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Induction of Mammary Tumors, Estrous Cycle Abnormalities and Endometrial Hyperplasia in Rats Exposed to Different Doses of *N*-Nitrosomethylurea*

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Abstract—*Groups of female Sprague–Dawley strain rats were given 3 i.v. injections of N-nitrosomethylurea in doses of 0.5, 1, 2, 3, 4 or 5 mg/100 g body weight at 4-week intervals. The first dose was given when they were 50 days old. By 23 weeks after the first injection, mammary tumors had developed in 0, 0, 33, 54, 72 and 100% of animals respectively. There was a direct relationship between the total dose of carcinogen administered and the degree of tumor anaplasia observed on histological examination. All of the tumors contained assayable amounts of estrogen and progesterone receptors, and the receptor concentrations were not related to the dose of carcinogen. Twenty-one rats, all exposed to the 4 highest doses of N-nitrosomethylurea, had arrest of the estrous cycle at the stage of estrus. In 15 of the 21 the walls of the uterine horns were thickened and grossly distended by fluid. Histological examination demonstrated the presence of endometrial hyperplasia. These uterine abnormalities were usually accompanied by polycystic disease of the ovaries. Both endometrial hyperplasia and abnormal estrous cycles without uterine changes were associated with elevated progesterone receptor to estrogen receptor ratios in the corresponding mammary carcinomas.*

INTRODUCTION

CONSIDERABLE interest has been shown in rat mammary carcinomas induced by *N*-nitrosomethylurea (NMU) since they were first described by Gullino *et al.* [1] as a model for human breast cancer. These tumors are virtually all carcinomas which frequently undergo regression after ovariectomy and contain estrogen, progesterone and prolactin receptors [2–4].

In a previous study we found that tumor incidence, latent period and histological characteristics were influenced by the dosage schedule of NMU administration [3]. Three injections of carcinogen, 5 mg/100 g body weight given 4 weeks apart, produced tumors in nearly all animals and with a relatively short latent period. These tumors were frequently of anaplastic histological appearance and had other features indicative of a high degree of malignancy. When the same dose of NMU was given on 2 occasions 1 week apart, tumor incidence was lower, the average latent period was longer and many of the tumors were very well differentiated cystic papillary adenocarcinomas.

The purpose of the investigation reported here was to compare the incidence, histological features, and estrogen receptor (ER) and progesterone receptor (PgR) content of tumors obtained when 6 different doses of NMU were administered on 3 occasions at 4-week intervals.

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MATERIALS AND METHODS

Animals and tumor induction

The NMU was purchased from ICN Pharmaceuticals Inc., Plainview, NY and was used without further purification [1]. Solutions at concentrations of 1, 2, 4, 6, 8 and 10 mg/ml were prepared just prior to use by dissolving

the carcinogen is distilled water acidified with 3% acetic acid. Female Sprague–Dawley strain rats (King Laboratories, Oregon, WI) were purchased when 45 days old and allocated randomly to 1 of the 6 treatment groups. There were initially 25 animals in each group. Three i.v. injections of NMU, in doses of 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 mg/100 g body weight, were given 4 weeks apart, commencing when the animals were 50 days old. Palpations were performed each week and the maximum diameters of the tumors were measured with calipers.

The observation period for each rat ended when 1 or more tumors had grown to a maximum diameter of approximately 2 cm or when 23 weeks had elapsed since the first NMU injection. At this time, vaginal smears were examined daily until diestrus occurred or when the absence of normal estrous cycles became apparent. The rats were then anesthetized with i.p. ketamine, blood taken for serum hormone assays and anesthesia maintained with ether during excision of the mammary tumors. A portion of each tumor was removed for histological examination and grading, and representative slices of the remainder placed immediately in iced buffer (Tris 10 mM, EDTA 1 mM, sucrose 0.25 M). These samples for receptor assays were quickly frozen and stored at −70°C. When all of the mammary tumors had been removed the animals were killed by deep ether anesthesia and necropsies performed.

Histology

Tissues were placed in WARF fixative solution [5], 5-μm sections prepared and stained with hematoxylin and eosin. A histological grading system was devised for the classification of NMU-induced mammary tumors according to the degree of anaplasia. Four categories were defined: 0, fibroadenoma; 1, fibroadenocarcinoma or cystic papillary adenocarcinoma; 2, mixed cystic papillary and medullary carcinoma; and 3, medullary carcinoma. Medullary carcinomas were characterized by a lack of any significant organoid structure, being composed of sheets of anaplastic tumor cells with a scanty connective tissue stroma. Gross pleomorphism, hyperchromasia and a high level of mitotic activity were often present, indicating the extreme anaplasia of some of these medullary carcinomas.

ER and PgR assays

The tumors were minced, placed in 10 mM Tris, 1 mM EDTA, 30% glycerol buffer, pH 7.4 (TEDG) buffer to give a 1 : 10 dilution (w/v) and homogenized with a teflon pestle Potter–Elvehjem No. 24 homogenizer immersed in an ice bath. Homogenization was usually completed with a single 5-sec pulse; very fibrous tissues required up to 4 pulses, each of 15 sec, with a 15-sec cooling interval between each pulse. Tumor supernatants were prepared by centrifugation at 1000 **g** for 20 min at 4°C. Both ER and PgR assays were performed on the same, freshly prepared supernatants, which gave similar results to those performed on a 100,000 g supernatant. Protein concentration was determined by the method of Lowry [6]. ER and PgR are expressed as fmol/mg supernatant protein.

Total ER binding was determined by adding to duplicate tubes 0.2 ml of tumor supernatant, 0.2 ml of 10 mM Tris, 1 mM EDTA buffer, pH 7.4 (TED) buffer and 0.2 ml (0.6 pmol) of 2,3,6,7-[^{3}H] estradiol (sp. act. 101 Ci/mmol; New England Nuclear, Boston, MA). Non-specific binding was determined by replacing the buffer with unlabeled 17β-estradiol (Sigma Chemical Co., St. Louis, MO) in 0.2 ml of TED at a concentration 100 times that of the [^{3}H] estradiol. Duplicate blanks contained 0.4 ml of TED buffer and 0.2 ml of [^{3}H] estradiol, while total count tubes contained 1.2 ml of TED, 0.2 ml TEDG and 0.2 ml of [^{3}H] estradiol. The reagents were mixed with a vortex mixer and incubated at 4°C for 6 hr. The binding incubation was stopped by the addition of 1 ml of dextran-coated charcoal (0.25% charcoal, 0.025% dextran) prepared by diluting a stock solution (Wein Laboratories, Succasunna, NJ) 1 : 10 (v/v) in TED buffer. One milliliter was added to each tube, mixed and a second incubation at 4°C carried out for 10 min. During this incubation the tubes were mixed once again to prevent settling out of the dextran-coated charcoal. They were then centrifuged at 1000 **g** for 15 min at 4°C, the supernatants decanted into vials containing 10 ml of ACS solubilizer (Amersham Corp., Arlington Heights, IL) and the radioactivity counted in the dual channel mode of a Tracor Analytic Mark III scintillation counter.

The PgR assay was similar and used [^{3}H] R5020 ([6,7-^{3}H]–17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione, sp. act. 85–87 Ci/mmol; New England Nuclear) and unlabeled R5020. Reagents were prepared in TEDG buffer. The labeled R5020 was added to the assay tubes at a concentration of 12 pmol in 0.2 ml and the unlabeled progestogen at a 100-fold concentration for the determination of non-specific binding. The total counts tubes contained 1.0 ml TED, 0.4 ml TEDG and 0.2 ml [^{3}H] R5020. Dextran-

coated charcoal, at the same concentration as that used in the ER assay, was added after incubation at 4°C for 2 hr. This was followed by a second incubation for 15 min, during which time the reagents were mixed 3 times.

RESULTS

All rats in the group given a total dose of 15 mg of NMU/100 g body weight had at least 1 tumor 14 weeks after the first of the 3 injections. No other dose level of carcinogen produced a 100% tumor incidence by the end of the 23-week observation period. The tumor incidence was clearly related to total NMU dose (Table 1). No tumors developed in the groups given the 2 lowest doses of carinogen. The average number of tumors per rat was also related to dose; an unpaired Student's t test demonstrated significant differences between the various groups except for the 9 and 12 mg/100 g body weight groups ($P < 0.01$; Table 1).

The higher the total dose of NMU given the larger the percentage of tumors classified as exhibiting histological features of anaplasia (Table 2). Thus 94.4% of the tumors from rats given 15 mg/100 g body weight and 87.5% of those from rats given 12 mg/100 g body weight were grades 2 and 3, while only 5.6 and 12.5% respectively were grades 0 or 1. In contrast, 58.3% of the tumors induced by 9 mg/100 g body weight and 33.2% induced by 6 mg/100 g body weight total dose were of the higher grades, and 41.7 and 66.8% respectively were classified as grades 0 or 1. These relationships were statistically significant at the $P < 0.01$ level (Kruskal–Wallis test).

Table 3 gives the ER, PgR and the ratio of PgR to ER. There were no significant differences in the receptor levels of tumors arising from the 4 carcinogen dosage regimens nor in the PgR/ER ratios.

Twenty-one rats, all exposed to the 4 highest doses of NMU, had abnormal estrous cycles characterized by vaginal smears showing arrest

Table 1. Influence of total NMU dose on tumor incidence and number

Total NMU dose mg/100g body weight	No. tumor-bearing rats / Total No. of rats*	(%)	Average No. of tumors per rat†
15	23/23	(100)	4.18
12	18/25	(72)	2.35
9	13/24	(54)	0.96
6	8/24	(33)	0.38
3	0/25	(0)	0
1.5	0/25	(0)	0

*At the start of the experiment there were 25 rats per group, but 2 given the 15 mg total dose and 1 each given the 6 and 9 mg doses died before the end of the 23-week observation period without developing tumors.

†Significant differences between all groups, Student's unpaired t test $P < 0.01$, except for the animals treated with 9 and 12 mg of NMU/100 g body weight.

Table 2. Classification of tumors according to total dose of NMU administered and histological grade

Total NMU dose mg/100g body weight	No. of tumors graded/group*	Histological grade, % of total tumors 0	1	2	3
15	89	1.1	4.5	58.4	36.0
12	40	2.5	10.0	52.5	35.0
9	24	0	41.7	33.3	25.0
6	9	11.0	55.8	11.0	22.2

*No tumors developed in rats given 1.5 mg or 3.0 mg/100 g body weight total NMU dose.

Table 3. Hormone receptor levels and the PgR/ER ratio in tumors classified according to total dose of NMU*

Total NMU dose mg/100g body weight	No. of tumors	ER (fmol/mg supernatant protein)	PgR (fmol/mg supernatant protein)	PgR/ER
15	66	77.9 ± 2.3	221.7 ± 4.8	3.6 ± 0.2
12	30	77.4 ± 7.0	342.0 ± 29.1	6.0 ± 0.6
9	19	76.4 ± 7.9	308.1 ± 42.6	4.9 ± 0.9
6	7	70.2 ± 15.3	282.5 ± 115.9	4.5 ± 2.2

*The values are the means ± 2 S.E.
The Kruskal–Wallis test showed no significant differences between the 4 groups for any of the receptor values, nor for the calculated geometric means of the ratios.

in estrus. Fifteen of these animals were found at necropsy to have extreme distension of the uterus by fluid which was sometimes blood-stained (Fig. 1; Table 4). On gross inspection the walls of these uteri were thickened. Histological examination confirmed the presence of endometrial hyperplasia (Fig. 3, 4), together with areas of squamous metaplasia (Fig. 5, 6). Chi-square tests showed that there were no significant differences in the occurrence of uterine or estrous cycle abnormalities between the NMU dose regimens ($P > 0.1$). In most cases uterine disease was accompanied by polycystic ovaries (Fig. 2). Examination of the animals without tumors did not show similar abnormalities of the estrous cycle nor the presence of endometrial hyperplasia.

Figure 7 shows the PgR/ER ratios plotted according to the presence or absence of uterine abnormalities and/or disturbed estrous cycles. Statistical analysis by the Mann–Whitney rank test determined that elevated PgR/ER ratios were associated with both uterine hyperplasia ($P < 0.0001$) and with abnormal cycles in the absence of detectable uterine changes ($P < 0.001$). In the group with prolonged estrus and uterine abnormalities the absolute tumor ER levels were reduced significantly ($P < 0.05$) and the PgR levels were elevated ($P < 0.001$) compared with animals lacking estrous or uterine changes. Tumor receptor levels in the animals with prolonged estrus but apparently normal uteri did not differ from those in normally cycling rats ($P > 0.1$).

DISCUSSION

The present investigation identifies a low dose of NMU below which mammary tumors failed to develop within 23 weeks of the first treatment when administered according to the schedule devised by Gullino *et al.* [1]. These data may prove useful for studying the promotional effects of dietary, hormonal and other factors of NMU-initiated rat mammary carcinogenesis. For example, Ip [7] has reported that a high-fat diet, perhaps by increasing circulating prolactin levels, enhances the development of mammary tumors in rats exposed to suboptimal doses of 7,12-dimethylbenz(a)anthracene. We have found that reserpine-induced

*Table 4. The occurrence of uterine abnormalities and prolonged estrus in NMU-treated, tumor-bearing, rats**

Total NMU dose mg/100g body weight	No. of tumor-bearing rats examined†	No. with estrous cycle and uterine abnormalities	No. with estrous abnormalities only
15	22	4	1
12	16	7	2
9	12	2	3
6	7	2	0

*Rats without tumors had no estrous cycle and uterine abnormalities.
†Five tumor bearing rats died during the study and no examination was possible.

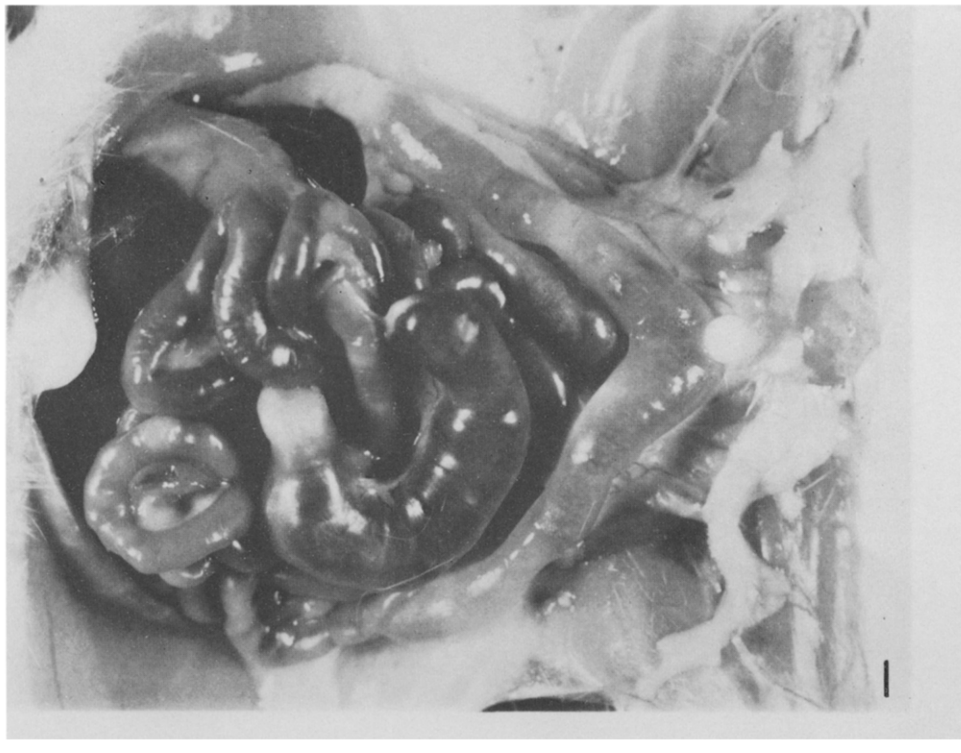

Fig. 1. *Fluid-distended uterus in a rat bearing mammary carcinoma after exposure to* 3 *injections of NMU,* 5 mg/100 g *body weight* 4 weeks *apart.*

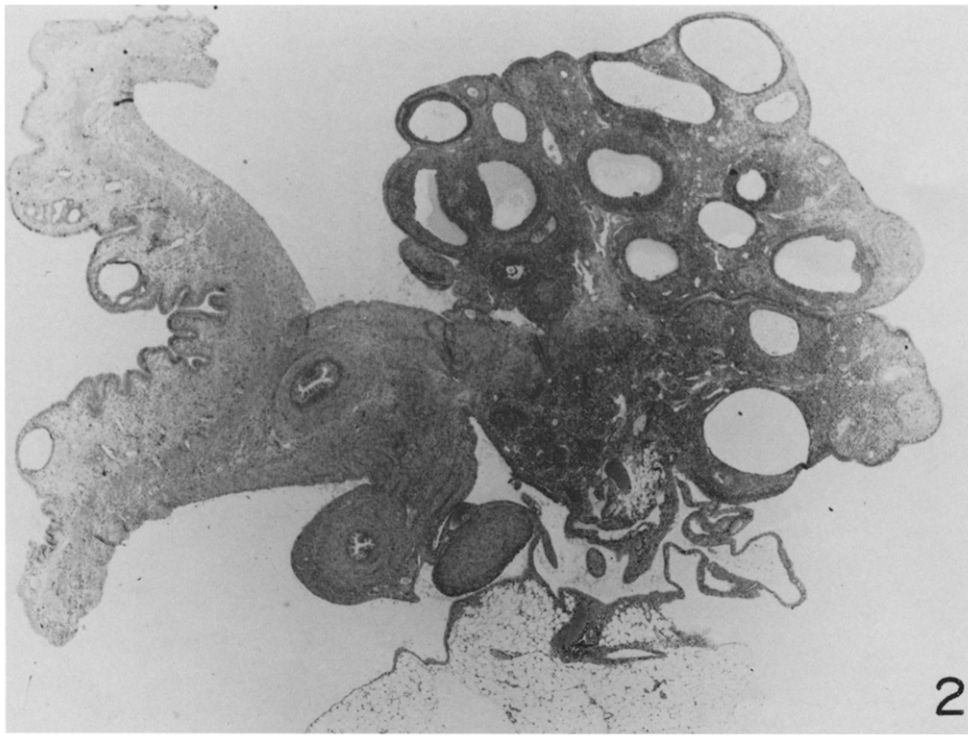

Fig. 2. *Polycystic ovaries from the rat showing uterine abnormalities in Figs* 3 *and* 4 (× 12.75).

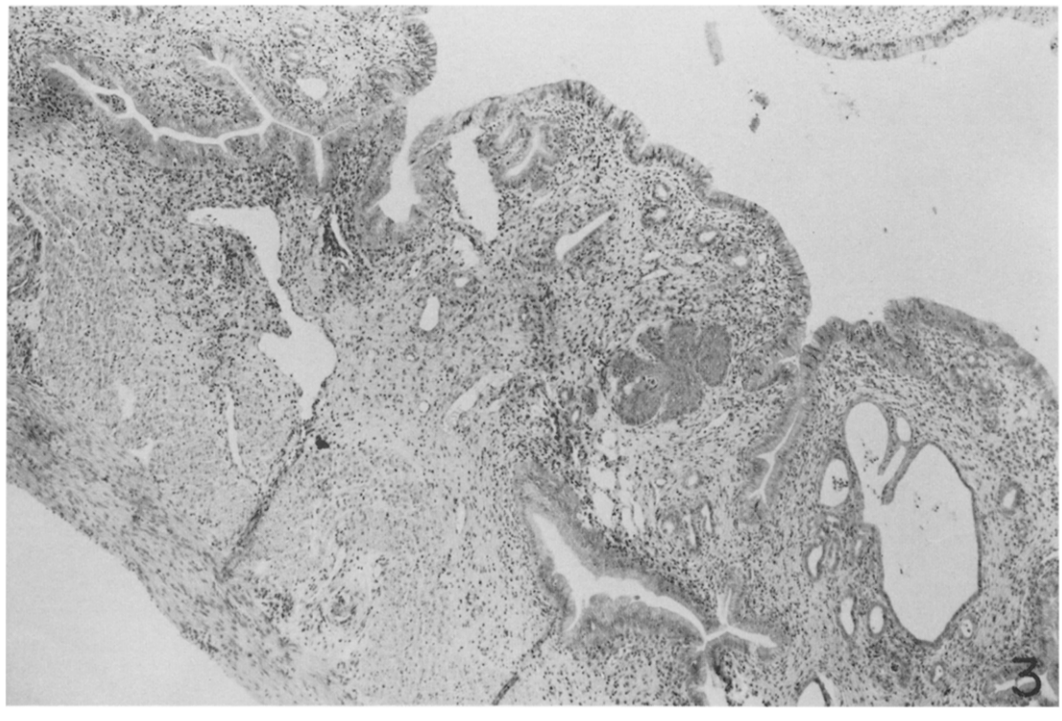

Fig. 3. Endometrial hyperplasia and cystic areas in a mammary tumor-bearing rat (× 102). The uterus was distended with fluid and there was arrest of the estrous cycle in estrus. All of the mammary carcinomas showed elevated PgR/ER ratios.

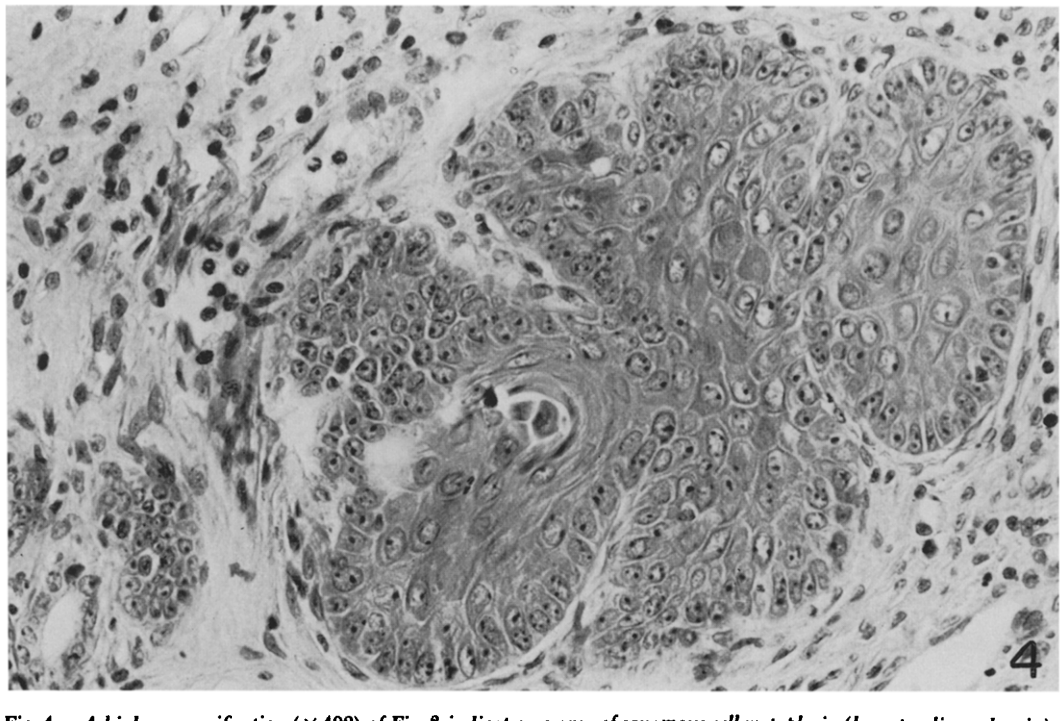

Fig. 4. A higher magnification (× 408) of Fig. 3 indicates an area of squamous cell metaplasia (hematoxylin and eosin).

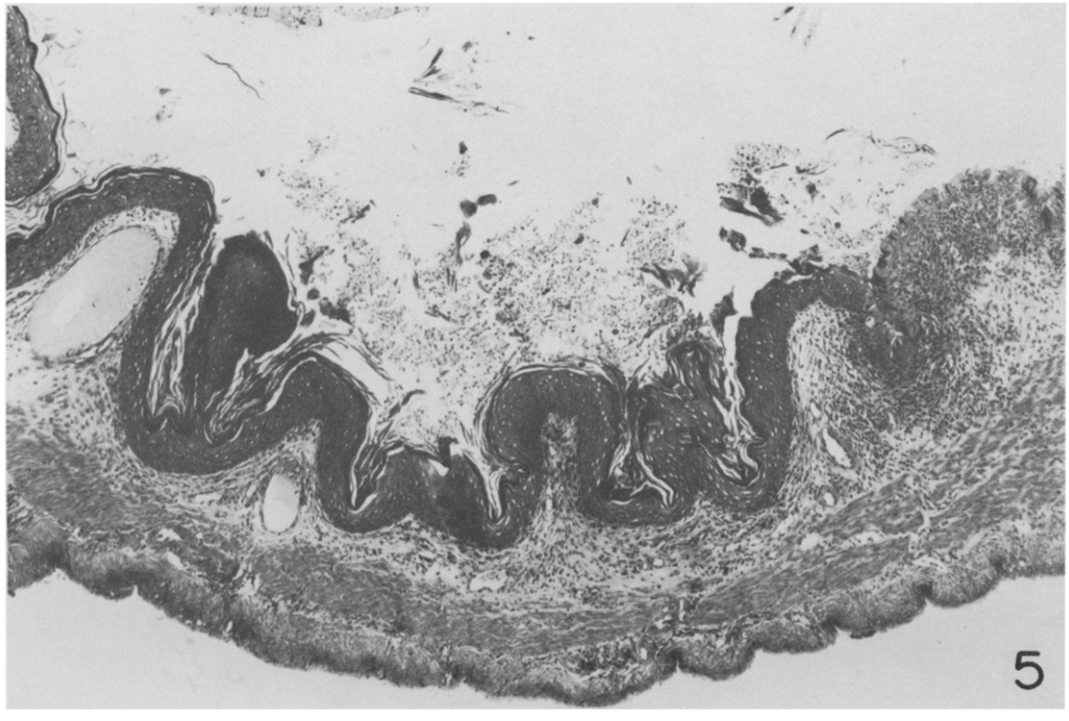

Fig. 5. *Transition of columnar epithelial cells into extensive squamous cell metaplasis of the luminal surface* (× 102) *stained with hematoxylin–phloxine–safran.*

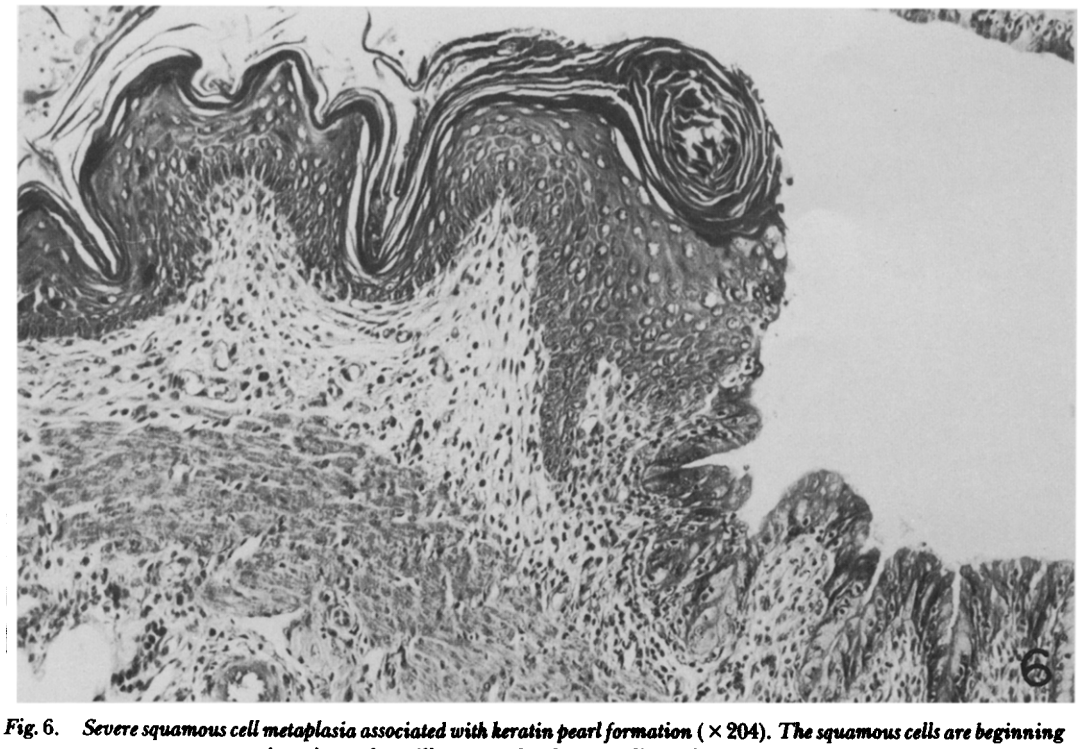

Fig. 6. *Severe squamous cell metaplasia associated with keratin pearl formation* (× 204). *The squamous cells are beginning to form inward papillary growths (hematoxylin–phloxine–safran).*

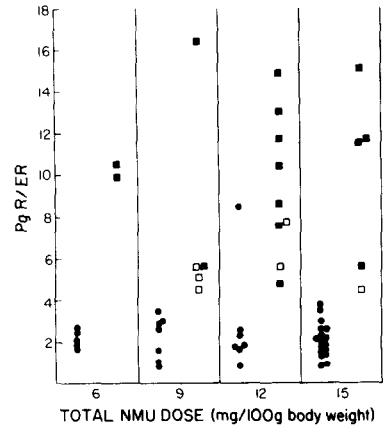

Fig. 7. The mammary tumor PgR/ER ratios in rats with normal uteri and estrous cycles (●), disturbed estrous cycles but normal uteri (□) and prolonged estrous with fluid-distended uteri (■).

hyperprolactinemia stimulates the rate of growth of mammary tumors in rats given 5 mg/100 g body weight of NMU[8].

In agreement with our previous report [3], the dose also determined the histological characteristics of NMU-induced rat mammary carcinomas but not the levels of tumor hormone receptors. This finding contrasts with the situation in human breast cancer, where the presence of ER is associated with well-differentiated tumors of high nuclear and low histologic grades [9, 10]. Perhaps this discrepancy is relevant to the failure of ER levels in biopsy samples to predict the response of NMU-induced tumors to ovariectomy or antiestrogen therapy [3].

There is an association between cancer of the breast and endometrium, the two occurring with a frequency greater than that expected by chance in the same patient [11]. Adenomatous hyperplasia of the endometrium is a premalignant condition which in its most extreme form may merge into a state of endometrial carcinoma *in situ* [12]. The Stein–Leventhal syndrome, characterized by infertility, menstrual irregularities or amenorrhea, and hirsutism in women with large polycystic ovaries is associated particularly with endometrial carcinoma occurring in women under the age of 40 years [13, 14].

Obvious parallels exist between these relationships and our finding of disturbed estrous cycles, endometrial hyperplasia and polycystic ovaries in rats exposed to doses of NMU which induce rat mammary carcinomas. It should be stressed that over the duration of this investigation these abnormalities were not seen in rats without mammary tumors. In our experience, under the animal room lighting conditions used, untreated Sprague–Dawley strain rats of a similar age do not develop these estrous or uterine abnormalities. Chronic unopposed exposure to excessive estrogenic activity due to low progesterone is believed to be responsible for endometrial carcinoma in association with polycystic ovarian disease; the production of androstenedione, an estrogen precursor, is increased in these patients [15]. Similarly, the high PgR/ER ratios which were observed in the mammary tumors from rats with prolonged estrus and endometrial hyperplasia are consistent with increased estrogen activity. In recently completed experiments we did find a pronounced reduction of serum progesterone levels in 20 NMU-treated animals, with a mean value (± S.E.) of 7.7 ± 2.66 ng/ml compared with 51.1 ± 10.81 ng/ml for 35 controls [16]. Parallel changes were not seen in the serum estrogens.

Statistical analysis demonstrated that the high ratios were due to both a low ER and a high PgR. High tumor PgR and low ER levels in the group with prolonged estrus and uterine abnormalities could be explained in part because rats with normal estrous cycles were killed at diestrus. Alternatively, the high PgR could be due to low serum progesterone and therefore greater availability of receptor sites during analysis. In addition, another study concerning tumor peroxidase [17] showed that rats with elevated ratios also had high tumor and uterine peroxidase activity. Estrogens cause translocation of ER from the cytoplasm into the nucleus, with resulting PgR (and peroxidase) synthesis [18], which could account for the elevated cytoplasmic PgR/ER ratios.

These observations were incidental to the primary purpose of the present investigation and require further study. They do suggest, however, that NMU can produce an abnormality of endocrine function which provides a model for the association between the gynecologic disease and human breast cancer.

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