Estradiol Stimulation and Inhibition of Cell Growth in New Estrogen-sensitive Cell Lines and Tumors Established from the MtTF4 Tumor

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ABSTRACT

Cell lines were established from the MtTF4 tumor, growth of which is inhibited by estradiol, in order to determine whether the effect observed in vivo was due to a direct action on tumor cells. Two different cell lines were obtained according to the medium in which tumor cells were dispersed and cultured. The F,P cells were obtained when the culture medium contained charcoal-treated fetal calf serum. The growth rate of these cells was slowed down by 17β-estradiol in animals and also in culture during the early passages. Thereafter, they became insensitive to 17β-estradiol in culture but remained negatively controlled in vivo. These cells, whatever their sensitivity to 17β-estradiol, secrete prolactin and carry functional D2 dopamine binding sites. The F2Z cells were established in medium containing fetal calf serum not treated with charcoal. The growth rate of these cells was stimulated by 17β-estradiol in animals but was 17β-estradiol insensitive in culture up to subculture 26. At this time, the growth rate of the subline also became stimulated by 17β-estradiol in culture, and this phenotype was still found at passage 108 (50% effective dose, 5 to 10 pmol; maximum stimulation, 180 to 300% of control). These cells neither secrete measurable amounts of prolactin nor have dopamine binding sites.

Thus, according to the medium in which cells were dispersed and cultured, two different cell strains were derived from a tumor in which growth is inhibited by 17β-estradiol. The point of interest is that the growth rate of one strain was inhibited by 17β-estradiol, while the other was stimulated. Convergent data suggest that MtTF4 tumor was heterogeneous and that selection had occurred during the dispersion or the culture of cells. Since the growth of one of these cell lines was slowed down transiently in culture we conclude that the inhibition of tumor growth could be due to a direct action of 17β-estradiol on tumor cells. However, the dissociation between the response to 17β-estradiol in culture and in the animal observed at some time of cell evolution suggests that environment affects the sensitivity of cells to 17β-estradiol.

INTRODUCTION

We have shown that 17β-estradiol inhibits the growth of the MtTF4 tumor of rat pituitary origin. Such an effect was surprising, since this tumor was induced by chronic treatment with the synthetic estrogen diethylstilbestrol. This drug was also shown to inhibit the growth of another rat pituitary tumor, the MtTW15 tumor (3), and that of a tumor developed from rat Leydig cells (4) and of pluripotent hemopoietic stem cells (5). Moreover, estrogen treatment has allowed objective remissions of breast tumors in postmenopausal women (6). Since the inhibitory effect of 17β-estradiol on cell growth does not appear just anecdotal and although, due to side effects, the estrogen treatment of breast cancer has been discontinued, we decided to analyze further the action of 17β-estradiol on MtTF4 tumor cells.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: FCS, fetal calf serum; CT-FCS, charcoal-treated fetal calf serum; PRL, prolactin; EDA50, 50% effective dose; PBS, phosphate-buffered saline; RIA, radioimmunoassay; IC50, 50% inhibitory concentration.
before the cells were maintained in culture only. For cloning, a diluted suspension of cells was dispensed in 96-well plates (Falcon No. 3072; Becton Dickinson, Oxnard, CA). Only single cell clones were allowed to grow. Cell lines were shown to be free of Mycoplasma contamination by DNA staining with the fluorescent dye bisbenzimidazol (Seromed).

Control of Cell Growth in Culture. Seeding was performed in 96-well plates (Falcon No. 3072) or 24-well plates (Falcon No. 3047). In six experiments (Fig. 1) the bottom of the wells had previously been covered with extracellular matrix produced by endothelial cells of bovine cornea (7, 8). Generally, cells were seeded and cultured, without any change, in RPMI 1640 medium supplemented with 5% CT-FCS and antibiotics. Hormones were added in ethanol. Ethanol concentrations were the same (±0.01%) in control and 17ß-estradiol-treated cells. All the results shown in the figures were obtained with phenol red-containing medium, but some experiments described in "Results" were performed in phenol red-free medium. The cells were harvested by trypsin-EDTA treatment and counted with a Coulter Counter (Model ZB-ZBI; Coultronics) in triplicate.

Control of Cell Growth in Animal. Cells (=2 × 10⁶) harvested at different subcultures or dispersed from tumors were injected s.c. into adult male Fisher rats. Rats were allocated randomly to two groups. The 17ß-estradiol-treated rats received an implant of Silastic tubing (1 cm long, catalogue No. 602-265; Dow Corning Corp., Midland, MI) filled with 17ß-estradiol. We assessed the interval between cell injection and the time at which a tumor became palpable at the site of injection, the ratio between rats with and without palpable tumors at increasing periods of time following cell injection, tumor weight, and finally the mitotic index. Due to the large number of experiments required to define the stability of the phenotype, we generally used a limited number of rats per group (n = 5). For mitotic index assays, tumor slices were applied to microscope slides, and the attached cells were fixed with ethanol/ether (1/1) and stained with May-Grünwald-Giemsa. One thousand cells were examined.

Estrogen Receptor Assays. Two methods were used. In culture, 17ß-estradiol receptors were assayed on whole cells (9). Briefly, cells were cultured in RPMI 1640 medium containing 5% CT-FCS for 3 days, harvested after trypsin-EDTA treatment, and carefully washed twice with RPMI 1640 medium. Cells in suspension (10⁶ cells in 0.8 ml) were incubated with increasing concentrations of 17ß-[³H]estradiol in the presence or absence of 100-fold excess of nonradioactive diethylstilbestrol (Sigma) for 1 h at 37°C in 95% air-5% CO₂. Then, cells were centrifuged for 5 min at 200 × g, dispersed in the same medium, and kept for 10 min at 37°C to allow dissociation of loosely bound hormones. A second run of centrifugation-wash was performed before counting radioactivity.

The 17ß-estradiol receptors contained in the cytosol prepared from tumors were measured (1). Results are given in fmol/mg of cytosol protein. Radioactivity was counted in Scintillator 299 (Packard) with an efficiency ±50%. The specific binding was the difference between the 17ß-[³H]estradiol bound in the absence and presence of diethylstilbestrol.

![Fig. 1. Phase-contrast microscopic features of F4Z cells (a to d) and F4P cells (e and f). a and b, heterogeneity of F4Z cells at passage 22 (arrow, myoid cell); c and d, two aspects of F4Z cells at passage 45; e, clusters of loosely attached F4P cells at passage 12 cultured on plastic; f, the same cells as in e cultured on plastic coated with extracellular matrix. × 150.](image-url)
Affinity Labeling of Estrogen Receptors. Estrogen receptors were covalently labeled and analyzed (10). Briefly, cytosol was incubated for 2 to 6 h at 4°C with [3H]tamoxifen aziridin (=2 to 20 nmol; Amersham International, Buckinghamshire, England) in the absence or presence of 1000-fold excess diethylstilbestrol. Proteins were precipitated with 66% acetone (v/v) or ammonium sulfate (30% saturation) and separated by 7.5% polyacrylamide gel electrophoresis in denaturing and reducing conditions. X OMAT S films (Kodak) was exposed to dried gels at -70°C for 13 to 20 days. The percentage of inhibition was calculated by taking as reference the number of cells cultured in control medium.

D2 Dopamine Receptor Assays. Two methods were used. In tumors, assays were performed on partially purified cell membranes (11). In culture, these receptors were assayed on whole cells (12). Briefly, cells harvested with PBS-EDTA were suspended in 120 mm NaCl:5 mm KCl:2 mm CaCl2:1 mm MgCl2:50 mm Tris-HCl (pH 7.4) and then were incubated with increasing concentrations of [3H]NPSpiperone in the presence or absence of 100-fold excess of (-)-butaclamol for 12 min at 37°C. Cells were recovered on filters (GF/C, 0.45 µm; Whatman) and washed at 4°C (3 times with 5 ml of incubation buffer). Radioactivity was counted as above.

Prolactin Assays. This hormone was measured (13) in serum of tumor-bearing rats (blood was collected after decapitation less than 2 min after the beginning of anesthesia with ether) and in conditioned medium stored at -20°C for less than 1 mo. The material and the protocol used were supplied by the Rat Pituitary Hormone Distribution Program (Dr. A. F. Parlow, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH.

Estradiol Assays. RIAs were performed by M. G. Forest (14) using as competitor the material extracted from 0.1 and 0.4 ml of FCS and CT-FCS.

Results are given as the mean ± SD, and statistical significance was calculated by Student’s t test.

RESULTS

Cells Established and Cultured in Medium Containing Charcoal-treated Fetal Calf Serum (F4P Cells). Six preparations of F4P cells were made, but the majority of the work was performed with F4P1 cells. Other F4P cells were used to confirm, complete, or extent observations made in F4P1 cells. These cells were round and grew slightly attached to the plastic (Fig. 1, e and f). They formed clumps which tended to be free-floating spontaneously or when the flask was slightly shaken as reported for other pituitary tumor cells (15). A confluent monolayer layer was never obtained. No evident morphological change was observed through serial subcultures. When attached to extracellular matrix they tended to have a spindle shape (Fig. 1f). The doubling time in the presence of the optimal concentration of CT-FCS (5%) was 42 h on a plastic surface and 36 h on an extracellular matrix (not shown).

The growth of F4P cells in culture was inhibited by 17β-estradiol (100 nmol) with an efficiency which decreased progressively until passage 13 (Fig. 2). A similar inhibition during the early subcultures was observed with five other preparations of F4P cells, but we did not attempt to follow the evolution of the response to 17β-estradiol of these cells through serial subcultures. The inhibition was dose dependent (ED50, 5.0 nmol between passages 2 and 5) and was prevented by 4-hydroxy-trans-tamoxifen (Fig. 3). 17β-Estradiol inhibited also the growth of cells cultured on extracellular matrix (Fig. 2, ▵ and ▼).

The growth of F4P tumors that developed at the site of injection of F4P cells was also slowed down by 17β-estradiol. Indeed, tumors appeared later in 17β-estradiol-treated rats than in controls (Table 1) regardless of the 17β-estradiol responsiveness of cell growth in culture (stimulation of cells at subcultures 3 to 12 or insensitivity of cells at subcultures 27 and 29). There was some variability in the time at which a tumor appeared from one experiment to another; e.g., no tumor was palpable during a 6-mo period after injection of cells at subcultures 7 and 11 in 17β-estradiol-treated rats, while a tumor was palpable 2 wk after injection of cells at passages 27 and 29. The second way to define the action of 17β-estradiol on tumor growth was to compare the weight of tumors at the same time after cell injection, in control and 17β-estradiol-treated rats (Table 2). The weight of the tumors that developed from either F4P cells maintained for an increasing number of subcultures or cells dispersed from F4P1 tumors was lower in 17β-estradiol-treated rats than in control rats. The action of 17β-estradiol on tumor growth was confirmed by the decrease of mitotic index in tumors of 17β-estradiol-treated rats (Table 2). A marked inhibition was also observed with other F4P strains (F4P2, Tables 1 and 2; F4P3, Table 2). Thus, according to three criteria, 17β-estradiol slowed down F4P tumor growth, and this phenotype was stable through both serial cell subcultures (up to 29) and serial tumor transplantations (up to 6 transplantations of a
between subcultures 10 and 40, the number of binding sites and relative binding affinity for various competitors. In whole cells sites with features of D2 dopamine receptors according to the dopamine receptors (11), we looked for such receptors in both receptor-negative cells. Figure 4 showed that the tumors grown from receptor-positive cells contained a number of binding sites (28 and 30 fmol/mg of protein) of high affinity (\(K_a = 52,000\)) estrogen receptors (Fig. 4). The cytosol of tumors containing \(\beta\)-estradiol was specifically bound to proteins of molecular weight 37,890, and dopamine were similar to those found (not shown) in tumors developed from cells between subcultures 10 and 29, the number of binding sites was 198 ± 390, and dopamine were similar to those of tumors grown from a Silastic implant, and the other group (control) was not treated. The ratio between rats with a palpable tumor and rats alive at the end of the experiment in the group was determined every week from 2 or 3 wk after cell injection. The absence of a ratio indicates that all the rats of the group were killed because all of them carried a tumor.

### Table 1 17β-Estradiol inhibition of F4P tumor growth (kinetics of appearance of a palpable tumor)

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Subculture no.</th>
<th>Passage no. in rats</th>
<th>Tumor wt (g)</th>
<th>Mitotic index (%)</th>
<th>Duration of treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4P1</td>
<td>3</td>
<td>Controls</td>
<td>15.4 ± 6.6 (5)</td>
<td>2.8 ± 2.6 (6)</td>
<td>46 ± 4 (3)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Estradiol treated</td>
<td>6 ± 2.4 (6)</td>
<td>4.3 ± 3.9 (5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Controls</td>
<td>8.1 ± 5.6 (6)</td>
<td>1.7 ± 1.1 (6)</td>
<td>52 ± 9 (6)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Estradiol treated</td>
<td>4.8 ± 2.1 (5)</td>
<td>1.9 ± 1.3 (6)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Controls</td>
<td>4.1 ± 13.5 (4)</td>
<td>10.8 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>Estradiol treated</td>
<td>11.4 ± 3.4 (5)</td>
<td>4.9 ± 1.7 (4)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>Controls</td>
<td>4.9 ± 2.3 (8)</td>
<td>1.8 ± 1.5 (9)</td>
<td>ND</td>
</tr>
<tr>
<td>F4P2</td>
<td>2</td>
<td>Controls</td>
<td>13.2 ± 8.7 (8)</td>
<td>3.9 ± 3.2 (6)</td>
<td>ND</td>
</tr>
<tr>
<td>F4P3</td>
<td>1</td>
<td>Estradiol treated</td>
<td>18.3 ± 10 (5)</td>
<td>0.68 ± 0.4 (5)</td>
<td>ND</td>
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</table>

* Mean ± SD.

### Table 2 17β-Estradiol inhibition of F4P tumor growth (tumor weight and mitotic index)

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Subculture no.</th>
<th>Passage no. in rats</th>
<th>Tumor wt (g)</th>
<th>Mitotic index (%)</th>
<th>Duration of treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4P1</td>
<td>10</td>
<td>Controls</td>
<td>15.4 ± 6.6 (5)</td>
<td>2.8 ± 2.6 (6)</td>
<td>46 ± 4 (3)</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>Estradiol treated</td>
<td>6 ± 2.4 (6)</td>
<td>4.3 ± 3.9 (5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>Controls</td>
<td>8.1 ± 5.6 (6)</td>
<td>1.7 ± 1.1 (6)</td>
<td>52 ± 9 (6)</td>
</tr>
<tr>
<td>F4P2</td>
<td>2</td>
<td>Controls</td>
<td>4.8 ± 2.1 (5)</td>
<td>1.9 ± 1.3 (6)</td>
<td>ND</td>
</tr>
<tr>
<td>F4P3</td>
<td>1</td>
<td>Estradiol treated</td>
<td>4.1 ± 13.5 (4)</td>
<td>10.8 ± 10</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of rats alive at the end of experiments.

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Tumor developed from cells at subculture 29).

Saturation experiments were used to look for estrogen receptors. High-affinity (\(K_a = 0.16 ± 0.10\) nmol), low-capacity (961 ± 357 sites/cell) (\(n = 4\)) 17β-estradiol binding was found in whole cells between subcultures 2 and 5, but we were not able to detect any saturable binding thereafter (\(n = 6\)). On this basis, cells were considered to be 17β-estradiol receptor positive or negative. Surprisingly, we found, in the cytosol of tumors that developed from receptor-negative cells, high-affinity (\(K_a = 0.16 ± 0.11\) nmol), low-capacity (34 ± 11 fmol/mg of cytosol protein) (\(n = 9\)) 17β-estradiol binding which was very likely due to the presence of estrogen receptors. Indeed, \(\beta\)tamoxifen aziridin was specifically bound to proteins of molecular weight similar to those of native (MW 68,000) or proteolysed (MW 52,000) estrogen receptors (Fig. 4). The cytosol of tumors grown from receptor-positive cells contained a number of binding sites (28 and 30 fmol/mg of protein) of high affinity (\(K_a = 0.24 ± 0.18\) nmol) similar to those of tumors grown from receptor-negative cells.

Since we had shown that the MtTF4 tumor contained D2 dopamine receptors (11), we looked for such receptors in both F4P1 cells and F4P2 tumors. Both cells and tumors contained a single class of high-affinity, low-capacity \(\beta\)spiperone binding sites with features of D2 dopamine receptors according to the relative binding affinity for various competitors. In whole cells between subcultures 10 and 40, the number of binding sites and \(K_a\) at 37°C were stable (139 ± 56 fmol/mg of protein, 0.18 ± 0.07 nmol, \(n = 12\)). The results of a typical saturation experiment are given in Fig. 5. The IC\(_{50}\) values of \(\beta\)-butaclamol, bromocriptine, the D1 antagonist SCH 23 390, and dopamine were, respectively, 0.02, 0.1, 3, and 5 \(\mu\)mol (Fig. 6). In crude membranes of F4P tumors that developed from cells between subcultures 10 and 29, the number of binding sites was 198 ± 54 fmol/mg of protein, and the \(K_a\) was 0.2 ± 0.1 nmol (\(n = 26\)). The IC\(_{50}\) values of \(\beta\)-butaclamol, bromocriptine, SCH 23 390, and dopamine were similar to those found (not shown) in cultured F4P cells (0.01, 0.5, 3, and \(\pm 5\) \(\mu\)mol). In order to determine whether D2 dopamine binding sites were functional we analyzed the effects of bromocriptine, a dopamine agonist, on cell growth and on PRL accumulation in conditioned medium. Cell proliferation was slowed down (Fig. 7A), and the accumulation of immunoreactive PRL-like material was decreased (Fig. 7B) in a dose-dependent fashion. The binding curve of labeled rat PRL in the presence of increasing amounts of conditioned medium paralleled the standard curve, and one of the major secreted proteins comigrated with ovine PRL in polyacrylamide gel electrophoresis in denaturing conditions (not shown). The inhibitory effects produced by bromocriptine at 1 nmol (Fig. 7A) or 100 nmol (Fig. 7B) were prevented by the addition of 100 \(\mu\)mol d-butaclamol. D2 receptors were still found at subculture 40.

Cells Established and Passed in Medium Containing Fetal Calf Serum Not Treated with Charcoal (F4Z Cells). F4Z cells

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* NE, not examined.
were established from one MtTF4 tumor. We assigned the designations F4Z1 to those which were 17β-estradiol insensitive in culture and F4Z2 to those which became 17β-estradiol responsive in culture. F4Z1 and F4Z2 grew firmly attached to plastic but presented different morphological features. The F4Z1 cell line (Fig. 1, a and b) was a mixture of two major types of cells: one with long and narrow extensions and the other one smaller, more refringent, and rounded. A few very large cells (arrow in Fig. 1a) were also found. The F4Z2 subline was also heterogeneous. It included mainly two types of cells. Some were small, dark, and angular (Fig. 1c), and others were amoeba-like with large nuclei and numerous nucleoli (Fig. 1d). The doubling time of F4Z2 cells in the RPMI 1640 medium containing 5% CT-FCS was 49 ± 7 h in the absence of 17β-estradiol and decreased to 39 ± 5 in the presence of 1 nm 17β-estradiol (n = 6 between subcultures 28 and 65).

In culture, the 17β-estradiol sensitivity of F4Z cell growth varied through serial passages. F4Z2 cells were insensitive to 17β-estradiol (between 0.01 and 100 nmol) whatever the culture conditions, i.e., RPMI 1640 medium without any supplementation or supplemented with 5% FCS, 5% CT-FCS, 5% gelding serum, or 1% steroid-free serum substitute (ULTROSER SF; IBF, Villeneuve-la-Garenne, France) in the presence or absence of extracellular matrix. At passage 26, without any detectable change in the cell environment, a subset of F4Z1 cells became stimulated by 17β-estradiol and was designated F4Z2. This stimulation was observed with RPMI 1640 medium supplemented with CT-FCS (Fig. 8B) but not after addition of FCS (Fig. 8A). The highest response was observed in the presence of 5% CT-FCS, and this concentration was thereafter used. In serum-free medium, 17β-estradiol had no effect on F4Z2 cells, while in the same conditions the proliferation of MCF-7 cells was slightly stimulated by 17β-estradiol (1.4-fold increase after 7 days of culture with 1 nmol). In standard conditions (5% CT-FCS and 7 days of treatment), the effect of 17β-estradiol was dose dependent (ED50, 5 to 10 pmol), and the plateau (180 to 300% of control) was reached between 0.1 and 1 nmol. A typical response is shown in Fig. 9. F4Z2 cells were found responsive to 17β-estradiol up to the current passage 108.
ESTROGEN RESPONSIVENESS OF PITUITARY TUMOR CELLS

Fig. 8. Action of 17β-estradiol (10 nmol) on F4Z2 cell growth: search for the best conditions for stimulation. Cells (5 × 10^4/well) at subculture 46 were seeded in 96-well plates, cultured in RPMI 1640 medium supplemented with increasing percentages of either PCS (A) or CT-FCS (B) for 7 days in the absence (•) or presence (○) of 17β-estradiol (10 nmol), and counted. Points, mean; bars, SD (n = 3).

Fig. 9. 17β-Estradiol (E2) stimulation of F4Z1 cell growth: dose response. Cells (5 × 10^4/well) at subculture 32 were seeded in 96-well plates, cultured in RPMI medium containing 5% CT-FCS in the absence (C, ○) or presence of increasing concentrations of 17β-estradiol (●) for 7 days, and counted. Points, mean; bars, SD (n = 3).

Fig. 10. 17β-Estradiol (E2) stimulation of F4Z2 cell growth: effect of one concentration of 4-hydroxytamoxifen on dose response. Cells (5 × 10^4/well) at subculture 39 were seeded in a 96-well plate, cultured in RPMI 1640 medium supplemented with 5% CT-FCS for 7 days in the presence of vehicle (C) or increasing concentrations of 17β-estradiol in either the absence (●) or presence of 4-hydroxytamoxifen (4-OH-TAM; 0.1 μmol, ○), and counted. Points, mean; bars, SD (n = 3).

However, we compared dose responses to 17β-estradiol of F4Z2 cells grown for 7 days in the absence and presence of phenol red (8 mg/ml). The presence of dye modified neither the amplitude of the stimulation nor the ED50 (results not shown of two series of experiments).

The growth of tumors that developed from F4Z1 cells (insensitive to 17β-estradiol in culture; Tables 3 and 4) or F4Z2 cells (stimulated by 17β-estradiol in culture; Table 5) was stimulated by 17β-estradiol. Indeed, in rats treated with 17β-estradiol, F4Z1 tumors appeared earlier (Table 3), and tumor weight and mitotic index were higher than in controls (Table 4). Similar effects on tumor weight were observed with 10 clones of the F4Z1 cells (not shown) and with F4Z2 cells (Table 5). The stimulation by 17β-estradiol of tumor growth was observed in all the experiments performed, but its magnitude was rather variable. However, this phenotype was stable since it was found in tumors serially transplanted from a tumor that developed from either F4Z1 cells at subcultures 9, 23, and 35 (Table 4) or F4Z2 cells at subculture 43 (Table 5).

Both F4Z1 and F4Z2 cells contained a single class of high-affinity 17β-estradiol binding sites, possibly estrogen receptors. There was no evident difference between the Ke (0.14 ± 0.03 versus 0.19 ± 0.11 nmol) in the two types of cells, but the number of binding sites per cell was significantly different (P ≤ 10^-4): 920 ± 300 in F4Z1 cells (n = 7) versus 6700 ± 4000 in F4Z2 cells (n = 15). Tumors that developed from the two types of cells contained a similar number of binding sites (32 and 25 fmol/mg of protein in F4Z1 tumors versus 74 ± 43 fmol/mg of protein in F4Z2 tumors) (n = 9) of similar affinity (0.14 nmol in two F4Z1 tumors versus 0.20 ± 0.12 nmol in F4Z2 tumors).

No D2 dopamine binding site was found in either F4Z1 cells or F4Z2 cells or in membranes of tumors that developed from either type of cells. PRL was below the level of detection in medium conditioned by these cells (0.1 ng/ml), and its concentration in serum of rats bearing tumors that developed from these cells was not significantly different from that of controls (35 ± 16 versus 18 ± 9 ng/ml, n = 8).

DISCUSSION

We shall address three aspects: the particularities of the two cell lines we established; the reasons why such different cells could be obtained; and the plasticity of the phenotype according to the cell environment.

Two cell lines (F4Z and F4P) were established from the MTTF3 experiments, 3 yr after primary culture. As expected, the 17β-estradiol effect was prevented by the antiestrogen 4-hydroxytamoxifen (Fig. 10). Since phenol red has an estrogen-like effect on MCF-7 cell growth (16) we compared the number of F4Z2 cells grown for 7 days in phenol red-free RPMI 1640 medium containing 5% CT-FCS with those grown in the same medium supplemented with increasing concentrations of phenol red (8 to 16 mg/ml). In two series of independent experiments the addition of phenol red did not increase the cell number. Moreover,
In the group treated with 17ß-estradiol in a Silastic implant, and the other group was not treated (controls). The ratio between rats with a palpable tumor and rats alive at the end of the experiment in the group was determined every week from 2 wk after cell injection. The absence of a ratio indicates that all of the rats of the group were killed because they carried a tumor.

<table>
<thead>
<tr>
<th>Subculture no.</th>
<th>Passage no. in rats</th>
<th>Tumor wt (g) Controls</th>
<th>17ß-Estradiol treated</th>
<th>Mitotic index (%) Controls</th>
<th>17ß-Estradiol treated</th>
<th>Duration of treatment (days)</th>
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<td>3.9 ± 3.0 (5)*</td>
<td>15.4 ± 4.8 (4)†</td>
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<td>11</td>
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<tr>
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<td>3.4 ± 1.2 (10)</td>
<td>8.8 ± 3.2 (10)</td>
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<tr>
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<td>35</td>
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<td>48 ± 5 (9)†</td>
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* Mean ± SD.
† Numbers in parentheses, number of rats alive at the end of the experiment.
‡ P ≤ 0.005.
§ ND, not determined.
* P ≤ 0.05.
† P ≤ 0.0005.

The experiment was similar to that described in the legend of Table 4 but performed with F4Z2 cells.

<table>
<thead>
<tr>
<th>Subculture no.</th>
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<th>Duration of treatment (days)</th>
<th>Tumor weight (g) Controls</th>
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</thead>
<tbody>
<tr>
<td>35</td>
<td>1</td>
<td>28</td>
<td>3.4 ± 2.7* (5)</td>
<td>6.68 ± 1.5 (5)‡</td>
</tr>
<tr>
<td>43</td>
<td>1</td>
<td>20</td>
<td>0.74 ± 0.5 (4)</td>
<td>2.09 ± 0.64 (3)†</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
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<td>3.9 ± 2.4 (5)†</td>
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<td>3</td>
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<td>26</td>
<td>1.02 ± 0.8 (5)</td>
<td>4.6 ± 3.2 (6)‡</td>
</tr>
</tbody>
</table>

* Mean ± SD.
† Numbers in parentheses, number of rats alive at the end of experiments.
‡ P ≤ 0.05.

The effects of 17ß-estradiol on tumor growth and mitotic index were also determined in Table 4. The tumor weight and mitotic index were measured at the end of treatment. The experiment was similar to that described in the legend of Table 4 but performed with F4Z2 cells.

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‡ P ≤ 0.05.

The reasons why such different cells (F4Z2 and F4P) could be established from the MtTF4 tumor remain to be elucidated. However, since at least one of their characteristics, the 17ß-estradiol sensitivity of their growth rate, D2 dopamine receptor concentration, and the ability to bind to plastic surfaces. The growth of F4P cells was transiently inhibited by 17ß-estradiol (ED50, 5 to 10 pmol), and this effect was also blocked by 4-hydroxytamoxifen. To the best of our knowledge this is the second cell line of pituitary origin in which growth is strongly stimulated by 17ß-estradiol. It was shown that GH4C1 cells, a subline of GH cells cloned from MtT15 tumor (19), were indeed stimulated by 17ß-estradiol in either a special gelding serum (20) or serum-free medium (21). This response to 17ß-estradiol is reminiscent of that of prolactinocytes in vivo, but F4Z2 cells neither secrete any measurable amount of PRL nor carry D2 dopamine receptors. Conversely, the cell line F4P in which growth was inhibited by 17ß-estradiol both in vivo and in vitro did secrete PRL and carry the dopamine binding sites. A single type of high-affinity binding sites for [3H]spiperone, a ligand known to bind preferentially to D2 dopamine binding sites, was found. In addition, the dopamine agonist bromocriptine inhibits both basal PRL release and cell proliferation. This is the first time that such a cell line has been described. Indeed, in the MMQ cell established from the 7315a rat pituitary tumor, dopamine inhibits the secretagogue-induced but not the basal PRL release (22), and the dopamine sensitivity of cell proliferation has not been shown. F4P cells and MMQ cells appear to be complementary to the transfected GH4C1 cells (23) in further studies on the mechanisms of action of dopamine.
estradiol sensitivity in rats, was stable through serial subcultures and since tumors were frequently heterogeneous, a selection of cells seems to be more probable than changes occurring early after cell dispersion from tumors. The heterogeneity of the MtTF₄ tumor cells has been suggested by in situ studies (13); e.g., while some cells were immunoreactive with PRL antibodies, others were not and, although 17β-estradiol dramatically decreased the mitotic index, residual mitoses were observed. The two protocols used in the present work might have led to the selection of two different populations of cells (24). The classic protocol, which includes the use of medium supplemented with FCS and the alternating passages of cells, in animal and in culture, allowed us to establish cells growing firmly attached to plastic where their growth is stimulated by 17β-estradiol. The protocol associating the use of medium supplemented with CT-FCS and the absence of alternating passages of cells in animal and in culture allowed us to establish cells growing loosely attached to plastic and inhibited by 17β-estradiol. It is impossible to determine whether charcoal treatment or cell injection into rats was responsible for cell selection in our study, but it is possible to examine if there is a correlation between 17β-estradiol concentration in culture medium and the type of cells obtained. The 17β-estradiol contained in medium supplemented with 5% FCS (2.5 pmol) might be involved in the selection of cells in which proliferation was stimulated by 17β-estradiol, since such a low concentration was able to stimulate cell proliferation (Figs. 9 and 10). However, in such a hypothesis other factors are, no doubt, involved since F₄Z₂ cells were not responsive to 17β-estradiol in serum-free medium and F₄Z cells, although stimulated by 17β-estradiol in vivo, were insensitive to 17β-estradiol in culture. Such a discrepancy between the 17β-estradiol responsiveness in culture and in vivo has already been reported, e.g., in MCF-7 cells (25). Concerning the F₄P cells it is improbable that the low 17β-estradiol concentration (0.9 pmol) in medium containing 5% CT-FCS was responsible for the selection of cells in which growth was inhibited by 17β-estradiol, since only a modest inhibition was observed at 10 pm 17β-estradiol (Fig. 3). Thus, there is no evident relationship between the 17β-estradiol concentration in culture medium and the type of cells obtained according to the protocol used. If charcoal treatment of serum is a key step for the selection of cells in which growth is inhibited by 17β-estradiol, the role of other factors adsorbed simultaneously with 17β-estradiol has to be elucidated.

One other observation was that the phenotype “17β-estradiol responsiveness of growth” was stable in rats and unstable when characterized in culture. The F₄P cells originally showed 17β-estradiol inhibition in culture and, at later passages, they became insensitive while their growth was still slowed down in rats. Since the alteration which occurred in culture was accompanied by the loss of measurable 17β-estradiol binding, we suggest that cells became resistant because they lost 17β-estradiol receptors. The recovery of 17β-estradiol responsiveness in vivo by these cells, when they gave rise to a tumor, may have been due to the induction of 17β-estradiol receptors. In addition, the growth of F₄Z cells, whatever their type (F₄Z or F₄Z₂) and passage number, was stimulated by 17β-estradiol in vivo. However, in culture, F₄Z₂ and not F₄Z cells were 17β-estradiol responsive. The major change in 17β-estradiol responsiveness which occurred when F₄Z₂ cells appeared was accompanied by a modest increase in 17β-estradiol receptor number. These findings are consistent with other reports suggesting that environmental factors may alter the hormone sensitivity of cell growth. It has indeed been reported that hormone sensitivity of mammary tumors decreased or disappeared when cells were cultured for a long period of time in hormone-free medium (26–28) and, in MCF-7 cells, the loss of 17β-estradiol responsiveness was accompanied by a 3-fold increase in 17β-estradiol receptors. Thus, the factors responsible for the phenotype instability and their intracellular targets remain to be determined.

In conclusion, features of cells that are established from a tumor depend strongly on the experimental conditions used to disperse and culture cells. Since one of the two cell lines established from the MtTF₄ tumor was, at least transiently, inhibited by 17β-estradiol in vitro, it is probable that the inhibitory action of 17β-estradiol on tumor growth was due, at least in part, to a direct action on tumor cells. These new cell lines appear of interest for studying the control of cell division not only by using 17β-estradiol and dopamine agonist but also other factors, such as glucocorticoids and thyroid hormones.

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