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Influence of estrogen and progesterone on the induction of mammary carcinomas by 7,12-dimethylbenz(a)anthracene in ovariectomized rats

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Summary. The influence of estrogen on mammary carcinogenesis was studied in female Sprague-Dawley rats ovariectomized at the age of 36 days and given injections of 17β-estradiol (group I:0, II:1, III:10, IV:100, V:1000 μg/2 days) between the ages of 36 and 250 days and a single oral dose of 20 mg of 7,12-dimethylbenz(a)anthracene (DMBA) at the age of 50 days. No palpable mammary carcinomas were detected up to the age of 135 days. At the age of 135 days, each group was divided into two subgroups (a and b). Rats of the second subgroup (Ib, IIb, IIIb, IVb and Vb) were given additional injections of progesterone (P; 4 mg/2 days) between the ages of 135 and 250 days. At the age of 250 days, the incidence of mammary carcinoma was significantly higher in rats from group IIIb than in groups Ib and IIIa, and that in group IVa was also higher than in group Ia. The incidence in group IVb was significantly lower than in group IVa. The carcinomas in group IIIb were palpable papillo-tubular adenocarcinomas and those in group IVa were secretory micro-adenocarcinomas. These results indicate that the induction of mammary carcinomas by DMBA is totally inhibited by ovariectomy and/or high doses of estrogen, but that mammary carcinomas are initiated by DMBA under hormonal conditions in which suitable levels of estrogen are present. They also suggest that the growth of DMBA-induced mammary carcinomas in the rats from group III were accelerated by additional injections of P and that those in rats from group IV were inhibited by additional P. The mammary glands of 50-day-old rats in groups III and IV contained more terminal ducts and terminal ends buds or lobules with BrdU-positive cells than those of rats in other groups.

Key words: Mammary carcinoma – 7,12-dimethylbenz(a)anthracene – Estrogen – Progesterone – Rat

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Introduction

The incidence of breast cancer in men and women ovariectomized before the age of 37 years is extremely low (Hirayama and Wynder 1962), suggesting that mammary carcinogenesis is influenced by hormonal conditions. However, despite extensive investigation, the hormonal circumstances that lead to the development of breast cancer have still not been elucidated. Recently, an extended life-span study of atomic bomb survivors from Hiroshima and Nagasaki demonstrated a high incidence of breast cancer among adult women who were aged under 10 years at the time of exposure (Tokunaga et al. 1987). These data suggested that the susceptibility of the female breast to carcinogenic stimuli under hormonal conditions where estrogen (E) is present and progesterone (P) absent may be high. The 7,12-dimethylbenz(a)anthracene(DMBA)-induced rat mammary carcinoma model has been an useful experimental system for studying the pathogenesis of mammary carcinomas (Murad and Haam 1972; Russo et al. 1977). In the present investigation, we have used this model to investigate the influence of estrogen on the development of the mammary gland and its susceptibility to carcinogenesis induced by DMBA administration.

Material and methods

Animals. All the animals were inbred Sprague-Dawley rats maintained in filtered air laminar flow on commercial rations and tap water ad libitum. Female rats were ovariectomized at 36 days old, divided into five groups and given injections of 17β-estradiol (E; group I:0, II:1, III:10, IV:100, V:1000 μg) dissolved in 0.05 ml of ethanol:sesame oil (1:4) every 2 days, between the ages of 36 and 250 days.

Induction of mammary carcinomas. At 50 days old, 12 rat in each group (I, II, III, IV and V) were given 20 mg 7,12-dimethylbenz(a)anthracene (DMBA, Wako Pure Chemical Co, Osaka, Japan) by a single intragastric intubation (Table 1). No palpable mammary carcinomas were detected up to the age of 135 days in rats from any of the five groups. Each group was then divided into two

subgroups (a and b). Rats of the second subgroup (Ib, IIb, IIIb, IVb and Vb) were given additional injections of P (4 mg) every 2 days between the ages of 135 and 250 days. All the rats given DMBA were examined by palpation to detect mammary tumors twice a week and were killed by ether anesthesia at the age of 250 days. DMBA-induced masses were fixed in 10% buffered formalin for histologic examination.

Morphology and 5-Bromodeoxyuridine staining. In order to clarify the morphologic and cell proliferation features of the mammary glands at initiation, inguinal mammary glands were excised from 50-day-old rats in groups I, II, III, IV and V, 1 h after 5-Bromodeoxyuridine (BrdU) was administered intraperitoneally at 20 mg/kg body weight. The mammary glands in the right inguinal region were fixed in 10% phosphate-buffered formalin, dehydrated, defatted, and stained with alum carmine before storage in ceder oil and examination under a stereomicroscope to study their terminal ducts (TD), terminal end buds (TEB), alveolar buds (AB) and lobules (L) (Russo et al. 1977; Russo et al. 1979). After examination, they were embedded in paraffin wax, sectioned at 5 μm, and restained with hematoxylin and eosin for light microscopy. The mammary glands in the left inguinal region were placed in 70% ethanol for 2 h and then routinely processed for immunohistologic examination with a monoclonal anti-5-Bromodeoxyuridine antibody (anti-BrdU) to detect cell proliferation. Avidin-biotin complex immunoperoxidase assays were performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). Deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol solution to quench endogenous peroxidase activity and were subsequently incubated at room temperature with the following reagents, with phosphate-buffered saline (PBS) washes in between: 1.0% normal bovine serum albumin (BSA) for 30 min, anti BrdU dilated in PBS with 1.0% BSA (1:50) for 30 min, biotinylated goat anti-mouse antiserum for 30 min, ABC complex for 30 min, and 3,3'-diaminobenzidine tetrachloride containing 0.05% hydrogen peroxide for 5 min. The sections were then washed, counterstained, dehydrated, cleared in xylene and mounted. Controls were set up with PBS containing 1.0% normal bovine serum albumin (BSA) instead of the primary antisera. Monoclonal anti-BrdU, purchased from Becton Dickinson Immunocytometry system (San Jose, Calif., USA) was derived from hybridization of mouse Sp2/0-Ag14 cells with spleen cells of BALB/c mice immunized with iodouridine-conjugated ovalbumin.

The incidence of tumors, the stereomicroscopic structure of the right inguinal mammary glands, and the proportions of BrdU-positive cells in each structure of the left inguinal mammary glands in each group were tested by Student's t-test and fourfold contingency tables (Mainland and Murray 1952).

Results

The incidence of mammary carcinomas induced by a single oral administration of DMBA in 250-day-old rats under various hormonal conditions is shown in Table 1. Mammary carcinomas occurred infrequently in rats treated with 1 μg E and 4 mg P (group IIb) or 10 μg E only (group IIIa), with a high frequency in rats given 10 μg E with 4 mg P (group IIIb) or 100 μg E only (group IVa), but not with any other combination. The carcinomas in group IIIb were palpable papillo-tubular adenocarcinomas (Fig. 1) characterized by papillary growth of atypical ductal epithelium. The carcinomas in group IVa were secretory micro-adenocarcinomas with homogeneous eosinophilic material within the small duct-like spaces formed by the tumor (Fig. 2). The morphology of the right inguinal mammary glands in each group is summarized in Tables 2, 3, 4 and 5. The number of TD, TEB and L was significantly higher in group III, groups II and III, and groups III, IV and V, respectively than that in group I (Table 2, 3, 4 and 5). The percentage of BrdU-positive cells in L of the left inguinal mammary glands was significantly higher in group IV than in the other groups (Table 5, Fig. 3, 4).

Discussion

In the present investigation, no palpable mammary carcinoma was detected in female rats less than 135 days old that had been ovariectomized at 36 days of age and given E (I:0, II:1, III:10, IV:100, V:1000 μg) every 2 days between the ages of 36 and 134 days and 20 mg DMBA at the age of 50 days. However, it is known that palpable mammary carcinomas develop between the ages of 78 and 109 days in all intact female rats given 20 mg

Table 1. Mammary carcinomas induced by DMBA in ovariectomized rats

Group and treatment		No. of rats	No. of rats with carcinoma (%)	No. of rats with palpable carcinoma (%)	No. of rats with microcarcinoma (%)
I-a		6	0 (0)	0 (0)	0 (0)
I-b	+P (4 mg)	6	0 (0)	0 (0)	0 (0)
II-a	E (1 μg)	6	0 (0)	0 (0)	0 (0)
II-b	E (1 μg) +P (4 mg)	6	1 (16.7)	1 (16.7)	0 (0)
III-a	E (10 μg)	6	1 (16.7)	0 (0)	1 (16.7)
III-b	E (10 μg) +P (4 mg)	6	6 (100.0)[1,2]	6 (100.0)[1,2]	0 (0)
IV-a	E (100 μg)	6	5 (83.3)[3]	0 (0)	5 (83.3)[3]
IV-b	E (100 μg) +P (4 mg)	6	0 (0)[4]	0 (0)	0 (0)[4]
V-a	E (1000 μg)	6	0 (0)	0 (0)	0 (0)
V-b	E (1000 μg)+P (4 mg)	6	0 (0)	0 (0)	0 (0)

All the rats were ovariectomized at the age of 36 days and given 20 mg DMBA by a single intra gastric intubation at the age of 50 days; E (1, 10, 100, 1000 μg) and P (4 mg) were given every 2 days by intramuscular injection between the ages of 36 and 250 days

[1] Differs from group Ib; $P<0.01$

[2] Differs from group IIIa; $P<0.05$

[3] Differs from group Ia; $P<0.05$

[4] Differs from group IVa; $P<0.05$

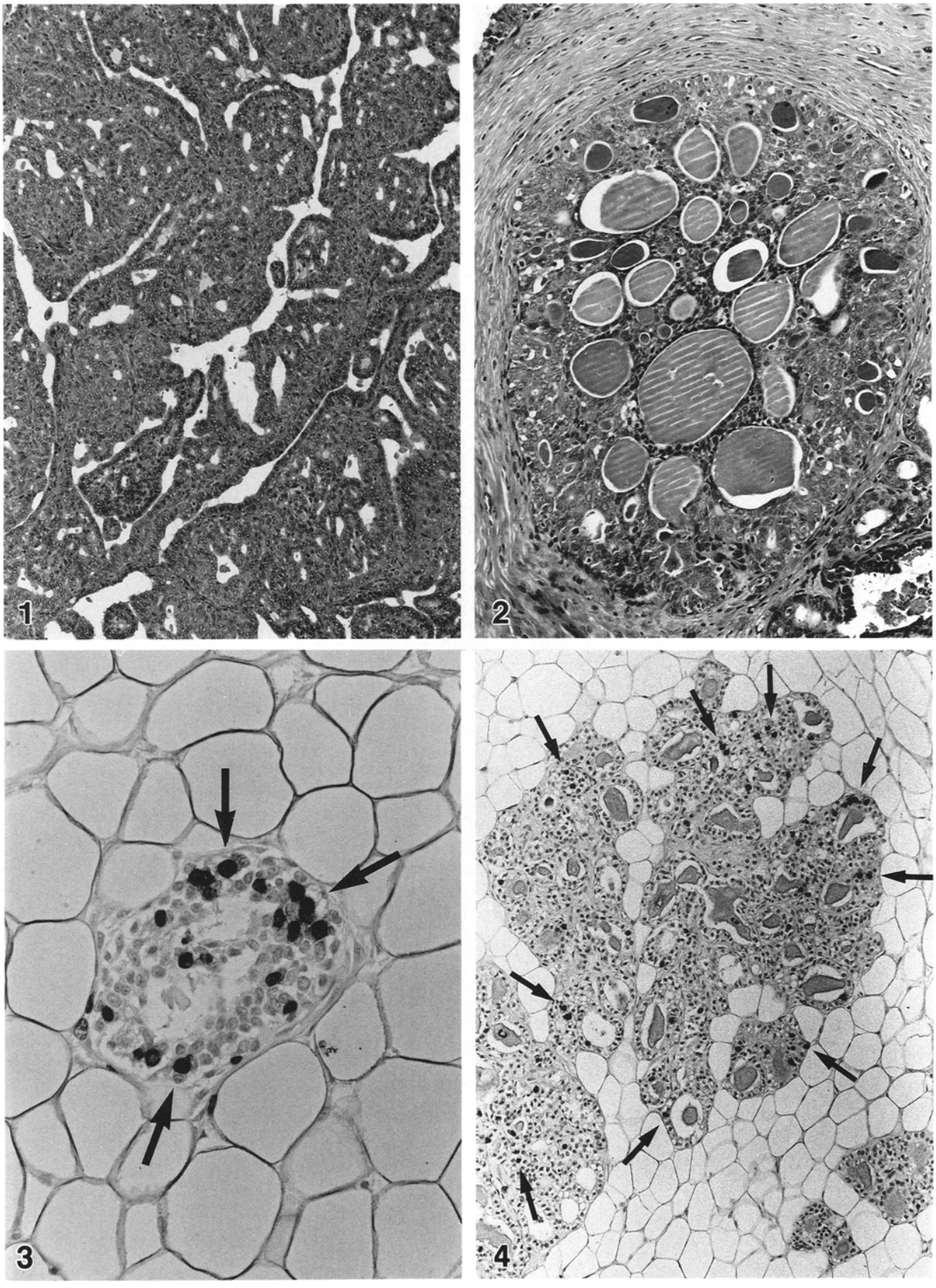

Fig. 1. DMBA-induced papillo-tubular adenocarcinoma in ovariectomized rats given injections of 17β-estradiol (10 μg), whose growth is stimulated by progesterone (Group IIIb). (hematoxylin and eosin stain, × 100)

Fig. 2. DMBA-induced secretory micro-adenocarcinoma in ovariectomized rats given injections of 17β-estradiol (100 μg) (Group IVa). (hematoxylin and eosin stain, × 150)

Fig. 3. BrdU-positive cells (*arrow*) in terminal end buds of mammary glands of ovariectomized rats given injections of 17β-estradiol (10 μg) (Group III). (× 400)

Fig. 4. BrdU-positive cells (*arrow*) in lobules of mammary glands of ovariectomized rats given injections of 17β-estradiol (100 μg) (Group IV). (× 200)

Table 2. Terminal ducts (TD) in the right inguinal mammary glands and BrdU-positive cells in the left inguinal mammary glands of ovariectomized rats given hormonal treatment

Group and treatment		No. of rats	Total No. of TD in group	No. of TD in a rat (means ± SD)	Percentage of BrdU positive cells in TD (means ± SD)
I		12	365	29.1 ± 15.8	11.0 ± 5.3
II	1 μg E	8	229	28.6 ± 16.1	13.5 ± 8.6
III	10 μg E	8	359	44.9 ± 23.0*	11.8 ± 3.8
IV	100 μg E	8	11	0.9 ± 1.6	3.0 ± 3.5
V	1000 μg E	7	0	0	–

All the rats were ovariectomized at the age of 36 days and given injections of 17β-estradiol (E) (I:0, II:1, III:10, IV:100, V:1000 μg/2 days) starting on the day of ovariectomy
* Differs from group I; $P < 0.05$

Table 3. Terminal end buds (TEB) in the right inguinal mammary glands and BrdU-positive cells in the left inguinal mammary glands of ovariectomized rats given hormonal treatment

Group and treatment		No. of rats	Total No. of TEB in group	No. of TEB in a rat (means ± SD)	Percentage of BrdU-positive cells in TEB (means ± SD)
I		12	91	7.2 ± 8.2	13.6 ± 5.9
II	1 μg E	8	104	14.3 ± 4.9[1]	16.6 ± 5.8
III	10 μg E	8	256	32.0 ± 20.4[2,3]	16.8 ± 3.6
IV	100 μg E	8	11	1.4 ± 1.8	14.2 ± 4.4
V	1000 μg E	7	0	0	–

All the rats were ovariectomized at the age of 36 days and given injections of 17β-estradiol (E) (I:0, II:1, III:10, IV:100, V:1000 μg/2 days) starting on the day of ovariectomy
[1] Differs from group I; $P < 0.05$ [2] Differs from group I; $P < 0.001$ [3] Differs from group III; $P < 0.005$

Table 4. Alveolar buds (AB) in the right inguinal mammary, glands and BrdU-positive cells in the left inguinal mammary glands of ovariectomized rats given hormonal treatment

Group and treatment		No. of rats	Total No. of AB in group	No. of AB in a rat (means ± SD)	Percentage of BrdU-positive cells in AB (means ± SD)
I		12	910	77.1 ± 24.2	8.1 ± 5.8
II	1 μg E	8	385	48.1 ± 26.3*	8.5 ± 4.6
III	10 μg E	8	785	98.1 ± 47.2	10.0 ± 3.1
IV	100 μg E	8	17	2.1 ± 4.6	3.7 ± 3.0
V	1000 μg E	7	0	0	–

All the rats were ovariectomized at the age of 36 days and given injections of 17β-estradiol (E) (I:0, II:1, III:10, IV:100, V:1000 μg/2 days) starting on the day of ovariectomy
* Differs from group I; $P < 0.05$

Table 5. Lobules (L) in the right inguinal mammary glands and BrdU-positive cells in the left inguinal mammary glands of ovariectomized rats given various hormonal treatments

Group and treatment		No. of rats	Total No. of L in group	No. of L in a rat (means ± SD)	Percentage of BrdU positive cells in L (means ± SD)
I		12	2	0.2 ± 0.6	1.9 ± 1.2
II	1 μg E	8	32	4.0 ± 7.2	1.4 ± 1.3
III	10 μg E	8	54	6.8 ± 7.6[1]	0.4 ± 0.2
IV	100 μg E	8	1347	168.4 ± 113.6[2]	5.0 ± 2.3[1]
V	1000 μg E	7	880	125.7 ± 38.9[2]	3.2 ± 1.9

All the rats were ovariectomized at the age of 36 days and given injections of 17β-estradiol (E) (I:0, II:1, III:10, IV:100, V:1000 μg/2 days) starting on the day of ovariectomy
[1] Differs from Group I; $P < 0.01$
[2] Differs from Group I and III; $P < 0.001$

DMBA (Huggins et al. 1961). In order to discover whether the failure to develop mammary carcinomas was due to a lack of initiation of tumors by DMBA under such hormonal condition or to growth inhibition of DMBA-induced tumor cells by the absence of progesterone, rats from each group was divided into two subgroups (a and b) at the age of 135 days. Animals in the first subgroup (a) were given E, and those in the

second (b) were given both E and P between the ages of 135 and 250 days. The present investigation showed that a large number of palpable papillo-tubular adenocarcinomas and secretory micro-adenocarcinomas developed in rats of group IIIb given 10 μg E and 4 mg P and in rats of group IVa given 100 μg E, but that very few or no mammary carcinoma developed in animals from the other subgroups (Table 1). This indicates that mammary carcinogenesis is totally inhibited by ovariectomy and/or pharmacological dosages of estrogen, as has been shown in previous studies (Kiang and Kennedy 1971; Huggins and Yang 1962; Bodwin et al. 1981). The results also show that mammary carcinomas are induced by DMBA under hormonal conditions in which suitable levels of estrogen are present but progesterone is absent. However, both the biological character and the morphologic features of the DMBA-induced mammary carcinomas differed according to the level of estrogen. The present observation that palpable mammary carcinomas developed rapidly in ovariectomized rats given DMBA and 10 μg E after additional injections of P (group IIIb) but not at all in rats from group IIIa that received no additional P, suggests that mammary carcinomas, whose growth is stimulated by P, may be initiated but continue to exist as non-palpable lesions long after the administration of DMBA under such hormonal conditions. Mammary carcinomas of this type seem very similar to those induced by DMBA in neonatally androgenized rats (Yoshida et al. 1980a, b, c).

On the other hand, the growth of secretory microcarcinomas initiated by DMBA in ovariectomized rats given high dosages of estrogen (100 μg/2 days) is inhibited by injections of P. We have established pharmacological dosages for an estrogen-dependent secretory carcinoma (TF15) which was derived from an ovary-dependent mammary carcinoma (TF4) (Yoshida et al. 1987). TF15 grows well in ovarectomized rats given injections of 1000 μg E but not in intact or ovarectomized rats given injections of 0, 1, 10, or 100 μg E. TF15 grows well in ovarectomized rats given 1000 μg E and 10 mg P, in which the mammary glands maintain lactation, but does not grow in ovarectomized rats given 10 μg E and 10 mg P, in which lactation is inhibited. According to these findings from our previous studies (Yoshida et al. 1987), DMBA-induced secretory microcarcinomas in ovarectomized rats given 100 μg E seem to be of the same type as TF15, although further studies are necessary to confirm this. The microcarcinomas in group IVa may fail to grow up into palpable carcinomas due to the lower level of E, and may disappear in group IVb because of the additional high dosages of P administered which inhibit lactation.

There are few reports on the induction of mammary carcinomas by DMBA in ovarectomized rats given DMBA and estrogen. Talwalker et al. (1964) reported a low incidence of palpable mammary carcinomas in rats under such treatment. In their previous study, mammary carcinomas were induced in 23% of ovarectomized rats given daily injections of 10 μg E for 7 days before and after DMBA administration. However, following DMBA treatment, tumors developed in all intact female rats, but none of the animals ovarectomized but not given E. The DMBA-induced mammary carcinomas detected in our previous study were thought to be hormone-independent because they developed in conditions in which estrogen and progesterone are absent. The discrepancy in the incidence of carcinomas between our present and previous studies seems to be due to the difference in the duration of E treatment and the absence of palpable or microcarcinomas whose growth might be stimulated by P. The results of the present experiment are consistent with those of epidemiological studies which have shown that the highest incidence of mammary carcinomas occurs in women exposed to atomic radiation at prepuberty, a developmental stage at which estrogen but no progesterone is present.

This study shows that the levels of estrogen and progesterone at the time of administration of a chemical carcinogen profoundly influences mammary carcinogenesis and suggests that prepuberty and/or the menopause may be critical periods for tumor initiation.

It is known that carcinogenic initiation involves the stable alteration of DNA molecules by carcinogens and that the oncogenic response is greater in dividing than nondividing cells (Nagasawa and Yani 1974; Banerjee and Rogers 1971; Nagasawa et al. 1976). According to the findings obtained in the present experiments using a monoclonal antibody to BrdU, the number of terminal ducts, terminal end buds and lobules with BrdU-positive cells, which are thought to be proliferative cells (Morstyn et al. 1983), was significantly higher in the high (groups III and IV) than in the low incidence groups. A previous study using intact rats has also shown that the highest incidence of DMBA-induced mammary carcinomas occurs when young animals are inoculated, at a time when the number of terminal ducts and terminal end buds and the level of DNA synthesis are at their highest (Russo and Russo 1978; Russo and Russo 1978, 1980a, 1980b; Russo et al. 1979, 1983). It therefore seems reasonable to assume that the terminal ducts and terminal end buds have a special susceptibility to the carcinogen. However, the incidence of mammary carcinoma was extremely low in ovariectomized rats (group I) and ovariectomized rats given injections of 1 μg E (group II) despite the high number of BrdU-positive cells in the mammary glands. This result suggests that both initiation and promotion of mammary carcinogenesis may be influenced by the level of estrogen. The origin of the secretory microcarcinomas in group IV is of obscure. However, numerous nodules of mammary dysplasia (rat fibrocystic disease) were also induced in this group (data not shown). Since mammary dysplasia is a lesion thought to originate in lobules according to our and other previous studies (Yoshida et al. 1980b, c; Russo et al. 1977), this may be a clue, but further observations at the cellular level are necessary to clarify the issue.

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