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Longterm Effects of Neonatal Treatment with Cortisol and/or Estrogen in the Female BALB/c Mouse (40396)

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Neonatal exposure of mice to estrogen, testosterone or progesterone produces changes in the hypothalamus and vaginal epithelium resulting in persistent vaginal cornification in the adult. The cornified vaginal and cervical epithelium undergoes a progression of changes from invasive epithelial hyperplasia appearing around 6 months of age to neoplastic transformation by 12 months of age (1–4). Analogous changes (vaginal and cervical adenosis, vaginal carcinoma) have been found in the genital tracts of young women exposed prenatally to the synthetic estrogen, diethylstilbestrol (5).

Newborn human infants exposed perinatally to glucocorticoid therapy show evidence of growth impairment, intraventricular hemorrhage, and central nervous system damage (6, 7). Similar observations have been made in neonatally treated rats and mice (8–11). However, the longterm effects of perinatal glucocorticoid treatment have received little attention.

Persistent vaginal cornification can be induced in mice injected neonatally with cortisone acetate followed by estrogen injections. Either early cortisone acetate or late estrogen alone do not have this effect (12). Cortisone acetate is believed to prolong the estrogen-sensitive critical period (4), but its mechanism of action is unknown. An involvement of the immune system has been suggested (12), as glucocorticoids are known to interfere with immunological development (8, 13). The present experiment was designed to examine the longterm morphological effects of neonatal cortisol treatment, with and without subsequent exposure to estrogen, on the female mouse reproductive and immune systems.

Materials and methods. Female BALB/c mice were given daily subcutaneous injections of 50 μg of cortisol in 0.02 ml vehicle (Steroid Suspension Vehicle; NIH, Bethesda, MD) or of vehicle alone for postnatal days 1–10. A new cortisol microsuspension was prepared daily to prevent loss of activity by spontaneous oxidation to 21-dehydrocortisol (14). Daily intraperitoneal injections of 20 μg of 17β-estradiol in 0.02 ml sesame oil or of oil vehicle alone were given during days 11–15.

All animals were weaned at 30 days of age, and housed 2–6 females per cage. Body weights were taken at 2 and 6 months of age. Vaginal smears were taken during days 70–89, 141–150, and 161–180. About one-half the mice from each treatment group were ovariectomized at 5 months of age. The ovaries were preserved in Bouin's fluid for histological examination. The chi-square independence test was used to determine if the number of abnormally cycling mice per group was dependent on hormone treatment.

The mice were killed by cervical dislocation at 6 months of age. The ovaries, uteri and vaginae were removed and fixed in Bouin's fluid. The spleen, thymus, both inguinal lymph nodes and both adrenals were weighed upon removal and also fixed. All organs were sectioned in paraffin at 7-μm thickness in step serial sections, and were stained with hematoxylin and eosin. Index organ weights and body weights were compared among groups by the one-sided multiple comparison test of Dunnett and by the Student "t" test.

Results: Vaginal Smears. Mice given estrogen injections during days 11–15 after birth (groups 1 and 3) included a significantly higher number of abnormally cycling animals than controls (group 4) at *ca.* 3 and *ca.* 5 months of age, with the same trends evident at *ca.* 6 months of age which did not reach significance owing to reduction of group size. The abnormal cycles were characterized by persistent diestrus or prolonged estrous cycles, the latter consisting of at least 6 consecutive days of cornified smears per cycle. The number of normally cycling animals treated

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TABLE I. VAGINAL SMEARS OF INTACT TREATED BALB/c MICE.

Group	Treatment	No. of mice	No. of mice with: Normal cycles (%)	Abnormal cycles: Pd[a]	Pe[b]	Pvc[c]	Total (%)	P^d
			Days 70–89					
1	F + E	22	5 (23)	5	12		17 (77)	<.005
2	F + oil	23	18 (78)	1	4		5 (22)	ns
3	ssv + E	22	9 (41)	5	8		13 (59)	<.005
4	ssv + oil	24	22 (92)		2		2 (8)	
			Days 141–150					
1	F + E	21	6 (29)	9	6		15 (71)	<.005
2	F + oil	23	17 (74)	4	2		6 (26)	ns
3	ssv + E	21	6 (29)	11	3	1	15 (71)	<.005
4	ssv + oil	24	23 (96)		1		1 (4)	
			Days 161–180					
1	F + E	11	4 (36)	3	4		7 (64)	ns
2	F + oil	12	11 (92)	1			1 (8)	ns
3	ssv + E	11	5 (45)	3	1	2	6 (55)	ns
4	ssv + oil	12	10 (83)		2		2 (17)	

[a] Persistent diestrus.
[b] Prolonged estrus: six or more consecutive days of cornified smears.
[c] Persistent vaginal cornification.
[d] Chi-square analysis of cycle normality in treated groups compared with controls.
F = 50 μg cortisol/day during days 1–10
E = 20 μg 17β-estradiol/day during days 11–15
ssv = 0.02 ml steroid suspension vehicle/day during days 1–10
oil = 0.02 ml sesame oil/day during days 11–15
ns = not statistically significant

with cortisol alone (group 2) did not differ from controls (group 4) at 3, 5, or 6 months of age (Table I).

Two of the mice treated with estrogen alone (group 3) exhibited persistent vaginal cornification by 5 months of age. The estrous cycles of all 45 mice ovariectomized at 5 months of age were completely eliminated, as determined by vaginal smears during days 161–180 (data not shown).

Body weights. The mean body weights of mice treated with cortisol and estrogen, or with cortisol alone (groups 1 and 2) were significantly lower than controls (group 4) at 60 days of age. By 6 months of age, only the animals given cortisol followed by estrogen (group 1) had lower body weights than controls. Ovariectomy did not alter the body weights at 6 months of age (Table II).

Organ weights. All intact animals neonatally treated with cortisol (groups 1 and 2) had significantly higher thymus weights at day 180 than controls (group 4). Ovariectomized mice did not have significantly elevated thymus weights. The spleen, inguinal lymph node, and adrenal weights of the treated groups were not significantly different from controls (group 4) at 6 months of age. This was true both for intact mice and for those ovariectomized at 5 months (Tables III and IV).

A comparison of organ weights was made between the intact and ovariectomized animals of each group by the Student "t" test. Ovariectomy did not appear to change the spleen, thymus, or inguinal lymph node weights. However, adrenal weights were significantly decreased by ovariectomy in Groups 1, 2 and 4 (Table III: $P < .01$, $< .01$, $< .05$, respectively).

Histology. The uteri of all animals were normal. Ovaries lacking corpora lutea and containing hypertrophied interstitial cells, which are characteristic of neonatally estrogenized mice, were frequently found in animals treated with cortisol followed by estrogen (group 1, 17/22, 77%) or estrogen alone (group 3, 17/21, 81%).

Areas of vaginal mucification were found in a few animals of every group, but could

TABLE II. MEAN BODY WEIGHTS OF MICE AT 60 AND 180 DAYS OF AGE.

		Day 60 Intact mice			Day 180 Intact mice			Day 180 Ovariectomized mice		
Group	Treatment	No. of mice	Mean body wt (g)[a]	P^b	No. of mice	Mean body wt (g)	P	No. of mice	Mean body wt (g)	P
1	F + E	22	18.7 ± 0.4	<.01	11	22.6 ± 0.5	<0.5	11	23.1 ± 0.7	<.05
2	F + oil	23	19.9 ± 0.5	<.01	12	23.5 ± 0.4	ns	11	24.0 ± 0.5	ns
3	ssv + E	22	20.4 ± 0.4	ns	11	23.4 ± 0.5	ns	11	24.7 ± 0.4	ns
4	ssv + oil	24	21.7 ± 0.3		12	24.5 ± 0.6		12	25.5 ± 0.5	

[a] ±SEM.
[b] Comparison of body weights between each treated group and control (group 4) by the one-sided multiple comparison test of Dunnett.
ns = not statistically significant.

TABLE III. THYMUS AND ADRENAL MEAN INDEX WEIGHTS[a] AT 6 MONTHS OF AGE.

		Thymus weights					Adrenal (2) weights			
		Intact mice			Ovariectomized mice		Intact mice		Ovariectomized mice	
Group	Treatment	No. of mice	Index wt	P^b	No. of mice	Index wt[c]	No. of mice	Index wt[c]	No. of mice	Index wt[c]
1	F + E	11	14.4 ± 0.4	<.05	11	14.4 ± 1.0	11	3.83 ± 0.09	11	2.94 ± 0.27
2	F + oil	12	14.9 ± 0.8	<.01	11	12.9 ± 1.3	12	3.59 ± 0.13	11	2.32 ± 0.34
3	ssv + E	11	13.4 ± 0.7	ns	11	11.0 ± 0.6	11	3.45 ± 0.28	11	2.99 ± 0.12
4	ssv + oil	12	11.8 ± 0.3		12	12.3 ± 0.7	12	3.67 ± 0.18	11	2.63 ± 0.43

[a] Index weight = organ wt (g)/body wt (g) × 10^4 ± SEM.
[b] Comparison of organ weights between each treated group and control (group 4) by the one-sided multiple comparison test of Dunnett.
[c] No significant differences between any treated group (1–3) and controls (group 4) as determined by the one-sided multiple comparison test of Dunnett.
ns = not statistically significant.

not be correlated with any specific neonatal treatment. Of the 14 mice with mucification, 12/14 (86%) had ovaries that appeared "estrogenized" or were at least lacking corpora lutea. More intact mice (12/14) showed mucification than did ovariectomized mice (2/14).

The spleen, thymus, inguinal lymph nodes, and adrenals of treated mice did not differ histologically from controls.

Discussion. Animals injected with cortisol followed by estrogen (group 1) did not exhibit persistent vaginal cornification as had been expected. This finding is not in accord with the results of Takasugi (4) and Takasugi and Tomooka (12) who induced persistent vaginal proliferation and keratinization in C57B1 and C3H mice neonatally treated for 10 days with cortisone acetate (20–50 μg) followed by 5 daily estradiol (1–20 μg) injections. The discrepancy may be due to a difference in the actual glucocorticoid used (cortisol vs. cortisone acetate), glucocorticoid vehicle (aqueous suspension vs. oil solution), or mouse strain (BALB/c vs. C57B1 or C3H).

Groups of mice treated with cortisol plus estrogen or estrogen alone had 70–80% more abnormally cycling animals than the control group at 3 and 5 months of age. Abnormal cycles consisted of persistent diestrus or prolonged estrous periods. The ovaries of animals from the day 11 to 15 estrogen-treated groups contained hypertrophied interstitial tissue and lacked corpora lutea, a condition usually characteristic of mice given estrogen injections during days 1–5 after birth (2). Early estrogen is considered to affect the hypothalamo-hypophysial axis so that normal gonadotropin control of the ovaries is disrupted and ovulation is prevented. The later estrogen treatment in the present experiment was able to produce longterm changes in the mouse estrous cycles and ovaries, indicating a lasting sensitivity of the hypothal-

TABLE IV. SPLEEN AND INGUINAL LYMPH NODE MEAN INDEX WEIGHTS[a] AT 6 MONTHS OF AGE.

		Spleen weights				Lymph node (2) weights			
		Intact mice		Ovariectomized mice		Intact mice		Ovariectomized mice	
Group	Treatment	No. of mice	Index wt[b]	No. of mice	Index wt[b]	No. of mice	Index wt[b]	No. of mice	Index wt[b]
1	F + E	11	55.0 ± 1.7	11	53.4 ± 1.5	11	3.71 ± 0.24	11	3.52 ± 0.23
2	F + oil	12	53.3 ± 1.6	11	54.6 ± 1.3	12	3.25 ± 0.22	11	3.96 ± 0.24
3	ssv + E	11	58.0 ± 1.8	11	54.4 ± 2.7	11	3.94 ± 0.24	11	4.06 ± 0.29
4	ssv + oil	12	55.7 ± 2.6	12	52.8 ± 2.4	12	3.73 ± 0.26	12	3.47 ± 0.26

[a] Index weight = organ wt (g)/body wt (g) × 10^4 ± SEM.

[b] No significant differences between any treated group (1–3) and control (group 4) as determined by the one-sided multiple comparison test of Dunnett.

amo–pituitary–ovarian axis to estrogen throughout postnatal days 11–15.

Neonatal injections of cortisol alone did not interfere with normal cycling. However, Turner and Taylor (15) found that rats given intraperitoneal corticosterone implants on postnatal day 3 showed 82% persistent diestrus at 1 month of age and 45% prolonged estrus by 2 months. A single injection of cortisol into 3-day-old female rats was reported to delay the onset of vaginal opening and first ovulation (16).

Fifty μg/day of cortisol was used in the present experiment because higher dosages proved lethal, and in a preliminary experiment 50 μg of cortisol/day for 10 days was found to cause severe thymic atrophy and growth inhibition in 11-day-old mice (Ways, unpublished). The 20 μg estradiol daily dose was chosen to examine possible ovary-independent vaginal changes after cortisol plus estrogen exposure, since this dose induces ovary-independent persistent vaginal cornification when given to neonatal mice during days 1–5.

Half of the mice from every group were ovariectomized at 5 months of age, to determine if persistent cornification was present only in certain areas of the vaginal parenchyma in any of the animals. A condition such as this could allow an animal to show normal, cyclic vaginal smears, and persistent cornification would not be detected. However, ovariectomy abolished all signs of cornification, as determined by histological study.

All mice treated neonatally with cortisol (groups 1 and 2) had lower body weights by 8–14% than controls at day 60 ($P < .01$). All exhibited "catch up" growth by 6 months of age, except those given cortisol plus estrogen (group 1). Glucocorticoids have previously been reported to retard growth in young animals (17, 18), and accelerated growth occurs after the cessation of hormone treatment (19).

Spleen and inguinal lymph node weights of treated animals were not different from controls at 6 months of age. Intact neonatally cortisol-treated mice (groups 1 and 2) had 25% higher thymus weights than controls at 6 months. Thymus weights were also found to be elevated at 60 days of age in a preliminary experiment (Ways, unpublished). Perinatal glucocorticoid treatment of rodents initially reduces spleen, thymus, and lymph node weights but complete recovery is gradually attained (8, 12, 20, 21). Overgrowth of the thymus has been reported to occur 7–42 days following cessation of glucocorticoid therapy in human infants (22). There is emerging evidence that neonatal exposure of mice to estrogens results in modifications of the immune system (21, 23; Blair, personal communication). This possible consequence of exposure of humans to diethylstilbestrol *in utero* needs examination.

Summary. Neonatal treatment of mice with cortisol (days 1–10) resulted in decreased body weights at 2 months of age and increased thymus weights at 6 months. The spleens, inguinal lymph nodes, and adrenals from cortisol-treated animals were normal histologically and their weights did not differ from controls at 6 months of age. The estrous cycles were normal, and the reproductive organs did not suffer any apparent abnormalities. Treatments with cortisol followed by estrogen or with estrogen alone (days 11–15) altered the estrous cycle by prolonging the period of estrus or by eliminating the cycles

altogether (persistent diestrus). The ovaries of these animals lacked corpora lutea and had hypertrophied interstitial tissue. The spleen, thymus, inguinal lymph nodes, and adrenals had normal weights and histological appearance. The body weights of all treated mice were lower than controls at 2 months of age, but only the weights of those given cortisol followed by estrogen remained depressed at 6 months.

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