The Immature Mouse Is a Suitable Model for Detection of Estrogenicity in the Uterotropic Bioassay

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The traditional rodent uterotropic response assay has been incorporated into the U.S. Environmental Protection Agency's screening and testing program for environmental endocrine-disrupting chemicals (EDCs). While much effort continues to focus on determining protocol variables, few studies compare uterotropic responses in rats, a species commonly used in toxicologic testing, with other rodent species. In this study, we compared uterine responses in immature outbred CD-1 mice and Sprague-Dawley rats. After three daily subcutaneous injections with 17β-estradiol (0.1–500 μg/kg/day), immature mice and rats demonstrated a similar dose–response increase in absolute uterine wet weight and uterine weight:body weight ratio. Further, morphologic and biochemical parameters of estrogenicity, including uterine epithelial cell height and number, gland number, and induction of estrogen-responsive proteins lactoferrin and complement C3, mirror wet weight increases. We conclude that mice are as well suited as rats for the uterotropic bioassay. Because of the advantages of using mice, including lower costs, less space required, and smaller amounts of compound needed for tests, mice should be given appropriate consideration in testing paradigms for EDCs. *Key words*: complement C3, dose–response modeling, endocrine disruptors, endocrine modulators, environmental estrogens, estrogens, lactoferrin, risk assessment extrapolations, uterotropic bioassay. *Environ Health Perspect* 109:821–826 (2001). [Online 1 3 August 2001] *http://ehpnet1.niehs.nih.gov/docs/2001/109p821-826banks/abstract.html*

Reports in the scientific literature and in the media have raised concerns that environmental chemicals, in particular those that persist in the environment, may produce adverse health effects in wildlife, domestic animals, and humans by interfering with the endocrine system (*1–4*). Some reported effects include reproductive and developmental abnormalities, increases in certain hormone-related cancers (breast, uterus, prostate, testis), and declines in wildlife populations. Although the issue of public health risks from exposure to endocrine-disrupting chemicals (EDCs) continues to be rigorously debated, it is noteworthy that the British Royal Society recently issued a report restating that the risks posed by these chemicals remain of great concern for people of many countries (*5*). Going a step further than an earlier report from the U.S. National Research Council (NRC) (*6*), the British Royal Society report (*5*) acknowledges that there is strong evidence to link EDC exposure to effects in some organisms; they further recommend that human exposure be minimized, especially exposure of pregnant women to EDCs such as plasticizers and insecticides. The British Royal Society's response is a poignant reminder of the continuing public health concerns about EDCs.

In 1998, the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) of the U.S. Environmental Protection Agency (U.S. EPA) issued their final report (*7*), which includes recommendations of tests for estrogenic and antiestrogenic environmental chemicals. The rodent uterotropic response assay, long considered the "gold standard" for determining estrogenicity, was identified as a preferred *in vivo* screen. Although much attention has been focused on identifying protocol variables and reproducibility between laboratories, direct comparison of uterine responses between rodent species has been rarely addressed. Rats are commonly used in toxicologic testing, and many studies have been performed in this species (*8–13*). Because of our experience with the CD-1 mouse and our previously published data on the uterotropic responses in this strain (*14–18*), we compared uterine responses in this mouse strain with responses in the Sprague-Dawley rat. We investigated the sensitivity of both species to varying doses of 17β-estradiol by comparing uterine wet weight increase and a number of morphologic and biochemical end points that are known estrogenic responses in both species (*19–24*). Because developing tissues are particularly susceptible to perturbation by hormones (*25,26*), we restricted our study to the use of immature rather than adult ovariectomized animals. Our study shows the sensitivity of the mouse compared to the rat to estradiol stimulation and suggests that the mouse is a suitable model for testing EDCs for estrogenic activity.

Materials and Methods

Immature mouse uterotropic bioassay. Timed-pregnant CD-1 mice [Crl:CD-1(ICR)], obtained from the breeding colony at the National Institute of Environmental Health Sciences (NIEHS), delivered their young on day 19 of gestation. At birth (day 1), all pups were sorted by sex and redistributed so that all litters were standardized to 10 female pups per dam. Male pups were used in another study. All animals were housed under controlled lighting (12 hr light:12 hr dark) and temperature (21–22°C) conditions. Mice were given fresh reverse-osmosis/deionized (RO/DI) water and NIH-31 rodent diet (an open formula, autoclavable, natural-ingredient rodent diet; Zeigler Brothers, Inc., Gardners, PA) *ad libitum*. All animal procedures complied with National Institutes of Health (NIH)/NIEHS animal care guidelines. Female pups were weaned on day 17 and housed five mice per cage. Starting on the day of weaning, immature mice (n = 5/treatment group) were subcutaneously injected with varying doses of 17β-estradiol (0, 0.1, 1, 10, 100, or 500 μg/kg/day; Sigma Chemical Company, St. Louis, MO) dissolved in corn oil. Mice were injected for 3 consecutive days and killed by cervical dislocation on the morning of the fourth day (20 days of age), and body weights were determined. After making an incision in the skin and the abdominal muscle, the uterine cervix was cut away from the vagina fornix. Because fluid imbibition is an estrogen response, care was taken to retain all uterine luminal fluid. The uterus was then removed by gently lifting tissue anteriorly and trimming away the mesometrium. A cut was made at the uterotubal junction, thus preserving the integrity of both uterine horns and avoiding loss of uterine fluid. The uterus was immediately weighed, placed on absorbent cardboard in order to maintain the original *in vivo* orientation, fixed in cold Bouin's fixative, and processed for histologic and morphometric examination. We then calculated the uterine wet weight:body weight ratios. Collection techniques and similar uterotropic responses and measurements

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in other groups of mice have been previously reported by this laboratory (*16,17*).

Immature rat uterotropic bioassay. Timed-pregnant rats [Tac:N(SD)fBR] were obtained from Taconic Laboratories (Raleigh, NC) on gestation day 15. At birth (day 1), all pups were sorted by sex and redistributed so that all litters were standardized to eight female pups per dam. All animals were housed in a temperature-controlled room (21–22°C) with a 12 hr light:12 hr dark cycle. Rats were provided fresh RO/DI water and NIH-31 lab chow *ad libitum*. All animal procedures complied with NIH/NIEHS animal care guidelines. Rats were weaned on day 23 and housed four per cage. Starting on the day of weaning, rats (n = 4/treatment group) were subcutaneously injected with varying doses of 17β-estradiol (0, 1, 10, 100, or 500 µg/kg/day) dissolved in corn oil for 3 consecutive days; lower doses were not tested in rats because reports in the literature showed no significant difference between lower doses and control values in uterine wet weight changes (*11*). Rats were euthanized by cervical dislocation on the day after the last injection (day 26). Body weights and uterine wet weights were determined as described for the mice. Uterine tissues were fixed in Bouin's fixative and processed for histologic and morphometric examination.

Uterine wet weight determination. Uterine wet weight:body weight ratios were calculated for each animal by dividing the uterine wet weight by the body weight and multiplying by 100. We used StatView SE + Graphics (ABACUS Concepts, Inc., Berkeley, CA) to determine the mean and SE and to perform analysis of variance (ANOVA) using the Fisher's test of significance ($p < 0.05$).

Uterine histologic observations. Uterine tissues were processed and embedded in paraffin with one horn placed longitudinally and the other placed cross-wise; sections were cut at 4 µm and stained with hematoxylin and eosin (H&E). We quantitated histologic responses using an Olympus BH-2 Microscope (Olympus Corp., New Hyde Park, NY) interfaced with an image analysis system (Southern Micro Instruments, Atlanta, GA) as described previously (*22,27*).

Cell height determinations. To determine cell height, we obtained three measurements from each of three areas from a minimum of three animals (at least 27 measurements per group). We measured cell height in longitudinally embedded tissues, always from the middle of the uterus and away from the oviduct or cervical segments. We calculated the mean for each area and then the mean for each animal. We then used these animal means to determine the mean ± SE for each treatment group.

Cell number determination. We determined the number of uterine epithelial cells per micrometer of basement membrane by obtaining three counts per area (in longitudinally embedded tissues, as described above) from three areas per animal from a minimum of three animals (at least 27 measurements per treatment group). We counted the epithelial cells in a length of approximately 100 µm uterine epithelium and calculated the number of cells per micrometer of basement membrane. We calculated the mean for each area and then the mean for each animal. We used these animal means to determine the mean ± SE for each treatment group.

Gland number determinations. We counted the number of glands in cross-sections of uterine tissues obtained from the middle region of the uterus. We counted glands in three sections per animal from a minimum of four animals per treatment group (a minimum of 12 counts per group). The counts for each animal were averaged, and these means were used to determine the mean ± SE for each group.

Table 1. Comparison of the immature mouse and rat in the uterotropic bioassay.

17β-Estradiol (µg/kg/day)	Body weight (g)	Uterine wet weight (g)		Uterine weight:body weight ratio	
		Actual	Fold induction over control	Relative	Fold induction over control
Mouse					
0	9.58 ± 0.374	0.011 ± 0.001	1.00	0.111 ± 0.006	1.00
0.1	9.28 ± 0.428	0.012 ± 0.001	1.09	0.131 ± 0.012	1.18
1	10.38 ± 0.504	0.025 ± 0.002*	2.27	0.242 ± 0.009	2.18
10	9.63 ± 0.809	0.044 ± 0.006*	4.00	0.450 ± 0.046	4.06
100	10.58 ± 0.301	0.062 ± 0.008*	5.64	0.578 ± 0.059	5.21
500	11.01 ± 0.221*	0.073 ± 0.007*	6.64	0.725 ± 0.068	6.54
Rat					
0	77.31 ± 1.87	0.047 ± 0.004	1.00	0.061 ± 0.006	1.00
0.1	ND	ND	ND	ND	ND
1	70.50 ± 3.48	0.085 ± 0.003*	1.81	0.121 ± 0.010	1.98
10	55.47 ± 2.02*	0.108 ± 0.008*	2.30	0.208 ± 0.014	3.41
100	55.57 ± 2.17*	0.150 ± 0.007*	3.19	0.283 ± 0.010	4.64
500	67.27 ± 0.56*	0.217 ± 0.017*	4.62	0.344 ± 0.028	5.64

ND, not determined. Numbers represent mean ± SE.
*Statistically significant at $p < 0.05$.

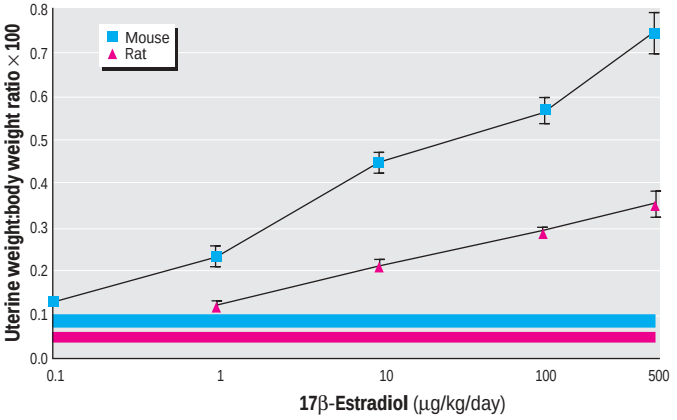

Figure 1. Uterine weight:body weight ratio of mice (n = 5) and rats (n = 4) that received varying subcutaneous doses of 17β-estradiol once daily for 3 days. See "Materials and Methods" for details. Values shown are the mean ± SE. The shaded areas represent control values (mean ± SE). The slope is similar for both mice and rats, but the sensitivity of mice is higher than that of rats at all doses tested.

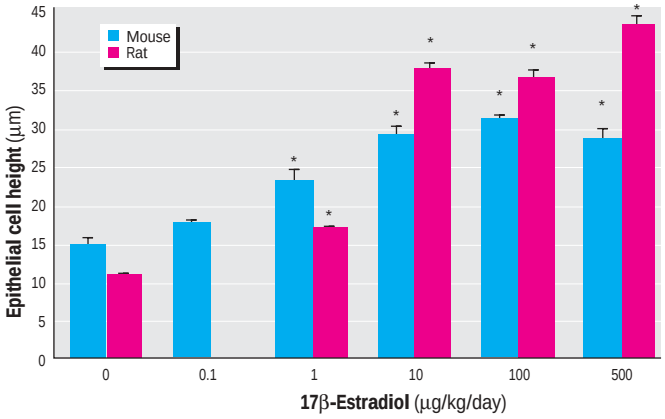

Figure 2. Epithelial cell height in the uterotropic bioassay of immature mice and rats. Values represent the mean ± SE of three measurements from three areas from three animals (total of 27 counts/group). For doses ≥ 1 µg/kg/day in both mice and rats, there was a significant increase over the corresponding control values.
*Statistically significant differences between treated mice and rats and their respective controls ($p < 0.05$).

Uterine biochemical observations. Lactoferrin (LF) and complement C3 have been shown to be estrogen-responsive proteins in the mouse and rat uterus (*18*). After exposure to estrogen, both proteins were induced in uterine epithelial cells, and LF was shown to increase with increasing doses of estrogen (*28*). Thus, we examined these two proteins to determine the sensitivity of each species to increasing estrogenic doses and to determine the expression of these two estrogen-inducible proteins.

LF immunocytochemistry. Mouse and rat uterine tissue sections were immunostained for the presence of LF using horseradish peroxidase immunocytochemistry as previously described (*28*). Briefly, sections were incubated with rabbit anti-mouse LF (*28*) for 2 hr at room temperature, or with normal rabbit IgG (Accurate Chemical Co., Westbury, NY) diluted 1:200 or without LF antibody for negative controls. We used the image analysis system (Southern Micro Instruments) to score LF intensity. We measured staining intensity in a minimum of three areas (three measurements per area) for each of three animals. The intensity scale ranged from 0 (no stain) to 50 (very intense stain).

Complement C3 immunocytochemistry. Mouse and rat uterine tissue sections were immunostained for complement C3. Tissue sections were incubated with goat anti-mouse complement C3 (primary antibody) diluted 1:5,000 (Cappel, Durham, NC) for 2 hr at room temperature. We used negative controls as described for LF immunolocalization. The immunostain was detected using an avidin:biotinylated enzyme complex (ABC) method. Briefly, tissue sections were rinsed with Tris-buffered saline containing 0.6% Tween-20 (TBS-T), incubated with biotinylated anti-goat antibody diluted 1:500 for 30 min at room temperature, rinsed with TBS-T, then incubated with ABC-AP (ABC-AP kit; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Tissue sections were washed with TBS-T and rinsed briefly with TBS (pH 9.5) before the alkaline phosphatase (AP) substrate was applied. We prepared the color enhancement according to directions in the AP substrate kit IV (blue; Vector Laboratories) and incubated the tissue sections for approximately 10 min. Tissues were rinsed in distilled water, dehydrated, and coverslipped. We determined the intensity of C3 intensity as described for LF.

Statistical analysis. We performed statistical analysis by ANOVA (StatView SE + Graphics); values were determined to be significant at $p < 0.05$.

Results

Uterotropic wet weight. The results of the uterotropic bioassays in mice and rats are shown in Table 1. Although there are differences in body weight in mice and rats after estradiol treatment, none of the animals appeared ill; thus, differences in body weight most likely did not have any confounding effect on this study. We observed a similar dose–response increase in uterine wet weight in both species with increasing doses of 17β-estradiol. We expressed data as the uterine weight to body weight ratio (Table 1, Figure 1). Doses ranging from 1 to 500 µg/kg/day were statistically significant for both mice and rats compared to their corresponding controls.

Uterine histologic observations. Epithelial cell height. Figure 2 shows the epithelial cell height results for the immature mouse and rat. The increase in cell height was higher in immature rats treated with ≥ 1 µg/kg/day 17β-estradiol than in their corresponding controls. Uterine epithelial cell height in the mouse followed the same pattern of increase as in the rat, but the rat demonstrated a greater increase over controls with this end point than the mouse at given doses. In Figure 3, photomicrographs show the effects of 17β-estradiol treatment (500 µg/kg/day) on epithelial cell height in mice (Figure 3B) and rats (Figure 3D).

Epithelial cell number. Figure 4 shows an increase in cell number in response to ≥ 10 µg/kg/day 17β-estradiol in both mice and rats. There was a greater increase over controls in mice than in rats at all doses. The increase in cell number is also shown in Figure 3.

Uterine gland number. The number of glands is increased in mice in response to ≥ 1 µg/kg/day 17β-estradiol (Figure 5). However, there is no apparent increase in gland number in rats after any dose of estradiol used in this study. Thus, there is a striking difference between these two species in the ability of 17β-estradiol to elicit gland formation in rodents of this age.

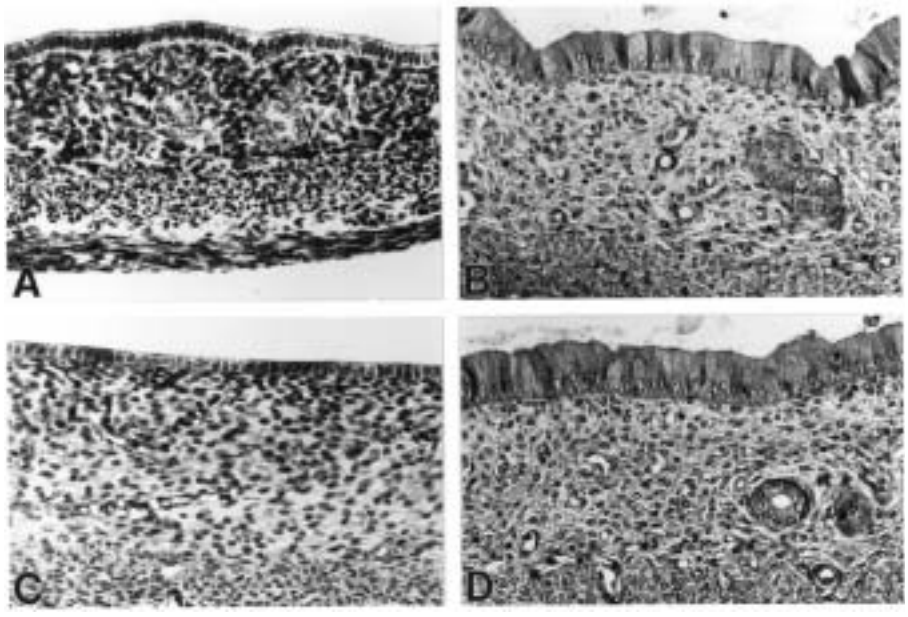

Figure 3. Photomicrograph of uterine tissues from immature mice (*A*,*B*) and rats (*C*,*D*). (*A*) The unstimulated uterus from an immature mouse shows a single layer of cuboidal or low columnar cells lining the uterine lumen; there is minimal differentiation in the stroma. (*B*) The stimulated uterus from an immature mouse treated with 17β-estradiol (500 µg/kg/day) for 3 days shows tall columnar epithelial cells; the stromal cells have round nuclei and more space between nuclei. (*C*) The unstimulated uterus from an immature rat shows low cuboidal epithelium lining the uterine lumen, and stromal cells are undifferentiated and unresponsive as in the immature mouse. (*D*) The stimulated uterus from an immature rat treated with 17β-estradiol (500 µg/kg/day) for 3 days shows tall pseudostratified columnar cells and differentiated responsive stromal cells. Magnification, 200× for all photomicrographs.

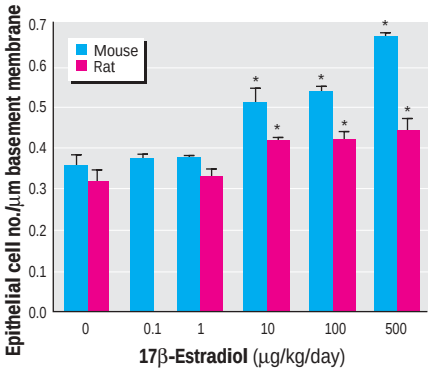

Figure 4. Epithelial cell number in the uterotropic bioassay of immature mice and rats. Values represent the number (mean ± SE) of epithelial cells per micrometer of basement membrane for three counts from three areas from three animals for each dose (total of 27 counts per group).
*Statistically significant differences between mice and rats and their respective controls ($p < 0.05$).

Uterine LF and C3 immunohistochemistry. The results of LF quantitation are shown in Figure 6. We detected LF in all of the 17β-estradiol–treated groups of immature mice and rats. We previously reported a dose-dependent increase in LF immunohistochemistry over a tighter dose–response curve than is shown in Figure 6 (*28*). For all of the doses tested, the highest measurements were seen in mice. Figure 7 shows the pattern of LF immunostaining for both mice and rats. The cellular distribution of LF in the stimulated mouse uterus (Figure 7B) is different from that in the stimulated rat uterus (Figure 7D): LF is evenly distributed over the entire uterine epithelial cell in the mouse (Figure 7B), whereas LF in the rat is more apical (Figure 7D). Negative control slides showed no staining.

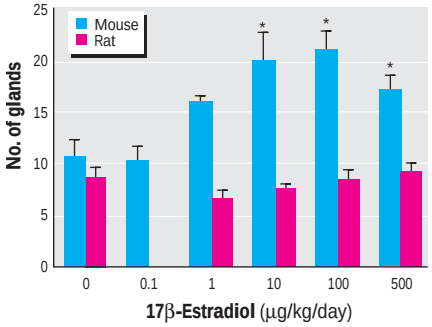

Figure 5. Number of uterine glands in the uterotropic bioassay of immature mice and rats. Values shown are the number of glands per section (mean ± SE) for three sections from three animals for each dose (total of 27 counts per group).
*Statistically significant differences between each mice and rats and their respective controls; there is a marked response in mice with this end point, whereas few uterine glands are present in the rat regardless of the 17β-estradiol dose ($p < 0.05$).

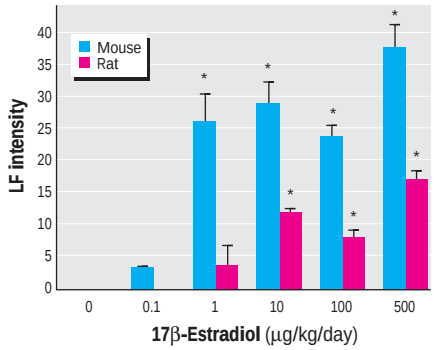

Figure 6. Quantitation of image analysis of LF immunostaining in immature mice and rats. Values represent the intensity of staining (mean ± SE) determined from measuring three readings from three areas from three animals (total of 27 counts per group). LF showed a consistently stronger response in mouse tissues than in rat tissues. Quantitation of C3 showed an identical response (data not shown).
*Statistically significant differences between rats and mice and their respective controls ($p < 0.05$).

Tissue sections immunostained for complement C3, another estrogen-inducible protein, are shown in Figure 8. Although 17β-estradiol induced C3 in the same cell type, uterine epithelial cells, the cellular distribution was different in mice and rats. C3 was expressed in the apical portion of luminal epithelial cells of the 17β-estradiol–treated mouse (Figure 8B), whereas C3 was more evenly distributed throughout the uterine epithelial cells of the 17β-estradiol–treated rat. Image analysis results of the C3 immunostained tissue sections were similar to those of LF-immunostained tissue (data not shown). Thus, both the mouse and the rat show a strong staining reaction for both LF and C3 in uterine epithelial cells in response to 17β-estradiol, but the pattern of staining varied between the two species. The differential distribution of these two proteins in mice and rats has been discussed in more detail in a recent report from our laboratory (*24*).

Discussion

The data presented in this paper shows the sensitivity and degree of uterine response of the CD-1 mouse and the Sprague-Dawley rat to 17β-estradiol in the *in vivo* uterotropic bioassay. Of particular interest is the difference in some end points between the strains, whereas other end points are similar. Some of these differences have been noted in the past and go back at least 40 years (*29*). However, with renewed interest in using the rodent uterotropic bioassay as a screen for endocrine-disrupting substances, a more direct comparison is of interest to laboratories desiring to use an *in vivo* assay to determine the estrogenicity of compounds. Therefore, we conducted the present study to compare the estrogenic responses in mice and rats and to provide additional morphologic and biochemical end points that may be useful for increasing the information that can be obtained from the uterotropic bioassay. For the purposes of this study, we compared the responses of rats and mice in the linear portion of the uterotropic dose–response curve; higher doses show an S-shaped response for uterine wet weight (*16*), as well as other estrogen-responsive end points (*30*).

LF and C3 are strongly expressed in uterine luminal epithelial cells of both rats and mice after 17β-estradiol treatment. It is interesting to note the specific pattern of expression of these two proteins and the cellular distribution of each protein within the epithelial cells of rats and mice. In the mouse, LF is distributed throughout the cytoplasm of the uterine epithelial cells, and C3 localizes primarily at the apical tip. In the rat, LF localizes at the apical tip, and C3 is distributed throughout the cytoplasm. This pattern of protein distribution agrees with an earlier report (*24*). The differential cellular distribution of both proteins in rats

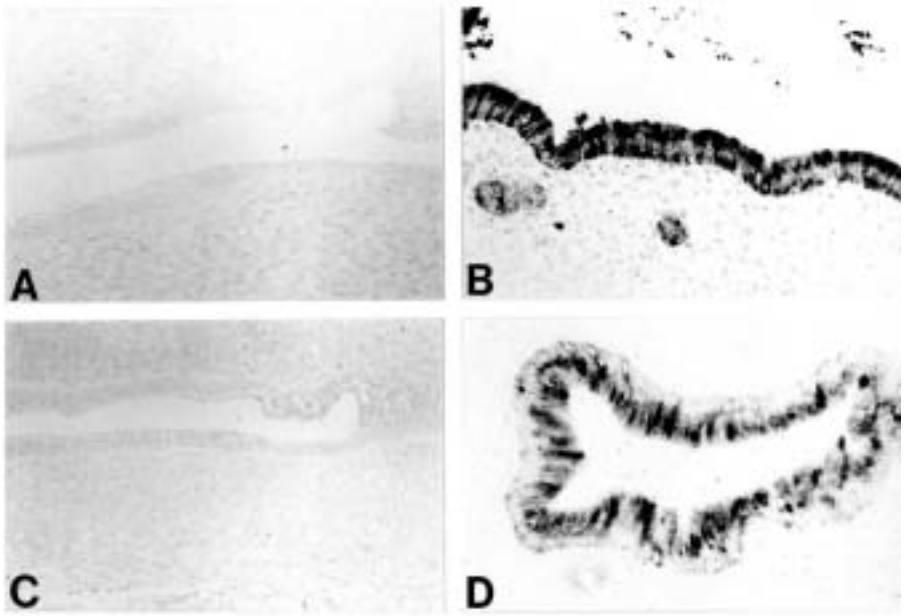

Figure 7. Immunohistochemical localization of LF in the uterotropic bioassay of immature mice and rats. (*A*) The unstimulated uterus from an immature mouse shows no apparent LF staining. (*B*) The stimulated uterus from an immature mouse treated 3 days with 500 μg/kg/day 17β-estradiol shows intense immunostaining in uterine epithelial cells; note the localization of LF throughout the cytoplasm. (*C*) The unstimulated uterus from an immature rat shows no apparent LF staining. (*D*) The stimulated uterus from an immature rat treated 3 days with 500 μg/kg/day 17β-estradiol shows specific LF staining in the luminal epithelial cells; there are fewer intensely stained cells than in the stimulated mouse, and the immunostaining is primarily at the apical surface of the epithelial cells. Negative controls, as described in "Materials and Methods," demonstrated no immunostaining. Magnification, 200× for all photomicrographs.

and mice may be because of different patterns of gene expression within each species.

Although most end points showed similarities between mice and rats, there was an increase in uterine gland formation after estrogen treatment in the mouse but not in the rat. Previous studies have shown that during postnatal development, uterine gland differentiation is sensitive to estrogen perturbation, resulting in inhibition of gland differentiation in both rats (*31*) and mice (*32*). However, after postnatal differentiation, the response of uterine glands to estrogen in the immature animal is quite different in rats and mice. Similar to humans, mouse uterine glandular epithelium responds to estrogen by becoming more convoluted and by increasing secretions.

Values for control and 17β-estradiol–treated rat tissues reported in this study are consistent with those from other laboratories (*33*). Furthermore, uterotropic responses in immature Alpk:AP rats (*8*) is similar to responses observed in Sprague-Dawley rats in this study. In the Alpk:AP rats (*8*), the uterine wet weight (mean ± SE) was 38.2 ± 7.3 mg in controls and 95.4 ± 17.0 mg in rats treated with 10 µg/kg/day 17β-estradiol. In the Sprague-Dawley rats in the present study, the uterine wet weight (mean ± SE) was 47 ± 4 mg in controls and 108 ± 8 mg in rats treated with 10 µg/kg/day 17β-estradiol. This suggests similar estrogenic responses in two different strains of rats. Our data are also consistent with control values reported in other studies using immature rats (*8,10,12*). Further comparison after estrogen treatment is not possible because these studies only reported "blotted" uterine weight instead of actual uterine wet weight, which includes uterine luminal fluid. We specifically included uterine luminal contents because fluid imbibition is an important early estrogenic response.

Although we did not compare different routes of exposure between mice and rats in our study, a previous report showed a similar magnitude of uterine response in rats between oral gavage and subcutaneous injection (*10*). Although we used a small number of rats in this study, the data are consistent with other studies in the literature; this leads us to believe that our data are representative of responses seen in rat uterotropic bioassays.

The present study was designed to compare species differences in uterine response between immature mice and rats. We did not compare immature and adult ovariectomized animals because earlier studies demonstrated that immature animals are more sensitive to stimulation with estrogens than adult ovariectomized animals (*34*). Kang et al. (*35*) has recently reconfirmed this. Thus, the age of the animals must be considered in designing the most sensitive screening methods for estrogenic chemicals.

As reported by other laboratories (*13,36*), there was a greater variation in body weights in all of the treatment groups in rats compared to mice. Also, rats showed a general decrease in body weight after 17β-estradiol treatment, and mice showed an increase with similar treatment. This greater variation in body weight in the rat can decrease the significance of the results and thus make the estrogenic effect of test chemicals less apparent, so that weak estrogens may not be detected. Therefore, decreased variability afforded by the mouse may result in decreased numbers of animals needed for testing. The potentially smaller numbers of animals and the certainty of lower animal husbandry costs and smaller amounts of compound needed for tests (due to the smaller body size of mice) support the use of mice in this screen until substitute *in vitro* models can be validated.

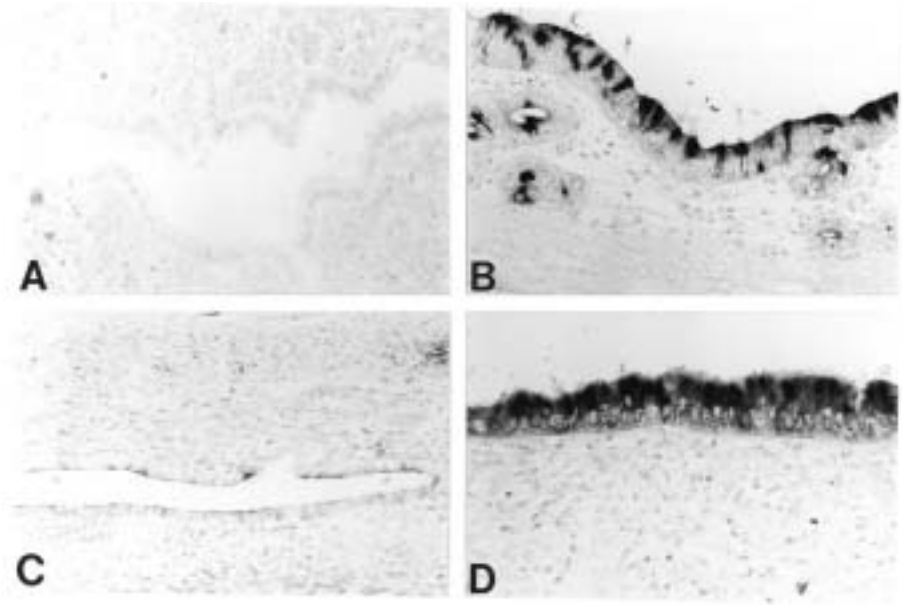

Figure 8. Immunolocalization of C3 in the uterotropic bioassay of immature mice and rats. (*A*) The unstimulated uterus from an immature mouse shows no apparent C3 staining. (*B*) The stimulated uterus from an immature mouse treated 3 days with 500 µg/kg/day 17β-estradiol shows intense immunostaining in uterine epithelial cells; note the variable staining pattern, with intense stain in the apical portion of a few cells. (*C*) The unstimulated uterus from an immature rat shows no apparent immunostaining. (*D*) The stimulated uterus from an immature rat treated 3 days with 500 µg/kg/day 17β-estradiol shows specific immunolocalization of C3 in the epithelial cells; there is uniform staining between the cells, and the stain seems to be evenly distributed throughout the cytoplasm. Negative controls, as described in Materials and Methods," demonstrated no immunostaining. Magnification 200× for all photomicrographs.

References and Notes

1. McLachlan JA. Estrogens in the Environment. New York:Elsevier Science, 1985.
2. Colborn T, Clement C, eds. Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton, NJ:Princeton Scientific Publishing, 1992.
3. McLachlan JA, Korach KS. Introduction: Symposium on Estrogens in the Environment, III. Environ Health Perspect 103(suppl 7): 3–4 (1995).
4. Colborn T, Dumanski D, Myers JP. Our Stolen Future. New York:Penguin Books, 1996.
5. Loder N. Royal Society warns on hormone disrupters. Nature 406:4 (2000).
6. Macilwain C. Endocrine disrupter verdict left open. Nature 400:607 (1999).
7. U.S. EPA. Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) Final Report. Washington, DC:U.S. Environmental Protection Agency, 1998.
8. Ashby J, Odum J, Foster JR. Activity of raloxifene in immature and ovariectomized rat uterotrophic assays. Regul Toxicol Pharmacol 25:226–231 (1997).
9. Odum J, Lefevre PA, Tittensor S, Paton D, Routledge EJ, Beresford NA, Sumpter JP, Ashby J. The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:176–188 (1997).
10. Ashby J, Tinwell H. Oestrogenic activity of burgen bread to female rats. Hum Exp Toxicol 17:598–599 (1998).
11. O'Connor JC, Frame SR, Biegel LB, Cook JC, Davis LG. Sensitivity of a Tier I screening battery compared to an in utero exposure for detecting the estrogen receptor agonist 17 beta-estradiol. Toxicol Sci 44:169–184 (1998).
12. O'Connor JC, Cook JC, Slone TW, Makovec GT, Frame SR, Davis LG. An ongoing validation of a Tier I screening battery for detecting endocrine-active compounds (EACs). Toxicol Sci 46:45–60 (1998).
13. Christian MS, Hoberman AM, Bachmann S, Hellwig J. Variability in the uterotrophic response assay (an in vivo estrogenic response assay) in untreated control and positive control (DES-DP, 2.5 microG/kg, bid) Wistar and Sprague-Dawley rats. Drug Chem Toxicol 21:51–100 (1998).
14. Korach KS, Metzler M, McLachlan JA . Estrogenic activity in vivo and in vitro of some diethylstilbestrol metabolites and analogs. Proc Natl Acad Sci USA 75:468–471 (1978).
15. Isenhower WD Jr, Newbold RR, Cefalo RC, Korach K, McLachlan JA. Absence of estrogenic activity in some drugs commonly used during pregnancy. Biol Res Pregnancy Perinatol 7:6–10 (1986).
16. Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* assays. Environ Health Perspect 104:1296–1300 (1996).
17. Jefferson WN, Padilla-Burgos E, Miller C, Newbold RR. Endocrine modulating effects of various phytoestrogens.

In: Human Diet and Endocrine Modulation: Estrogenic and Carcinogenic Effects (Dunaif GE, Olin SS, Scimeca JA, Thomas JA, eds). Washington, DC:ILSI Press, 1998;286–289.
18. Jefferson WN, Newbold RR. Potential endocrine-modulating effects of various phytoestrogens in the diet. J Nutr 16:658–662 (2000).
19. Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, Lyttle CR. Estrogen regulation of tissue-specific expression of complement C3. J Biol Chem 264:16941–16947 (1989).
20. Teng CT, Pentecost BT, Chen YH, Newbold RR, Eddy EM, McLachlan JA. Lactotransferrin gene expression in the mouse uterus and mammary gland. Endocrinology 124:992–999 (1989).
21. Teng C. Mouse lactoferrin gene: a marker for estrogen and epidermal growth factor. Environ Health Perspect 103(suppl 7):17–20 (1995).
22. Newbold RR, Teng CT, Beckman WC Jr, Jefferson WN, Hanson RB, Miller JV, McLachlan JA. Fluctuations of lactoferrin protein and messenger ribonucleic acid in the reproductive tract of the mouse during the estrous cycle. Biol Reprod 47:903–915 (1992).
23. Newbold RR, Hanson RB, Jefferson WN. Ontogeny of lactoferrin in the developing mouse uterus: a marker of early hormone response. Biol Reprod 56:1147–1157 (1997).
24. Jefferson WN, Padilla-Banks E, Newbold RR. Lactoferrin is an estrogen responsive protein in the uterus of mice and rats. Reprod Toxicol 14:103–110 (2000).
25. Bern H. The fragil fetus. In: Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992;9–16.
26. McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. Environ Health Perspect 101:386–387 (1993).
27. Jefferson WN, Voelker CR, Newbold RR. Increased sensitivity of Western blots using various secondary antibody detection methods. Toxicol Methods 3:100–108 (1993).
28. Jefferson WN, Hanson RB, Newbold RR. Detection of lactoferrin in paraffin-embedded sections of mouse uterus: comparison of fixation and immunohistochemical techniques. Cell Vis 3:329–334 (1996).
29. Hisaw FL. Comparative effectiveness of estrogens on fluid imbibition and growth of the rat's uterus. Endocrinology 54:276–289 (1959).
30. Newbold RR, Jefferson WN, Padilla Banks E, Walker VR, Pena DS. Cell response endpoints enhance sensitivity of the immature mouse uterotropic assay. Reprod Toxicol 15:245–252 (2001).
31. Branham WS, Sheehan DM, Zehr DR, Ridlon E, Nelson CJ. The postnatal ontogeny of rat uterine glands and age-related effects of 17 beta-estradiol. Endocrinology 117:2229–2237 (1985).
32. Maier DB, Newbold RR, McLachlan JA. Prenatal diethylstilbestrol exposure alters murine uterine responses to prepubertal estrogen stimulation. Endocrinology 116:1878–1886 (1985).
33. Fail PA, Hines JW, Zacharewski T, Wu ZF, Borodinsky L. Assessment of polystyrene extract for estrogenic activity in the rat uterotropic model and an in vitro recombinant receptor reporter gene assay. Drug Chem Toxicol 21(suppl 1):101–121 (1998).
34. Martin L, Claringbold PJ. The mitogenic action of oestrogens in the vaginal epithelium of the ovariectomized mouse. J Endocrinol 20:173–186 (1960).
35. Kang K-S, Kim H-S, Ryu D-Y, Che J-H, Lee Y-S. Immature uterotropic assay is more sensitive than ovariectomized uterotropic assay for the detection of estrogenicity of *p*-nonylphenol in Sprague-Dawley rats. Toxicol Lett 118:109–115 (2000).
36. Tinwell H, Soames AR, Foster JR, Ashby J. Estradiol-type activity of coumestrol in mature and immature ovariectomized rat uterotrophic assays. Environ Health Perspect 108:631–634 (2000).