Antiestrogen Modulation of the Growth and Properties of Ovarian-Autonomous and Ovarian-Dependent Mammary Tumors in Rats

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ABSTRACT

Studies were undertaken to examine the effects of antiestrogens on the growth of two ovarian-autonomous mammary tumors in rats, namely, some dimethylbenz(a)anthracene (DMBA)-induced and ovarian-autonomous tumors in Sprague-Dawley rats and the ovarian-autonomous R3230AC mammary tumor in Fischer 344 rats. Of the approximately 15% of DMBA-induced tumors that continue to grow upon ovariectomy or antiestrogen treatment (250 μg daily s.c. of U 23,469, cis-(3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol) in 0.15 M NaCl, almost all fail to have their growth retarded by subsequent antiestrogen treatment or ovariectomy. However, some DMBA-induced tumors whose growth is stabilized by ovariectomy or by antiestrogen treatment may be further benefited by subsequent treatment with the complementary therapy.

Antiestrogen treatment markedly depresses the growth of the ovarian autonomous R3230AC mammary tumor. Administration of the antiestrogen U 23,469, cis(3-[p(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol) (20 to 250 μg daily s.c. in 0.15 M NaCl) and two related antiestrogens U 11,100A, 1-((p-[3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl)pyrrolidine hydrochloride and CI628 α-[4-4-pyridinidroethoxy]phenyl-4-methoxy-a'-nitrostilbene, beginning at the time of tumor transplantation into Fischer 344 host rats results in a 2 to 4-fold depression in tumor growth rate, and the degree of growth reduction is related to the dose of antiestrogen. 17β-Estradiol (15 μg) also depresses R3230AC tumor growth, while growth is at or slightly above the control rate in ovariectomized hosts. This diminished tumor growth by the antiestrogens cis-(3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol) and 1-((p-[3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl)pyrrolidine hydrochloride, cytoplasmic estrogen receptor levels in tumor are below those of the control, and nuclear estrogen receptor levels are elevated. Also, the pro lactin and estradiol concentrations in serum do not differ between antiestrogen-treated and control groups. Ovarian-dependent DMBA-induced mammary tumors regressing under antiestrogen treatment show high levels of nuclear and low levels of cytoplasmic estrogen receptor and a marked (approximately 50%) reduction in prolactin receptor level. Ovariectomy also results in a great decrease in the prolactin receptor level of regressing DMBA tumors. These studies document the fact that antiestrogens interact with the estrogen receptor system in R3230AC mammary tumors and slow tumor growth and suggest that antiestrogens may provide palliative benefit in the case of some ovarian-unresponsive but estrogen-sensitive breast cancers.

INTRODUCTION

Breast cancer probably consists of a spectrum of diseases in terms of its hormonal dependence, as evidenced by the responses of patients to endocrine therapy. It is generally assumed that hormone-dependent breast cancers, which respond to ovariectomy, would also respond to antiestrogen therapy. Studies in humans indicate a good correlation between the presence of estrogen receptor and response to hormone ablative (ovariectomy) or additive hormonal or antihormonal (such as antiestrogen) therapy (28). Yet it is occasionally found (16) that some patients, whose tumors appear to lack estrogen receptors (based upon cytoplasmic receptor-binding assays), do respond to antiestrogen therapy and, likewise, some patients that do not respond to ovariectomy respond to subsequent antiestrogen treatment or vice versa.

Antiestrogens are nonsteroidal triphenylethylene-type compounds which antagonize a variety of estrogen-stimulated processes (18). Previous studies have shown that these compounds are capable of controlling the growth of hormone-dependent DMBA-induced rat mammary tumors (8, 14, 17, 21, 34), some human breast cancer cell lines (26, 36), and human breast cancers (2, 12, 13) in clinical trials. Little information is available, however, on the possible effects of antiestrogens on the growth of mammary tumors which are recalcitrant to endocrine ablation. Hence, the aim of this study was 2-fold: (a) to determine whether DMBA-induced mammary tumors unresponsive to ovariectomy would be caused to regress by antiestrogens and, conversely, whether tumors unresponsive to antiestrogen treatment would regress upon ovariectomy; and (b) to compare the effects of antiestrogens on the growth and biochemistry of ovarian-dependent antiestrogen-sensitive R3230AC mammary adenocarcinoma (15).

We find that in the DMBA mammary tumor system, although the majority of tumors are effectively regressed by ovariectomy or antiestrogen treatment, the tumors that respond partially to antiestrogen treatment or ovariectomy, i.e., are growth arrested or stabilized, may be further benefited by subsequent treatment.
with the complementary therapy. In the R3230AC mammary tumor system, where ovariectomy does not alter tumor growth, antiestrogens are found to evoke a dose-related and marked diminution in tumor growth rate. This paper explores some possible mechanisms of this antiestrogen-evoked depression of tumor growth in these 2 systems.

MATERIALS AND METHODS

DMBA-induced Rat Mammary Tumors. The maintenance of rats (virgin female Sprague-Dawley rats, from the Holtzman Company, Madison, Wis.), administration of DMBA, palpation for tumor appearance and sizing, and bilateral ovariectomy were performed exactly as previously described (34). When the DMBA-induced tumors in a given rat reached a minimum of 0.6 cm in each diameter (length and width; usually about 60 days after DMBA), the rats were divided into groups with comparable tumor number and size. Groups of rats received s.c. daily injections of 0.5 ml of 0.15 m NaCl alone (control group) or 0.5 ml 0.15 m NaCl containing various antiestrogens (U 23,469, U 11,100A, or Cl-628) or estrogens (17β-estradiol or estradiol benzoate); another group of animals was ovariectomized. Tumor size and number were monitored twice each week during the treatment period. Rats in the control group were killed on diestrus (staged by vaginal morphology), and the rats in the antiestrogen- and estrogen-treated groups were killed 19 to 23 hr after the last injection. All rats were sacrificed by rapid decapitation. Mammary tumor samples were collected, quickly frozen in liquid nitrogen, and stored until assayed.

In another experiment, the effects of antiestrogen (U 23,469) on the growth of ovariectomy-nonresponsive DMBA tumors were studied; also, the effect of ovariectomy on antiestrogen-treated but nonresponding tumors was investigated. One-half of the rats bearing tumors with a minimum size of 0.6 cm in each diameter (approximately 60 days after DMBA) were given daily injections of 250 μg of U 23,469, and the other half of the rats were bilaterally ovariectomized (see below). Tumor development was recorded carefully. Ovariectomy was subsequently performed on rats that were bearing tumors not completely regressed (less than 0.15 sq cm in area, length x width) after at least 8 weeks of U 23,469 treatment. Tumor area was then followed for another 4 weeks after ovariectomy. In the rats that had been initially ovariectomized to achieve tumor regression, tumor growth patterns were monitored; in those cases where the area of a single tumor was not reduced to less than 0.15 sq cm at 4 weeks after ovariectomy, the rats bearing such tumors then received U 23,469 injections (250 μg daily for 8 weeks) to determine if the tumors would be suppressed by subsequent treatment with antiestrogen. The response of DMBA tumors to antiestrogen and/or ovariectomy was classified as: (a) grow (tumor has consistently with time increased in area, by at least 50%, compared to its original area, i.e., length x width, before treatment); (b) stabilize (tumor growth is arrested so that tumor area remains approximately the same as at the start of the treatment period); (c) regress (tumor area is decreased to less than one-half of the original); and (d) disappear (tumor is no longer detectable by palpation).

R3230AC Rat Mammary Tumors. Forty-day-old female Fischer 344 rats (approximately 70 g body weight; Charles River Breeding Laboratories, Inc., North Wilmington, Mass.) received s.c. axillary transplants of R3230AC tumor via a sterile trocar technique under light sodium pentobarbital anesthesia. Twenty-one-day posttransplanted R3230AC tumors were removed from donor rats (supplied by Mason Research Institute, Worcester, Mass.), minced, and kept in iced, sterilized Medium 199 (containing penicillin and streptomycin) during tumor transplantation. Groups of rats either were ovariectomized 5 days before receiving tumor transplants or intact rats received vehicle (0.15 m NaCl containing 4% ethanol) or various antiestrogens (U 23,469, 20, 100, or 250 μg; U 11,100A, 250 μg; and Cl-628, 100 μg) within 24 hr of tumor transplantation. The various compounds were given s.c. daily in 0.5 ml 0.15 m NaCl containing 4% ethanol. Tumor palpation started a week after tumor transplantation. At 25 days after tumor transplantation, some of the R3230AC tumor-bearing rats in some groups were sacrificed in order to obtain their mammary tumors and serum samples for assay. The other rats were maintained, and their tumor growth was followed until 45 days after tumor transplantation.

Chemicals and Reagents. [2,4,6,7-3H]Estradiol (91 Ci/mmol) was purchased from Amersham Radiochemical Center (Arlington Heights, Ill.); [2,4,6,7-3H]estradiol (115 Ci/mmol) and carrier free Na125I (17 Ci/mg) were obtained from New England Nuclear, (Boston, Mass.). The radioactive estradiol was checked for radiochemical purity by thin-layer chromatography. Bovine serum albumin, diethylstilbestrol, 17β-estradiol, 17β-estradiol 3-benzoate, and phenylmethylsulfonylfluoride were obtained from Sigma Chemical Co. (St. Louis, Mo.). The antiestrogens U 23,469 and U 11,100A (nafinoxidone HCl) were kindly provided by the Upjohn Co., (Kalamazoo, Mich.); Cl-628 was kindly provided by the Parke-Davis Co., Ann Arbor, Mich. DMBBA (15% fat emulsion) was a gift from Upjohn. Trasylol was from Mobay Chemical Co., New York, N. Y. The charcoal: dextran slurry and hydroxylapatite slurry were prepared as described previously (5).

Iodination of Prolactin. Ovine prolactin (NIH P-S12; obtained from the Hormone Distribution Office, National Institute of Arthritis, Metabolism, and Digestive Diseases) was iodinated by the chloramine-T method of Hunter and Greenwood (11) exactly as extensively modified and described by Kuo-Jang and Ramirez (24). The iodinated prolactin was separated from free 125I and damaged hormone by chromatography on a Sephadex G-75 column (1.0 x 12 cm), that had been equilibrated with 0.02 m Tris-HCl buffer, pH 7.6, and presaturated with 2 ml of 1% bovine serum albumin. The column was eluted with 0.025 m Tris-HCl buffer, pH 7.6, and 0.5-ml fractions were collected into polystyrene tubes containing 0.5 ml 0.1% bovine serum albumin (prepared in 0.02 m Tris-HCl buffer, pH 7.6). The fractions from the descending shoulder of the iodinated protein peak were pooled and stored at −20°C and were repurified by gel filtration on Sephadex G-75 before each assay. Approximately 80% of the 125I-labeled prolactin was bound by an excess of liver membranes from mature female rats. The specific activity of 125I-labeled prolactin ranged from 49 to 83 μCi/μg as determined by the method of Shiu and Friesen (31).

Preparation of Membrane Fractions and Prolactin-binding Assay. The preparation of a single membrane particle fraction from tumors and the procedure for prolactin receptor assay were similar to those described (5, 6) with slight changes. All the steps were performed at 4°C. Briefly, a 200- to 300-mg portion of homogenously minced tumor tissue (from tumor that had been frozen in liquid nitrogen) was homogenized in
0.025 M sodium phosphate:0.15 M NaCl, pH 7.0, with Kontes all-glass tissue grinders at approximately 100 mg/ml. The homogenate was filtered through organza cloth (wet with buffer); the mesh and tube were washed with 0.0005 M CaCl₂, and the filtrate was brought to a total volume of 8 ml. The diluted homogenate was then spun at 150 x g for 20 min. The resulting supernatant was centrifuged at 100,000 x g for 50 min to obtain the membrane fraction. The pellet was resuspended in 0.6 ml of freshly prepared Buffer A (0.025 M sodium phosphate, 0.01 M MgCl₂, 0.1% bovine serum albumin, and 10 μg gentamicin per ml (pH 7.0)) using the Kontes homogenizer. One hundred μl of suspended membrane particle containing approximately 300 μg of membrane protein were added to 100 μl of Buffer A containing approximately 100,000 cpm 125I-labeled oxine prolactin (approximately 2 ng of hormone) with or without 1 μg of unlabeled prolactin. The incubation mixture in duplicate was brought up to 0.5 ml with Buffer A in a 12- x 75-cm polystyrene tube (previously rinsed with Buffer A) and was incubated at 22° for 16 hr with shaking. Ice-cold Buffer A (2.5 ml) was added to each tube to end the incubation. The particles were sedimented at 20,000 x g for 15 min, washed once with 0.01 M sodium phosphate buffer (pH 7.0), dissolved in 0.5 ml of 0.1 M NaOH, and counted in a Nuclear Chicago 1185 Series automatic gamma counter with 86% counting efficiency.

The time course of specific binding of 125I-labeled prolactin to tumor membrane particles was monitored previously at several temperatures (4°, 22°, and 37°) and was found to be very similar to that reported (5, 22, 24) so that a 16-hr incubation at 22° was routinely used. The Kₐ of prolactin binding to tumor receptors was determined with 125I-labeled prolactin (100,000 cpm) and increasing concentrations of unlabeled prolactin and was estimated to be 2 x 10⁻¹⁰ M. Nonspecific binding routinely accounted for 4 to 6% (4000 to 6000 cpm) of the total added iodinated prolactin. Specific prolactin binding refers to the amount of radioactivity that could be displaced by excess unlabeled prolactin and is expressed as the percentage of total radioactivity added to each incubation tube. Although the concentration of 125I-labeled prolactin used in routine assays was 6 to 10 x 10⁻¹¹ M and hence did not saturate receptor sites, the percentage of specific binding should provide a relative measure of prolactin receptor concentration. This assumes that the Kₐ of prolactin receptor interaction remains constant during the endocrine manipulations; although we have not verified this in all treatment groups, this is probably the case, inasmuch as the Kₐ for prolactin-receptor interaction remains unchanged during DMBA-induced tumor regression by androgens (5).

Determination of Nuclear and Cytosol Estrogen-binding Sites. Minced tumor tissue (approximately 200 mg) was homogenized in 2 ml of iced TEA buffer. The nuclear exchange and cytosol-binding assays were performed as described previously (20, 34) except that both assays utilized 5 nm [³H]-estradiol in the presence and absence of 10⁻⁶ M diethylstilbestrol to correct for low affinity binding. These exchange protocols have been validated previously for DMBA tumors (34) and in the course of these studies for R3230AC tumors to enable optimal determination of nuclear and cytosol receptor sites that are occupied by estrogen or antiestrogen. In some cases, tissue from growing or from regressing tumors was divided and homogenized in TEA buffer alone, or TEA buffer containing 500 or 5000 kallikrein-inactivating units of Trasylol per ml or 0.1 mM phenylmethylsulfonylfluoride. Similar values for nuclear or cytosol receptor levels were determined in the presence or absence of these protease inhibitors, suggesting that in vitro proteolytic activity did not influence our receptor measurements.

Radioimmunoassays. Plasma prolactin was determined by double antibody radioimmunoassay using the National Institute of Arthritis, Metabolism, and Digestive Diseases radioimmunoassay kit with rat prolactin RP-1 reference preparations as standard. All the samples were run in duplicate in one assay. Plasma was assayed for 17β-estradiol according to the procedure reported by Bahr (1), with the exception that a highly specific 17β-estradiol antisera was used. The 17β-estradiol antiserum 244, supplied by Dr. G. D. Niswender, was prepared against 6-bovine serum albumin:17β-estradiol. The antiserum was used at a final dilution of 1:12,000, which bound 50% of 24,000 cpm of 17β-[³H]estradiol. All samples were chromatographed on a Sephadex LH-20 column using a solvent system of benzene:methanol, 90:10 (v/v) (3), and the estradiol region was collected and assayed. Addition of 16, 33, and 66 pg of cold 17β-estradiol to rat plasma resulted in an average recovery of 83.4 ± 6.3% (S.E.). Data were calculated using RIAPAC, a program developed for the Wang calculator by Dr. S. Glenn (The University of Texas, Houston, Texas).

Statistics. The significance of differences between treatment groups and the control group was examined by Student’s t test.

RESULTS

The Responses of DMBA-induced Mammary Tumors to Ovariectomy or Antiestrogen Treatment. As reported previously by us (34) and others (8, 14, 17, 21), approximately 85% of DMBA-induced mammary tumors regress in response to ovariectomy or antiestrogen administration. However, some of the tumors that disappear completely in response to ovariectomy or antiestrogen treatment reappear and grow at a later date (Chart 1, A and B), and approximately 15% of tumors show either no response to ovariectomy or antiestrogen treatment (Chart 1, C and D) or only a partial response to these treatments (i.e., growth is arrested or tumor size is decreased, but response is not complete) (Chart 1, E and F). It is with these latter tumors that we wished to determine whether tumors unresponsive or only partially responsive to ovariectomy would be caused to regress by antiestrogen treatment and, conversely, whether tumors unresponsive or only partially responsive to antiestrogen treatment would be further reduced in size by subsequent ovariectomy.

Representative responses of tumors to a subsequent course of antiestrogen treatment or ovariectomy are depicted in Chart 1, C to F. In some cases, tumors unresponsive to ovariectomy (C) or antiestrogen administration (D) did not respond to subsequent antiestrogen treatment or to ovariectomy. Other tumors showing either a partial response (E) or growth stabilization (F) upon ovariectomy or antiestrogen treatment were found to disappear completely upon subsequent antiestrogen or ovariectomy.

These data are quantitated in Table 1. Of the 95 tumors in 45 rats that were ovariectomized, 59% disappeared completely and 15% showed considerable regression within 4 weeks of the ovariectomy. Fourteen % of the tumors continued to grow,
and 12% of the tumors had their growth stabilized or arrested after ovariectomy. Of the 5 tumors growing in ovariectomized hosts that were monitored further, all continued to grow upon subsequent antiestrogen (U 23,469) treatment. Of the 10 tumors whose growth was arrested or stabilized by ovariectomy (Group 1B), 2 were stimulated, 2 remained growth arrested, 2 regressed, and 4 disappeared completely during antiestrogen treatment. Of the tumors regressing after ovariectomy (Group 1C), 30% (3 out of 10) continued to regress further or disappeared during the antiestrogen treatment period, whereas the growth of 6 tumors was enhanced during this period. Finally, of 20 tumors that disappeared following ovariectomy, 90% remained undetectable during the 8-week antiestrogen treatment period, whereas 10% (2 out of 20) reappeared and grew.

Table 1 indicates the responses of DMBA tumors to an initial course of antiestrogen treatment. Fifty % of the tumors disappeared and 15% regressed during the treatment period, whereas 19% of the tumors continued to grow and 16% had their growth stabilized. Of the 4 growing tumors that were monitored further (Group 2A), 3 continued to grow after ovariectomy while the growth of one was arrested. Of 4 tumors stabilized by antiestrogen treatment (Group 2B), subsequent ovariectomy caused the regression or disappearance of 3 of these. Two of 3 regressing tumors (Group 2C) disappeared following ovariectomy, whereas all 6 tumors that disappeared during U23,469 treatment (Group 2D) remained undetectable following ovariectomy.

Although the numbers of tumors are small, the following generalizations seem to emerge. Almost all DMBA-induced tumors that continue to grow following ovariectomy or antiestrogen are unresponsive to subsequent antiestrogen or ovariectomy (Groups 1A and 2A), and some tumors whose growth is stabilized by ovariectomy or by antiestrogen treatment regress or disappear following the other treatment regimen (Groups 1B and 2B).

Influence of Hormonal Treatments on DMBA Tumor Development and Receptor Levels. Table 2 presents data on the efficacy of several nonsteroidal antiestrogens in eliciting tumor regression, as well as the efficacy of high doses of estrogens (17β-estradiol or estradiol benzoate) or ovariectomy. All of the treatments elicited the regression or disappearance of many of the tumors within 2 weeks.

In tumors regressing due to these different treatments (Table 3), prolactin receptor levels are significantly depressed below that of the control. The reduction in the tumor prolactin receptor

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Table 1

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Group</th>
<th>Response after ovariectomy</th>
<th>Response after U 23,469</th>
<th>Repeat and grow</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1A</td>
<td>Grow 12/95 (14)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1B</td>
<td>Stabilize 12/95 (12)</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>1C</td>
<td>Regress 14/95 (15)</td>
<td>6/10</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>1D</td>
<td>Disappear 56/95 (59)</td>
<td>18/20</td>
<td>2/20</td>
</tr>
</tbody>
</table>

Table 2 presents data on the efficacy of several nonsteroidal antiestrogens in eliciting tumor regression, as well as the efficacy of high doses of estrogens (17β-estradiol or estradiol benzoate) or ovariectomy. All of the treatments elicited the regression or disappearance of many of the tumors within 2 weeks.

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---

Table 2

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Group</th>
<th>Response after U 23,469</th>
<th>Response after ovariectomy</th>
<th>Repeat and grow</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>2A</td>
<td>Grow 14/74 (19)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>Stabilize 12/74 (16)</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>Regress 11/74 (15)</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>Disappear 37/74 (50)</td>
<td>6/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rats were given injections of DMBA at 47 to 50 days of age. Animals bearing tumors with a minimum size of 0.6 cm in each dimension (length and width) were ovariectomized, and tumor growth was monitored for 4 weeks; or animals were given injections of the antiestrogen U 23,469 (250 μg s.c. in 0.15 M NaCl daily) for 8 weeks and tumor growth responses were monitored.

<sup>b</sup> Number of tumors showing each response out of the total number of tumors assayed.

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Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>No. of rats</th>
<th>Total no. of tumors</th>
<th>Response after 2 wk treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>12</td>
<td>50</td>
<td>Disappear: 0/50 (10)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16</td>
<td>40</td>
<td>Regress: 4/40 (10)</td>
</tr>
<tr>
<td>U 23,469 (250 µg/day)</td>
<td>3</td>
<td>4</td>
<td>15</td>
<td>Stabilize: 23/40 (58)</td>
</tr>
<tr>
<td>U 11,100A (250 µg/day)</td>
<td>4</td>
<td>11</td>
<td>36</td>
<td>Grow: 9/40 (22)</td>
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<tr>
<td>CI-628 (200 µg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β-Estradiol (50 µg/day)</td>
<td>5</td>
<td>9</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Estradiol benzoate (50 µg/day)</td>
<td>6</td>
<td>5</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>7</td>
<td>6</td>
<td>21</td>
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</tr>
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</table>

- Rats were given injections of DMBA at 47 to 50 days of age. Animals bearing tumors with a minimum size of 0.6 cm in each dimension (length and width) received s.c. injections of antiestrogen, estrogen, or 0.15 M NaCl daily for 2 weeks or were ovariectomized and followed for 2 weeks.
- Number of tumors showing each response out of the total number of tumors monitored.
- Numbers in parentheses, percentage of tumors showing each response.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>E-RNa b</th>
<th>E-Rc</th>
<th>E-RNa + E-Rc</th>
<th>E-Ra + E-Rc (% specific binding)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>80 ± 11c (16)</td>
<td></td>
<td></td>
<td>300 ± 33 (16)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>180 ± 62d (6)</td>
<td></td>
<td></td>
<td>214 ± 62 (6)</td>
</tr>
<tr>
<td>U 23,469 (250 µg/day)</td>
<td>3</td>
<td>120 ± 22e (8)</td>
<td></td>
<td></td>
<td>47 ± 11 (8)</td>
</tr>
<tr>
<td>U 11,100A (250 µg/day)</td>
<td>4</td>
<td>63 ± 7f (4)</td>
<td></td>
<td></td>
<td>109 ± 11 (4)</td>
</tr>
<tr>
<td>CI-628 (200 µg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18 ± 1 (5)</td>
</tr>
<tr>
<td>17β-Estradiol (50 µg/day)</td>
<td>5</td>
<td>75 ± 12g (8)</td>
<td></td>
<td></td>
<td>235 ± 30 (8)</td>
</tr>
<tr>
<td>Estradiol benzoate (50 µg/day)</td>
<td>6</td>
<td>18 ± 10h (5)</td>
<td></td>
<td></td>
<td>66 ± 16 (5)</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>7</td>
<td>132 ± 02i (6)</td>
<td></td>
<td></td>
<td>1.3 ± 0.4 (8)</td>
</tr>
</tbody>
</table>

- Each rat received s.c. injections of antiestrogen or estrogen in 0.5 ml 0.5 M NaCl daily for 2 to 4 weeks. Controls received 0.15 M NaCl alone. The tumors were assayed 19 to 23 hr after the last injection.
- E-Ra, nuclear estrogen receptor; E-Rc, cytosol estrogen receptor; PROL-R, prolactin receptor.
- The difference in bound 125I-labeled prolactin with or without excess cold prolactin expressed as the percentage of total radioactivity added in each tube.
- Numbers in parentheses, number of tumors assayed.
- p < 0.05 versus control value.
- Regression tumors were assayed approximately 2 weeks after ovariectomy.

Development and Receptor Levels. Antiestrogen treatment was found to depress markedly the growth of the ovarian-autonomous but estrogen-sensitive R3230AC rat mammary tumor. Chart 2 shows the average tumor (R3230AC) area per rat in animals receiving different hormonal treatments. U 11,100A (250 µg), CI-628 (100 µg), 3 dose levels of U 23,469 (20, 100, and 250 µg), or estradiol (15 µg) was administered to female Fischer 344 rats within 24 hr after they received R3230AC tumor transplants, and the treatments were continued throughout the entire experimental period. The rats in the ovariectomy group were bilaterally ovariectomized 5 days before receiving the tumor transplant. In animals receiving only 0.15 M NaCl injections (control group), R3230AC tumors began to be palpable in almost all rats 10 days after tumor transplantation, and they grew rapidly thereafter so that the total tumor area per rat reached an average of 20 sq cm by 30 days posttransplant. The rate of tumor growth was reduced markedly by various antiestrogen treatments (U 11,100A, U 23,469, and...
CI-628) or by estradiol administration. In contrast, tumors grew at or above the control rate in ovariectomized hosts, as has been reported previously (16) for this ovarian-independent tumor system. The depressive effect of U 23,469 on tumor growth rate appeared to be dose related, with the highest dose (250 μg) being the most effective, especially at the later stages of tumor growth (30 days after tumor transplantation). Although tumor growth rates differed markedly between groups, body weights were not significantly different between treatment groups or the control, all of which increased from approximately 70 to 140 g over the 45-day period of assay.

In the hopes of determining the possible bases for the depressive effect of antiestrogen and estradiol on tumor growth, estrogen receptor levels were determined in some tumors harvested at 25 days after transplantation. As seen in Table 4, antiestrogen treatments resulted in some changes in estrogen receptor distribution similar to those seen in the DMBA mammary tumor system. For example, treatment with U 23,469 or U 11,100A resulted in a significant reduction of cytoplasmic estrogen receptors and an increase in nuclear estrogen receptor sites, so that over 60% of total receptors were in the nucleus at 19 to 24 hr after the last injection of these antiestrogens. (Note that the control receptor level in R3230AC tumors is approximately 20% that of control DMBA tumors.) Estradiol treatment likewise reduced the level of cytoplasmic receptor very markedly without a corresponding increase in nuclear receptor content, so that total tissue estrogen receptor content was only approximately one-half that of the control level; a similar effect of estradiol treatment was seen in DMBA tumors (Table 3, Group 6). In contrast to the situation seen in DMBA tumors (Table 3, Group 4), however, the antiestrogen CI-628 did not affect the estrogen receptor distribution pattern in the R3230AC tumors, although it did significantly depress tumor growth rate. Ovariectomy (Table 4, Group 6) had no effect on estrogen receptor levels, in agreement with the report of Wittliff et al. (35).

There was also no significant (p < 0.05) difference in plasma prolactin and estradiol levels between any of the treatment groups and the controls (Table 5). Since we found the level of prolactin receptor in control R3230AC mammary tumors to be quite low (approximately 12% that of DMBA tumors), we were not able to measure reliably any possible changes in tumor prolactin receptors in the different treatment groups.

**DISCUSSION**

The studies reported here with DMBA and R3230AC mammary tumors indicate significant differences in their spectrum and degree of responsiveness to additive (hormonal or antihormonal) therapy and endocrine ablative therapy. Antiestrogens depress growth of the R3230AC mammary tumor, although the growth of this tumor is not affected by ovariectomy. Likewise, we find that high levels of estrogen also depress growth, as reported previously by Hilf et al. (15). This tumor is also sensitive to other hormones, with high levels of androgen (15), insulin (4), and prolactin (33) also reducing growth. How-

![Chart 2. The influence of treatment with antiestrogens or estrogen on the growth of R3230AC mammary tumors. Tumors were transplanted into intact female Fischer 344 host rats, and, beginning on the day after tumor transplantation, rats received the indicated daily doses of antiestrogens or estradiol (E2). In one group, tumors were transplanted into host rats that had been ovariectomized (OVX). The antiestrogens utilized are U 23,469 (U-23), U 11,100A (UA), and CI-628.](image-url)
ever, although antiestrogens markedly reduce the growth of R3230AC tumors, these agents do not elicit tumor regression or the complete suppression of tumor development, as is seen in the DMBA tumor system (this report and Ref. 34). Also, R3230AC tumor growth is suppressed most effectively when treatment is started at the time of tumor transplantation; growth is slowed only slightly if antiestrogen administration is begun at 2 weeks after tumor transplantation. This is in keeping with the observation of Hilf et al. (15) that sensitivity to estrogen treatment decreases as R3230AC tumors become established.

In contrast to the R3230AC mammary tumors, the large majority (approximately 85%) of DMBA-induced mammary tumors are ovarian dependent for their growth, and many are effectively suppressed by antiestrogen treatment or by large doses of estrogen (Table 2). However, it is well known that DMBA tumors show considerable variability in terms of their hormonal dependence or responsiveness (9, 25, 30). Of the small proportion of DMBA tumors that are ovarian unresponsive (Table 1, Group 1A), our data, with a small number of such tumors, indicate that they usually do not respond to subsequent antiestrogen and, likewise, that antiestrogen-insensitive tumors (Table 1, Group 2A) rarely are arrested by subsequent ovariectomy. In addition, some tumors that are growth arrested or stabilized by ovariectomy (Table 1, Group 1B) or antiestrogen treatment (Table 1, Group 2B) may be further benefited by subsequent antiestrogen or endocrine ablative treatment. The extent to which antiestrogens are capable of inducing decreases in tumor size beyond that achieved by ovariectomy may be a reflection of their ability to antagonize the low levels of endogenous estrogens (e.g., adrenal estrogens) that still may be present after ovariectomy.

An aim of this study was to investigate the possible mechanisms underlying the effects of antiestrogens on tumor growth modulation in the DMBA and R3230AC mammary tumor systems. During DMBA tumor regression by the 3 antiestrogens we have studied, prolactin receptor was reduced in all cases [as also reported by Kelly et al. (21) for the steroidal antiestrogen RU 16117]. Likewise, estrogen receptor distribution is perturbed by antiestrogens in a manner such that much of the receptor is found in the nucleus with low levels of cytoplasmic receptor. Of interest, also, is the observation that continuous exposure to estradiol appears to decrease the total tumor content of estrogen receptor (Table 3), a finding reported also in studies by Manni et al. (27). Hence, changes in both estrogen receptors and prolactin receptors may account for growth inhibition in this tumor system. Kledzik et al. (22) and Smith et al. (32) have also reported that high levels of estrogen depress prolactin receptors in this tumor.

In R3230AC tumors, however, growth is markedly depressed but not completely eliminated by antiestrogens or estrogen. With 2 of the antiestrogens (U 23,469 and U 11,100A), there is the suggestion that estrogen receptor distribution changes (Table 4) parallel those seen in the DMBA tumor system. However, this was not always the case, inasmuch as Cl-628, which effectively reduced tumor growth (Chart 2), did not alter the subcellular distribution of estrogen receptor (Table 4). There was also no effect of any of these antiestrogen treatments on the blood level of estradiol and little, if any, decrease in the blood level of prolactin.

Smith et al. (33) have reported that very high doses of estradiol valerate (7.5 mg/kg/week in oil) slightly decrease (by 20 to 25%) prolactin receptor levels in R3230AC tumors. However, lower doses of estradiol valerate (0.5 mg/kg/week) which do not reduce prolactin receptor levels (33) are effective in converting the tumor to a lactation-like morphology (23). Hence, the data suggest that the basis of the ovarian autonomy, yet estrogen responsiveness and antiestrogen sensitivity, of the R3230AC mammary tumor is likely complex with possibly subtle interrelationships between levels of estrogen and prolactin receptors and blood levels of these hormones. It is also possible that other hormones, such as insulin (4), which may be involved in growth regulation of breast cancer, may influence the effects of antiestrogens in this tumor.

One hypothesis that has been raised to account for the differences in hormonal dependence of mammary tumors suggests that hormonal dependence is related to the level of hormone receptors in the tumor. Earlier studies have suggested that the markedly lower levels of estrogen receptor (found to be approximately 20% that of DMBA tumors in this report and in Ref. 29) and prolactin receptor (found to be approximately 12% that of DMBA tumors in this report and in Refs. 32 and 33) in R3230AC tumors, as compared with the DMBA-induced mammary tumor, may account for the ovarian autonomy yet estrogen responsiveness of this tumor. This concept is also supported by the report of reduced prolactin receptor and estrogen receptor levels in a hormone-independent transplantable mammary tumor (MTW9) compared with its hormone-dependent counterpart (7). However, even in the DMBA mammary tumor system where prolactin and estrogen receptor levels are, as a rule, considerably higher than in R3230AC tumors and where there is generally a good correlation between receptor levels and hormonal responsiveness, absolute correlations between levels of estrogen and prolactin receptors and hormonal dependence frequently do not occur (9). In addition, our observations that antiestrogens and estrogen interact with estrogen receptors and modulate the subcellular distribution and level of estrogen receptors in R3230AC tumors, while ovariectomy does not appear to influence these parameters, may explain the estrogen and antiestrogen sensitivity but ovarian autonomy of this tumor.

Since human breast cancers cover a broad spectrum in terms of their hormonal dependence, the DMBA and R3230AC mammary tumor systems appear to represent at least 3 portions of the spectrum: Class 1, ovarian-dependent tumors which are effectively regressed by antiestrogens; Class 2,
ovarian-independent tumors [such as some DMBA tumors (Table 1, Group 1A)] which are largely unresponsive to antiestrogens; and Class 3, ovarian-independent tumors (such as the R3230AC tumor) whose growth is depressed by antiestrogens. It is possible that tumors of Class 3, represented in this study by the R3230AC tumor model, may derive some palliative benefit from antiestrogen treatment despite a lack of responsiveness to endocrine ablative therapy.

ACKNOWLEDGMENTS

We are grateful to Dr. Bogden of the Mason Research Institute for supplying us with donor rats bearing R3230AC tumors, to Drs. Janice Bahr and Victor Ramirez for assistance with estradiol and prolactin radioimmunoassays, to the Parke-Davis and Upjohn Companies for providing us with antiestrogens, and to the Upjohn Company for the supply of DMBA emulsions. We also thank the National Institute of Arthritis, Metabolism, and Digestive Diseases Hormone Distribution Program for providing us with ovine prolactin.

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Antiestrogen Modulation of the Growth and Properties of Ovarian-Autonomous and Ovarian-Dependent Mammary Tumors in Rats

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