exposed, genitally intact animals.

Additional research is required in order to fully understand the morphological and physiological differences

observed between ACI females exhibiting intact and unilateral agenesis genital morphologies. It is suggested by these data that some type of hormonal alteration is in effect in the organisms of the animals exhibiting this inherited urogenital tract impairment. In any event, the differences between INT and UA animals provide justification for removing them from the analysis and interpretation of the effects of DES exposure on mammary gland development, which was the original intent of this thesis.

CONCLUDING STATEMENT

The results of the present study suggest that transplacental exposure to DES induces atypical mammary gland morphology in the peripubertal ACI rat. Furthermore, DES-exposed glands appear to be refractory in their response to local stimulatory molecules such as DES and E2, while sialoadenectomy does not appear to affect the mammary gland development pattern observed in DES-exposed or vehicle-exposed animals.

We suggest that DES could be acting as an initiator of carcinogenesis on the embryonic mammary gland and that this initiation is manifested as a morphological glandular defect well before maturity. The mammary mesenchymal cells in addition to the mammary epithelial cells, are the possible sites of the proposed intrauterine initiation of mammary gland cells. Thus affected embryonic mammary mesenchymal cells could then direct the altered epithelial morphology and sensitivity patterns to local stimulation observed here. Furthermore, we suggest that endogenous submandibular gland EGF does not appear to play an important role in the development of the mammary gland in the peripubertal ACI rat.

Finally, in view of the physiological and morphological differences observed between peripubertal ovariectomized ACI rats with intact genital tracts and those displaying unilateral agenesis of the urogenital system we suggest that

some hormonal differences exist between these two groups of animals. Although the nature of this hormonal alteration was not apparent by our observations, we would like to indicate that these differences should be taken into account when using this strain in experimentation.

APPENDIX

The mammary gland is the target tissue of many hormones and growth factors. Ovarian steroids are responsible for ductal growth and branching, while lobulo-alveolar development during pregnancy requires prolactin (for review see "Introduction"). In addition, high affinity receptors for epidermal growth factor (EGF) have been identified in the mammary glands of mice.

This appendix summarizes the preliminary experiments performed to determine the appropriate amounts of synthetic and natural estrogens, progesterone , prolactin and EGF, that would elicit a local proliferation effect in the mammary glands of female ACI rats. Local administration of these mammogens was achieved by surgical implantation of DES, estradiol 17B, progesterone, prolactin or EGF-containing Elvax 40P pellets (for procedure see, "Materials and Methods" Chapter 2). This implant material causes no inflammatory response, and is capable of slow local release of biologically active molecules.

A series of experiments investigating the conditions that would optimize the manifestation of these localized effects was also performed. These conditions included: the appropriate mammary gland pair for pellet implantation, developmental state of the mammary gland prior to implantation, exact animal age for implantation of the above pellets, and optimum length of time that the mammogen-

containing implant would remain implanted by the mammary gland. All of the above experiments are summarized in the following sections.

Effect of Elvax 40P copolymer on the morphology of the ACI rat mammary gland.

In order to ensure that Elvax 40P does not, by itself, have any proliferative effects on the morphology of the ACI mammary gland, 4 adult non-ovariectomized ACI rats were implanted with Elvax 40P pellets. The fifth and sixth right mammary gland pairs were used as the target tissues. The contralateral glands were left unaffected. The same Elvax preparation and surgical procedure was used as the one described in Chapter 2. Pellets were left in the rats for 4 days. Following that time, the animals were sacrificed and both mammary gland pairs were removed and stained, according to the procedure described in the previous chapters, for histological examination. No difference in the degree of glandular development was noted between the pellet-bearing glands and the contralateral controls. It was concluded, that Elvax 40P, nor the surgical procedure had any effects on the morphology of the ACI rat mammary gland.

Effect of DES-containing Elvax 40P pellets on the mammary glands of adult non-ovariectomized ACI rats.

Having established that Elvax 40P, by itself, does not

cause any change in the morphology of the ACI mammary gland, we proceeded to investigate the effect of DES-containing pellets on the development of these glands. In this experiment, the right fifth mammary glands of 3 virgin adult, non-ovariectomized ACI rats were implanted with Elvax pellets containing 360 μg of DES. An additional 3 animals, were implanted with pellets containing 3.6 μg of DES. The contralateral glands of these animals were implanted with Elvax-control pellets of equal weight (2mg). The pellets were left in the animals for 12 days in both cases. Upon sacrifice the fifth mammary gland pairs were removed, for histological evaluation. In addition, the fourth and sixth mammary gland pairs of each animal were also removed to check for possible systemic effects of DES. It was found that, all three of the mammary gland pairs in each animal in both dosage categories exhibited extensive degrees of lobulo-alveolar development, which is not characteristic of this state of the animal's life. These results were interpreted as a possible systemic effect of both of these doses of the DES pellets on the mammary glands of animals in this age group.

A concomitant trial of these doses with the pellets in place for 5 or 2 days showed once again that there was a systemic effect of the DES pellet on the mammary glands of these rats.

Lower doses of DES pellets (0.36 μg and 0.036 μg of DES/2 mg pellet), implanted on the right fifth mammary gland on even

younger virgin animals (4 weeks of age), resulted in a similar degree of proliferation on both the right and left mammary glands. These effects were observed to be independent of the time that the pellet was left in the animals. In this series of experiments, pellets were left on the mammary glands for 2, 4, 5, 6, or 8 days. The neighboring glands, namely the fourth and sixth mammary gland pairs, were left unaffected. Similar experiments performed on 2 and 3 week old animals yielded the same types of results. When an even lower dose of DES/pellet (0.0036 μg DES/pellet i.e. 3.6 ng) was used on 23 day old rats, a non-systemic effect of the pellet was noted.

In order to produce a more easily noticeable effect of this dose of DES/pellet, on the mammary glands of these animals, it was decided to preform ovariectomies on the animals prior to implantation. Ovariectomy would keep the mammary gland at a relatively more undifferentiated state, so that the effects of the implant would be more profound. A new series of experiments was undertaken to determine the proper age of the animals at which ovariectomy would be performed. In addition the third right mammary gland was chosen as the implantation site. We found that this was a more preferable site since the pellets seemed to stay better in place, and since this gland pair appeared to be consistently somewhat less differentiated than the fifth pair. The length of time that the implant would stay in the animal was also varied in these experiments. It was concluded from the morphology of

the glands examined, that the optimum time for ovariectomy was at 34 days of age and for implantation 15 days later, at 49 days of age. At that time the mammary gland of the ACI rats seemed to have proliferation scores equivalent to Class II or Class III. Furthermore, the most clear proliferation results from this dose of DES pellet were seen when the pellets were left in the animals for 4 days.

Effects of EGF-containing Elvax-40P pellets on the Morphology of the Mammary Glands in the Prepubertal Ovariectomized ACI Rat.

In a different set of experiments, the effect of EGF-containing Elvax 40P pellets on the development of the ovariectomized, prepubertal ACI rats was investigated. In these experiments, the dose of EGF/pellet was varied in addition to the length of time that the pellet was left in the animals. Because EGF is a protein and the amount was very small, serum albumin was used as a carrier to insure equal dispersion of the EGF through the pellet, per method of Silberstein and Daniel (1987). Both ovariectomized and non-ovariectomized animals were used in these studies. Rats that were ovariectomized, were operated on at 34 days of age. The third right mammary glands of both ovariectomized and non-ovariectomized animals were implanted with 1, 2, or 4 μg of EGF/ pellet, two weeks later. The contralateral glands of each animal were implanted with control pellets.

The results obtained from these experiments, have proved to be non-conclusive or contradictory. It is possible that the gland's receptors were saturated with endogenous EGF, and this state of saturation rendered our efforts fruitless.

Alternatively, it is possible that EGF is not a potent proliferation stimulator in this stage, since removal of the primary source of endogenous EGF, the submandibular glands, in these animals did not alter the proliferation pattern seen in the mammary glands of the prepubertal ovariectomized female ACI rats (Chapter 3). Additional possibilities include uneven dispersion of the EGF in the pellet or degradation of its bioactivity in the course of pellet preparation.

Effect of Estradiol 17B (E2), Progesterone and Prolactin-containing Elvax 40P pellets on Mammary Morphology in the Peripubertal Ovariectomized ACI Rat.

In this series of experiments, we sought to determine the correct combination of the above three mammogenic hormones that would elicit a localized proliferative effect on the mammary glands of the prepubertal ovariectomized rat. It was found that, a combination of 1.1 μg of prolactin plus 0.11 μg of progesterone plus 0.011 μg of E2 /pellet resulted in a localized proliferative effect on the mammary glands of these animals. In addition, a combination of 11 μg of prolactin plus 0.11 μg of progesterone plus 0.011 μg of E2 / pellet resulted in a systemic effect on these mammary glands.

On the other hand, Elvax 40P pellets containing 1.1 μg of prolactin or 0.1 μg of progesterone alone were not capable of inducing any visible local morphological change in the mammary glands of these rats. It is possible that prolactin can act synergistically with these ovarian steroid hormones to induce a proliferative effect on the mammary glands of prepubertal ACI rats. Further work is necessary to determine whether such combination pellets would have differential effects on the DES-exposed mammary gland.

BIBLIOGRAPHY

Barnajee, M.R., Wood, B. G., Kinder, D. L. 1973. Whole mammary gland organ culture: Selection of appropriate gland. In Vitro., 9: 129-133.

Barret, I.C., Wong, A., McLachlan, J.A. 1981. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. Science., 212: 1402-1404.

Bergman, B., and Boylan, E.S. 1982. Autoradiographic analysis of DNA and RNA synthesis in the neonatal rat mammary gland after prenatal exposure to diethylstilbestrol. Proceedings of the 64th Annual Meeting of the Endocrine Society, Abstract 1404, San Francisco: The Endocrine Society, p. 430.

Bern, H., Talamantes, F. 1981. Neonatal mouse models and their relation to disease in the human female. In: Developmental effects of Diethylstilbestrol (DES) in pregnancy, A.L. Herbst and H.A. Bern (eds.), New York, Thieme-Stratton, pp. 129-147.

Boylan, E.S. 1978. Morphological and functional consequenses of prenatal exposure to diethylstilbestrol in the rat. Biol. Reprod., 19: 854-863.

Boylan, E., Calhoon, R. 1979. Mammary tumorigenesis in the rat following prenatal exposure to DES and postnatal treatment with 7,12 Dimethylbenz(a)anthracene. J. Toxicol. Environ. Health., 5:1059-1071.

Boylan, E.S., Calhoon. R. 1983. Transplacental action of DES on mammary carcinogenesis in female rats given one or two doses of 7,12 dimethylbenz(a)antracene. Cancer. Res., 43: 4879-4884.

Boylan, E.S., Calhoon, R., Vonderhaar, B.K. 1983. Transplacental action of DES on reproductive and endocrine organs, mammary glands and serum hormone levels in two and nine month old female rats. Cancer. Res., 43: 4872-4878.

Buenaventura, S. K., Jacobson-Kram, D., Dearfield, K.C., Williams, J. R. 1984. Induction of sister chromatid exchange in metabolically competent hepatoma cell lines but not in fibroblasts. Cancer Res., 44: 3851-3855.

Carpender, G., Cohen, S. 1979. Epidermal growth factor. Annu. Rev. Biochem., 48: 193-216.

Ceriani, R.L. 1970. Fetal mammary gland differentiation in vitro in response to hormones II. Biochemical findings. Dev Biol., 21: 530-546.

Closer, H., and Cerni, C. 1984. Increase of sister chromatid exchange formation induced by diethylstilbestrol. Oncology., 41: 285-288.

Coleman, S., Daniel, C. W. 1990. Inhibition of mouse mammary ductal morphogenesis and down regulation of the EGF receptor by epidermal growth factor. Dev. Biol., 137: 425-433.

Coleman, S., Silberstein, G. B., Daniel, C. W. 1988. Ductal morphogenesis in the mouse mammary gland: Evidence supporting a role for epidermal growth factor. Dev. Biol., 127: 304-315.

Cramer, D., Gill, T. J. 1975. Genetics of the urogenital abnormalities in the ACI inbred rats. Teratology., 12: 27-32.

Curtis, G. R., Bullock, F. D., Dunning, W.F. 1931. A statistical study of the occurrence of spontaneous tumors in a large colony of rats. Am. J. Cancer., 15: 67-121.

Daniel, C. W., Silberstein, G. B., 1987. Postnatal development of the rodent mammary gland. In: Mammary gland: development, regulation and function, M.C. Neville, and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 3-36.

Daniel, C. W., Silberstein, G. B., Strickland, P. 1987. Direct action of 17β-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. Cancer Res., 47: 6052-6057.

Dembinski, T. C., Shiu, P. C. 1987. Growth factors in mammary gland development and function. In: The mammary gland: development, regulation and function, M.C. Neville and C. W. Daniel (eds.), New York and London, Plenum Press, pp. 355-381.

Dickson, R. B., Huff. K. K., Spencer, E. M., Lippman, M.E. 1985. Induction of EGF-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinol., 116: 138-142.

Dodds, E. D., Goldberg, L., Lawson, W., Robinson, R. 1938. Oestrogenic activity of certain synthetic compounds. Nature., 141: 247.

Duax, W. L., Weeks, C. M. 1980. Molecular basis of estrogenicity: X-ray crystallographic studies. In: Estrogens in the environment, J.A. McLachlan (ed.), Elsevier/North Holland, New York, pp. 11-32.

Dulbecco, R., Allen, W.R., Bologna, M., and Bowman, M. 1986. Marker evolution during the development of the rat mammary gland: Stem cells identified by markers and the role of myoepithelial cells. Cancer Res., 46: 2449-2456.

Dunning, W. F., Curtis, M. R. 1946. The respective roles of longevity and genetic specificity in the occurrence of spontaneous tumors in hybrids between two inbred lines of rats. Cancer. Res., 6: 61-81.

Dunning, W.F., Curtis, M.R., and Segaloff, A. 1947. Strain differences in response to diethylstilbestrol and the induction of mammary gland and bladder cancer in the rat. Cancer. Res., 7: 511-521.

Dunning, W. F., Curtis, M. R. 1952. The incidence of diethylstilbestrol-induced cancer in reciprocal F1 hybrids obtained from crosses between rats of inbred lines that are susceptible and resistant to the induction of mammary cancer by this agent. Cancer Res., 12: 702-706.

Durnberger, H., Kratochwil, K. 1980. Specificity of tissue interactions and origin of mesenchymal cells in the androgen response of the embryonic mammary gland. Cell., 19: 465-471.

Edery, M., Pang, K., Larson, L., Colosi, T., Nandi, S. 1985. Epidermal growth factor receptor levels in mouse glands in various physiological states. Endocrinol., 117: 405-411.

Forsberg, J., Kalland, T. 1981. Neonatal estrogen treatment and epithelial abnormalities in the cervicovaginal epithelium of adult mice. Cancer Res., 41: 721-734.

Fischer, L., Weissinger, J., Ricket, D., Hintze, K. 1976. Studies on the biological disposition of diethylstilbestrol in rats and humans. J. Toxicol. Environ. Health., 1: 587-605.

Furth, J., Clifton, K. H. Experimental pituitary tumors. In: The pituitary gland. G.W. Harris, B.T. Donovan (eds.), Butterworths, London, pp.460-497.

Frantz, A. G. 1977. The assay and regulation of prolactin in humans. In: Advances in experimental medicine and biology. Vol. 80, Comparative endocrinology of prolactin (H.D. Dellman, J.A. Johnson, and D.M. Klachko (eds.), Plenum Press, New York and London, pp. 95-133.

Freeman, C. S., Topper, Y. J. 1978. Progesterone is not essential to the differentiative potential of mammary epithelium in the male mouse. Endocrinol., 103: 186-192.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., Sporn, M. B. 1983. Purification and initial characterization of a type β transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA., 80: 3676-3680.

Fujikura, T. 1970. Kidney malformations in fetuses of AxC line 9935 rats. Teratology., 3: 245-250.

Gottsclich, R., Metzler, M. 1984. Oxidative metabolites fo the teratogen and transplacental carcinogen diethylstilbestrol in the fetal Syrian golden hamster. J. Environ. Pathol. Toxicol. Oncol., 5(4-5): 329-338.

Greene, R., Burrill, M., Ivy, A. 1939. Experimental intersexuality: modification of sexual development of the white rat with a synthetic estrogen. Proc. Soc. Exp. Biol. Med., 41: 169-179.

Greene, R., Burrill, M., Ivy, A. 1940. Experimental intersexuality: the effects of estrogens on the antenatal sexual development in the rat. Am. J. Anat., 67: 305-312.

Gottschlish, R., Metzler, M. 1984. Oxidative metabolites of the teratogen and transplacental carcinogen diethylstilbestrol in the fetal Syrian golden hamster. J. Environ. Pathol. Toxicol. Oncol., 5 (4-5): 329-338.

Haslam, S. Z. 1987. Role of sex steroid hormones in normal mammary gland function. In: The mammary gland: development, regulation and function, M.C. Neville, and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 499-533.

Haslam, S. Z. 1988. Local versus systemcally mediated effects of estrogen on normal mammary epithelial cell deoxyribonucleic acid synthesis. Endocrinol., 122: 860-867.

Haslam, S. Z. 1988. Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. Endocrinol., 122: 464-470.

Henry, E.C., Miller, R.K., Baggs, R.B. 1984. Direct fetal injections of diethylstilbestrol and 17-β estradiol: A method for investigating their teratogenicity. Teratol., 29: 297-304.

Herbst, A.L., Ulfelder, H., Poskanzer, D.C., 1971. Adenocarcinoma of the vagina: association of maternal stilbestrol therapy with tumor appearence in young women. New England. J. Med., 284: 878-881.

Herbst, A. L., Kurman, R. J., Scully, R.E. 1972. Clear cell adenocarcinoma of the genital tract in young females: Registry report. New Engl. J. Med., 287: 1259-1264.

Herbst, A. L. 1981. The epidemiology of vaginal and cervical clear cell adenocarcinoma. In: Developmental effects of diethylstilbestrol (DES) in pregnancy. A. L. Herbst, H. A. Bern (eds.), New York, Thieme-Stratton, pp. 63-70.

Herbst, A. L. 1984. Diethylstilbestrol exposure-1984. New Engl. J. Med., 311: 1433-1434.

Heuberger, B., Fitzka, I., Wasner, G., and Kratochwil, K. 1982. Induction of androgen receptor formation by epithelial-mesenchymal interaction in embryonic mammary gland. Proc. Natl. Acad. Sci. USA., 79: 2957-2961.

Hill, A., Wolf, S. 1982. Increased induction of sister chromatid exchange by diethylstilbestrol in lymphocytes from pregnant and premenopausal women. Cancer Res., 42: 893-896.

Hoshino, K. 1979. Hormonal teratogenesis in mammary glands of the mouse. In: Advances in the study of birth defects. T. Persaud (ed.), Lancaster, MTP Press, pp. 139-160.

Igushi, T., Takasugi, N., Bern, H., Mills, K.T. 1986. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. Teratology., 34: 29-35.

Inui, T., Tsubura, A., Morii, S. 1989. Incidence of precancerous foci and growth rate of transplantable mammary cancers in sialoadenectomized mice. J. Natl. Cancer Inst., 81(21): 1660-1663.

Jean, C. 1969. Evolution post-natale des anomalies mammaires produites chez les descendants par un traitment oestrogenique de la souri gestante. Comptes rendu Soc. Biol., 163: 1126-1131.

Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Derynk, R., Dickson, R.B. 1987. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell, 48: 417-428.

Korach, K. S., Levy, L. A., Sarver, P. J. 1985. Stereochemical analysis of stilbene estrogens: receptor binding and hormone responsiveness. In: Estrogens in the environment II. Influences on development. J. A. McLachlan (ed.), New york, Elsevier, pp. 43-66.

Kratochwil, K. 1969. Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. Dev. Biol., 20: 46-71.

Kratochwil, K. 1971. In vitro analysis of the hormonal basis for sexual dimorphism in the embryonic development of the mouse mammary gland. J. Embryol. Exp. Morphol., 25: 141-153.

Kratochwil, K. 1975. Experimental analysis of the prenatal development of the mammary gland. Mod. Probl. Paediat., 15: 1-15.

Kratochwil, K., Schwartz, P. 1976. Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue for testosterone. Proc. Natl. Acad. Sci. USA., 73: 4041-4044.

Kurachi, H., Okamoto, S., Oka, T. 1985. Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc. Natl. Acad. Sci., 82: 5940-5943.

Lu, K. H., Meites, J. 1973. Effects of serotonin precursors and melatonin on serum prolactin release in rats. Endocrinol., 93: 152-155.

Lyons, W. R. 1958. Hormonal synergism in mammary growth. Proc. R. Soc. Lond., B149: 303-325.

Lyons, W. R., Li, C, H., Johnson, R. E. 1958. The hormonal control of mammary growth and lactation. Recent Progr. Horm. Res., 14: 219-248.

Maier, B., Newbold, R.R., and McLaclan, J.A. 1985. Prenatal diethylstilbestrol exposure alters murine uterine responses to prepubertal estrogen stimulation. Endocrinology., 116: 1878-1886.

Maydl, R., Newbold, R.R., Metzler, M., McLachlan, J.A. 1983. Diethylstilbestrol metabolism by the fetal genital tract. Endocrinology., 113: 146-151.

McGrath, C. M. 1983. Augmentation of response of normal mammary epithelial cells to estradiol by mammary stroma. Cancer Res., 43: 1355-1360.

McLachlan, J.A. Newbold, R.R., Bullock, B.C. 1980. Long term effects on the female mouse associated with prenatal exposure to diethylstilbestol. Cancer Res., 40: 3988-3999.

McLachlan, J. A., Wong, A., Degen, G. H., Barrett, J. C. 1982. Morphological and neoplastic transformation of Syrian golden hamster embryo fibroblasts by diethylstilbestrol and its analogs. Cancer Res., 42: 423-448.

McLachlan, J.A., Korach, K.S., Newbold, R.R., and Degen, G.H. 1984. Diethylstilbestrol and other estrogens in the environment. Fundamental and Applied Toxicol., 4: 686-691.

McLachlan, J. A., Shah, H. C., Newbold, R.R., Bullock, B. C. 1975. Effect of prenatal exposure of mice to diethylstilbestrol on the reproductive tract function in the offspring. Toxicol. and Applied Pharmacol., 33: 190.

Mehta, R, D., von Borstel, R. C. 1981. Genetic activity of diethylstilbestrol in Saccharomyces cerevisiae. Enhancement of mutagenicity by oxidizing agents. Mut. Res., 92: 49-61.

Meites, J., Clemens, J. A. 1972. Hypothalamic control of prolactin secretion. Vitam. Horm., 30: 165-222.

Metzler, M., McLachlan, J.A. 1978. Oxidative metabolism of diethylstilbestrol and steroidal estrogens as a potential factor in their fetotoxicity. In: Role of pharmacokinetics in prenatal and perinatal toxicology. (eds. D. Neubert, H. Nau, J. Langman), Stuttgart, Georg Thieme Publishers, pp.157-164.

Metzler, M. 1981a. The metabolism of diethylstilbestrol. CRC Critical Rev. Biochem., 10: 171-21.

Metzler, M. 1981b. Diethylstilbestrol metabolism in humans and experimental animals. In: Developmental effects of diethylstilbestrol (DES) in pregnancy. A. L. Herbst, H. A. Bern (eds.), New York, Thieme-Stratton, pp. 158-166.

Metzler, M. 1982. Role of metabolic activation in the transplacental carcinogenicity of diethylstilbestrol. Biol. Res. in Pregn., 3: 103-107.

Metzler, M. 1984. Metabolism of stilbene estrogens and steroidal estrogens in relation to carcinogenicity. Arch. Toxicol., 55: 104-109.

Miller, R. K., Baggs, R. B., Odoroff, C.L., McKenzie, R. C. 1982. Prenatal exposure to diethylstilbestrol (DES) : vaginal tumors and malformations in the rat. Pharmacologist., 24: 236-241

Miller, R. K., Heckmann, M. E., McKenzie, R.C. 1982. Diethylstibestrol placental transfer, metabolism, covalent binding and fetal distribution in the Wistar rat. J. Pharmacol. Exp. Ther., 220: 358-365.

Morgan, C. W. 1953. Inherited congenital kidney absence in an inbred strain of rats. Anat. Rec., 115: 635-639.

Myers, J.A. 1917. Studies on the mammary gland II. The fetal development of the mammary gland of the female albino rat. Am. J. Anat., 22: 195-223.

Narbaitz, R., Stumpf, W.E., and Sar, M. 1980. Estrogen receptors in mammary gland primordia of fetal mouse. Anat. Embryol., 158: 161-166.

Nagasawa, H., Yanai, R., Shodono, M., Nakamura, T., Tanake, Y. 1974. Effect of neonatally administered estrogen or prolactin on normal and neoplastic mammary growth and serum estradiol-17β level in rtas. Cancer Res., 34: 2643-2646.

Nagasawa, H., Mori, T., Nakajima, Y. 1980. Long term effects of progesterone or diethylstilbestrol with or without estrogen after maturity on mammary tumorigenesis in mice. Eur. Exp. J. Cancer., 16: 1583-1589.

Napalkov, N. P., Anisimov, V. N. 1979. Transplacental effect of diethylstilbestrol in female rats. Cancer Lett., 6: 107-114.

Newbold, R. R., McLachlan, J. A. 1981. Vaginal adenosis and adenocarcinoma in mice treated prenatally with diethylstilbestrol (DES). Proc. AACR., 22: 82.

Newbold, R. R., Bullock, B. C., McLachlan, J. A. 1982. Exposure to diethylstilbestrol during pregnancy permanently alters the ovary and oviduct. J. Reprod., 28: 735-744.

Newbold, R. R., McLachlan, J. A. 1982. Vaginal adenosis and adenocarcinoma in mice exposed prenatally or neonatally to diethylstilbestrol. Cancer Res., 42: 2003-2011.

Newbold, R. R., Bullock, B. C., McLachlan. J. A. 1985. Carcinoma of the rete testis in mice exposed prenatally to diethylstilbestrol: The male counterpart of vaginal adenocarcinoma? Proc. AACR., 26: 196.

Nicoll, C. S., Fiorindo, R. P., McKenee, C. T., Parsons, J. A. 1970. Assay of hypothalamic factors which regulate prolactin secretion. In: Hypophysiotropic hormones of the hypothalamus: assay and chemistry, J. Meites (ed.), Williams and Wilkins, Baltimore, pp. 115-144.

Nomura, T., Kanzaki, T. 1977. Induction of urogenital anomalies and some tumors in the progeny of mice receiving diethystilbestrol during pregnancy. Cancer. Res., 37: 1099-1104

Nomura, T., Masuda, M. 1980. Carcinogenic and teratogenic activities of diethylstilbestrol in mice. Life Sciences., 26: 1955-1962.

Oka, T., Topper, Y. J. 1971. Hormone-dependent accumulation of rough endoplasmic reticulum in mouse mammary epithelial cells in vitro. J. Biol. Chem., 246: 7701-7707.

Okamoto, S., Oka, T. 1984. Evidence for physiological function of epidermal growth factor: Progestational sialoadenectomy of mice decreases milk production and increases offspring mortality during lactation period, Proc. Natl. Acad. Sci. USA., 81: 6059-6063.

Orenberg, C. 1981. DES- What, Where, Why, When, Who and How. In: DES: the complete story. New York, St. Martin's Press, pp. 10-33.

Plapinger, L., Bern, H. 1979. Adenosis-like lesions and other cervicovaginal abnormalities in mice treated perinatally with estrogen. J. Natl. Cancer Inst., 63: 507-515.

Proper, A. 1968. Relations epidermo-mesodermiques dans la differencation de l'ebauche mammaire d'embryon de lapin. Ann. Embryol. Morphol., 1: 151-160.

Prosser, C. G., Topper, Y. J. 1986. Changes in the rate of carrier-mediated glucose transport by mouse mammary epithelial cells during ontogeny: Hormone dependence delineated in vitro. Endocrinol., 119: 91-96

Radvin, P. M., Van Beurden, M., Frintz, N. F. 1987. Effect of submandibular gland removal on carcinogen induced mammary tumours. Eur. J. Cancer Clin. Oncol., 23: 449-450.

Raynaud, A., Raynaud, J. 1954. Les diverses malformations mammaires produites chez le foetus de souris par l' action des hormones sexuelles. C.R. Soc. Biol. Paris., 148: 963-968.

Reznic-Shuller, H. 1979. Carcinogenetic effects of diethylstilbestrol in the Syrian Golden hamsters. J. Natl. Cancer Inst., 62: 1083.

Rhine. W. D., Hsieh, D. S., Langer R. 1980. Polymers for sustained macromolecule release: Procedures to fabricate reproducible delivery systems and control release kinetics. J. Pharmac. Sci., 69 (3): 265-270

Rose, S. P., Stahn, R., Passovoy, D.S., Herschman, H. Epidermal growth factor enhancement of skin tumor induction in mice. Experientia., 32: 913-915.

Rothschild, T.C., Boylan, E.S., Calhoon, R., Vonderhaar, B.K. 1987. Transplacental effects of diethylstilbestrol on mammary development and tumorigenesis in the female ACI rat. Cancer. Res., 47: 4508-4516.

Rothschild, T.C., Calhoon, R.E., Boylan, E.S. 1988. Effects of diethylstilbestrol exposure in utero on the genital tracts of female ACI rats. Exp. and Mol. Pathol., 48: 59-76.

Rothschild, T.C., Calhoon, R.E., Boylan, E.S. 1988. Genital tract abnormalities in female rats exposed to diethylstilbestrol in utero. Reprod. Toxicol., 1: 193-202.

Rudiger, H. W., Haenisch, F., Metzler, M., Oeslh, F., Glatt, H. R. 1979. Metabolites of diethylstilbestrol induce sister chromatid exchange in human cultured fibroblasts. Nature (Lond.), 281: 392-394.

Russo, I. H., Russo, J. 1978. Developmental stage of the rat mammary gland as a determinant of its succeptibility to 7,12-dimethylbenz[a]anthracene. J. Natl. Cancer. Inst., 61: 1451-1459.

Russo, I. H., Russo, J. 1987. Development of the human mammary gland. In: Mammary gland: development, regulation and function, M.C. Neville, and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 67-93.

Rustia, M., Shubik, P. 1976. Transplacental effects of diethylstilbestrol on the genital tract of hamster offspring. Cancer. Lett., 1: 139-146.

Rustia, M., Shubik, P. 1979. Effects of transplacental exposure to diethylstilbestrol on carcinogenetic susceptibility during postnatal life in hamster progeny Cancer Res., 39: 4636-4644.

Sakakura, T., Sakagami, Y., Nishizuka, Y. 1979. Acceleration of mammary cancer development by grafting fetal mammary mesenchymes in CH3 mice. Gann, 70: 459-466.

Sakakura, T., Sakagami, Y., Nishizuka, Y. 1979. Persistance of responsiveness of adult mammary gland to the induction of embryonic mesenchyme. Dev. Biol., 72: 201-210.

Sakakura, T., Sakagami, Y., Nishizuka, Y. 1982. Dual origin of mesenchyme tissues participating in the mouse mammary gland embryogenesis. Dev. Biol., 91: 202-207.

Sakakura, T. 1987. Mammary embryogenesis. In: The mammary gland: development, regulation and function, M.C. Neville, and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 37-62.

Salomon, D. S., Zwiebel, J. A., Bano, M., Lobonczy, I., Feechnel, P., Kidwell, W. R. 1984. Presence of transforming growth factors in human breast cancer cells. Cancer Res., 44: 4069-4077.

Segaloff, A., Maxfield, W.S. 1971. The synergism between radiation and estrogen in the production of mammary cancer in the rat. Cancer. Res., 31: 166-168.

Shah, H.C., McLachlan, J.A. 1976. The fate of diethylstilbestrol in the pregnant mouse. J. Pharmacol. Exp. Ther., 197: 687-696.

Sheffield, L. G., Sinha, Y. N., Welsch, C. W. 1985. Cholera toxin treatment increases in vivo growth and development of the mouse mammary gland. Endocrinol., 117: 1864-1869.

Shellabarger, C., Soo, V. 1973. Effects of neonatally administered sex steroids on 7,12-dimethylbenz[a]anthracene-induced mammary neoplasia in rats. Cancer Res., 33: 1567-1569.

Shellabarger, C. J., Stone, J. P., Holtzman, S. 1978. Rat differences in mammary tumor induction with estrogen and neutron radiation. J. Natl. Cancer Inst., 61(6): 1505-1508.

Shellabarger, C. J., McKnight, B., Stone, J.P., Holtzman, S. 1980. Interaction of dimethylbenz[a]anthracene and diethylstilbestrol on mammary adenocarcinoma formation in female ACI rats. Cancer Res., 40: 1808-1811.

Shyamala, G., Ferenzy, A. 1984. Mammary fat pad may be potential site for initiation of estrogen action in the normal mouse mammary glands. Endocrinol., 115: 1078-1081.

Silberstein, G. B., Daniel, C. W., Trumboces, V., Coleman, S. 1984. In vivo cAMP stimulates growth and morphogenesis of mouse mammary ducts. Proc. Natl. Acad. Sci. USA., 81: 4950-4954.

Silberstein, G. B., Daniel, C. W. 1987. Investigation of mouse mammary ductal growth regulation using slow-release plastic implants. J. Dairy. Sci., 70: 1981-1990.

Silberstein, G. B., Daniel, C.W. 1987. Reversible inhibition of mammary gland growth factor-β. Science, 237: 291-293.

Stone, J.P., Holtzman, S., Shellabarger, J. 1979. Neoplastic responses and correlated plasma prolactin levels in diethylstilbestrol-treated ACI and Spague_Dawley rats. Cancer..Res., 39: 773-778.

Stoscheck, C. M., King, L. E. 1986. Role of epidermal growth factor in carcinogenesis. Cancer Res., 46; 1030- 1037.

Takasugi, N., Kimura, T., Mori, T. 1970. Irreversible changes in mouse vaginal epithelium induced by early postnatal treatment with steroid hormones. In: The postnatal development of phenotype. S. Kazda, V. Denenberg (eds.), Prague, Academia, pp. 229-251.

Takehani, Y., Oka, T. 1982. Epidermal growth factor stimulated cell proliferation and inhibited functional differentiation of mouse mammary epithelial cells. Endocrinol., 113: 871-877.

Takehani, Y., Oka, T. 1983. Possible physiological role of epidermal growth factor in the development of the mouse mammary gland during pregnancy. Febs. Lett., 152: 256-260.

Thordarson, G., Talamantes, F. 1987. Role of the placenta in mammary gland development and function. In: The mammary gland: development. regulation and function, M.C. Neville and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 459-498.

Tomooka, Y., Bern, H.A. 1982. Growth of mouse mammary glands after neonatal sex hormone treatment. J. Natl. Cancer. Inst., 69: 1347-1352.

Tonelli, Q, J., Sorof, S. 1980. Epidermal growth factor requirement for development of cultured mammary gland. Nature, 285: 250-252.

Topper, Y. J., Freeman, C. S. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Phys. Rev., 60: 1049-1106.

Tsutsui, T., Maizumi, H., McLachlan, J.A., Barrett, J.C. 1983. Aneuploidy induction and cell transformation by diethylstilbestrol: a possible mechanism in carcinogenesis. Cancer. Res., 43: 3814-3821.

Tsutsumi, O., Tsutsumi, A., Oka, T. 1987. Importance of epidermal growth factor in implantation and growth of mouse mammary tumor in female nude mice. Cancer Res., 47: 4651-4653

Tucker, R.W., Barrett, J.C. 1986. Decreased numbers of spindle and cytoplasmic microtubules in hamster embryo cells treatment with a carcinogen, diethylstilbestrol. Cancer Res., 46: 2088-2095.

Turkington, R. W., 1969. The role of epidermal growth factor in mammary gland development in vitro. Exp. Cell. Res., 57: 79-85.

Turner, C.W., Gomez, E.T. 1933. The normal development of the mammary gland of the male and female albino mouse. I. Intrauterine. M. Agric. exp. Res. Bull., 182: 3-20.

Valverde, R. C., Chieffo, V., Reichlin, S. 1972. Prolactin-releasing factor in porcine and rat hypothalamic tissue. Endocrinol., 91: 982-993.

Vassilacopoulou, D., Boylan, E.S. 1990. Atypical mammary gland development after prenatal exposure to diethylstilbestrol in female ACI rats. Proc. AACR., 30: 215.

Vonderhaar, B. K. 1987. Prolactin: Transport, function and receptors in mammary gland development and differentiation. In: The mammary gland: development, regulation and function, M.C. Neville and C.W. Daniel (eds.), New York and London, Plenum Press, pp. 383-438.

Vorherr, H. 1974. Development of the female breast. In: The breast, H. Vorherr (ed.), New York, Academic Press, pp. 1-18.

Vorherr, H., Messer, R.H., Vorherr, V., Jordan, S.W., Kornfield, M. 1979. Teratogenesis and carcinogenesis in the rat offspring after transplacental and transmammary exposure to diethylstilbestrol. Biochem. Pharmacol., 28: 1865-1871.

Walker, B. E. 1981. Tumors of female offspring of mice exposed prenatally to diethylstilbestrol. J. Natl. Cancer Inst., 73: 133-137.

Walker, B. E. 1983. Uterine tumors in old female mice exposed prenatally to diethylstilbestrol. J. Natl. Cancer Inst., 73: 133-137.