Receptor Status and Subsequent Sensitivity of Subclones of MCF-7 Human Breast Cancer Cells Surviving Exposure to Diethylstilbestrol

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ABSTRACT

The estrogen receptor (ER)-positive human breast cancer cell line MCF-7 was incubated continuously in the presence of pharmacological concentrations of diethylstilbestrol (DES) in an attempt to correlate receptor status with DES sensitivity. It was consistently observed that cytotoxicity occurred at DES concentrations > 5 \times 10^{-6} \text{ M}; however, a small percentage of cells, both from the wild-type MCF-7 line and from subclones derived in soft agar from single MCF-7 cells, survived, with altered morphology, up to 4 months of continuous exposure to DES concentrations ranging from 5 \times 10^{-4} to 1 \times 10^{-4} \text{ M}.

Characterization of seven regenerated surviving cell populations suggested that they remained ER positive; no evidence could be found for a block in the pathway of hormonal activation, as determined by progesterone receptor induction, to explain the ability of these cells to survive DES.

Three regenerated cell populations were reexposed to DES. Two remained as sensitive to growth inhibition as untreated parent cells from which they were derived; however, one of these, designated MCF-7\(_{35-11}\), was found to have autonomously high progesterone receptor (463 \pm 94 \text{ fmol/mg of cytosol}; K_d = 1.8 \pm 0.2 \times 10^{-9} \text{ M}) which was not significantly stimulated by the addition of 1 \times 10^{-6} \text{ M} 17\beta\text{-estradiol for 72 hr.} The third population, designated MCF-7\(_{35-3b}\), which survived initial exposure to 5 \times 10^{-6} \text{ M} DES for 109 days and which remained ER positive (27 \pm 3 \text{ fmol/mg of cytosol}; K_d = 0.8 \pm 0.2 \times 10^{-10} \text{ M}) and progesterone receptor inducible, demonstrated significantly decreased sensitivity (p = 0.025) on reexposure to DES; conversely, significantly increased sensitivity (p < 0.03) to the antiestrogen tamoxifen was observed.

The mechanisms by which some MCF-7 cells survive prolonged exposure to DES are not certain; the data suggest that there is no clear relationship between ER status and sensitivity to DES and that there is no way of predicting the ultimate status of cells surviving DES treatment.

INTRODUCTION

The discovery in 1971 by Jensen et al. (4) that some breast cancers contain ER\(^3\) and that the presence of these receptors may predict clinical response has led to a resurgence of interest in the use of synthetic estrogens such as DES and antiestrogens such as TAM in the treatment of breast cancer (6). However, the mechanisms by which pharmacological concentrations of these compounds exert their effects on tumor cells and by which tumor cells become resistant to DES and TAM are unclear. For example, a recent National Surgical Adjuvant Breast Program clinical study indicates that the response to TAM in women 65 years of age and over may be independent of ER and PGR status (1), indicating that perhaps TAM is cytotoxic to breast cancer cells by some alternate mechanism.

In an attempt to further elucidate the relationship between receptor status and sensitivity to synthetic estrogenic and antiestrogenic compounds, we studied the effects of prolonged exposure to pharmacological concentrations of DES on various populations of MCF-7 human breast cancer cells in vitro. The results reported here suggest that (a) cells surviving DES exposure for up to 4 months remain ER positive, (b) some, but not all, surviving cell populations retain their pretreatment sensitivity to DES on subsequent exposure, (c) quantitative changes may occur in the level of ER or PGR in some surviving cell populations, and (d) sensitivity to DES may be distinct from that to TAM.

MATERIALS AND METHODS

Cell Culture. Experimental media used for all growth experiments consisted of DMEM (Grand Island Biological Co., Grand Island, N. Y.) supplemented with insulin (10 \mu g/ml; Sigma Chemical Co., St. Louis, Mo.), glucose (3.5 mg/ml; Fisher Scientific Co., Pittsburgh, Pa.), and either 1 or 2% DCC-stripped FCS (Grand Island Biological Co.). The MCF-7 cell line, derived from a patient with metastatic breast carcinoma and described by Soule et al. (9), was grown in 75-sq cm tissue culture flasks (Coming Glass Works, Coming Medical and Scientific, Coming, N. Y.); cells were passaged as necessary following treatment with trypsin-EDTA (Grand Island Biological Co.) and were incubated at 37° in 5% CO\(_2\).

Cloning of MCF-7 Cells in Soft Agar. To obtain subpopulations homogeneous for ER, 500 \mu l of suspension, each containing 100 to 500 MCF-7 wild-type cells, were dispersed in DMEM containing 0.24% agar and overlaid on 2.5 ml of 0.5% agar in 35-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.). Isolated single cells were identified under the inverted microscope, and their location was marked. Over a period of 14 to 21 days, colonies of 30 to 50 cells were observed to develop from these single progenitors with a cloning efficiency of approximately 70%; the colonies were then physically removed under direct vision using a fine needle attached to a tuberculin syringe and transferred to 35-mm plastic Petri dishes (Falcon) containing 2.5 ml of DMEM plus 10% FCS.

Preparation of Cytosol. Cells were harvested in 150-sq cm flasks (Coming) at an approximate density of 3.5 \times 10^7 cells/flask. Detachment of cells was facilitated by incubation with 1 \times 10^{-3} \text{ M} EDTA in Hanks' balanced salt solution (Grand Island Biological Co.) without calcium and magnesium for 10 min at 37°, after which an equal volume of DMEM was added to equilibrate pH. All subsequent steps were performed at...
4° unless otherwise stated. Cell suspensions for ER determination were centrifuged at 700 × g for 5 min, washed with Hanks’ balanced salt solution, centrifuged, washed with phosphate buffer, and centrifuged again. The resulting cell pellet was resuspended in 3 to 4 ml of Na2HPO4-EDTA-dithiorethiol buffer containing 10 × 10⁻⁴ M Na2HPO4 (BDH Pharmaceuticals Ltd., Toronto, Ontario, Canada), 1.5 × 10⁻³ M EDTA (Fisher), and 0.5 × 10⁻³ M dithiorethiol (Sigma) to which was added 20 × 10⁻³ M sodium molybdate (J. T. Baker Chemical Co., Phillipsburg, N. J.) for receptor stabilization (5) and adjusted to pH 7.4. Cell pellets for PGR determinations were resuspended in Na2HPO4-EDTA-dithiorethiol buffer to which were added 10% glycerol and 20 × 10⁻⁴ M sodium molybdate. Cells were homogenized using a Polytron homogenizer (Brinkman Instruments, Inc., Rexdale, Ontario, Canada). The homogenate was centrifuged at 80,000 × g for 35 min, and the supernatant was retained. Cytosolic protein concentrations were determined by the Bio-Rad protein assay method (Bio-Rad Laboratories, Richmond, Calif.).

**ER Assay.** Forty-eight hr prior to ER determination, cells were washed once with DMEM plus glucose (3.5 mg/ml) and then incubated in the same medium. To measure ER, cytosols were incubated for 1 hr at 20° to determine free receptor and for 3 hr at 30° to determine total receptor (free plus bound). A microplate adaptation of the standard DCC assay, as described by McGuire et al. (7), was used in the presence of 17β-2,4,6,7-estradiol (114 CI/mmol; New England Nuclear, Boston, Mass.) at concentrations ranging between 0.2 and 5 × 10⁻⁴ M with and without a 100-fold excess of cold DES (Sigma) to estimate nonspecific binding. Concentration of ER was calculated by Scatchard analysis (8) and expressed as the number of fmol/mg of crude cytosol protein.

**PGR Assay.** Measurement of PGR was carried out by incubating cytosols for 4 hr at 4° in the presence of the labeled synthetic progestogen R5020 (New England Nuclear) in concentrations ranging between 0.2 and 5 × 10⁻⁹ M with and without a 100-fold excess of unlabeled DES (Sigma) to estimate nonspecific binding. PGR activation was intact, the induction of PGR by 1 × 10⁻⁸ M 17β-estradiol for 4 hr at 4° in the presence of the labeled synthetic progestogen R5020 (New England Nuclear) in concentrations ranging between 0.2 and 5 × 10⁻⁹ M with and without a 100-fold excess of unlabeled DES (Sigma) to estimate nonspecific binding. Culture conditions for this assay are described in detail by McGuire et al. (7).

**Long-Term Cell Culture in DES.** Seventy-five-sq cm tissue flasks (Corning) containing DMEM plus 10% FCS were seeded with either MCF-7 parent cells or with subclones derived from isolated cells in soft agar. When the cells reached 75% confluent monolayer growth, DES concentrations ranging from 1 × 10⁻⁶ M to 1 x 10⁻⁴ M were added to fresh prewarmed DMEM plus 1 or 2% DCC-stripped FCS. Total cell number was determined by Coulter Counter or by phase microscopy; morphology of surviving cells was photographed, and the viability was assessed by exclusion of trypan blue dye. Following continuous exposure to DES, surviving cell populations were allowed to recover in the absence of hormone in DMEM supplemented with 10% FCS.

**Measurement of Relative Growth Rate in the Presence of DES and TAM.** Cells were seeded into 9.62-sq cm tissue flasks (Linbro, Flow Laboratories, Inc., McLean, Va.) at a cell density of 1 × 10⁴ cells/well. After 24 hr of growth in DMEM plus glucose, in which logarithmic cell growth was attained, fresh prewarmed DMEM plus 1% or 2% DCC-stripped FCS, supplemented with insulin (10 μg/ml) and glucose (3.5 mg/ml) and containing various concentrations of either DES or TAM, was added. Concomitant control wells contained the same cell concentrations in medium and vehicle alone (0.25% ethanol). Hormone-treated cells and their controls were washed free of either drug or vehicle every 48 to 72 hr and removed from the wells by treatment with 1 × 10⁻³ M EDTA; total cell number was determined by Coulter Counter or by phase microscopy. Cell number was plotted logarithmically as a function of time for each point ± 2 S.D. in the relative growth rate curves.

**RESULTS**

**ER Status of Cloned MCF-7 Cells.** A total of 50 subclones was obtained from single wild-type MCF-7 cells in soft agar. Eleven of these populations have been tested at random for the presence of ER (Table 1). All subclones tested so far have been ER positive; 5 of the clones have been tested on 2 or more occasions, revealing that there is an intertest S.E. for ER of 23% of the mean.

**Effects of Long-Term Incubation in the Presence of DES.** In addition to the MCF-7 wild-type line, the ER-positive subclone MCF-7 (35) was arbitrarily chosen for long-term incubation in the presence of DES; it has been tested for ER concentration on multiple occasions, with values ranging between 28 and 69 [52 ± 12 (S. E.)] fmol/mg cytosol at 20°. The Kd for the subclone is 4.5 ± 0.5 × 10⁻¹⁰ M. MCF-7 (35) is also PGR positive with a basal PGR concentration of 111 ± 16 fmol/mg cytosol; this value increased to 314 ± 12 fmol/mg cytosol after 72 hr of incubation with 1 × 10⁻⁶ M 17β-estradiol (p < 0.02), thus demonstrating PGR inducibility. Incubation of both the MCF-7 (35) subclone and the parent MCF-7 wild-type cell line with increasing concentrations of DES showed a similar dose-response effect over time (Chart 1). The wild-type MCF-7 parental cell line and the MCF-7 (35) subclone were incubated for periods ranging from 7 days to 4 months in the presence of DES, varying in concentration from 1 × 10⁻⁶ M to 1 x 10⁻⁴ M. For both the parental line and subclone, cell loss was seen in all cases at DES concentration ≥ 5 × 10⁻⁶ M starting between 24 and 72 hr after incubation; maximal cell loss occurred in all flasks at approximately 7 to 10 days. However, in each case, a small number of cells (approximately 1 x 10³) survived treatment, irrespective of the length of exposure to DES; these cells did not appear to divide and were altered in their morphology, demonstrating a rounding of their shape with loss of cytoplasmic extensions. The nuclei were large and often contained 2 to 5 nucleoli; some cells were binucleate (Fig. 1).

**Table 1**

<table>
<thead>
<tr>
<th>Subclone</th>
<th>ER status of subclones derived in soft agar from a single wild-type MCF-7 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 (35)</td>
<td>213 ± 28²</td>
</tr>
<tr>
<td>MCF-7</td>
<td>218 ± 69</td>
</tr>
<tr>
<td>MCF-7 (26)</td>
<td>264 ± 58</td>
</tr>
<tr>
<td>MCF-7 (36)</td>
<td>86 ± 21</td>
</tr>
<tr>
<td>MCF-7 (34)</td>
<td>52 ± 12</td>
</tr>
<tr>
<td>MCF-7 (28)</td>
<td>57²</td>
</tr>
<tr>
<td>MCF-7 (27)</td>
<td>80</td>
</tr>
<tr>
<td>MCF-7 (25)</td>
<td>31</td>
</tr>
<tr>
<td>MCF-7 (24)</td>
<td>67</td>
</tr>
<tr>
<td>MCF-7 (23)</td>
<td>75</td>
</tr>
<tr>
<td>MCF-7 (22)</td>
<td>27</td>
</tr>
</tbody>
</table>

² Mean ± S. E. for 2 or more determinations.

² Single determinations only.
Receptor Status in MCF-7 Cells Surviving DES

That these apparently dormant cells were viable was judged by their ability to exclude trypan blue dye and by the fact that they slowly regained their normal morphology (Fig. 2) following removal of hormones from the medium; after 7 to 14 days, cell division once again occurred, and the cells regained confluence over a period of between 3 and 6 weeks.

Receptor Status of Regenerated Cells. Following DES exposure, the regenerated cells were again tested for ER and also for PGR inducibility by 17β-estradiol (Table 2). In 7 such populations, all derived from the MCF-7(35) subclone, ER ranged from 24 ± 4 fmol to 138 ± 13 fmol with 3 populations [MCF-7(35-2), MCF-7(35-5), and MCF-7(35-6)] demonstrating an ER concentration significantly greater (p < 0.05) than the parent MCF-7(35) subclone. Two others, MCF-7(35-3) and MCF-7(35-7), demonstrated a significantly lower ER (p < 0.05), while that of MCF-7(35-4) was not significantly different from the MCF-7(35) subclone (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Cell population</th>
<th>20° (fmol/mg of cytosol protein)</th>
<th>30° (fmol/mg of cytosol protein)</th>
<th>Kd (x 10^-10 M)</th>
<th>PGR (4°) (fmol/mg of cytosol protein)</th>
<th>Duration of DES exposure (days)</th>
<th><a href="kJ">DES</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7(35-5)</td>
<td>52 ± 12</td>
<td>56 ± 13</td>
<td>4.5 ± 0.5</td>
<td>111 ± 16</td>
<td>7</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-1)</td>
<td>60 ± 4</td>
<td>76 ± 16</td>
<td>0.4 ± 0.1</td>
<td>463 ± 94</td>
<td>105</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-6)</td>
<td>100 ± 13</td>
<td>114 ± 19</td>
<td>1.4 ± 0.4</td>
<td>59 ± 11</td>
<td>30</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-5)</td>
<td>27 ± 3</td>
<td>37 ± 6</td>
<td>0.8 ± 0.2</td>
<td>13 ± 4</td>
<td>109</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-5)</td>
<td>63 ± 6</td>
<td>91 ± 42</td>
<td>2.5 ± 0.2</td>
<td>84 ± 11</td>
<td>30</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-5)</td>
<td>115 ± 12</td>
<td>152 ± 25</td>
<td>1.1 ± 0.1</td>
<td>54 ± 11</td>
<td>109</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-5)</td>
<td>138 ± 13</td>
<td>172 ± 45</td>
<td>1.2 ± 0.1</td>
<td>109 ± 22</td>
<td>32</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>MCF-7(35-5)</td>
<td>24 ± 4</td>
<td>33 ± 16</td>
<td>2.0 ± 0.3</td>
<td>141 ± 16</td>
<td>80</td>
<td>5 x 10^-4</td>
</tr>
</tbody>
</table>

*Mean ± S.E. for ER and Kd on a minimum of 2 separate determinations.

**Mean ± S.E. of the intercept for PGR on one determination.

†Parental subclone not exposed to DES (control).

‡Not significant versus Footnote f; significance versus Footnotes g and h, p < 0.05.

§Mean ± S.E. of the intercept for PGR on 3 separate determinations.
All but one of the regenerated populations demonstrated basal PGR concentrations of >20 fmol with the exception of MCF-7(35-3), which, however, demonstrated significant PGR inducibility by 17β-estradiol (Table 2). In addition, MCF-7(35) demonstrated the emergence of autonomously high PGR levels which were not significantly increased by the addition of 1 x 10^-8 M 17β-estradiol for 72 hr (Table 2).

Two regenerated MCF-7 wild-type populations surviving 30 days of exposure to 5 x 10^-6 M DES also remained ER positive (180 ± 25 fmol) and PGR inducible (data not shown).

### Effect of Reexposure to DES of Recovered Sublines

The regenerated sublines MCF-7(35-1) ("unchanged" ER, autonomously high PGR), MCF-7(35-2) ("higher" ER, "normal" PGR), and MCF-7(35-3) ("lower" ER, "lower" PGR) were reexposed to various concentrations of DES in 1% DCC-stripped FCS, and their relative growth rates, which take into account observed differences in doubling time (Table 3), were compared to that observed for a sample of the parental MCF-7(35) line which had not been exposed previously to DES. No significant difference in sensitivity was observed for the MCF-7(35-1) or MCF-7(35-2) sublines (Chart 2); however, there was a significant decrease (p = 0.025) in sensitivity for MCF-7(35-3) (Chart 2), and this change in sensitivity has persisted for 1 year, despite continuous passage.

### Effect of Exposure of MCF-7(35-3) to TAM

Conversely, exposure of this same MCF-7(35-3) subline to increasing doses of TAM demonstrated a significantly increased sensitivity (p < 0.03) to this antiestrogen as compared to MCF-7(35) cells which had not been exposed previously to DES (Chart 3).

### DISCUSSION

The results indicate that a small fraction of MCF-7 cells, whether from the wild-type population or derived from a single MCF-7 cell in soft agar, consistently survives exposure to DES at high concentrations for periods of up to 4 months. Moreover, the regenerating cell populations have been found to retain their ER-positive status.

However, in at least one case, DES exposure has led to an alteration in PGR status, in that the MCF-7(35-1) surviving subline developed an autonomously high level of PGR which was not significantly increased by incubation with 1 x 10^-8 M 17β-estradiol. This finding parallels that for the T47D human breast cancer cell line which also demonstrates autonomously high PGR concentrations (3); however, the T47D line, unlike the MCF-7(35-1) population, has no cytoplasmic ER, a low concentration of apparently occupied nuclear sites (3), and, in addition, is insensitive to estrogens. The possibility that the high autonomous PGR concentration in conjunction with ER positivity in the MCF-7(35-1) subline represents a biochemical defect in receptor mechanisms which allowed these cells to survive initial DES exposure requires further investigation.

Of the remaining 2 sublines which were reexposed to DES, MCF-7(35-3) was found to have a significant decrease in ER as compared to the MCF-7(35) population from which it was derived. These findings suggest that long-term exposure to DES may in some cases lead to changes in ER concentration over time; furthermore, the MCF-7(35-3) cells demonstrated significantly decreased sensitivity on reexposure to DES, despite remaining ER positive.

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**Table 3**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0% FCS</td>
</tr>
<tr>
<td>MCF-7(35) (parental line)</td>
<td>1.5 ± 0.1a</td>
</tr>
<tr>
<td>MCF-7(35-1)</td>
<td>4.2 ± 0.5a</td>
</tr>
<tr>
<td>MCF-7(35-2)</td>
<td>2.3 ± 0.2'</td>
</tr>
</tbody>
</table>

* A sample of this subclone was not exposed to DES and served as control.
* Significance versus Footnotes d and f, p < 0.001.
* Not significant versus Footnote e.

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**Chart 3.** Relative growth rate in the presence of TAM at various concentrations for the MCF-7(35) parental subclone and for the MCF-7(35) population surviving 109 days of DES exposure (5 x 10^-6 M). Measurement of R μ% is the same as for Chart 2. Points, mean of replicate samples in 2 separate determinations; bars, S.D.
positive and PGR inducible. Of great clinical interest is the finding that, conversely, the MCF-7(35-3) subline was significantly more sensitive to TAM than a population of its parental MCF-7(35) line not exposed previously to DES. This supports the possibility that estrogens and antiestrogens may act through different receptors (10) or at different loci in the cell and may explain why some patients respond to TAM after failing with DES or vice versa. However, the finding that the MCF-7(35-3) subline may be more sensitive to estrogen inducibility of PGR (11-fold versus 3-fold for MCF-7(35); Table 2) suggests an alternative mechanism for increased TAM sensitivity, as PGR may correlate more closely with clinical response to TAM in some patients (1).

It may be hypothesized that, in all cases, surviving “dormant” cells were in G0 at the time of DES exposure and possibly nonspecifically immune to the effects of any cytotoxic agent; with prolonged exposure to DES, as in the experiments reported here, true drug resistance might emerge, as possibly exemplified by MCF-7(35-3). The in vitro results could also suggest that intermittent pulsing of hormone in vivo, rather than continuous daily administration, might allow a “resistant” residue of ER-positive cells to recover from a dormant state, concomitant with renewed DES sensitivity and before some form of permanent resistance emerges.

Finally, this study suggests that the relationship between ER status and sensitivity to DES and TAM is not clear. MCF-7 cells surviving the cytotoxic effects of DES may demonstrate many findings, including an increase in ER [MCF-7(35-2)], a decrease in ER with a possible increase in PGR inducibility [MCF-7(35-3)], or no change in ER in the presence of an autonomously high PGR [MCF-7(35-1)]. On subsequent exposure to DES, the cells may remain sensitive [MCF-7(35-1) and MCF-7(35-2)] or demonstrate decreased sensitivity, possibly correlating with a decrease in ER [MCF-7(35-3)]. Thus, the results suggest that there may be no way of predicting the ultimate status of cells surviving DES treatment and that, clinically, subsequent therapies must be considered with caution.

REFERENCES
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