The Influence of Sex Hormones on Mammary Tumors
Induced by 2-Acetaminofluorene*

A. Cantarow, M.D., J. Stasney, M.D., and K. E. Paschkis, M.D.

(From the Departments of Biochemistry, Pathology, Physiology and Medicine, Jefferson Medical College, Philadelphia 7, Pa.)

(Received for publication December 2, 1947)

There is abundant evidence that estrogens enhance the development of mammary tumors in high-cancer strains of mice, with the possible exception of the Marsh-Buffalo strain. The inhibitory effect of testosterone in this connection is also well known. Although similar studies in species other than the mouse have yielded conflicting results, there is evidence that estrogens enhance the development of mammary tumors in rats.

The present report deals with the influence of estrogen, androgen and progesterone upon the development of mammary tumors in rats receiving 2-acetaminofluorene in their diet.

MATERIAL AND METHODS

Observations were made on 179 virgin female and 196 male rats about 2 months old; the majority were of the Sherman strain, the remainder Wistar descendants. We have encountered no significant difference between these two groups in their response to acetaminofluorene or to the various sex hormones employed in this and previous studies (8, 21). All received 0.03 per cent 2-acetaminofluorene in the following diet (protein content 16 per cent) to the time of sacrifice: corn meal, casein, alfalfa, linseed oil, bone ash, and NaCl, with supplements of brewers' yeast and cod liver oil. They were divided into the following experimental groups:

Control Group.—57 females and 79 males received no additional treatment.

Endogenous Estrogen Group.—17 females received 25 I. U. of pregnant mare serum (PMS) gonadotrophin (in aqueous solution) intramuscularly 3 times weekly.

Exogenous Estrogen Group.—36 females and 52 males received 0.125 mgm. of estradiol dipropionate (in sesame oil) intramuscularly 3 times weekly.

Exogenous Androgen Group.—32 females and 51 males received 0.5 mgm. of testosterone propionate (in sesame oil) intramuscularly 3 times weekly.

Progesterone Group.—26 females, 11 castrated females and 14 males received 0.5 mgm. of progesterone intramuscularly 3 times weekly.

Treatment was continued in all instances to the time of death or sacrifice. The control animals were examined weekly and the hormone-treated animals at the time of injection for the presence of mammary tumor. Vaginal smears were made repeatedly during the experimental period and in every instance at the time of sacrifice. The mammary tumors were fixed in Bouin's solution and sections were stained with hematoxylin-eosin and by Masson's trichrome method.

RESULTS

The incidence of malignant tumors of the breast in the several groups is indicated in Table I. The time of appearance of the first palpable tumor in each group, subsequently confirmed histologically, was as follows: female control, 159 days; female-estradiol, 161 days; female-gonadotrophin, 285 days; female-progesterone, 93 days; male-estradiol, 177 days; male-testosterone, 141 days.

It is interesting that, in contrast to liver tumors (8, 21), there was apparently no tendency for the incidence of breast tumors to increase with increasing duration of treatment in any of the experimental groups.

The tumors were either single or multiple, unilateral or bilateral. When small, they were usually firm; when large, they were often cystic, containing either hemorrhagic or creamy, gray-yellow fluid. The smaller tumors tended to be encapsulated and could be shelled out readily. Larger tumors were usually attached to the skin, which was often ulcerated. The surface was irregularly lobulated, pale gray, frequently with bluish cysts. The cut surface was irregularly lobulated, mottled yellow-gray in color, with cystic spaces containing bloody or creamy fluid.

There were certain differences in the histologic
TABLE I: INCIDENCE OF MAMMARY CARCINOMA

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Estradiol</th>
<th>Gonadotrophin</th>
<th>Progesterone</th>
<th>Castrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEMALE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of rats</td>
<td>Tumor</td>
<td>No. of rats</td>
<td>No. of rats</td>
<td>No. of rats</td>
<td>No. of rats</td>
</tr>
<tr>
<td>92-200</td>
<td>25</td>
<td>9</td>
<td>18</td>
<td>3*</td>
<td>2</td>
</tr>
<tr>
<td>201-250</td>
<td>8</td>
<td>2</td>
<td>9</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>251-300</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>301-350</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>351-400</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>17</td>
<td>36</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Estradiol</th>
<th>Gonadotrophin</th>
<th>Progesterone</th>
<th>Castrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MALE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of rats</td>
<td>Tumor</td>
<td>No. of rats</td>
<td>No. of rats</td>
<td>No. of rats</td>
<td>No. of rats</td>
</tr>
<tr>
<td>92-200</td>
<td>34</td>
<td>0</td>
<td>36</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>201-250</td>
<td>23</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>251-300</td>
<td>14</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>301-350</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>351-400</td>
<td>79</td>
<td>0</td>
<td>52</td>
<td>3</td>
<td>51</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>0</td>
<td>52</td>
<td>3</td>
<td>51</td>
</tr>
</tbody>
</table>

* One sarcoma

characteristics of the tumors in the various experimental groups.

**Control Group.**—The malignant proliferation involved both epithelial and connective tissue cells, their relative proportions varying in different portions of the tumor, with epithelial proliferation generally dominant. The pattern most commonly observed was that of adenocarcinoma (Fig. 1). This was characterized by acini of varying size, lined by cuboidal or low columnar epithelial cells with dark nuclei and vacuolated or granular cytoplasm, occasionally containing pink homogeneous material in their lumen. Groups of these acini were separated by connective tissue septa containing lymphocytes and fibrocytes.

The type of lesion next in order of frequency in the control group was papillary cystadenoma. This was characterized by irregular cystic spaces, containing blood or albuminous fluid, lined by low, dark, cuboidal cells, with numerous papillary infoldings comprising a connective tissue core covered by layers of tall columnar epithelium (Fig. 3). Another lesion seen frequently in this group was characterized by papillary projections into dilated ducts, resembling intraductal papilloma (Fig. 2). Ductal carcinoma (comedo) (Fig. 4) occurred less frequently than adenocarcinoma and papillary cystadenoma.

Areas of necrosis and hemorrhage were frequently seen in the larger nodules, as well as greatly distended blood vessels filled with red blood cells. Lesions of all types contained numerous mitotic figures and their malignant nature was evidenced by invasion of adjacent muscle (Fig. 6), infiltration of the capsule and metastasis to the liver and lungs. The amount of connective tissue varied, but at times the proliferation of connective tissue was so extensive as to resemble the scirrhous form of mammary carcinoma (Fig. 5). In such cases the tissue was usually quite cellular, containing numerous lymphocytes and fibrocytes, with scattered, compressed groups of dark-staining epithelial cells in acinous arrangement.

**Hormone-Treated Groups.**—The mammary tumors in females receiving estradiol, gonadotrophin or progesterone were almost exclusively of the adenocarcinoma and papillary cystadenoma varieties, both of which occurred practically always in the same tumor. A few in the progesterone group (but none in the estrogen series) contained also lesions characteristic of ductal carcinoma. There was no essential difference between the histologic characteristics of the lesions in the estrogen-treated and progesterone-treated females. Both differed from the control group, however, in that connective tissue proliferation was relatively slight except in one animal receiving progesterone (Fig. 5). One mammary sarcoma occurred in the estrogen group. The single mammary tumor in the testosterone-treated males differed in no respect from those seen in the estrogen and progesterone female groups, except that it, and also the tumors in the estrogen-treated males, exhibited only acinar epithelial proliferation, with no evidence of duct involvement.

The acinar cells in the hormone-treated groups were cuboidal or columnar, their cytoplasm often...
being vacuolated or foamy. The lumen of the acini
and cysts consistently contained pale-blue amorphous or pink granular and vacuolated material and desquamated cells. The stroma of the tumors in the progesterone-treated animals contained areas which appeared edematous or myxomatous. Not only was the tumor incidence much higher in this group (85 per cent), but also the growth of the tumors was much more rapid than in the other groups.

DISCUSSION

There is considerable variation in the reported incidence of mammary carcinoma in female rats of various strains receiving 2-acetaminofluorene, as follows: Bielschowsky (2, 3), Wistar 64 per cent, piebald 4 per cent; Harris (11), Wistar 62 per cent; Wilson, De Eds, and Cox (24), Slonaker 8 per cent; Dunning, Curtis, and Madsen (9), Marshall none, Copenhagen none, August 10 per cent, Fischer 20 per cent, A X C 10 per cent. The difference may be attributable in some instances to differences in diet and in dosage of the carcinogen, but this is not the case with the two strains employed by Bielschowsky (2, 3) or the four strains studied by Dunning, Curtis, and Madsen (9). However, the fact that there were only 10 animals in each of the groups reported by the latter raises some question as to the statistical significance of the observed variation (0 to 20 per cent). Bielschowsky (3) reported the occurrence of mammary cancer in 1 of 11 female castrates (9 per cent) and in 3 of 41 males (4 per cent). No breast tumors in acetaminofluorene-treated male rats have been reported by other investigators and none has been observed by us in male rats receiving the carcinogen alone. In our group of female controls (which received only acetaminofluorene) the incidence was 30 per cent, with no significant difference between Sherman and Wistar animals.

The well-established formula for development of spontaneous mammary cancer in inbred strains of mice includes three factors: (a) genetic susceptibility, (b) the milk agent and (c) hormonal stimulation of mammary growth. Studies of the induction of breast tumors in mice by methylcholanthrene and 1,2,5,6-dibenzanthracene indicate that the first two of these factors are not essential under these circumstances (14, 15, 20, 22). Although certain strains are more resistant than others to the induction of mammary cancer by methylcholanthrene, this variation could not be correlated with susceptibility to development of spontaneous mammary cancer (14). The hormonal factors are, however, operative. It would appear that the breast must attain a certain degree and type of growth before either the milk agent or the carcinogens mentioned above can evoke the neoplastic reaction (20).

All stocks of rats studied have a very low incidence of spontaneous mammary cancer. Administration of even large amounts of estrogen usually results in the development of only benign lesions. Nevertheless, there are a few reports of the induction of mammary cancer in rats exposed to estrogen for prolonged periods, e.g., by means of pellet implantation (10, 17). It has been suggested that the apparently high resistance of rats in this connection may be due to lack of a proper genetic background or to the possibility that the estrogen requirement for induction of mammary cancer is much higher for rats than for mice.

The sex difference in incidence of breast tumors following administration of 2-acetaminofluorene is in accord with these observations and with prevalent views regarding the necessity for hormonal, \textit{i.e.} presumably estrogenic, stimulation of mammary growth as a prerequisite for mammary carcinogenesis. The inhibitory influence of testosterone and castration in females was also to have been anticipated on this basis. However, in the case of spontaneous tumors in high-tumor strains of mice, with few exceptions, the incidence is low in non-breeding as compared to breeding females, increasing to that in the latter after administration of estrogen (20). Similarly, in the case of methylcholanthrene-induced breast tumors in mice, the incidence has been reported to be low in non-breeding as compared to breeding females (16), although Orr (19) reported a high incidence in non-breeding females of low-tumor strains (IFS and CBA). The findings in acetaminofluorene-treated rats are not in agreement with the former (16), inasmuch as the females were virgins and this carcinogen is not estrogenic (2). The high incidence of mammary tumors in non-breeding females, viewed in the light of their absence in males and in castrate and testosterone-treated females, suggests that whereas estrogen is essential, only relatively small amounts are required for the development of such tumors under the influence of 2-acetaminofluorene. If this be the case, one cannot invoke the hypothesis of a high requirement, in this connection, of estrogen to account for the failure of estradiol (22 per cent) and gonadotrophin (18 per cent) to increase the tumor incidence in females and the relatively slight effect of estrogen in males.
adenoma in rats (12, 18) have revealed either no capable of inducing mammary tumors. We are aware of no previous reports that the combined action of estrogen and progesterone is necessary for maximum proliferation of the alveolar system in the mouse and rat. How is readily apparent. There are several observations of a relatively high incidence of mammary tumor in progesterone-treated pregnant mice of a low-tumor strain (23). It seems noteworthy, also, that in this group, as in the other experimental groups, the incidence of tumor did not increase with prolongation of the period of treatment. Other studies of the influence of this agent upon spontaneous mammary cancer in mice (5, 7, 13) and mammary fibroadenoma in rats (12, 18) have revealed either no effect or, more frequently, inhibition of growth of the tumors. We are aware of no previous reports of its use in conjunction with chemical carcinogens capable of inducing mammary tumors.

No satisfactory explanation of this phenomenon is readily apparent. There are several observations that the combined action of estrogen and progesterone is necessary for maximum proliferation of the alveolar system in the mouse and rat. However, there is no clear evidence that administration of progesterone alone produces a significant stimulating effect upon the mammary gland of normal female rats. The absence of breast tumors in progesterone-treated males and castrate females indicates that some estrogen is required for the production of the stimulating effect of progesterone upon mammary carcinogenesis under the influence of acetaminofluorene. As noted previously, the one instance of tumor in the testosterone-treated males is difficult to explain, although there is evidence that testosterone stimulates lobule growth in immature male and castrate female rats (1). The remarkable tumor-stimulating effect of progesterone in females, viewed in conjunction with the lack of such effect of estrogen in females and its minimal effect in males (6 per cent tumors), suggests, as indicated previously, that in the presence of a necessary, but relatively small amount of estrogen, the quantity of progesterone is the factor that determines the development of mammary cancer by 2-acetaminofluorene. Studies are in progress of the effect of administration of estrogen and progesterone simultaneously to males and castrate females receiving 2-acetaminofluorene. Data obtained in these experimental groups should indicate whether or not this hypothesis is valid.

Although the nature of our material does not permit any conclusion as to the histogenesis of the tumors observed, certain points of similarity to and difference from other types of experimental and spontaneous breast tumors seem worthy of mention. The histologic characteristics of the tumors in our control (AAF) group conformed to those described by Bielschowsky (4), who stated that (a) nearly all were adenocarcinoma, (b) malignant intraductal papilloma occurred more commonly than in spontaneous mammary tumors in mice, (c) metastases occurred only when tumors had reached considerable size, and (d) squamous metaplasia was seen rarely (not at all in our series) and scirrhous tumors infrequently. Papillary cystadenoma apparently occurred more commonly in our series.

It has been pointed out in studies of mammary tumors in mice that these differ from human breast cancer in the infrequency of true duct carcinoma and intraductal papilloma (6). On the other hand, duct carcinoma was observed frequently by Nelson (17) and Astwood, Geschickter, and Rausch (1) in estrogen-induced mammary tumors in rats. In view of these observations, the high incidence of these ductal lesions in rats receiving 2-acetaminofluorene alone and its absence in those receiving estrogen simultaneously suggest that there is some fundamental difference not only in the mode of action of these two agents but also in the effect of each in the presence of the other. The lesions induced by acetaminofluorene differed also from those induced in mice by methylcholanthrene (15) in that the squamous metaplasia commonly observed in the latter was not encountered in any experimental group in our series.

**SUMMARY**

The incidence of mammary carcinoma in non-breeding female Sherman and Wistar rats receiving 2-acetaminofluorene was not increased by simultaneous administration of estrogen but was increased enormously by progesterone. Breast tumors, absent in carcinogen-treated males, appeared in a small percentage receiving estrogen and in one receiving testosterone.

The data suggest that small amounts of estrogen are probably necessary for the development of breast carcinoma in Sherman and Wistar rats treated with 2-acetaminofluorene, but that the
quantity of progesterone may be the limiting hormonal factor in this connection.

The morphologic characteristics of these lesions are compared with those of spontaneous and carcinogen-induced tumors in mice and estrogen-induced tumors in rats.

ACKNOWLEDGMENT

We are indebted, for material employed in this study, to Dr. E. Schwenk of the Schering Corporation, and Drs. E. Oppenheimer and C. R. Schols of the Ciba Pharmaceutical Products, Inc., for estradiol dipropionate, testosterone propionate, and progesterone; and to Dr. M. H. Kuizenga of the Upjohn Co. for PMS gonadotrophin (Gonadogen).

REFERENCES


DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Adenocarcinomatous portion of the mammary tumor of male rat treated with testosterone for 141 days. Note the presence of pale vacuolated cells and pink granular material in the lumen of the dilated acini. Mag. × 100.

FIG. 2.—Portion of mammary tumor of a control female rat (carcinogen alone) treated for 159 days. Note the numerous ductal papillomata. Mag. × 50.

FIG. 3.—Papillary-cystadenocarcinomatous portion of a mammary tumor of a female rat treated with progesterone for 108 days. Mag. × 70.

FIG. 4.—Portion of duct or "comedo" carcinoma from a control female rat (carcinogen alone) treated for 340 days. Mag. × 125.

FIG. 5.—Portion of the mammary tumor of female rat treated with progesterone for 291 days. Note the marked proliferation of the connective tissue compressing the adenomatous structures. This type of lesion was more common in the control animals and resembles scirrhous adenocarcinoma. Mag. × 60.

FIG. 6.—Another portion of the tumor of Fig. 3, revealing the infiltrative growth between the adjacent skeletal muscle fibers. Mag. × 100.
The Influence of Sex Hormones on Mammary Tumors Induced by 2-Acetaminofluorene

A. Cantarow, J. Stasney and K. E. Paschkis