MINIREVIEW

Developing a Laboratory Animal Model for Perinatal Endocrine Disruption: The Hamster Chronicles

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At the biomedical, regulatory, and public level, considerable concern surrounds the concept that inappropriate exposure to endocrine-disrupting chemicals, especially during the prenatal and/or neonatal period, may disrupt normal reproductive tract development and adult function. The intent of this review was to 1. Describe some unique advantages of the hamster for perinatal endocrine disruptor (ED) studies, 2. Summarize the morphological and molecular consequences of exposure to the established perinatal ED, diethylstilbestrol, in the female and male hamster, 3. Present some new, histomorphological insight into the process of neonatal diethylstilbestrol-induced disruption in the hamster uterus, and 4. Introduce recent efforts and future plans to evaluate the potency and mechanism of action of other putative EDs in the hamster experimental system. Taken together, the findings indicate that the hamster represents a unique and sensitive *in vivo* system to probe the phenomenon of endocrine disruption. The spectrum of candidate endpoints includes developmental toxicity, neoplasia, and more subtle endpoints of reproductive dysfunction. Exp Biol Med 227:709–723, 2002

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Numerous laboratory and field studies are generating an expanding list of synthetic chemicals and even some natural products that are known or suspected to be environmental estrogens or xenoestrogens (1, 2). At the biomedical, regulatory, and public level, considerable concern surrounds the concept that inappropriate exposure to such agents, especially during the prenatal and/or neonatal period, may disrupt normal reproductive tract development and adult function (3–5). The reported consequences of such exposures include an increased incidence of hormone-dependent cancers plus the induction of infertility in both human and wildlife populations (6–8). The recognized term for this now high-profile topic is "endocrine disruption." However, it is only proper to acknowledge that it remains a very controversial topic with large knowledge gaps (9–14). Our long-term involvement with the topic began before it was called endocrine disruption and was prompted by the consequences of a now discredited obstetric use of the synthetic estrogen, diethylstilbestrol (DES).

Based on the medical catastrophe it caused, DES can be viewed as a prototype endocrine disruptor (ED). From the 1940s to the 1960s, it was prescribed heavily in the mistaken belief that it would protect against miscarriage during high-risk and even normal pregnancies (15). Consequently, it is estimated that 1 to 4 million offspring in the United States alone were exposed prenatally to this agent (16). The first negative consequences of this practice emerged in 1971, when Herbst *et al.* (17) described the very early occurrence of a rare cancer, vaginal clear-cell adenocarci-

noma, in seven young women who had been exposed to DES *in utero*. Numerous clinical and experimental animal studies have since demonstrated that perinatal DES exposure results in fertility deficits plus developmental toxicity and neoplasia throughout the male and female reproductive tract (18, 19). Because of the scope of this problem, it received major clinical, legal, and media attention and became commonly known as the DES syndrome (18).

Many of the manifestations of the clinical DES syndrome have been replicated in various experimental animal systems (18–21). However, the hamster possesses some characteristics that make it especially well suited for mechanistic studies of the phenomenon. The intent of this review is to 1. Describe the advantages of the hamster for perinatal ED studies, 2. Summarize the morphological and molecular consequences of exposure to the established perinatal ED, DES, in the female and male hamster, 3. Introduce some exploratory, histomorphological observations in the neonatally DES-exposed hamster uterus in the hope of attracting new collaborators, and 4. Outline our ongoing efforts to evaluate the potency and mechanism of action of other putative EDs in the hamster experimental system.

Advantages of the Hamster System for Perinatal Endocrine Disruptor Studies

A point of convenience that led us to choose Syrian golden hamsters (*Mesocricetus auratus*) as the test animal for our project was that they have a shorter and more predictable gestation period (exactly 16 days) than that of mice or rats. Because of the relative immaturity of hamster neonates, we predicted that the very early developmental stage of their reproductive tracts would be comparable with the situation in humans who were exposed *in utero* to DES. Thus, we could target that stage with a neonatal rather than a prenatal treatment regimen and so avoid the possibility of 1) indirect effects on fetuses as a result of a treatment-induced alteration in pregnancy outcome and 2) dosage uncertainties associated with maternal metabolism and placental transfer of a prenatally administered agent.

For the bulk of our studies, the standard treatment regimen has been a single subcutaneous (sc) injection of 100 μg/neonate of DES (≈33 mg/kg or 124 μmol/kg body weight) within 6 hr of birth. That dose level is high but not unreasonable considering that women ingested as much as 150 mg daily and 18.2 g total of the drug during their pregnancy (22). The hamster treatment regimen had no significant effect on the animals' general health or growth before puberty; however, 100% of the treated animals, both male and female, developed dramatic and progressive abnormalities throughout the reproductive tract during adulthood (20, 21, 23–26). In the adult female, gross abnormalities included massive enlargement of the uterine horns plus inflammatory lesions of the oviduct and ovarian bursa (salpingitis; reference 21). The latter phenomenon (salpingitis) was first reported in mice after both prenatal and neonatal treatment with DES (27, 28).

Another special attribute of the hamster is its cheek pouches, which were used as early as 1951 as tissue transplantation sites (29). Since then, a multitude of studies have confirmed that the pouch represents an immunologically privileged site that will accept and support the growth and development of most normal and neoplastic tissues of both allogeneic and xenogeneic origin. Other practical advantages that became obvious during the past five decades of cheek pouch transplantation experiments are the following: 1. The surgical procedures are very simple and require no special equipment or animal maintenance facilities, 2. The transplant grows in a structurally compliant and physiologically normal environment, 3. The transplant can be evaluated repeatedly (measured, photographed, etc.) by simple eversion of the pouch from an anesthetized animal, and 4. The transplant will respond to systemically delivered agents.

Our experience (discussed below) shows that the above claims about the hamster cheek pouch system are true. In fact, for the majority of experimental tissue transplantation studies, the cheek pouch system offers a number of significant advantages compared with the now-popular nude mouse system. For instance, the nude mouse is an inherently abnormal and fragile animal that must be maintained by specially trained personnel in a pathogen-free environment. Thus, their use is quite costly compared with normal rodents. Furthermore, they provide little opportunity for evaluating the ongoing status of a transplant and little flexibility in determining the proper time point to harvest and analyze it. That is especially true for transplants made to the kidney capsule site. Hopefully, this review will make the biomedical community more aware of the hamster cheek pouch as a convenient and cost-effective option for their tissue transplantation needs.

For example, the cheek pouch system allowed us to probe a fundamental aspect of ED action. An observation made in the hamster and other rodent systems is that the consequences of perinatal exposure to DES and other putative EDs become progressively more severe after puberty. Furthermore, it is well known that rodents that are perinatally exposed to either an estrogen or an androgen are likely to have an altered hypothalamic-pituitary-gonadal axis. Consequently, they enter a persistent estrus state (anovulatory and cystic ovaries) in adulthood that is characterized by an abnormal endocrine milieu of high estradiol-17β (E_2) levels but little or no progesterone (20, 30). That fact prompted us to consider the following alternative working hypotheses: 1. Perinatal ED exposure permanently alters the developing reproductive organs (gonad and/or tract) such that they respond inappropriately to normal endocrine control factors (gonadotropins and/or steroids) in adulthood (direct mechanism) or 2. Perinatal ED exposure results in persistent estrus and thus abnormal levels and/or patterns of endocrine control factors (gonadotropins and/or steroids) that drive the morphogenesis and function of reproductive organs (gonad and/or tract) in adulthood (Indirect Mecha-

nism). To test those hypotheses, we used the cheek pouch system to swap control and neonatally ED-exposed tissues between the normal endocrine environment that develops in control animals and the abnormal endocrine environment that develops in neonatally ED-exposed animals. That approach generated conclusive evidence (discussed in the next section) that neonatal DES exposure disrupts the hamster uterus by the direct mechanism.

As mentioned above, evidence from the clinical literature plus that from the hamster and other rodent experimental systems shows that high-dose perinatal exposure to the potent synthetic estrogen, DES, can result in overt developmental toxicity and neoplasia in the reproductive tract. However, lower, environmentally relevant levels of exposure to less potent xenoestrogens or phytoestrogens are likely to have more subtle disruptive effects. For instance, some preliminary results (see following sections) indicate that such effects in women could include an altered onset of menarche, menstrual disturbances, abnormal hormonal patterns, subfertility/infertility, and accelerated entry into the perimenopause/menopause. In experimental rodent systems, such end points can be modeled by monitoring the onset, regularity, and final cessation of the estrous cycle in adult animals. Again, the hamster has an advantage over other rodents for such studies because the normal duration of its estrous cycle is very regular (exactly 4 days) and can be easily monitored by the appearance of a distinctive, preovulatory vaginal discharge that is the hallmark of cycle day 1 (31, 32).

Summary of Findings in the Neonatally DES-Exposed Hamster Uterus

In the prepubertal uterus, neonatal DES exposure induced a rapid and profound pattern of morphogenic disruption. It included both acute and persistent changes in mitotic activity, organization, and dimensions of individual tissue compartments, as well as precocious development of endometrial glands (21, 24, 33). In mature (postpubertal) hamsters, 100% of the neonatally DES-exposed uteri developed endometrial hyperplasia, of which a large proportion (≈40%) progressed to neoplasia (endometrial adenocarcinoma; references 20, 21, 34). Prepubertal ovariectomy reduced but did not completely reverse uterine abnormalities in DES-exposed animals, although it did completely prevent tumor development (20, 21, 34). Furthermore, sustained E_2 replacement (from an E_2-filled Silastic® implant that maintains serum E_2 levels at ≈200 pg/ml for at least 5 months; reference 20) after prepubertal ovariectomy induced severe endometrial hyperplasia plus an even higher level of tumor incidence (>80%) in DES-exposed uteri but did not do so in control uteri (20, 21, 34). Thus, the uterine abnormalities that developed in the DES-treated animals were not simply a result of the persistent estrous syndrome commonly encountered in rodents after they are perinatally treated with either estrogens or androgens (30). A hypothesis consistent with those results is that the neonatal DES insult somehow alters the ability of the natural estrogen, E_2, to stimulate the adult hamster uterus. That hypothesized change in estrogen responsiveness does not appear to be due to any alteration in the physicochemical or functional properties of the uterine estrogen receptor system (35).

The cheek pouch transplantation approach was used to test the above hypothesis. The precedent for this strategy was a 1965 report that the cheek pouch could maintain the morphology and endocrine responsiveness of uteri transplanted into it from adult hamsters (36). When we took uteri from early postnatal (day 7) normal donors and transplanted them into prepubertal (day 21) normal female hosts, they grew, differentiated, and underwent estrogen-responsive morphogenic changes that were quantitatively and qualitatively consistent with that of the host's own uterus (37). Next, early postnatal uteri from control or neonatally DES-treated donors were cross-transplanted into the prepubertal cheek pouches of E_2-replaced control and neonatally DES-treated female hosts. Four months later, transplant masses and host uteri were harvested and processed for histological analysis. Among the four ectopic (cheek pouch) groups, a characteristic pattern of histopathological lesions was limited almost exclusively to the two groups that consisted of neonatally DES-exposed uteri (34). The virtual absence of lesions in control uteri transplanted to DES hosts eliminated host systemic factors as causative agents. If considered relative to the two-step model of carcinogenesis, the above observations are consistent with the hypothesis that: 1) neonatal DES treatment directly and permanently alters the developing hamster uterus (initiating event) such that 2) it responds abnormally later in life to stimulation (promoting event) with E_2.

The characteristic histopathological lesions observed in the hamster uterus after neonatal DES exposure and later stimulation with E_2 were most conspicuous in the endometrial epithelial cell compartment and included clear evidence of hyperplasia (34). Interestingly, that hyperplastic epithelium also displayed intense apoptosis according to morphological (apoptotic bodies), biochemical (internucleosomal DNA fragmentation), and histochemical (*in situ* labeling of free 3' DNA ends) evidence (21, 34). Those morphological responses were accompanied by the altered expression of several protooncogenes that are implicated in the regulation of both cell proliferation (c-*jun*, c-*fos*, c-*myc*) and apoptosis (*bax*, *bcl*-2, *bcl*-x; reference 38). Also observed was greatly enhanced expression of the glycoprotein product of the lactoferrin gene (24), which is a very sensitive and directly upregulated marker of estrogen action in the normal endometrium (39, 40) and overexpression of which is associated with malignant transformation of the human endometrium (41). Furthermore, strong signals for various transforming growth factor (TGF) isoforms (TGFα, $TGF\beta_1$, $TGF\beta_2$) were detected in the apoptotic cells that accumulate in the endometrial epithelium of DES-exposed uteri (21).

Although the morphological and molecular correlations noted above are intriguing, the actual mechanistic link be-

tween altered estrogen responsiveness and apoptotic activity in the neonatally DES-exposed hamster endometrium remains to be determined. Perhaps future studies should investigate the possibility raised by evidence in other tissues that estrogen-regulated apoptosis is determined by estrogen receptor subtype (anti-apoptotic ERα versus. pro-apoptotic ERβ) and the Fas/Fas ligand system (42, 43). However, such initiatives would need to keep in mind that the normal rodent and human endometrium is rich in ERα but very poor in ERβ (44–46).

Tissue Interactions and Neonatal DES-Induced Uterine Disruption

An ongoing project is focusing on the role of epithelial-stromal (mesenchymal) interactions in the mechanism of neonatal DES-induced uterine disruption. The general rationale for that effort is the wealth of evidence generated by Cunha *et al.* (47, 48) that the intercellular communication between epithelial cell and stromal cell tissue compartments plays key roles in the development of reproductive tract form and function. The key to the success of those investigators was a combination of tissue separation, recombination, and ectopic transplantation techniques. Particularly relevant to this section are the results of two very elegant applications of that approach. One is the demonstration in the mouse uterus that estrogen-induced proliferation of cells in the epithelial compartment requires factors released from the stromal compartment (49). The other showed that human prostatic carcinoma-associated fibroblasts could direct tumor progression of initiated but non-tumorigenic human prostate epithelial cells (50).

Another set of alternative hypotheses that is consistent with our previous observations and considers the possible role of tissue interactions is that the key DES-induced lesion occurs either: 1) in the developing stromal cell compartment such that its estrogen-regulated production of inductive signals becomes abnormal, or 2) in the developing epithelial cell compartment such that its ability to respond to the normal inductive signals from the stroma becomes abnormal. Again, the hamster system seemed well suited to test those hypotheses. Based on a previously tested approach (37, 51), early postnatal uteri from control (C) and neonatally DES-treated (D) donors are enzymatically separated into epithelial (E) and stromal (S) tissue fractions that are mixed to form homo-recombinants (CE:CS; DE:DS) plus hetero-recombinants (CE:DS; DE:CS) in short-term tissue culture and then the recombinants are transplanted into the cheek pouches of prepubertal female hosts that are chronically stimulated with E_2 (37). Even after this involved procedure, viability rates are now better than 90% for transplants that are maintained in the cheek pouch for as long as three months. Such long-term transplant success bodes well for our ability to determine the tissue-specific site of the direct and permanent change that neonatal DES treatment induces in the hamster uterus.

The logic for interpreting the results of the project will be as follows. Because the whole-uterus transplantation results were consistent only with the direct action hypothesis (34), the immediate DES-induced change must reside either only in stromal cells, only in epithelial cells, or in both cell populations. In the control homotypic recombinants, characteristic histopathological lesions should develop in DE:DS but not in CE:CS tissues. If DES directly affects developing stromal cells such that they later elaborate an inappropriate inductive signal, endometrial lesions will develop in CE:DS but not in DE:CS tissues. Conversely, If DES directly affects developing epithelial cells such that they later respond inappropriately to a normal inductive signal, endometrial lesions will develop in DE:CS but not in CE:DS tissues. If both tissue compartments are directly affected by DES, results intermediate to those just described may be obtained. The information derived from this project should greatly simplify any subsequent attempts to identify and study the key genetic factor or regulatory pathway that is targeted by DES (and perhaps other EDs) in the neonatal hamster uterus. In fact, evidence from related studies (52) suggests that the products of homeobox and Wnt genes are involved in the interface between epithelial-stromal interactions and perinatal endocrine disruption.

Comparison of Neonatal DES Versus E_2-Induced Effects in the Hamster

The observations in the hamster are consistent with other clinical and experimental evidence (18, 19) that perinatal DES-induced teratogenesis and neoplasia is confined to estrogen-target tissues in the reproductive tract. Thus, the ability of DES to bind to the ER is clearly important to the tissue specificity of its action as a perinatal ED. Surprisingly, however, few direct comparisons have been made between DES and E_2 with regard to their relative potency as perinatal EDs. The advantage of doing such a comparison in the hamster is based on the nature of its alpha-fetoprotein (AFP), a serum glycoprotein that is produced and circulates at high levels during fetal/neonatal stages but then declines drastically as mammals mature (53). Specifically, hamster AFP is like human AFP in that it does not bind E_2 (54, 55), rather than like rat and mouse AFP that does bind E_2 (53, 56). This fact underscores the significance of our findings (summarized below) that, at the same dose level (100 μg/animal), E_2 was much less potent than DES as a perinatal ED in the hamster.

Uterus. One analysis of the differential effects of neonatal DES versus E_2 exposure focused on the female tract (24). It showed that overall growth of the prepubertal tract (primarily the uterus) was quickly (peak on day 5) and persistently enhanced to a much greater extent in the DES-treated group than in the E_2-treated group. The DES-specific effects of precocious gland development and altered epithelial histology (hypertrophic and hyperplastic columnar cells with fenestrations that contained apoptotic cells) were observed as early as day 9 of life. During adulthood, reproductive tract weight was increased to a much

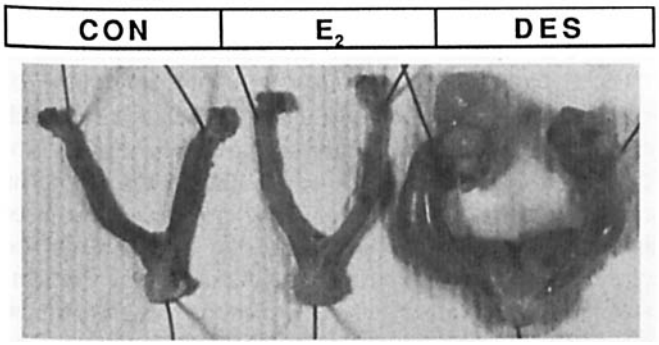

Figure 1. Effect of neonatal treatment with DES versus E_2 on reproductive tract gross morphology in adult female hamsters. Animals were injected on the day of birth with 50 µl of corn oil vehicle either alone (control, CON) or containing 100 µg of either E_2 or DES. At 4 months of age, reproductive tracts (cervix, uterine horns, oviducts, and ovaries) were removed and photographed.

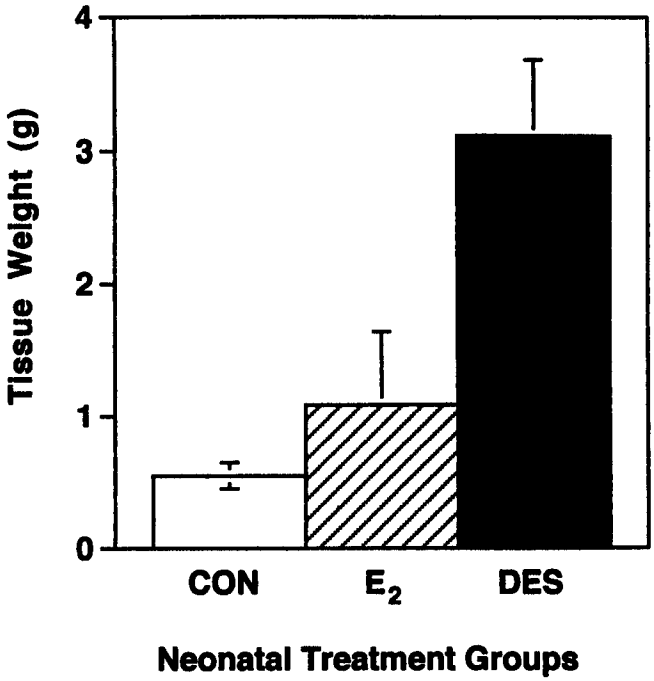

Figure 2. Effect of neonatal treatment with DES versus E_2 on reproductive tract weight in adult female hamsters. Neonatal treatments and tract harvesting age was the same as described for Figure 1. The data represent the mean ± SEM ($n = 4$) and is taken from (24). The mean value for the DES group is significantly different ($P < 0.05$) from those both for the CON and E_2 groups according to factorial ANOVA followed by Tukey's honestly significant differences for multiple comparisons.

greater extent in the neonatally DES-treated group than in the neonatally E_2-treated group. Examples of that response are shown at the qualitative level in Figure 1 and at the quantitative level in Figure 2. As can be appreciated from Figure 1, the DES-specific response was due to a combination of enhanced uterine mass and accumulation of inflammatory products in the oviduct/ovarian bursa (salpingitis). Also during adulthood, sustained blood levels of E_2 stimulated a much greater uterotropic response in the neonatally DES-exposed group than in the neonatally E_2-exposed group. The characteristic histopathological profile (hypertrophic and hyperplastic columnar cells with fenestrations that contained apoptotic cells) that is consistently observed in the endometrial epithelium of neonatally DES-exposed uteri (see above) was not observed in the neonatally E_2-exposed uteri. Another difference observed among the same three groups of adult animals was that levels of the estrogen-inducible protein lactoferrin were hyperinduced only in the neonatally DES-exposed uteri. That observation plus other reports (57–62) suggest that lactoferrin induction rather than organ weight response is a more sensitive or specific endpoint of endocrine disruption in the uterus.

Ovary. A recent histological study[1] showed that neonatal DES treatment also induces marked developmental, morphological, and physiological alterations in the hamster ovary that were not induced by neonatal E_2 treatment. Specifically, DES-treated animals were anovulatory and exhibited a state of persistent estrus. The mature ovaries in DES-exposed animals often contained large preovulatory follicles and luteinized unruptured follicles but no corpora lutea, thereby suggesting ovulation failure. The granulosa cells and oocytes of developing primary, secondary, and tertiary follicles appeared normal. However, the ovarian stroma exhibited marked hypertrophy, as did the theca interni of developing follicles. Developmentally, the ovarian stroma of prepubertal animals exposed neonatally to DES exhibited marked atypia relative to controls and E_2-exposed animals. The stromal atypia was characterized by premature organization and association with small follicles. A major developmental anomaly in prepubertal ovaries of animals neonatally exposed to DES was the presence of numerous polyovular follicles (POF) originating in the central cortical region. Such follicles were much less numerous in control and E_2-exposed animals. Among the developmental time points investigated, the DES-induced POF were first observed on day 9, became relatively numerous on days 15 and 21, and then underwent a marked decline in numbers by day 28. The presence of these unusual follicles suggests an abnormal interplay between somatic and germ cells during early stages of follicle development, perhaps as early as the primordial stage. That the number of POF declined as the animals aged suggests rapid elimination of the abnormal follicles.

Some of the perinatal DES-induced disruptive effects in the hamster ovary, especially POF, have been reported in other experimental systems (27, 63–66). However, the novel aspects of the hamster study should be noted. One of them is the direct comparison showing that, at the same dose level, DES but not E_2 was a potent perinatal disruptor of the hamster ovary. Again, the significance of that observation is emphasized by the non-E_2-binding nature of hamster AFP

[1] May JV, Rueda BR, Hendry WJ III, Differential ovarian disruption following neonatal exposure of hamsters to diethylstilbestrol versus estradiol-17β, submitted for publication.

(see above). Another novel aspect of the study was how early (day 9) disruptive effects became evident in the neonatally DES-exposed hamster ovary. Lastly, it is important to note that, even when the neonatal DES dose was reduced 10,000-fold to 10 ng/animal (3.3 μg/kg or 12.4 ηmole/kg body weight), disruption of the adult gonad was still observed at the gross and histological level in both male (67) and female hamsters.[2]

Male Reproductive Tract. A comparison of the endocrine disruptive effects of neonatal treatment with 100 μg/animal of DES versus E_2 also was conducted in the male hamster (25). Neither neonatal DES nor E_2 treatment had any significant effect either on testicular and accessory organ weight or on serum testosterone levels in pubertal (42 days of age) animals. Histological evaluation of testicular tissue from pubertal males also indicated normal initiation of spermatogenesis in the seminiferous tubules of both the E_2 and DES-treated animals. In contrast, 100% of DES-treated animals (n = 22) sacrificed at 90 days of age exhibited multiple lesions in the reproductive tract. The lesions included cryptorchidism, tumors composed of fibroblast-like cells in the interstitial compartment of the testes, multiple epididymal cysts, and involution of accessory organs. The seminiferous tubule had no developing germ cells (showing disruption of spermatogenesis), and the interstitial cells were abnormally organized as a sheath around the tubule. The epididymis had an epithelial layer of greatly reduced cell height and with a preponderance of multinucleated cells. The seminal vesicles exhibited evidence of apoptotic and/or necrotic changes. Although these pathologies suggest alterations in androgenic stimulation, neither the DES nor E_2 neonatal treatment regimen had any effect on the circulating levels of testosterone in the mature animals. Furthermore, the neonatally E_2-treated animals exhibited none of the alterations and lesions listed above. Thus, in the male hamster, DES also acts in a specific manner as a perinatal ED to induce permanent developmental lesions. These are manifested in adult animals as multiple abnormalities throughout the male reproductive tract and appear to reflect normal androgen levels acting on DES-altered cells. This very curious pattern and chronology of male tract disruption differs considerably from what we observe in the female tract (see previous sections). Thus, fundamentally different mechanisms may be operative in the two sexes.

Commentary. The results of the neonatal DES versus E_2 studies summarized above raise the provocative possibility that potency as a perinatal ED at the *in vivo* level may depend on characteristics other than an agent's relative estrogenicity as is commonly measured by *in vitro* assays of binding affinity to the ER and/or transactivation of ER-responsive reporter genes. In fact, this possibility is supported by a recent report (68) that focused on a variety of putative ED agents. Of course, the relative bioavailability of DES, E_2, and other agents may be a key determinant of perinatal ED potency. The practical consequence of such findings and considerations is that a comprehensive program to identify and study putative EDs or xenoestrogens must include both *in vivo* and *in vitro* strategies. Another important point to be made is that the overall disruption phenomena induced by neonatal DES exposure in the hamster provides a valuable positive control scenario for the evaluation of any other putative, perinatal ED agent.

Highlights of Recent Histological and Ultrastructural Observations in the Neonatally DES-Exposed Hamster Uterus. To gain a more detailed morphological insight into the phenomenon of neonatal DES-induced uterine disruption, we initiated a collaboration to perform ultrastructural analyses. One objective was to follow up some previous electron microscopy (EM) observations. Another objective was to determine whether the uterine disruption phenomenon involves destabilization of the subepithelial, extracellular matrix structure known as the basement membrane. Unfortunately, the project was abbreviated because of the unexpected closing of the collaborating EM facility. Despite the limited and thus preliminary nature of what we did accomplish, certain aspects deserve consideration.

Methodology. All animal maintenance, treatment, and surgical procedures were as described previously (33–35, 38). Briefly, animals were injected sc within 6 hr of birth with 50 μl of corn oil vehicle either alone (control) or containing 100 μg of DES. At 21 days of age (prepubertal), animals were either ovariectomized only or they were ovariectomized and E_2 replaced. At 1 and 2 months of age, uteri from two animals (from separate litters) in each of the four treatment groups were processed for histology by high-resolution light microscopy (LM) and for ultrastructure analysis by transmission electron microscopy (TEM) according to procedures used previously to investigate cell interactions in the human endometrium (69–72).

Histology. We evaluated tissue section histology by high-resolution LM for general quality control and to provide perspective for the TEM analysis that followed. The low-magnification histology in Figure 3 confirms previous tissue weight evidence (34) that the neonatal DES insult permanently increases uterine size under conditions of both E_2 withdrawal and sustained E_2 stimulation. This differs somewhat from the consequences reported after hamsters were prenatally exposed to DES and then uteri from the intact (non-ovariectomized), adult animals were inspected at various ages (73). In that study, uterine size was reduced compared with controls at 150 days of age and then became progressively bigger than controls after 300 days of age. The different pattern of uterine disruption between the two studies is likely related to differences in the DES treatment regimens (prenatal versus neonatal) and/or the postpubertal

[2] May JV, Hendry WJ III, unpublished observations.

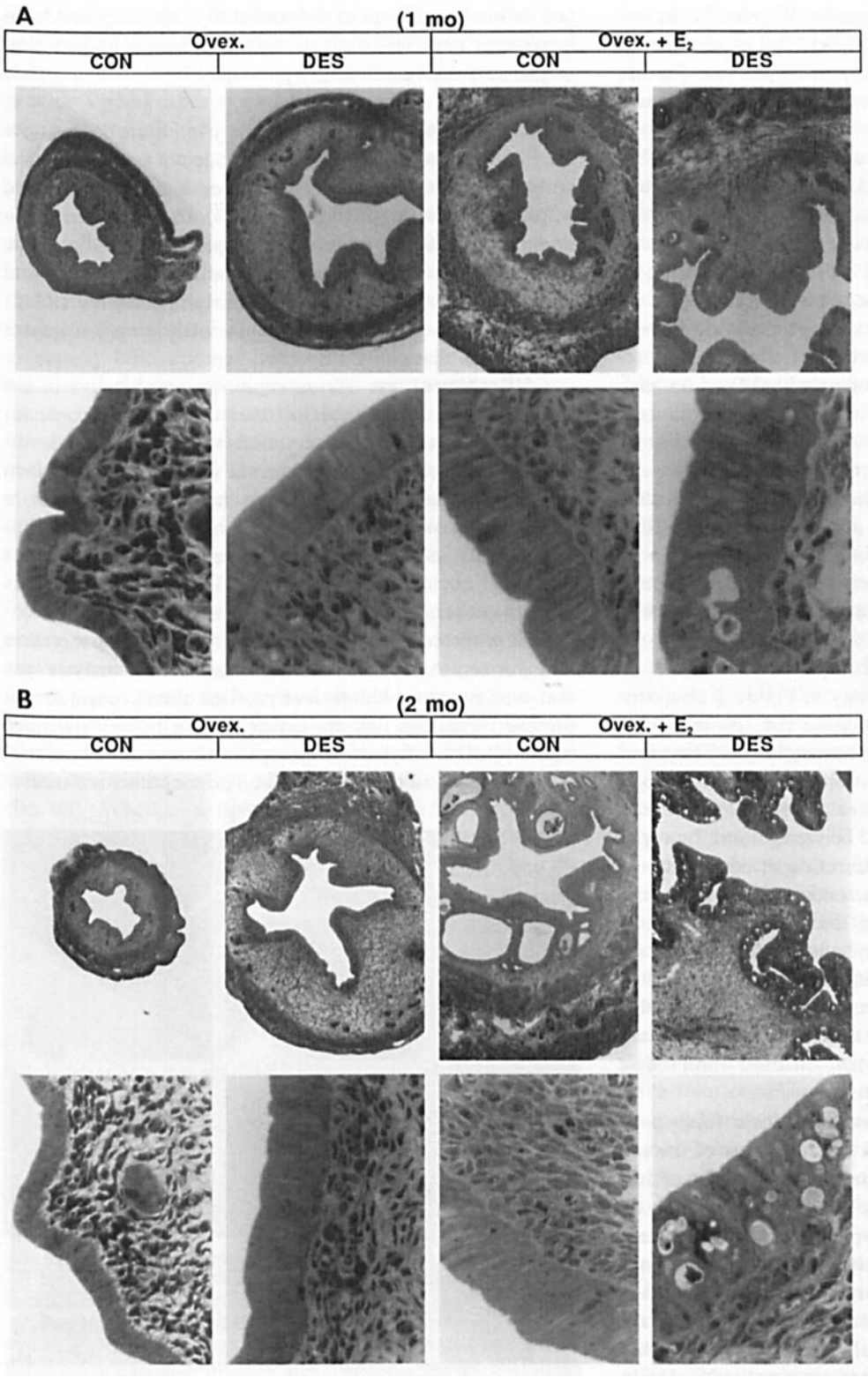

Figure 3. Effects of neonatal DES treatment on endometrial histology in E_2-withdrawn and E_2-stimulated adult uteri. At 21 days of age, control (CON) and neonatally DES-treated animals were either ovariectomized only (Ovex.) or they were ovariectomized and also received an E_2 implant (Ovex. + E_2). Before the plastic blocks were cut for TEM (see legend to Fig. 4), thick cross-sections (2 to 3 μm) of uterine horns from 1-month old (A) and 2-month old (B) animals were stained with Paragon (a contrast stain of basic fuschin and toluidene blue) and photographed by LM at low (×63, upper frames in A and B) and high (×400, lower frames in A and B) magnification.

states of the animals (intact versus ovariectomized) used in the two studies. For instance, timing of DES treatment may influence the potency of the drug in terms of its ability to directly and permanently disrupt (teratogenesis) the developing uterus and in terms of its ability to influence the onset or severity of neuroendocrine dysfunction that can indirectly disrupt postpubertal uterine morphogenesis. Such considerations may be related to similar differences reported in other rodent species. For instance, in the rat uterus, neonatal DES exposure resulted in a hypotrophic/hypoplastic condition in adulthood, even though a hypertrophic response occurred soon after treatment (74, 75). In

the mouse uterus, prenatal DES exposure elicited a hypertrophic/hyperplastic response while neonatal exposure elicited a hypotrophic/hypoplastic response (76–78). Further studies are required to determine the reasons for such differences.

Another neonatal DES-related response shown in Figure 3, that of sustained endometrial epithelial cell hypertrophy in adult, ovariectomized hamsters, was also noted previously (34). However, the animals in that study were ovariectomized postpubertally. This is noteworthy because, as happens for mice and rats after perinatal treatment with an estrogen (30), neonatally DES-treated hamsters do enter a persistent estrous state after puberty (20). Thus, the hypertrophy response seen in the previous study (34) could have been due to the brief, postpubertal exposure to the abnormal endocrine environment that is a hallmark of the persistent estrus condition. Such an indirect mechanism cannot explain the hypertrophic response shown in Figure 3 because the animals were ovariectomized at a prepubertal age (day 21) when ovarian steroidogenesis is still inconsequential and unaffected by neonatal DES exposure (20). Rather, the histology shown in Figure 3 supports the hypothesis that DES acts by a direct mechanism to permanently disrupt the developing, neonatal hamster uterus.

The low-magnification histology in Figure 3 also confirms the standard histological evidence (24, 34) that postpubertal E_2 stimulation of the neonatally DES-exposed hamster endometrium promotes an aberrant histopathology with distinctive hyperplastic features. Whereas cystic endometrial gland structures developed between 1 and 2 months of age in control animals, a proliferation of adenomatous/papillary structures occurred in the endometrium of neonatally DES-treated animals during the same period of E_2 stimulation. A similar hyperplastic response of the endometrium to postpubertal estrogen stimulation was reported in the study where hamsters were exposed prenatally to DES (73) and those investigators noted that the histopathological state resembles the carcinoma *in situ* situation observed in human endometria (79, 80).

The relevance of such experimental animal findings to the clinical situation is unclear. A recent review of the pathology, hormonal aspects, and molecular genetics of human endometrial cancer supports the view that it can be parsed into two types (81). The type I tumors are estrogen-related (sex steroid receptor positive) endometrioid adenocarcinomas that generally arise from the precursor condition of endometrial hyperplasia, whereas the type II tumors are nonendometrioid (serous, clear-cell) adenocarcinomas that are sex steroid receptor negative and apparently develop in a hormone-independent manner. Accordingly, the neonatal DES-initiated and then E_2-promoted phenomenon of endometrial hyperplasia/dysplasia/neoplasia in the hamster uterus more closely mimics the type I tumor features described for humans. That conclusion may not be valid for other experimental animal species since the evidence of increased estrogen responsiveness at both the histological and biochemical level in the neonatally DES-exposed hamster uterus (24, 34) contrasts with evidence of reduced estrogen responsiveness in the prenatally DES-treated mouse (78, 82)

The high-magnification histology in Figure 3 illustrates the E_2-promoted disruption that develops in the endometrial epithelium of neonatally DES-exposed hamsters. As noted in previous histology studies (24, 34), the advanced DES-disrupted epithelium appears: 1) hyperplastic to dysplastic because the cells are extremely tall, disorganized, and poorly demarcated from the underlying stroma and 2) "foamy" because it is riddled with infiltrating leukocytes and cavities that contain apoptotic cells.

Ultrastructure. As stated above, an objective of the short-lived collaborative effort was to follow up some interesting ultrastructural observations made during the developmental stage of our experimental system (20). One such observation that was replicated in the recent ultrastructure analysis (Fig. 4) was that many abnormal endometrial epithelial cells in the DES-exposed and E_2-stimulated uteri contained nuclei with complex profiles (nuclear pleomorphism), a characteristic that is commonly linked with neoplastic progression (83, 84). Another previous observation that was replicated in the recent ultrastructure analysis was that such pleomorphic nuclear profiles often contained distinctive inclusions that are called nuclear bodies and have been linked with hyperestrogenic stimulation of endometrial epithelial cells in the rat (85, 86). These entities are receiv-

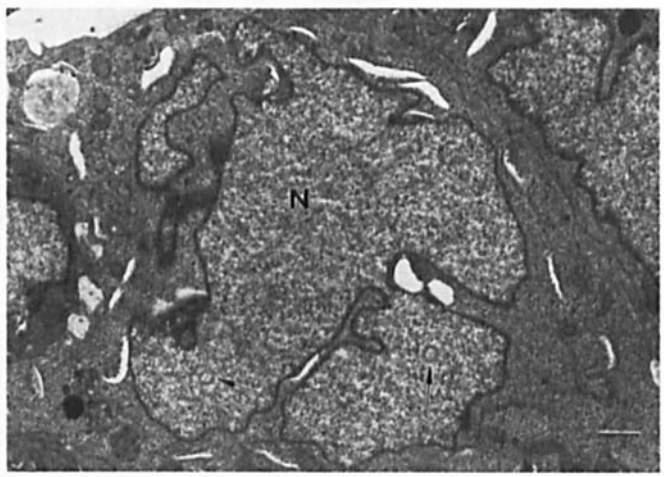

Figure 4. Nuclear ultrastructure in a severely dysplastic epithelial region of the endometrium in a neonatally DES-exposed and E_2-stimulated uterus. The tissue sample was from a 2-month-old animal that had been neonatally treated with DES and then was ovariectomized, and also received an E_2 implant on day 21. For this and the after TEM figures, midregion segments of the uterine horns were placed in phosphate-buffered 3% glutaraldehyde for 1 hr, rinsed in Millonig's buffer, and postfixed in phosphate-buffered 2% osmium tetroxide for 1 hr. After dehydration through a graded ethanol/propylene oxide series, the tissue was embedded in Epon-Araldite resin. Ultrathin (50 to 60 nm) sections were stained in uranyl acetate for 5 min and then in lead citrate for 5 min. Specimens were mounted on copper grids and examined with a Phillips CM-10 transmission electron microscope. The area shown here was photographed at ×10,725 magnification. Note the two nuclear bodies (arrows) within the convoluted nuclear (N) profile. Bar at lower right is 1 μm.

ing renewed interest (87, 88) that has generated tantalizing new insight into their function and biochemical makeup (89–91). The neonatally DES-exposed hamster uterus may provide a good experimental system to investigate the identity and physiological role of nuclear bodies, particularly with respect to estrogen-dependent carcinogenesis.

Previous histology studies (21, 34) also commented on enhanced leukocytic infiltration, likely eosinophils, in the neonatally DES-exposed hamster uterus. The recent ultrastructure analysis supports the contention that the enhanced infiltration phenomenon is a form of eosinophilia. For instance, Figure 5 shows that many of the infiltrating cells in neonatally DES-exposed and then E_2-stimulated uteri had multilobulated to ring-shaped nuclei with abundant aggregated clumps of chromatin in the nuclear periphery plus numerous and very electron-dense cytoplasmic granules with a round to oval shape; diagnostic characteristics of eosinophils (92). Although eosinophilia is known to be estrogen regulated (92–94), its role in the spectrum of hormone-responsive endpoints such as growth of the uterus remains unresolved (95).

Observations derived from use of the hamster cheek pouch, not as a tissue transplantation site but as a site for experimental oral carcinogenesis, may be relevant to the above topic. Those studies showed that eosinophils are recruited to sites of developing tumors, that they predominantly associate with malignant epithelium, and that most tumor-associated eosinophils express the cytokine TGFα (96–98). Whether a neonatal DES-induced disruption in such dynamics is relevant to the endometrial neoplastic response that develops in our experimental system will be assessed in future studies.

A histological feature noted above (in relation to Fig. 3) and previously (24, 34) is that the advanced, DES-disrupted epithelium appears poorly demarcated from the underlying stroma. Consequently, our recent ultrastructure initiative planned to focus on that region and the fact that the interface between the two tissue compartments consists of a distinct extracellular matrix structure. That structure is called the basement membrane by histologists even though it is well beyond the resolving power of the light microscope, whereas it is called the basal lamina under the electron microscope, where it can be resolved with uranium and lead staining into a lamina rara (low electron density, adjacent to epithelial cell membrane) and a lamina densa (high electron density, adjacent to stromal extracellular matrix; Ref. 99). Altered basement membranes are reported in many pathological states, including those that result from 1) metabolic disorders such as diabetes mellitus, 2) immune-mediated disorders such as glomerulonephritis, 3) infectious disorders such as Chagas' disease, and 4) various neoplastic disorders (99).

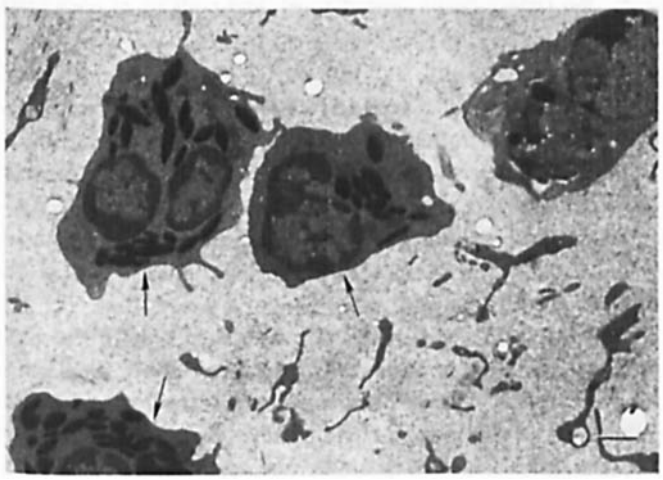

Figure 5. Ultrastructure of infiltrating eosinophils in the endometrial stroma of a neonatally DES-exposed and E_2-stimulated uterus. The tissue sample was from a 2-month-old animal that had been neonatally treated with DES and then on day 21 it was ovariectomized and also received an E_2 implant. The area shown here was photographed at ×10,725 magnification. Within the extracellular stromal matrix are shown three cells (arrows) with the oblong cytoplasmic granules that are characteristic of tissue eosinophils. Bar at lower right is 1 μm.

Our preliminary ultrastructure inspection of the subepithelial region revealed evidence of altered basement membrane integrity as neonatal DES-induced uterine disruption progressed. In E_2-withdrawn uteri, the lamina densa appeared less distinct and less uniform in the DES-exposed group (Fig. 6B) than in the control group (Fig. 6A). In E_2-stimulated uteri, the lamina densa remained continuous and uniformly organized in the control group (Fig. 7A) but exhibited extensive gaps beneath the most intense areas of endometrial hyperplasia/dysplasia in the neonatally DES-exposed group (Fig. 7B).

Such observations deserve further attention because recent studies indicate that: 1) changes in basement membrane integrity can influence the balance between epithelial cell proliferation or apoptosis (100, 101) and 2) the rheostat-like setting between proliferation and apoptosis (102, 103) can determine whether epithelial differentiation will follow either a normal or a pathological pathway (104, 105). Furthermore, basement membrane defects and discontinuities have been correlated with pathological changes in the human endometrium (106, 107). Also, histological images of tumor masses in the neonatally DES-exposed hamster uterus were devoid of cavities with apoptotic cells even though adjacent regions of hyperplastic endometrial epithelium were riddled with such elements (20, 21, 24). Those observations are consistent with the view that apoptosis serves to eliminate mutated cells that develop as a result of abnormal proliferative activity and frank neoplasms erupt at sites where apoptotic activity is either lost or overwhelmed (108). Thus, the hamster experimental system is likely to be a source of valuable new insight into the links between basement membrane biology, apoptosis, and neoplasia.

Commentary. Our abbreviated ultrastructure project did confirm some previous observations and also yielded preliminary evidence that basement membrane aberrations occur during the neonatal DES-induced uterine disruption phenomenon. Further assessment of the significance and

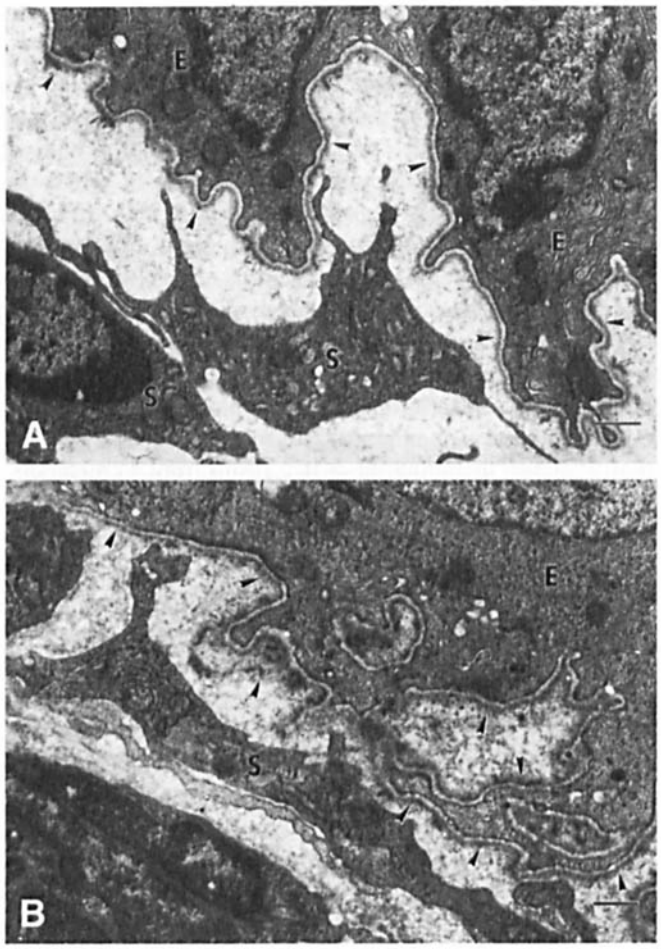

Figure 6. Ultrastructure of the epithelial:stromal interface in E_2-withdrawn uteri. From 2-month old control (A) and neonatally DES-treated animals (B) that were ovariectomized at 21 days of age, areas of uterine cross-sections were processed for TEM and photographed at ×23,720 magnification. The endometrial regions shown contain the basal aspect of epithelial (E) cells, the lamina densa (arrowheads), and portions of underlying stromal (S) cells. Bars at lower right are 0.5 μm.

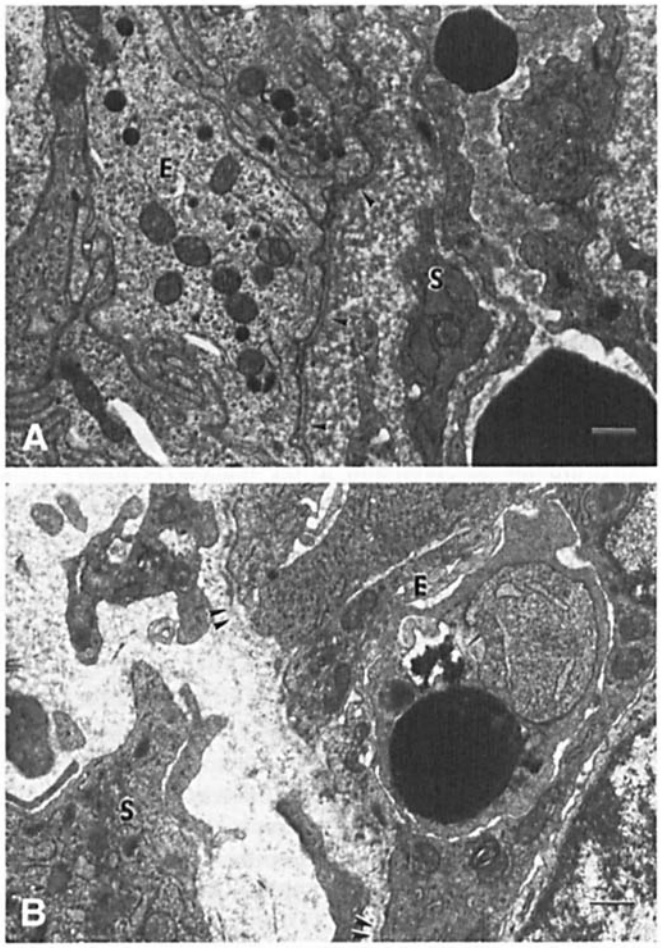

Figure 7. Ultrastructure of the epithelial:stromal interface in E_2-stimulated uteri. From 2-month-old control (A) and neonatally DES-treated animals (B) that were ovariectomized and also received an E_2 implant at 21 days of age, areas of uterine cross sections were processed for TEM and photographed at ×23,720 magnification. The endometrial regions shown contain the basal aspect of epithelial (E) cells, the lamina densa (arrowheads), and portions of underlying stromal (S) cells. Bars at lower right are 0.5 μm.

biochemical basis of those observations should provide new insight into the following: 1. The general phenomenon of perinatal endocrine disruption, 2. How estrogen regulates normal uterine growth and morphogenesis, and 3. How the latter process can degenerate to the unregulated neoplastic state. Hopefully, this review will attract the attention of future collaborators with the interest and means to participate in a comprehensive ultrastructural analysis of the neonatally DES-disrupted reproductive tract in both male and female hamsters. We also hope it helps other investigators to "rediscover" several of the unique but currently underutilized attributes of the hamster that can be applied to a variety of experimental topics.

Effects of Other Putative Perinatal ED Agents on the Hamster Ovary. We are now assessing additional agents for perinatal ED activity in the hamster. One preliminary study (109) focused on the ovary and it compared DES (positive control) with a group of agents that have been scrutinized in other experimental systems. Those agents included the phytoestrogen and protein tyrosine kinase inhibitor genistein, the plasticizer bisphenol A, and the insecticides chlordane and toxaphene. After injection (sc) on the day of birth with 100 μg of the agents, ovaries were harvested from prepubertal (21 days of age), pubertal (28 days of age), and adult (3 months of age) animals and processed for standard histology.

Confirming previous observations (see previous sections), DES treatment induced numerous POF in the cortex of prepubertal and pubertal ovaries. A similar but somewhat less intense POF-induction response was observed in all the other agent treatment groups. The agents also disrupted ovarian histology at the adult (3-month) time point, but the disruption pattern differed among the agents. The disruption end points in the adult ovary included luteinized unruptured follicles (all agents), lack of corpora lutea (DES, genistein, chlordane, toxaphene), vascular pooling (genistein, bisphenol A), cystic structures (bisphenol A, chlordane), and intrafollicular clear cells (chlordane, toxaphene).

The preliminary results presented above indicate that all of the agents tested induced extensive histological aberrations in the hamster ovary. However, the differential range of disruptive effects linked to each agent suggests different mechanisms of action among them. We hypothesize that, like DES, the other agents affected follicular organization during ovarian organogenesis leading to a reduction in ovarian follicles and impaired ovarian function. As discussed above, the hamster is well suited to test this hypothesis because the periodicity of its estrous cycle is normally very regular (exactly 4 days) and easy to monitor. Lastly, it is important to note that testes harvested from litter-mates in the various ED treatment groups described above exhibited no obvious signs of morphological or functional disruption.[3] That finding indicates that dramatic sex-specific differences in the potency of putative perinatal EDs may exist. Such a phenomenon might be expected considering the developmental differences between testes and ovaries. For instance, testes function early to produce testosterone that drives attainment of the male phenotype, whereas ovaries develop by default and so their early functioning is not required for final attainment of the female phenotype. In any case, careful follow-up studies of our observed sex-related differences are needed; especially in view of the contentious findings in other experimental systems about inverted U-shaped dose response curves and animal strain-specific differences for the potencies of various putative, perinatal EDs (110, 111).

Ovarian Transplantation Project. Published results plus recent trials indicate that the cheek pouch transplantation system can be used to test another set of alternative hypotheses that is fundamental to understanding the mechanism by which perinatal ED exposure disrupts ovarian development and function. That is, whether the proximate action of a candidate perinatal ED is 1) on the early, developing ovary itself (direct mechanism) or 2) at the level of the hypothalamus/pituitary so as to alter neuroendocrine programming of the ovary and thus produce the persistent estrus state (indirect mechanism).

The precedent for the proposed ovarian transplantation studies actually appeared several decades ago. In 1966, it was reported that normal estrous cycle function would occur in ovaries that were transplanted from adult donor hamsters into the cheek pouches of ovariectomized host hamsters (112). In a recent trial of the approach, ovaries were removed from prepubertal (day 21) animals, transplanted into the same animal's cheek pouches (one into each side), and evaluated two months later. Part of the evaluation was an assessment of uterine status in the hosts. As shown in Figure 8, it proved that prepubertal ovaries could develop steroidogenic function after they were transplanted into prepubertal hosts. To the extreme left is most of the reproductive tract

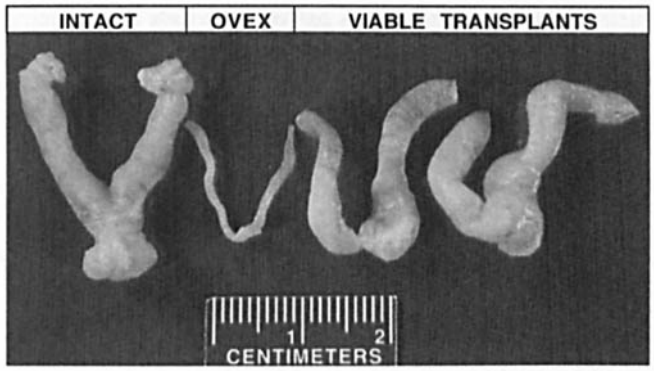

Figure 8. Dimensions of the female hamster reproductive tract under various endocrine states. Reproductive tract tissues were isolated from a normal adult hamster (intact), from a hamster that was ovariectomized on day 21 and killed 2 months later (ovex), and from two hamsters (host A on the left and host B on the right) that had their ovaries transplanted into their cheek pouches on day 21 and were killed 2 months later (viable transplants).

(cervical junction, ovarian horns, oviducts, and ovaries) that was isolated from a normal, adult hamster. Next to it is the cervical junction and uterine horns from a hamster that was ovariectomized on day 21 and killed two months later. The atrophic state of that tissue specimen illustrates the consequences of its withdrawal from the stimulatory influence of normal ovarian E_2 secretion. The two specimens on the right were from animals that also were ovariectomized on day 21 but did have viable ovarian transplant masses in their cheek pouches when they were killed 2 months later. Note that uterine horn dimensions in the latter two specimens match those of the tract on the extreme left that was isolated from a non-ovariectomized animal. Examples of the appearance in the cheek pouch of viable ovarian transplants whose steroidogenic function in the ectopic location was sufficient to support normal uterine dimensions are shown in Figure 9. Note the extensive vascularization of the transplant masses. These positive preliminary results support the feasibility of the cross-transplantation strategy to test the direct versus indirect mechanism of perinatal ED action on the hamster ovary.

Summary and Conclusions

A primary objective of this review was to present the salient advantages of the hamster as an *in vivo* system to

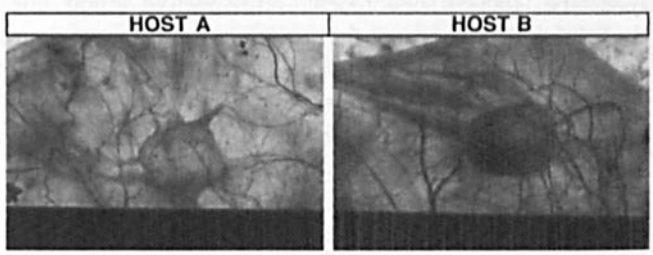

Figure 9. Examples of well-vascularized masses in cheek pouch sites that had received an ovary 2 months earlier. Shown is one of the two viable masses in each of the two hosts from which the stimulated uteri shown in Fig. 8 were taken. They were photographed after the pouches had been everted and pinned to a paraffin plate. The scale at the bottom of each frame is marked in millimeters.

[3] Walsh LP, Hendry WJ III, Stocco DM, Khan SA, Disruptive effects of neonatal exposure to diethylstilbestrol in the hamster testes are not induced by other estrogenic compounds, submitted for publication.

screen the potency of agents as perinatal EDs and/or determine their mechanism of action. Those advantages include the following: 1) a very short and precise gestation period, 2) an estrous cycle that is normally very predictable and easy to monitor, and 3) cheek pouches for convenient, long-term transplantation studies.

In the hamster system, a surprising observation in both the male and female reproductive tracts was the dramatic difference in disruptive effects after neonatal DES versus. E_2 exposure. That raises the provocative possibility that potency as a perinatal ED at the *in vivo* (whole organism) level may depend on characteristics other than an agent's relative estrogenicity as measured by *in vitro* assays of binding affinity to the ER and/or transactivation of ER-responsive reporter genes. Some obvious biological dynamics and pharmacokinetic considerations that are relevant to that topic but have not yet been fully probed in the hamster system include: 1) the method of administration (e.g., sc injection compared to the more 'natural' oral route, 2) the influence of serum binding proteins other than AFP (e.g., albumin, sex hormone-binding globulin, etc.), and 3) metabolic activation/clearance. Although further efforts along those fronts are needed, it is clear that the hamster model shows some fundamental differences from standard rat and mouse models. Thus, it represents another species for which dosing data will be important in defining the potency range of a variety of estrogenic chemicals for use in risk assessment.

Investigations in the hamster uterus showed that the established perinatal ED, DES, directly and permanently alters the developing organ (initiating event) such that it responds abnormally later in life to stimulation (promoting event) with E_2. Preliminary evidence indicates that the latter phenomenon involves alterations in cell:cell and cell:extracellular matrix interactions. Further assessment of the functional and biochemical basis of such alterations should provide new insight into: 1) how estrogen regulates normal uterine growth and morphogenesis and 2) how the latter process can degenerate to the unregulated neoplastic state. However, it is important to note that what we have observed in the hamster experimental system should not be viewed as necessarily predictive of the situation in the clinical DES syndrome. Indeed, one recent, retrospective report concluded that DES exposure during human pregnancy was not associated with risk of ovarian, endometrial, or other cancer (113), whereas another did support a risk of endometrial carcinoma (114). Furthermore, an even broader evaluation of the topic of intrauterine exposure to DES and long-term effects in humans (115) suggested that surveillance bias must also be considered (i.e., greater ease in women of assessing the lower reproductive tract [vagina and cervix] than the upper reproductive tract [uterus, oviduct, and ovary] and the historically greater focus on DES-exposed women than on DES-exposed men). In other words, it would be inappropriate at this point to frighten the already traumatized cohort of DES-exposed humans by implying that they are unavoidably destined to suffer all the problems we see in the neonatally DES-exposed hamster. On the other hand, it is not unreasonable to recommend that future surveillance of the aging cohort of DES-exposed humans should factor in some of our experimental results. For instance, the observation of altered estrogen responsiveness in the neonatally DES-exposed hamster uterus is particularly noteworthy considering the ongoing controversy about the risk/benefit of estrogen replacement therapy in the general population of women (116–118).

Regarding perinatal exposure to other classes of putative EDs, some of our recent studies suggest that they can have a much more potent disruptive effect on the female gonad than on the male gonad. The possible reproductive endpoints in women that could be affected by such exposures include onset of menarche, menstrual disturbances, altered hormonal patterns, subfertility/infertility, and entry into the perimenopause/menopause. Taken together, the findings indicate that the hamster represents a sensitive *in vivo* system to screen candidate agents for ED activity in terms of the induction of overt pathology in the reproductive tract (teratogenesis and neoplasia) and more subtle types of disrupted reproductive function. For agents that prove positive, the next step will be to combine the unique advantages of the hamster system with new genomic/proteomic technologies to elucidate the cellular and molecular mechanism of their disruptive action. For instance, interesting mechanistic possibilities are suggested by the reports that: 1) toxaphene and chlordane are antagonists for the estrogen-related receptor alpha-1 orphan receptor (ERRα-1Rc) (119) and 2) ERRα-1Rc interacts with the coactivator SRC1a or GRIP1 and constitutively activates the estrogen response elements of the human lactoferrin gene and perhaps other estrogen-responsive genes (120).

In conclusion, the special attributes of the hamster experimental system offer some unique opportunities to study the regulatory mechanisms that drive normal reproductive tract morphogenesis and function, and alterations of which are responsible for various pathologies and loss of fertility. The system is particularly useful for investigating the topic of perinatal endocrine disruption. In that regard, the convenient transplantation studies that can be performed in the system provide a means to discern between direct and indirect effects and to evaluate the role of epithelial-stromal interactions. We hope that the information in this review stimulates an expanded use of the system by the biomedical research community.

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