Regression of an Adrenal Cortical Carcinoma by Estradiol Treatment[1]

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SUMMARY

The effect of estradiol on a transplantable adrenal cortical carcinoma was studied in BALB/c mice. Estradiol treatment (10 μg daily) prior to inoculation of the tumors precluded successful transplantation and, when started after the tumors were palpable, resulted in their regression. Estradiol-treated tumors showed arrest of mitoses and accumulation of lipid and cholesterol in the cytoplasm.

The mode of action of estradiol was considered to be direct on tumor cells rather than through inhibition of pituitary hormones because regression of the tumors was observed in hypophysectomized, pair-fed mice treated with estradiol. In addition, estradiol treatment (20 μg/ml in modified Eagle's medium) of the tumors in organ culture inhibited and retarded their subsequent growth in isologous hosts. This *in vitro* effect of estrogen appeared to be specific on the adrenal tumor since a reticulum cell sarcoma of BALB/c mice similarly treated did not show this behavior.

INTRODUCTION

Adrenal cortical tumors have been induced by gonadectomy in various experimental animals (19, 27, 54). The induction of tumors could be prevented by the administration of estrogens (55) or androgens (34). Conversely, adrenal cortical tumors which occur spontaneously are rare in intact animals and are usually found in old females (13, 30, 58); this was correlated with the physiologic decline in ovarian function with aging (33). In all probability, the pituitary gland is involved in adrenal tumorigenesis, since the postgonadectomy adrenal tumors could be prevented by hypophysectomy (18). It has been postulated that prolonged stimulation of the adrenal cortex by pituitary gonadotropic hormone may be the initiating factor (56).

Depending upon the strain of animal gonadectomized, the lesion in the adrenal gland may be hyperplasia, adenoma, or carcinoma (20, 23, 29). The resulting tumors exhibited marked variation in biologic characteristics. Some adrenal tumors preserved the function of the parent tissue and secreted various steroid hormones (12, 20), while others appeared to be nonfunctioning (8, 13, 28). The tumors were either hormone-dependent and grew only in gonadectomized hosts (5, 6) or were

relatively autonomous and grew in intact (25, 57) or even hypophysectomized hosts (50).

The effect of hormones on transplantable adrenal cortical tumors has received relatively little attention. The only study of this nature reported so far is that of Browning (5–7), who transplanted a hormone-dependent tumor into the anterior chamber of castrated mice and found that the growth of the transplants could be retarded by estradiol and inhibited by testosterone. However, there is little information on the effect of sex hormones on the autonomous variant of these tumors. Furthermore, the morphologic features of these tumors following hormonal treatment has not yet been investigated.

The present manuscript describes the effect of estradiol on the growth and morphologic features of a transplantable adrenal cortical carcinoma and considers the mechanism of action of the hormone. It was postulated that estrogens could act directly on the tumor or indirectly through inhibition of the pituitary gland. To determine which mechanism was operative, the growth of the tumor was studied in hypophysectomized, estradiol-treated mice, and subsequently the direct effect of the hormone was studied on the adrenal tumor in tissue culture.

MATERIALS AND METHODS

Inbred female BALB/c mice were used throughout. These mice, obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, were 6–8 weeks old and weighed 18–22 gm. The animals were kept at 72°F, fed a standard diet of Purina chow pellets, and given drinking water *ad libitum*.

Two transplantable tumors of BALB/c mice were used: an adrenal cortical carcinoma, obtained from Dr. T. Dunn of the NIH, Bethesda, Maryland, and a reticulum cell sarcoma from Dr. M. Potter of the NIH, Bethesda, Maryland. The tumors were transplanted subcutaneously by sterile trocar technic (26). In each experiment, all implants were derived from a single parent tumor.

Estradiol-17B, obtained from Mann Research Laboratories, Inc., New York, was first dissolved in absolute ethyl alcohol at a concentration of 10 mg/ml, then diluted 1:1000 in physiologic saline (0.89% NaCl). This suspension was kept at 4°C and shaken before use. The mice received daily subcutaneous injections of 10 μg estradiol. For controls, mice received daily injections of an equal volume of the vehicle (1% ethyl alcohol in physiologic saline).

Hypophysectomy was performed using the parapharyngeal approach (16). After the operative procedure, the mice were kept at 80°F, given 5% glucose in normal saline solution *ad libitum*, and pair-fed. For the pair feeding, animals of the experimental and control groups were caged separately and fed on

powdered Purina chow *ad libitum* until one group began to decrease its food intake. From then on, the control was fed the same amount of food eaten by the paired hypophysectomized animal on the preceding day. The weights of animals were recorded every third day. Completeness of hypophysectomy was checked by histologic examination of the region of the sella turcica. After fixation in Bouin's fluid, the skulls were decalcified by an ion-exchange resin method (14). Each skull was trimmed coronally before and behind the pituitary fossa; paraffin sections were cut in a coronal plane, 6 μ thick at intervals of 30 mm, and stained with hematoxylin and eosin.

For evaluation of the hormonal effects on the tumors, three criteria were employed: (a) Tumor inhibition: estradiol treatment was started 24 hours before transplantation and the tumors were observed for failure of growth. (b) Tumor regression: hormonal treatment was started after the tumors had become palpable. The effect of treatment was measured by the failure of the tumor to grow, regress, or disappear. The volumes of the tumors were estimated from the measured diameters using the following formula of Chambers and Scott (9)

$$V = \sqrt{(d_1 d_2)^3}$$

where V is the volume in cu mm, and d_1 and d_2 are the smallest and largest diameters of the tumor in mm. At the termination of each experiment, the weights of the animals and tumors were determined. The ratio of tumor weight in mg to the body weight in gm was chosen for statistical analysis in the experiments which showed a significant variation in the weights of animals under treatment. (c) Histologic study: the tumors were fixed in Bouin's fluid and stained with routine hematoxylin and eosin dyes, periodic acid-Schiff reagent, Wilder's silver impregnation, and Masson's trichrome technic (24). For the identification of lipid, frozen sections were stained with Oil Red O (24) and the cholesterol content of the tumors was also studied polarimetrically as well as by Schultz (43) and Romieu (39) reactions.

For the tissue culture studies, mice were sacrificed by decapitation, and explants of tumor were prepared by cutting the neoplastic tissue into fragments about 1.5 mm in diameter with Baso-Parker blades (No. 10). All explants were derived from a single tumor and several samples of the tumor were studied to determine the initial histologic features. The basic culture medium used was Eagle's double strength amino acid medium (15) enriched with 20% calf's serum. Incorporated in the medium were: penicillin G, 100 units per ml; glucose, 1 mg per ml; and L-glutamine, 0–29 mg per ml. Estradiol-17B was dissolved in propylene glycol at a concentration of 2 mg per ml. This stock was sterilized by autoclaving at 120°C for 15 minutes; a 1:1000 dilution of the stock in the culture medium gave a final concentration of 20 μ per ml. Two control culture media were used: a modified Eagle's medium containing 1% propylene glycol and the same medium supplemented with cholesterol (20 μg per ml). Cholesterol was added to one of the media to serve as a control for the steroid ring of estradiol. The method of organ culture employed was a modification of procedures previously described by others (10, 49). In the present study, cellulose-acetate sponge (Dupont) covered with lens paper was used to support the tumor explants. Pieces of cellulose sponge 5 x 10 x 15 mm were cut, cleaned (40), then sterilized by autoclaving at 120°C for 15 minutes. Two of the prepared cellulose

sponges were placed in a sterile plastic tissue culture dish, 35 x 10 mm (Falcon plastics, B-D laboratories, Inc.), and 4 ml of the feeding medium were added. Two tumor explants were placed on each lens paper by a sterile, fine, curved forceps. The cultures were incubated for 72 hours at 35°C in an atmosphere of 5% carbon dioxide in air. The pH of the medium was maintained at 7.4 and the medium was not changed during that period of culture. When cultures were terminated, one-half of the explants was transplanted subcutaneously into mice (one tumor explant per animal). The other one-half was studied histologically. They were fixed in Bouin's fluid, embedded in paraffin, sectioned serially at 6 mm, and stained with hematoxylin and eosin.

RESULTS

Characterization of the Adrenal Tumor

The original tumor arose spontaneously in the adrenal cortex of an 18-month-old female BALB/c inbred mouse. In the present study the tumor was in its 32nd–43rd generation. It grew in about 98% of untreated mice in all transplant generations, and the growth rate was enhanced after subsequent passages. In the 35th transplant generation, the tumors were palpable 3–5 days after transplantation. The neoplasms grew to an average diameter of 1.0 cm, in 15 days after inoculation. No evidence of estrogenic or adrenocortical activity could be observed by studying the appropriate target organs of the host animals (17). About 2 months after inoculation, tumor-bearing mice usually died with metastases, cachexia, or ulceration of the tumor through the skin. Spontaneous regression of the tumor was not observed, but ulcerated infected tumors were found to regress locally.

About 20 days following inoculation, the subcutaneous implants were encapsulated, roughly spheric in shape, and measured 13 x 15 x 18 mm (Fig. 1a, *inset*). On cross-section, they were grayish-brown in color, well vascularized, and somewhat friable. The central areas of large tumors were necrotic and hemorrhagic. Histologically, the neoplastic cells were round, polyhedral, and rarely elongated in shape (Fig. 1a) with eosinophilic, homogeneous, or slightly granular cytoplasm contained within ill-defined cell borders. Little cytoplasmic lipid or cholesterol was present (Fig. 2a). The nuclei were round or oval in shape, slightly hyperchromatic, and of rather uniform size; chromatin granules were numerous and often peripheral in arrangement; one or two nucleoli were present. Three to six mitoses were usually found in every high-power field (Fig. 1a).

The pattern of this tumor did not resemble any particular zone of the normal adrenal cortex. For the most part, the cells were loosely arranged in sheets separated by narrow strands of vascular connective tissue stroma. A few reticular fibers were demonstrated in the stroma, but these were not observed to penetrate into the neoplastic tissue or to surround individual cells (Fig. 3a). Basement membrane material was demonstrated immunohistochemically in this tumor by Pierce (45). This material appeared as a feltwork of fine fibrils between stromal and tumor cells and was thought to be secreted by the neoplastic cells.

The incidence and the size of metastases appeared to depend on the duration of tumor growth. Intravascular tumor emboli were found in the lungs in 20% of mice 20 days following im-

plantation of the tumor. However, in tumor-bearing mice that survived more than 60 days, metastatic neoplastic nodules were observed in 80% of the mice, most commonly in the lungs (Fig. 1c), and at times in the liver and peritoneum.

The Effect of Estradiol on the Tumor

Estradiol administration, dose of 10 μg for 20 days, starting 24 hours prior to tumor inoculation resulted in complete inhibition of tumor transplantability with no evidence of growth for a period of 5 months. At necropsy, no neoplastic tissue was identifiable at the site of transplantation. Tumors grew successfully in untreated mice. Conversely, estradiol treatment, 10 μg daily, for 17 days starting 72 hours after tumor inoculation caused complete regression of 30% of the tumors and significant regression in the others (Table 1). One of the tumors that regressed in size after estradiol treatment was successfully transplanted into 7 mice and grew in the untreated hosts. This indicated that the effect of estradiol on the tumor was reversible.

Grossly, adrenal tumors from estradiol-treated mice were small in size and appeared lobulated (Fig. 1b, inset). They were firm, yellow, and showed a striking absence of hemorrhage and necrosis. After estrogen treatment, tumor cells were more closely packed and had vacuolated cytoplasm and well-defined cell borders (Fig. 1b). The nuclei were vesicular in appearance, with well-preserved chromatin. Mitotic figures were rare. Abundant stainable lipid was demonstrated in the cytoplasm (Fig. 2b). Cholesterol and its esters were also increased in the treated tumors as indicated by histochemical and polarimetric studies. The stroma of estrogen-treated tumors contained few blood vessels and showed marked fibroblastic proliferation with abundant collagen. Fibroblasts and fine reticular fibers penetrated from the stroma into the tumor tissue and surrounded individual tumor cells (Fig. 3b). The peripheral portions of the tumors were sharply demarcated by a thick fibrous capsule which often contained lymphocytic infiltrates (Fig. 1d). Metastases were not observed in the treated group but were present in 20% of the untreated controls.

The Mechanism of Action of Estradiol on the Tumor

The Effect of Hypophysectomy. The adrenal tumor in its 40th transplant was transplanted into 67 female BALB/c mice.

TABLE 1
The Effect of Estradiol on Transplanted Adrenal Cortical Carcinoma in BALB/c mice

Group	No. of mice	Av. weight/ mouse (gm ± S.D.)[a]	P[b]	No. of mice with complete tumor regression	Tumor weight (mg ± S.D.)	P
Control	20	19.3 ± 3.7			468.6 ± 151.5	
Estradiol-treated	20	18.3 ± 2.4	NS[c]	6	26.7 ± 27.0	<0.001

[a] All results are means ± S.D.

[b] P = significance of difference between experiment and control groups.

[c] NS = no significant difference from control ($P > 0.05$).

Seven days later, 37 of these mice were hypophysectomized and a sham operation was performed on the other animals. The mice were then divided into four main groups: Group C, sham-operated tumor-bearing controls; Group HC, hypophysectomized tumor-bearing controls. Group E, sham-operated mice treated with estradiol; Group HE, hypophysectomized estradiol-treated mice.

Estradiol treatment (10 μg daily) was started 24 hours after operation and continued for 12 days. The controls were similarly treated with the vehicle (0.1 ml of 1% ethanol in normal saline). In this experiment 5 mice expired after hypophysectomy and 8 mice that showed pituitary remnants were excluded. The rate of growth of the adrenal tumors in the various experimental groups is presented in Chart 1. Each point in these curves represents a mean volume of 12–16 tumors. By comparing Groups HE and HC, it was evident that the tumor had undergone regression following estradiol treatment even in the absence of the pituitary gland. In addition, there was no significant difference between the tumor-weight/animal-weight ratios of Groups C and HC (Table 2). Similarly the tumor/animal weight ratios of Groups E and HE were not significantly different (Table 2). Histologic study of tumors from estradiol-treated hypophysectomized mice showed arrest of mitosis
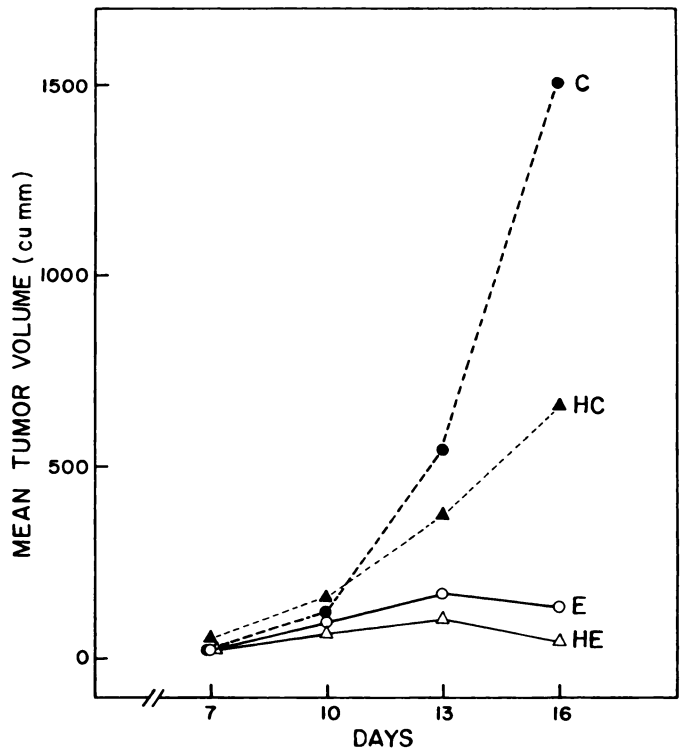

CHART 1. The effect of hypophysectomy and estradiol treatment on adrenal cortical tumor transplanted into BALB/c mice. Hypophysectomy was performed 7 days after transplantation of the tumor. Estradiol treatment, 10 μg daily, was started 24 hours after operation and continued for 12 days. Each value represents the mean volume of 12–16 tumors. C, sham-operated tumor-bearing controls; HC, hypophysectomized tumor-bearing controls; E, sham-operated mice treated with estradiol; HE, hypophysectomized, estradiol-treated mice.

TABLE 2

The Effect of Hypophysectomy and Estradiol Treatment on the Growth of Transplanted Adrenal Cortical Tumor

Group	No. of mice	Av. weight/mouse (gm ± S.D.)[a]	Tumor weight (mg ± S.D.)	Tumor wt./animal wt. (mg/gm ± S.D.)[b]	P[c]
(C) sham-operated control	14	22.6 ± 1.1	434.2 ± 244.5	19.1 ± 8.5	C and HC
(HC) hypophysectomized control	12	18.6 ± 1.9	324.8 ± 160.3	17.2 ± 7.6	NS[d]
(E) sham-operated estradiol-treated	16	21.6 ± 1.7	49.6 ± 26.6	2.1 ± 1.1	E and HE
(HE) hypophysectomized estradiol-treated	12	16.9 ± 1.7	28.6 ± 14.3	1.7 ± 0.9	NS

[a] All results are means ± S.D.
[b] Mean ratio of weights of tumors (mg) to weights of animals (gm) followed by standard deviation.
[c] P = significance of difference between tumor weight/animal weight ratios of the indicated groups.
[d] NS = no significant difference ($P > 0.05$).

TABLE 3

The Effect of Estradiol Treatment of Tumor Explants in Vitro *on Their Subsequent Growth in Mice*

Group	No. of mice	Av. weight (gm ± S.D.)[a]	P[b]	No. of mice with inhibition	Tumor weight (mg ± S.D.)	P
Adrenal tumor						
(AC) unsupplemented medium	10	22.5 ± 1.6			221.7 ± 82.5	
(ACC) cholesterol-treated	10	22.6 ± 1.1	NS[c]	8	239.8 ± 111.8	NS
(AE) estradiol-treated	20	21.3 ± 0.9	NS		18.3 ± 27.1	<0.001
Reticulum cell sarcoma						
(RC) cholesterol-treated	8	23.0 ± 1.9			1547.5 ± 254.2	
(RE) estradiol-treated	10	23.9 ± 1.3	NS		1732.7 ± 180.3	NS

[a] All results are means ± S.D.
[b] P = significance of difference between experiment and control groups.
[c] NS = no significant difference from the control ($P > 0.05$).

cytoplasmic accumulation of lipid, and fibroblastic proliferation in the stroma.

It is concluded from this study that the pituitary hormones were not essential for the maintenance of tumor growth. In addition, the estrogen-induced regression of the tumor was not mediated through inhibition of the pituitary gland.

Tissue Culture Studies

This experiment was designed to investigate whether estradiol has a direct effect on the adrenal tumors grown *in vitro*. The growth behavior of the variously treated tumor explants following transplantation into mice is presented in Table 3. The mean weight of tumors 15 days after implantation was used for the evaluation of the effect of estradiol on the adrenal tumor *in vitro*. As shown, 40% of the estradiol-treated tumor implants (Group AE) were inhibited and failed to grow. The mean weight of tumors of this group was significantly different from each of the controls (AC and ACC). This result indicated that estradiol treatment of the adrenal tumor *in vitro* caused a direct inhibition of its growth potentiality. To ascertain the specific response of adrenal tumor explants to estradiol, it was considered important to repeat the same experiment on a different type of tumor. A reticulum cell sarcoma (RCS) of BALB/c mice was chosen, a

tumor known to be unresponsive to estradiol treatment *in vivo* (17). It was shown that estradiol treatment of RCS tumor explants (Group RE) did not inhibit their subsequent growth in mice as it did with the adrenal tumor (Table 3). This suggested that the effect of estradiol on the adrenal tumor *in vitro* is rather specific in nature.

Histologic study of estradiol-treated tumor explants demonstrated a reasonable degree of cell viability and proliferation (Figs. 4a–c). The explants of the adrenal tumor were composed largely of well-developed epithelial cells not unlike those described *in vivo* (Fig. 4b). The central portion of the explant showed focal necrosis, whereas the peripheral portion remained viable and was covered with a layer of proliferating cuboidal and squamous epithelial cells several layers in thickness. (Fig. 4a). Estradiol-treated explants were compared with the controls for the degree of necrosis, cell proliferation, and amount of stroma, but no striking morphologic difference could be found between these groups.

DISCUSSION

In these experiments, the carcinostatic effect of estradiol on the adrenal cortical carcinoma was demonstrated by: (a) estradiol inhibition of tumor transplantability; (b) partial or complete

regression of estradiol-treated tumors; (c) retardation or inhibition of the growth of the tumors *in vivo* following estradiol treatment *in vitro*. These effects are probably examples of modulation rather than differentiation. Modulation may be defined as reversible changes in the structure and function of cells resulting from a variation of environmental conditions (53). In contrast, cellular differentiation implies a stable and apparently irreversible change in cellular morphologic features and function (1). The effect of estradiol on the adrenal cortical carcinoma was reversible since an estradiol-treated tumor was successfully transplanted and grew progressively in untreated isologous hosts; this indicates that the effect of estrogen is transient and suggests tumor modulation as the mechanism.

The main histologic alterations in the adrenal tumor following estradiol treatment were the arrest of mitosis and cytoplasmic accumulation of lipid. These features were also observed in tumors from hypophysectomized estradiol-treated mice, suggesting that pituitary hormones were not involved in producing these changes. Estrogens are known to influence mitotic activity of normal and neoplastic cells (3). Pettersson demonstrated that a small dose of estradiol increased mitoses, but a large dose inhibited cellular growth by blocking cell division in metaphase (44). The mechanism responsible for the increased cytoplasmic lipid is difficult to explain. It may be the result of cellular neosynthesis (41) or the result of degenerative changes (32, 47) with increase of stainable lipid (4). Finally, it is possible that inhibition of steroidogenesis by estradiol may lead to the accumulation of steroid precursors. McKerns and Bell (38) have shown that estrogen administration decreased the activity of adrenal glucose-6-phosphate dehydrogenase, an enzyme required for steroidogenesis. In any event, the effect of estrogens on the normal adrenal cortex appears to be paradoxic to its effect on the adrenal tumor. Estrogens caused hyperplasia and depletion of lipid in the adrenal cortex in rats (48, 52). These changes are mainly due to stimulation of pituitary adrenocorticotropic hormone (35).

In the case of tumors of endocrine glands, pituitary hormones frequently have a direct role in the growth of hormone-dependent tumors (21). Examples of this phenomenon are the dependence of thyroid tumors on thyroid-stimulating hormone (2), ovarian tumors on gonadotropins (59), and mammary tumors on pituitary mammotropic hormone (32). Kim and Furth (32) demonstrated that the regression of mammary adenocarcinoma of the rat by estrogen administration is indirect and mediated through inhibition of the pituitary gland. Conversely, other tumors of endocrine gland appear to be independent of pituitary hormones for progressive growth. Thus, Gardner described testicular tumors in mice that did not regress following hypophysectomy (22). Also, Snell and Stewart (50) as well as Cohen *et al.* (12) successfully grew adrenal cortical carcinomas in hypophysectomized mice. In addition, hypophysectomy had no effect on the growth of tumors in patients with adrenal cortical carcinoma (36, 37). In the present study, the growth of the adrenal tumor was the same in hypophysectomized animals as in the controls which indicated that the tumor was independent of pituitary hormones for maintenance of its growth. Furthermore, the action of estradiol on the tumor appeared to be independent of the pituitary secretion. Regression of tumors was also observed in hypophysectomized mice treated with estradiol.

The direct effect of estradiol on the tumor was also shown in tissue culture studies. Estradiol treatment of tumor explants *in vitro* inhibited and retarded their subsequent growth *in vivo*. Such a response might be the result of either a specific or cytotoxic effect of estradiol on the tumor *in vitro*. Rivera *et al.* (46) studied the toxic effects of steroid hormones on organ cultures of mouse mammary tumors and demonstrated that estradiol was only toxic in the concentration of 200 μg/ml of culture medium. It is unlikely that the dose of estradiol employed in the present study (20 μg/ml) was toxic because a reticulum cell sarcoma similarly treated *in vitro* was unaffected by this concentration of estradiol. It is concluded from these studies that estradiol acted directly and specifically on the adrenal tumor.

The mechanism of action of estrogens at molecular level is still unknown, but general concepts are beginning to emerge. Recent work on this problem has emphasized the fact that hormones can specifically alter the configuration of certain proteins and, in doing so, can modify their function. Thus estrogens may act by changing the permeability of cell membranes (11, 42), altering the catalytic activity of enzymes (51), or binding with genetic repressors, hence the induction or repression of specific enzymes (31, 41).

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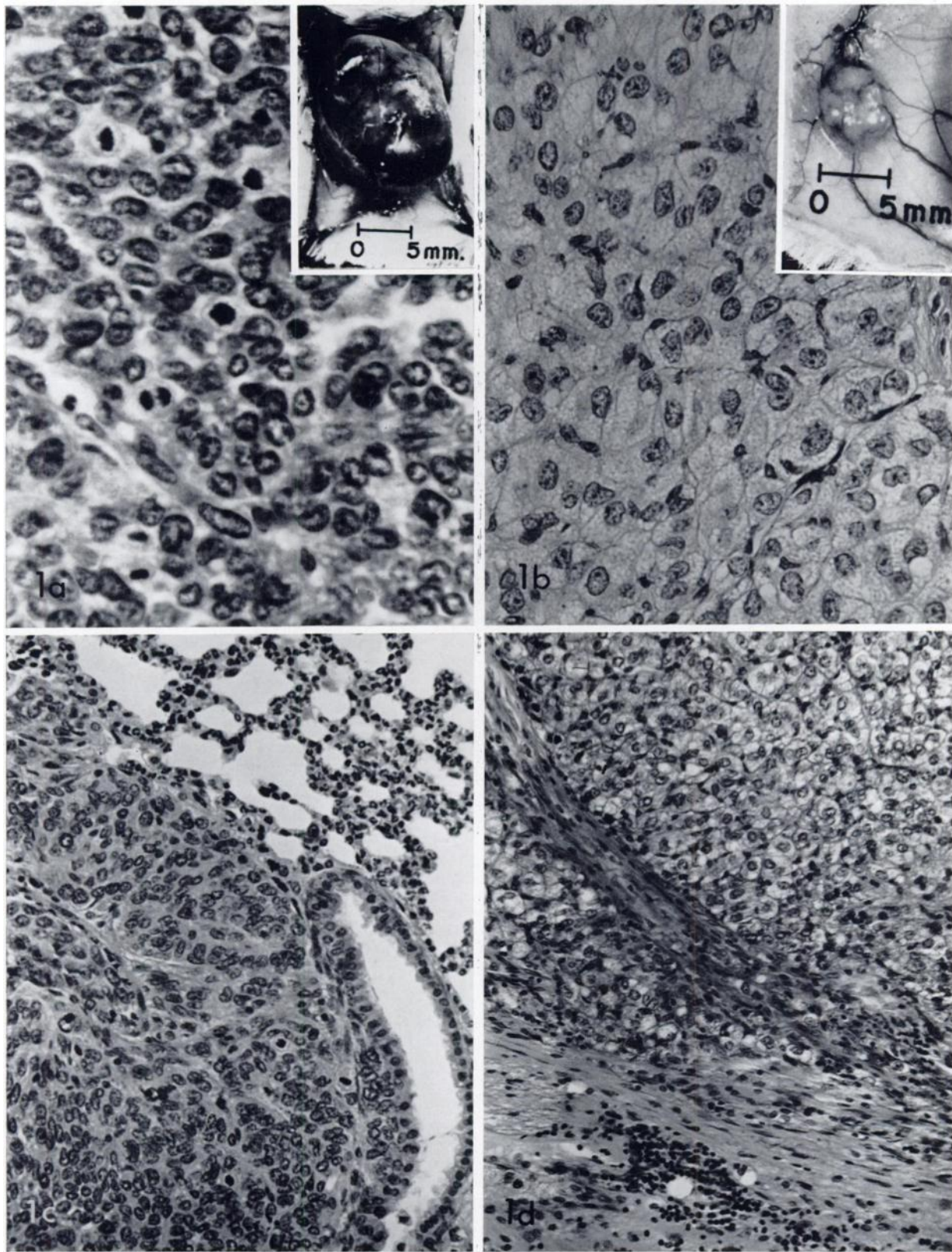
FIG. 1. Morphologic features of the adrenal cortical carcinoma. *a*, untreated tumor showing numerous mitoses. H & E, × 375. *Inset*, gross appearance of subcutaneous implant of the tumor. *b*, estradiol-treated tumor demonstrating the absence of mitoses, vacuolated cytoplasm, and well-defined cell boundaries. H & E, × 375. *Inset*, gross appearance of subcutaneous implant. *c*, pulmonary metastasis from the adrenal carcinoma. H & E, × 240. *d*, fibrous stromal proliferation and lymphocytic response in estradiol-treated tumor. H & E, × 375.
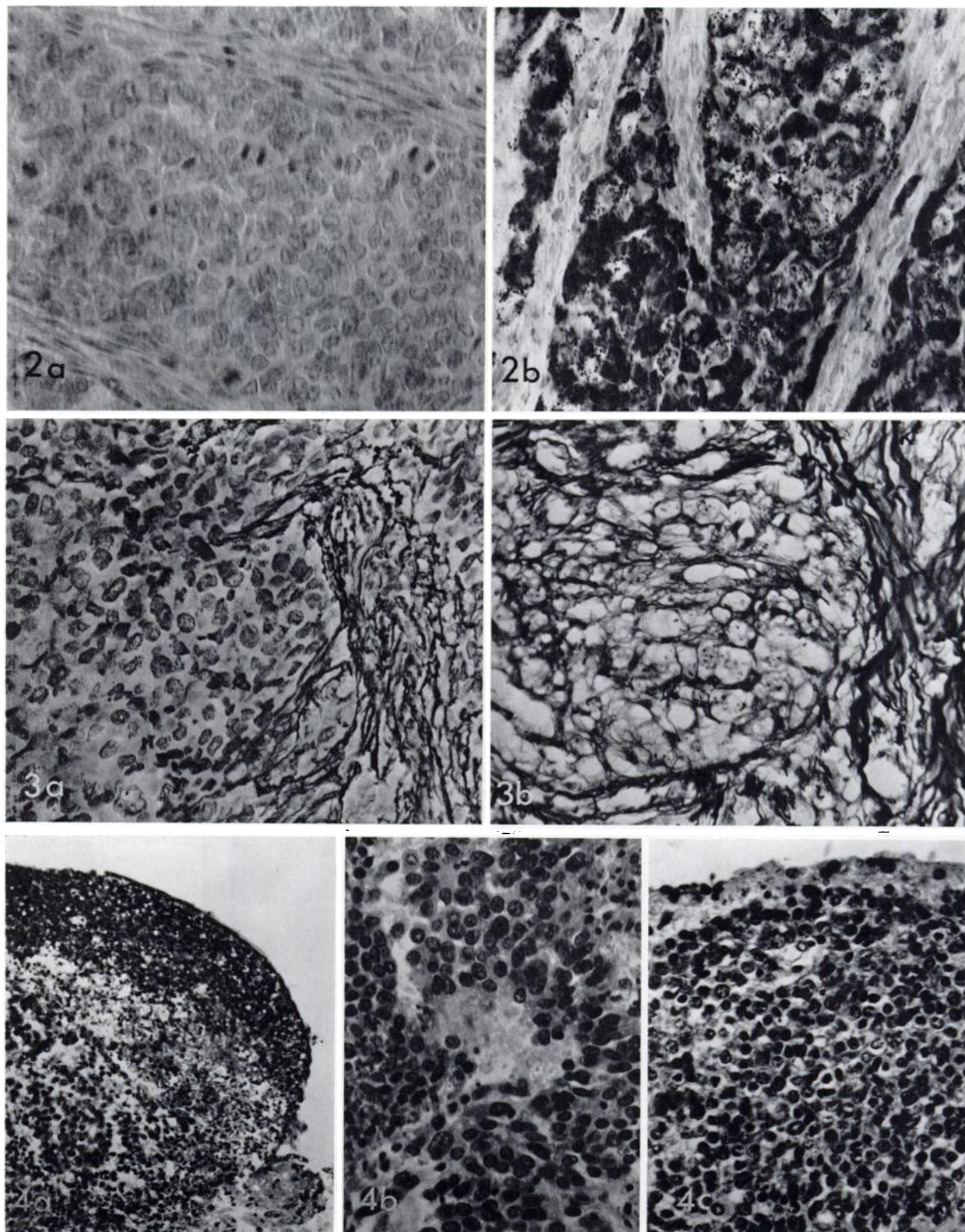
FIG. 2. Stainable lipid in the adrenal cortical carcinoma. Oil Red O, × 300. a, absence of lipid in untreated tumor; b, abundant lipid in estradiol-treated tumor.

FIG. 3. Reticulum in the stroma of the adrenal cortical carcinoma. Wilder's silver impregnation, × 300. a, untreated tumor showing reticular fibers around groups of tumor cells. b, intercellular reticulum in estradiol-treated tumor.

FIG. 4. Histologic features of estradiol-treated tumor explants. a, explant of adrenal cortical carcinoma showing peripheral layer of proliferated cells. H & E, × 120. b, preservation of organized epithelial structure in an explant of the adrenal tumor. H & E, × 300. c, estradiol-treated explant of reticulum cell sarcoma. H & E, × 300.

Regression of an Adrenal Cortical Carcinoma by Estradiol Treatment

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