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Cervicovaginal and Mammary Gland Abnormalities in BALB/cCrgl Mice Treated Neonatally with Progesterone and Estrogen, Alone or in Combination[1]

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ABSTRACT

Neonatal female BALB/cCrgl mice (mammary tumor virus unexpressed) were given a daily injection of estradiol and/or progesterone for 5 days, beginning within 36 hr after birth. About one-half of each group was ovariectomized when 40 days old, and all mice were killed when between 18.5 and 26 months of age. Neonatal progesterone leads to ovary-dependent persistent vaginal cornification and hyperplasia. In addition, 16 of the 24 progesterone-treated mice had genital tract lesions, and 4 of these showed predominantly glandular features. No such lesions were observed in either oil-treated or untreated mice. Lesions were also observed in both intact and ovariectomized mice treated with estrogen and estrogen-progesterone combinations, but most of the lesions were not as severe as those seen in mice treated neonatally with progesterone alone, and they were predominantly squamous in appearance. Although mammary tumors were not observed in either the control or the neonatally steroid-treated intact mice, many in the latter groups possessed hyperplastic alveolar-like mammary nodules and other abnormalities.

INTRODUCTION

The use of DES[4] in the first trimester of human pregnancy for the prevention of premature births has been associated with unexpected long-term consequences for the offspring exposed *in utero* (1, 9, 10). These DES-exposed daughters show a low but significant incidence of clear cell adenocarcinoma of the vagina; benign vaginal and cervical lesions, termed adenosis, appear as well. There have been reports of an association between maternal treatment with progestin and/or estrogen and congenital malformations in the newborn involving cardiovascular, skeletal, gastrointestinal, and genitourinary systems (27). Recently, the use of a progestin, 17α-hydroxyprogesterone caproate, has been proposed for the prevention of premature births (14).

In the early 1960's Dunn and Green (3) and Takasugi and Bern (36) demonstrated that female mice exposed antenatally or neonatally to both synthetic and natural estrogen injections showed continuous proliferation and cornification of the vaginal epithelium after cessation of estrogen treatment. The changes were shown to be irreversible at high doses, frequently culminating in the formation of precancerous and cancerous lesions in mice over 6 months old (3, 18, 19, 22, 36, 37). Female hamsters exposed *in utero* to DES also develop hyperplastic and neoplastic lesions in their reproductive system (32).

Although the long-term effects of neonatal treatment with estrogen and with androgen have been studied in mice and hamsters, little information is available on the long-term effects of exposure of the newborn mouse to progesterone. Recent studies in BALB/cfC3H/Crgl mice (MTV-expressed) indicate that neonatal administration of progesterone alone results in the appearance of an occasional vaginal and cervical lesion by 12 months of age (15). In addition, progesterone, as well as estrogen and androgen, results in a mammary tumor incidence significantly higher and with a lower mean age of onset than in controls (15, 23, 40). The present experiments were planned to study the long-term effects of neonatal progesterone administration to BALB/cCrgl mice (MTV-unexpressed) on the vagina, uterine cervix, and mammary gland. In addition, progesterone was also administered neonatally in association with low and high doses of estrogen. Whereas experiments with BALB/cfC3H mice were terminated by 12 months of age owing to the high mammary tumor incidence, BALB/cCrgl (henceforth referred to as BALB/c) mice could be maintained for an additional 10 months or so, owing to the absence of mammary tumor development, permitting further development of cervicovaginal lesions.

MATERIALS AND METHODS

A total of 314 female BALB/c mice was divided into 13 groups (Table 1). Members of newborn litters were randomly distributed among several groups. Experimental mice received daily s.c. injections of 5 or 20 μg 17β-estradiol, with or without 100 μg progesterone, in 0.02 ml sesame oil (the vehicle) for 5 days beginning within 36 hr after birth. Neonates receiving progesterone before 16 hr after birth had a high mortality rate; therefore, all steroid treatments were begun after 16 hr. Data from mice receiving their first injection of progesterone, alone or in combination with estradiol, before 24 hr and those receiving it between 24 and 36 hr were not significantly different (χ^2 method and Student's *t* test); hence, these 2 subgroups were combined. All mice were weaned at 25 days of age. About one-half of each treatment group was ovariectomized on Day 40. Vaginal smears were taken for a period of 25 days, beginning when the mice were 40 to 50 days old. When vaginal concretions occurred, they were removed with blunt forceps.

Treated mice were killed and autopsied when between 18.5 and 26 months old; the mean age of oil-treated controls was 21.9 months and the age of experimentals was 22.6 months. In addition, 35 untreated virgin mice from our stock colony

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[4] The abbreviations used are: DES, diethylstilbestrol; MTV, mammary tumor virus.

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Table 1

Histological features of vagina and cervix of intact and ovariectomized BALB/c mice subjected to neonatal treatment with estrogen and/or progesterone

Neonatal treatment (μg daily dose)							No. of mice showing vaginal changes at autopsy			No. of mice bearing lesions at autopsy		
Estradiol	Progesterone	No. of mice examined by vaginal smear	Av. age at autopsy (mos.)	Ovariectomy at Day 40	No. showing persistently cornified vaginal smears by Day 65–75	No. showing vaginal concretions during life	Cornification	Stratification and/or mucification	Atrophy	Cervical	Vaginal	Both
	100	36 (24)[a]	22.8 ± 1.6[b]	No	36 (100)[c]	13	24 (100)[c]	0	0	7	2	7
	100	34 (28)	23.0 ± 1.7	Yes	0 (0)	0	8 (29)	20	0	1	1	0
5		18 (13)	21.9 ± 1.5	No	18 (100)	15	13 (100)	0	0	2	2	3
5		14 (10)	22.5 ± 3.0	Yes	9 (64)	2	10 (100)	0	0	0	0	0
5	100	23 (12)	23.5 ± 2.2	No	12 (52)	11	12 (100)	0	0	1	1	4
5	100	25 (17)	21.9 ± 1.8	Yes	5 (20)	1	16 (94)	1	0	1	1	0
20		25 (11)	21.6 ± 1.2	No	25 (100)	12	11 (100)	0	0	2	2	2
20		19 (10)	22.8 ± 0.9	Yes	19 (100)	9	10 (100)	0	0	0	1	0
20	100	27 (10)	22.0 ± 3.0	No	6 (22)	10	10 (100)	0	0	3	0	5
20	100	26 (20)	22.1 ± 0.9	Yes	4 (15)	2	16 (80)	4	0	0	3	0
Sesame oil		15 (10)	22.0 ± 1.7	No	0 (0)	0	3 (30)	7	0	0	0	0
Sesame oil		17 (13)	21.8 ± 1.0	Yes	0 (0)	0	0 (0)	0	13	0	0	0
No treatment		0 (35)	22.3 ± 2.6	No		0	8 (23)	27	0	1[d]	1[d]	0

[a] Numbers in parentheses, number examined at autopsy.
[b] Mean ± S.E.
[c] Numbers in parentheses, percentages.
[d] Small "lesions" histologically distinct from those in steroid-exposed mice.

were killed when between 19 and 25 months old (mean, 22.3). Ovaries, adrenals, uteri, and one-half of the sagittally cut cervicovaginal tract were fixed in Bouin's fluid and parasagittally sectioned in 7-μm-thick sections; sample sections were stained with Harris's hematoxylin and eosin. Mammary glands were stained as whole mounts with iron hematoxylin.

RESULTS

Vaginal Smears. Initial vaginal changes were studied by examining daily vaginal smears for 25 days, beginning when mice were between 40 and 50 days old. Vaginal cycles in mice are frequently irregular (34). Therefore, mice were considered to have developed persistent vaginal cornification only if they showed continuous presence of cornified cells for 25 days; intermittent appearance of small quantities of leukocytes, nucleated epithelial cells, and/or mucus did not affect our interpretation. All mice treated with either 5 or 20 μg of estradiol for the first 5 days of life showed persistent vaginal cornification by about 40 days of age. Ovariectomy at Day 40 did not reduce the incidence of persistent vaginal cornification in mice treated neonatally with 20 μg of estradiol, and only marginally in mice treated neonatally with 5 μg of estradiol, judging from vaginal smears.

Vaginal smears after neonatal progesterone treatment showed the continuous presence of cornified cells and leukocytes. The pattern differed from that exhibited by mice treated neonatally with estradiol by the occurrence of some cyclic changes in the proportion of cornified cells to leukocytes. Ovariectomy eliminated the presence of cornified cells. When treated with progesterone and estradiol in combination, both intact and ovarectomized mice showed a reduced incidence of persistent vaginal cornification, compared with those receiving estradiol alone.

Vaginal Concretions. Vaginal concretions were found in all hormone-treated groups, with the exception of ovariectomized mice treated neonatally with progesterone. Intact mice receiving 5 μg estradiol had the highest incidence of vaginal concretions (Table 1). No concretions were found in control mice.

Vaginal Histology. In most mice treated neonatally with estradiol, the urethra was observed to open into the vaginal lumen, which involved fusion of urethral and vaginal epithelia. This phenomenon was more frequent in estradiol-treated mice than in those treated neonatally with estradiol-progesterone combinations, and it was never seen in mice treated neonatally with progesterone alone.

Progesterone alone produced cornification in all intact mice at termination. Ovariectomy at Day 40 did not abolish cornification in 10 of 28 mice examined at autopsy. Along with the presence of cornification, the vaginas in these castrated mice had areas showing varying degrees of parakeratosis and mucification (Figs. 1 and 2). This histological pattern was also observed in those ovariectomized animals treated neonatally with progesterone in combination with estradiol, which displayed predominantly cornified vaginas.

Vaginal and Cervical Lesions. Hyperplastic cervicovaginal lesions were observed in at least one member of all hormone-treated groups (Table 1), with the exception of those mice treated with 5 μg estradiol and then ovariectomized. Neonatal treatment with progesterone alone induced genital tract lesions in 16 of 24 intact mice. The lesions observed in these mice histologically ranged from those resembling squamous cell carcinomas (Fig. 3) to those (4 mice) with predominantly glandular features (Fig. 4); most of the lesions were a mosaic of both (Fig. 5). Seven of 46 mice treated with progesterone or estrogen-progesterone showed predominantly adenocarcinomatous features; the lesions in 5 of these mice also had squamous cell features. Although the lesions induced by neonatal progesterone treatment were generally the most severe, a genital tract lesion in one intact animal treated with 5 μg estradiol was the most extensive. This lesion invaded the vaginal wall (Fig. 6) to form a large mass around the neck of the bladder (Fig. 7).

No comparable lesions were observed in control mice. How-

ever, in 2 untreated mice, distinctive genital tract epithelial abnormalities were observed (Fig. 8). These abnormalities differed from the hyperplastic and neoplastic lesions observed in the hormone-treated animals in their restricted development. The epithelium bordering the lumen and that lining the basement membrane remained intact and showed no evidence of invasion. Calcium deposits appeared to be present.

Mammary Gland. Although mammary tumors were observed in neither control nor hormone-treated intact mice, many of the intact hormone-treated mice possessed hyperplastic alveolar-like nodules and other dysplasias (Table 2; Figs. 9 to 11) characterized by relatively prominent leukocytic infiltration (Fig. 12). It is emphasized that these hyperplastic alveolar-like nodules were present despite the absence of expressed MTV. In general, the mammary glands of neonatally steroid-treated mice showed evidence of secretory stimulation, dilated ducts, and abnormal lobuloalveolar development (Figs. 9 and 10).

A few hormone-treated ovariectomized mice also displayed abnormal lobuloalveolar development, dilated ducts, and dysplasia (Table 2). The highest incidence was among those mice treated neonatally with 20 μg estradiol plus 100 μg progesterone, in which 10 of 20 animals showed some ductal dilation, with 4 of the 10 showing lobuloalveolar development and 3 of the 10 having abnormalities resembling hyperplastic alveolar nodules.

Other Organs. Although the epithelium of the upper uterus proper proliferated abnormally (primarily squamous metaplasia) in neonatally estrogenized mice in which there were vaginal changes, no hyperplastic lesions of the uterus were observed. The uterine lumen of all progesterone-treated mice was distended, but the epithelium consisted of a single layer of cuboidal cells. The ovaries of all hormone-treated mice showed a few follicles but lacked corpora lutea. However, in mice receiving progesterone alone or in combination with estrogen, luteinlike cells were observed. These cells, which could be remnants of a small corpus luteum, were generally associated with degenerating follicles. The ovaries of all mice showed some degree of ceroid deposition and interstitial cell hyperplasia, most extensively among those mice neonatally treated with steroids. Massive ceroid deposition in the circummedullary zone of the adrenal and some proliferation of spindle-shaped cells in the outer cortex were observed in all mice. However, these abnormalities were more extensive in the steroid-treated animals. The presence of nodular hyperplasia, characterized by B-cells discussed by Woolley (39), was more frequent in the steroid-treated mice, intact or ovariectomized, than in the respective control animals. The pituitary of both the control and steroid-treated animals was morphologically normal in appearance.

DISCUSSION

Earlier studies have described the responses of the vaginal and cervical regions of the reproductive tract, as well as the mammary gland, to neonatal estrogen and androgen treatment, including the appearance of genital tract lesions (3, 5, 7, 18, 21, 23, 34). Recently, we have reported that progesterone administered neonatally resulted in ovary-dependent persistent vaginal cornification and the occurrence of an occasional genital tract lesion in BALB/cfC3H mice by 12 months of age (15). The results reported herein in neonatally progesterone-treated mice at around 2 years of age indicate that the incidence and severity of cervicovaginal tract lesions may be related to the age at which the animal is examined.

In BALB/c mice treated neonatally with progesterone alone, the vaginal smears showed an atypical persistent vaginal cornification. After ovariectomy, the neonatally progesterone-treated mice exhibited diestrous vaginal smears. However, at autopsy, 8 of the ovariectomized neonatally progesterone-treated mice possessed cornified vaginal epithelium. Although untreated ovariectomized BALB/c mice did not show any cornified epithelium at autopsy, Perry (28), Smith (33), and Pilgrim (30) have reported cornified vaginal epithelium in long-term ovariectomized C3H mice. It has been suggested that the postovariectomy adrenal in these mice secretes ovarian hormones under the influence of hypophysial gonadotropins (8, 29, 30). It is possible that the neonatal progesterone treatment modified the pituitary-adenocortical axis to result in adrenal estrogen secretion in some cases.

The average incidence of cervicovaginal lesions in all intact

Table 2

Occurrence of abnormalities at 18.5 to 27 months of age in mammary glands of female BALB/c mice treated neonatally with steroids

Neonatal treatment (μg daily dose)						No. of mice showing alveolar development						Incidence of mammary dysplasias (lesions)	
Estrogen	Progesterone	No. of mice	Av. age at autopsy	Ovariectomy	No. of mice with dilated ducts	−	±	+	++	+++	++++	No. of mice showing lesions	No. of lesions/mouse with lesions
	100	24	22.8 ± 1.6[a]	No	18	3	4	4	10	3	0	17	10
	100	28	23.0 ± 1.7	Yes	3	26	2	0	0	0	0	1	2
5		14	22.0 ± 1.5	No	10	4	1	3	2	2	2	7	6
5		10	22.5 ± 3.0	Yes	2	10	0	0	0	0	0	0	0
5	100	9	23.4 ± 2.5	No	8	0	1	2	4	1	1	6	7
5	100	17	21.9 ± 1.8	Yes	1	16	1	0	0	0	0	3	1
20		9	21.7 ± 1.3	No	4	3	1	0	5	0	0	4	4
20		10	22.8 ± 1.8	Yes	0	10	0	0	0	0	0	0	0
20	100	8	22.5 ± 2.7	No	6	0	3	1	2	0	2	5	12
20	100	20	22.1 ± 0.9	Yes	10	16	4	0	0	0	0	3	4
Sesame oil		10	22.0 ± 1.7	No	0	10	0	0	0	0	0	0	0
Sesame oil		10	21.8 ± 1.0	Yes	0	10	0	0	0	0	0	0	0
No treatment		35	22.3 ± 2.6	No	0	35	0	0	0	0	0	0	0

[a] Mean ± S.E.

hormone-treated groups of BALB/c mice was 67% by 26 months of age, compared with 22% in BALB/cfC3H mice by 12 months of age (15). Neonatal progesterone administration resulted in a genital tract lesion incidence of 67%. The genital tract lesions observed in this group differed from those found in mice neonatally treated with either estrogen or androgen, in that some lesions observed in neonatally progesterone-treated mice had adenocarcinomatous as well as squamous cell carcinomatous features (16). Forsberg (5–7) has reported adenosis-like areas in the cervix and vagina of adult mice treated neonatally with estradiol and DES. Plapinger and Bern (31) have recently shown that the failure to find adenosis-like lesions in perinatally estrogenized mice from this laboratory to date might be due to the sagittal plane of sectioning used. By examining the cervicovaginal tract of experimental animals sectioned transversely, they observed adenosis-like lesions primarily within the vaginal fornices. However, to our knowledge, experimental induction of genital tract tumors with adenocarcinomatous features has not been previously reported in mice thus treated, although recently highly suggestive lesions have been observed[5] after neonatal DES treatment. Several of our genital tract lesions have proven to be transplantable and have maintained their original histological pattern over several transplant generations[6]. Both Dunn and Green (3) and Takasugi (35) have transplanted vaginas of mice treated neonatally with estrogen into syngeneic hosts, which later developed tumors. However, the tumors which developed in Takasugi's host animals appear anaplastic and bear little resemblance to the tissue of origin.

Although vaginal concretions were found only among those mice showing persistent vaginal cornification at days 65 to 75, no correlation was observed between the formation of these concretions and the development of lesions in intact or ovariectomized mice. This is in agreement with previous published studies (15, 18). However, among older BALB/c mice neonatally treated with progesterone in this study, a greater proportion had concretions than was previously reported for younger BALB/cfC3H animals (15). Since no neonatally progesterone-treated animal was observed to have the vaginal lumen connected with the urethra, as occurs in most neonatally estrogenized mice, we have no explanation for the presence of the small vaginal concretions. The lack of urethral fusion with the vaginal lumen and the observed ovary dependence of the persistent vaginal cornification lead us to believe that neonatal progesterone administration results primarily in alteration of the hypothalamohypophysioovarian axis, at least at the dose given and during the period used. Rabbit and other mammalian fetuses of both sexes exposed to progestin *in utero* show stimulation of the caudal end of the Mullerian ducts prior to birth. Mullerian epithelium was encountered in areas from which it is normally absent (4, 26). Although Ainslie and Kohrman (2) have recently reported that neonatal treatment with 17α-hydroxyprogesterone caproate induced both ovary-dependent and ovary-independent vaginal changes in ICR mice, Iguchi and Takasugi (13) found that female C57BL/Tw mice treated with 100 μg progesterone daily for the first 10 days after birth showed no ovary-independent changes in the vagina at 90 days of age. Nevertheless, the possibility remains that, in addition to effects mediated by the ovary, in some species, including some mouse strains, perinatal progestin treatment may have a direct effect on the developing genital tract.

Although no genital tract lesions identical to those found in the neonatally hormone-treated animals were observed in the controls, 2 control animals possessed epithelial abnormalities. The papers of Heston (11) and Heston *et al.* (12) reported the occurrence of squamous cell lesions in untreated old BALB/cHe mice. These lesions resemble those found in our hormone-treated mice but not those found in our controls. In addition, the frequency of cervicovaginal lesions in Heston's control mice was much greater than we have observed (52 of 163 as compared with 2 of 45). The presence of genital tract lesions in normal old mice raises the possibility that the effect of neonatal sex steroid hormone exposure is to increase the risk of their occurrence rather than "induce" them *de novo*.

In earlier experiments, Takasugi and Bern (36) reported that neonatally estrogenized BALB/c mice autopsied by 12 months of age showed no evidence of mammary gland stimulation. However, Dunn and Green (3) and Kimura and Nandi (18) observed that older BALB/c mice treated neonatally with either estrogen or androgen showed distended mammary ducts and some alveolar development. Neonatal progesterone treatment, along with other steroid hormone treatments, caused similar mammary gland changes as observed by previous investigators (3, 18). Moreover, the neonatally progesterone-treated group had the highest incidence of mammary gland dysplasias, averaging 10/animal showing mammary gland abnormalities. Previous studies using MTV-bearing mice have shown that neonatal administration of androgen, estrogen, progesterone, or estrogen-progesterone results in a significant increase in mammary tumor incidence and in the number of mammary nodules by 12 months of age (15, 20, 21, 23, 38). It has been suggested that the effect of neonatal administration of sex steroid hormones on the incidence of mammary tumors in MTV-bearing mice may be at least in part a consequence of sustained prolactin secretion resulting from continued low-level estrogen secretion (24), as judged by mammary gland morphology, pituitary cytology, and plasma prolactin levels (17, 24, 40). In addition, prolactin levels in old BALB/c mice neonatally exposed to estrogen and/or progesterone are higher than in control mice of a comparable age (25).

Distended mammary ducts and some alveolar development were observed in a few mice from all ovariectomized hormone-treated groups, with the exception of that group treated neonatally with 20 μg 17β-estradiol. The presence of mammary gland stimulation in some of our ovariectomized, neonatally hormone-treated mice differs from previous published results (15, 18, 23). The difference may lie in the older age of our mice at the time of observation and in possible postcastrational adrenal sex steroid secretion in the old animals (8, 29, 30).

The long-term effects of neonatal progesterone treatment on BALB/c mice can be summarized as follows: (*a*) progesterone alone causes hyperplastic as well as neoplastic changes in both the vaginal and cervical epithelium, primarily as a result of an effect on the hypothalamohypophysioovarian axis; (*b*) the cervicovaginal lesions have histological features which resemble both squamous cell carcinoma and adenocarcinoma; (*c*) estradiol, alone and in combination with progesterone, causes both hyperplastic and neoplastic changes in the vaginal and cervical epithelium; (*d*) in the mammary gland, progesterone

[5] J-G. Forsberg, personal communication.
[6] L. A. Jones and R. Pacillas-Verjan, Cancer Research, in press, 1979.

and estradiol, alone or in combination, resulted in the appearance of hyperplastic alveolar-like nodules and other dysplasias of unknown neoplastic significance, despite the absence of expressed MTV.

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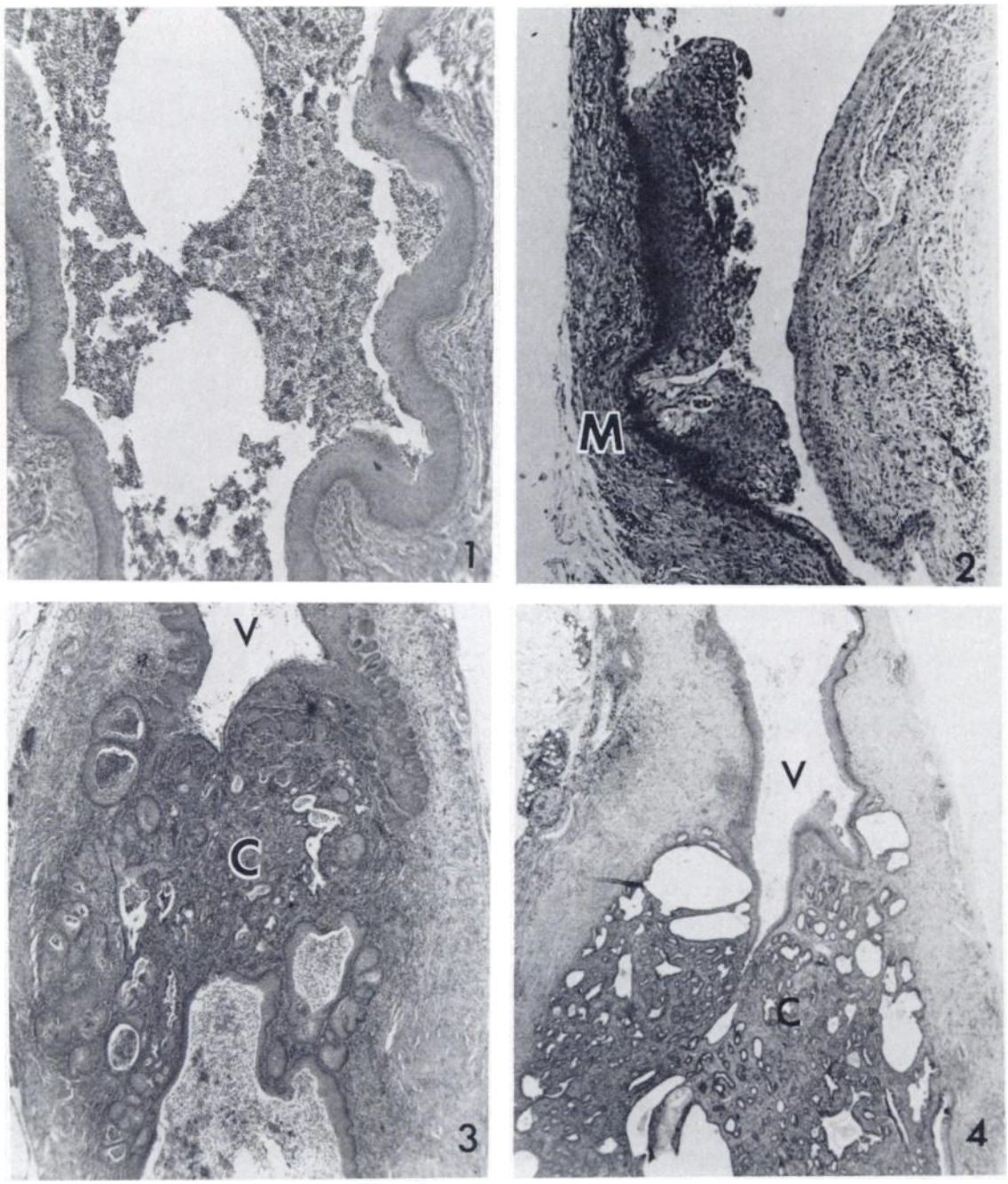

Fig. 1. Vagina from a 25-month-old ovariectomized mouse treated neonatally with 100 μg progesterone. Note parakeratosis, lymphocytes in the wall, and leukocytes in the lumen. H & E, × 40.

Fig. 2. Vagina from a 25-month-old ovariectomized mouse treated neonatally with 100 μg progesterone. Note the parakeratotic hyperplastic area and mucification (*M*). H & E, × 40.

Fig. 3. Hyperplastic lesion of the upper vagina (*V*) and cervix (*C*) from a 23.5-month-old mouse treated neonatally with 100 μg progesterone. Note squamous cell appearance of the lesion. H & E, × 40.

Fig. 4. Hyperplastic lesion of the upper vagina (*V*) and cervix (*C*) from a 23.5-month-old mouse treated neonatally with 100 μg progesterone. Note glandular appearance of the lesion. H & E, × 40.

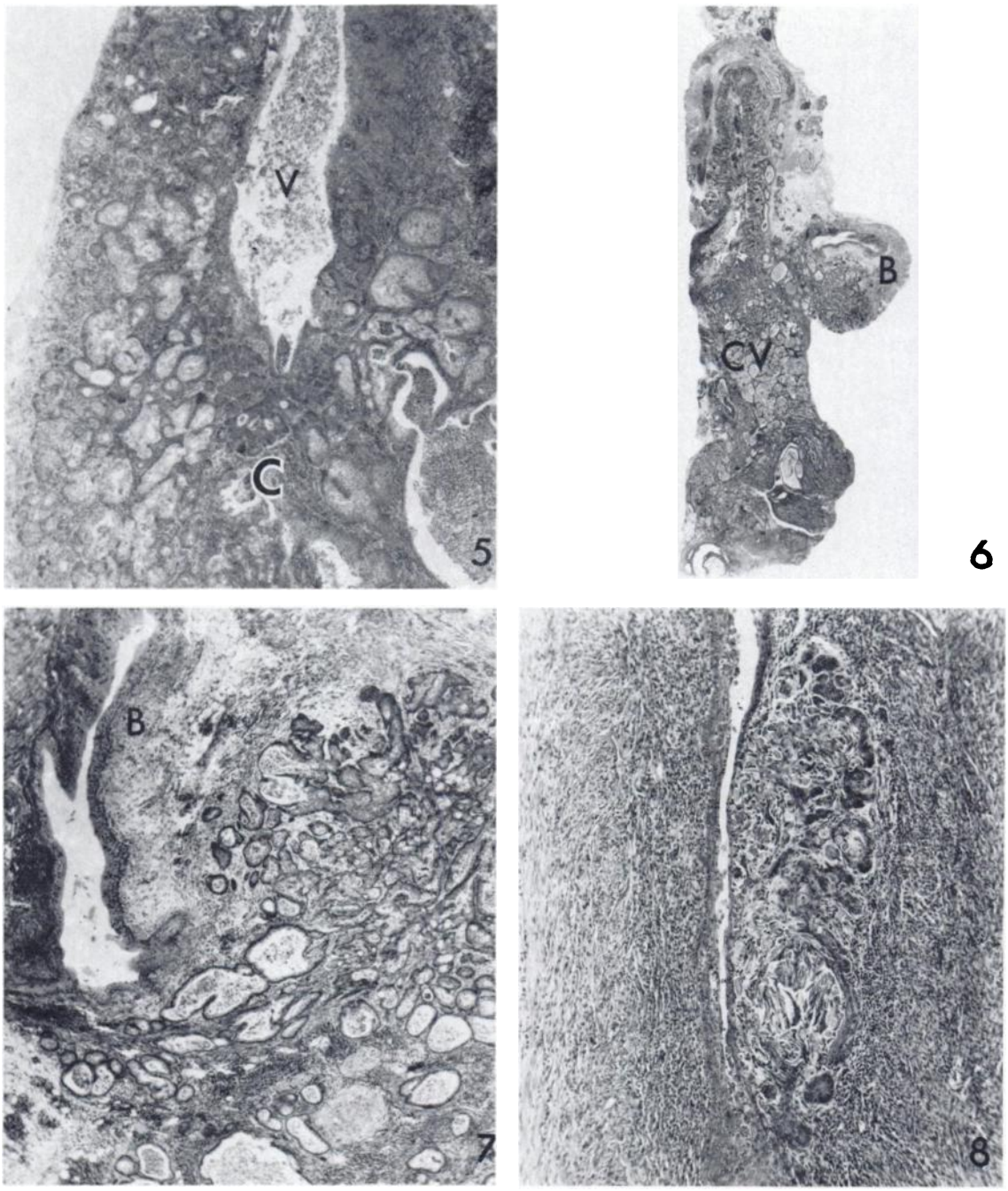

Fig. 5. Lesion of the upper vagina (*V*) and cervix (*C*) from a 23.5-month-old mouse treated neonatally with 100 μg progesterone. Note the adenosquamous appearance of the lesion. H & E, × 40.

Fig. 6. Cervicovaginal (*CV*) tumor from a 24-month-old mouse treated neonatally with 5 μg 17β-estradiol. Note the invasion of the tumor into the bladder wall (*B*). H & E, × 10.

Fig. 7. High magnification of Fig. 6. Note squamous cell lesion invading wall of the bladder (*B*). H & E, × 100.

Fig. 8. Vaginal dysplasia from a 21-month-old untreated mouse. Note the continuous basal lamina. H & E, × 40.

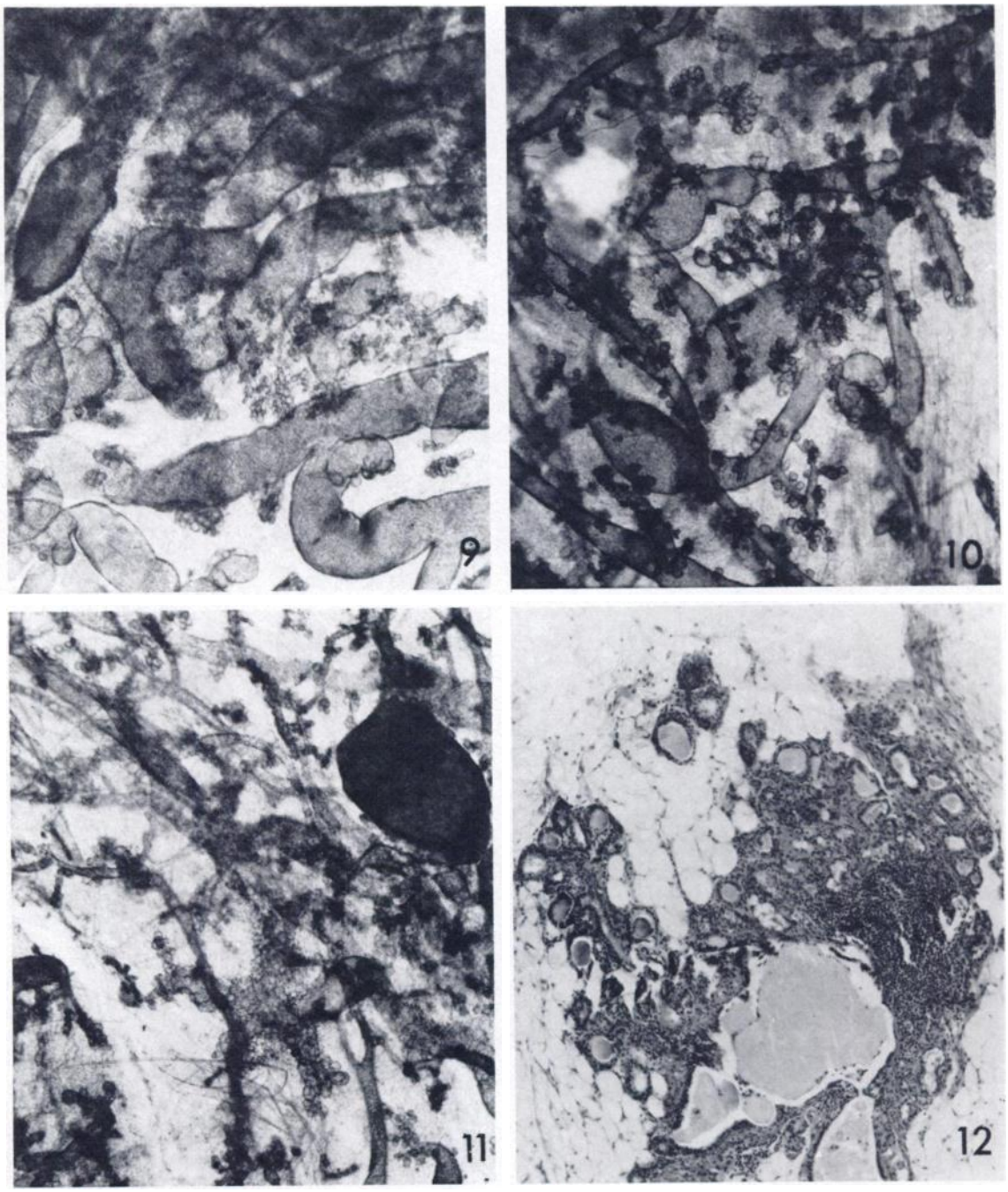

Fig. 9. Mammary gland dysplasia from a 20-month-old mouse treated neonatally with 100 μg progesterone. Note alveolar development and hyperplastic alveolar nodule-like lesion. Whole mount; iron hematoxylin, × 21.

Fig. 10. Mammary gland dysplasia from a 21-month-old mouse treated neonatally with 100 μg progesterone. Note dilation of the ducts. Whole mount; iron hematoxylin, × 21.

Fig. 11. Mammary gland dysplasia from a 23-month-old mouse treated neonatally with 100 μg progesterone. Note the hyperplastic alveolar nodule-like lesion. Whole mount; iron hematoxylin, × 21.

Fig. 12. Histological section of Fig. 11. Note the presence of secretion in the duct and prominent lymphocytic infiltration. H & E, × 100.