Cell Proliferation Kinetics of MCF-7 Human Mammary Carcinoma Cells in Culture and Effects of Tamoxifen on Exponentially Growing and Plateau-Phase Cells

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ABSTRACT

MCF-7 human mammary carcinoma cells were inoculated into 150-sq cm flasks at 3 \times 10^6 cells/flask, and after a lag period of about 48 hr, these cells grew exponentially for 5 days with a mean population doubling time of about 24 hr. During exponential growth, 80 to 90% of cells were in the "rapidly cycling" pool, the clonogenic fraction was 50 to 60%, and the mean percentage of cells in the G0-G1, S, and G2 + M phases of the cell cycle was 48.9 \pm 0.6% (S.E.), 39.4 \pm 0.6%, and 11.6 \pm 0.3%, respectively. These parameters changed rapidly between Days 7 and 13 when plateau phase was reached. Between Days 13 and 18, 74.8 \pm 0.7% of cells were in G0-G1, 15.3 \pm 0.4% were in S, and 9.8 \pm 0.8% were in G2 + M phase. Only about 30% of these cells were cycling rapidly, and the clonogenic fraction had fallen to less than 10%.

Tamoxifen induced a dose-dependent decrease in the growth rate of exponentially growing cells, which was accompanied by a dose-dependent increase in percentage of G0-G1-phase cells, and a decline in percentage of S-phase cells. At doses >10 \mu M, a 24-hr pulse of tamoxifen was cytotoxic to exponentially growing cells. Plateau-phase cells were less sensitive to these effects of tamoxifen.

In an attempt to define the kinetic basis of the G0-G1 accumulation induced by tamoxifen, asynchronous MCF-7 cells were pretreated for 42 hr with various doses of tamoxifen, and the rate of efflux of cells from the G0-G1 phase was assessed by blocking their reentry into G1 with ICRF 159. Following treatment of control cultures with ICRF 159, two populations of cells were distinguished by their rates of efflux from G0-G1 phase. The majority of cells left G0-G1 rapidly with a mean t0 of 2.3 hr ("rapidly cycling" cells). However, about 18% of cells had a much slower rate of exit with a mean t0 of about 30 hr ("slowly cycling" cells). Pretreatment with tamoxifen resulted in a dose-dependent decrease in the proportion of rapidly cycling cells and an increase in the proportion of cells with slow G1 transit times. Although this appeared to be the predominant effect, tamoxifen also decreased the rate at which the slowly cycling cells traversed G1. Simultaneous treatment with estradiol returned these parameters to control values at doses of tamoxifen \textless 5 \mu M, partially reversed the effect of 7.5 \mu M tamoxifen, but was without effect on the arrest of cell cycle progression induced by 10 \mu M tamoxifen. It is concluded that cells accumulate in G0-G1 following tamoxifen treatment due to an increase in the proportion of slowly cycling cells at the expense of a population of rapidly cycling cells, which appear to be relatively uninfluenced by the drug.

INTRODUCTION

The ER3-positive human mammary carcinoma cell line MCF-7 (33) has been used extensively for studies on human breast cancer, especially those aimed at understanding hormonal control mechanisms in this disease. The widespread use of this model system can be attributed to the fact that MCF-7 was the first ER-positive (7) and estrogen-responsive (22, 24) human breast cancer cell line to be documented. In addition, it has specific receptors and responsiveness to a wide range of other low-molecular-weight hormones, e.g., androgens, progestins, and glucocorticoids; vitamin D3; thyroid hormones (9, 15, 19, 20, 23, 25, 26, 43); polypeptide hormones, e.g., prolactin, insulin, and calcitonin; and epidermal growth factor (10, 17, 27, 29–32).

When initiating studies on the effects of estrogens and antiestrogens on the proliferation and cell cycle kinetics of this and other human mammary carcinoma cell lines, it became apparent that there were no detailed data on these basic characteristics of MCF-7 cells during unperturbed growth in vitro. Since it is likely that some hormones and antiestrogens will have differential effects on cells in different growth phases, experiments were undertaken to document the growth kinetics of MCF-7 cells before embarking on studies with hormones and their antagonists.

There are several studies documenting the direct effects of the synthetic nonsteroidal antiestrogen tamoxifen on the growth of human mammary carcinoma cells in vitro (2, 3, 10, 11, 22, 24, 34, 36). These studies demonstrate that tamoxifen and other structurally related antiestrogens inhibit growth, decrease incorporation of 35P, and tritiated thymidine into DNA, and inhibit DNA polymerase activity in ER-positive breast cancer cells (1–3, 10, 11, 14, 22, 24, 34, 36, 42, 43). The biochemical and cell kinetic basis of these effects has yet to be elucidated.

In this paper, the changes in proliferation kinetics during unperturbed growth of MCF-7 cells in monolayer culture are reported as is the kinetic basis of tamoxifen-induced inhibition of MCF-7 cell growth.

MATERIALS AND METHODS

Cells. MCF-7 cells in their 299th passage were supplied by Dr. Charles M. McGrath, Meyer L. Prentiss Cancer Center, Detroit, Mich. Following 3 passages in Roswell Park Memorial Institute Tissue Culture Medium 1640 [containing arginine (0.34 g/liter), asparagine (0.63 g/liter), and folic acid (0.04 g/liter) and supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 14 mM sodium bicarbonate, 5 mM L-glutamine, gentamicin (20 \mu g/ml), insulin (10 \mu g/ml), and 10% FCS], aliquots of 10^6 cells in 2 ml medium containing 200 \mu dimethyl sulfoxide were frozen and stored in liquid nitrogen.

Frozen cells were thawed, inoculated into two 150-sq cm flasks


2 To whom requests for reprints should be addressed.

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(Corning Glass Works, Corning, N. Y.), allowed to grow until confluent, and inoculated at 2 x 10^6 cells/150-sq cm flask. The inoculation density was gradually reduced to 3 x 10^6 cells/150 sq cm during the next 4 to 6 weekly passages. Stock cells were subsequently passaged at this inoculation density once a week for 25 to 30 weeks, when they were discarded, and a new vial of frozen cells was revived.

Growth Curves. Cells (3 x 10^9) from Day 7 cultures (see Chart 1A) were inoculated into 150-sq cm flasks in 50 ml of medium containing 5 or 10% FCS. At daily intervals thereafter, cells were harvested from the monolayer with 0.05% trypsin-0.02% EDTA in 1.5 mM KH2PO4-8.1 mM NaH2PO4-2.7 mM KCl-140 mM NaCl and counted under phase contrast on a hemocytometer, and a sample was stained for flow cytometry. Some cells were diluted in medium and replated at 2 x 10^6 cells/25-sq cm flask for regrowth and drug sensitivity experiments or plated into 5-cm-diameter plastic Petri dishes for the clonogenic assay.

After Day 6, some 150-sq cm flasks had their medium replenished daily, and the number of cells shed into the medium per day was recorded.

Flow Cytometry. Approximately 10^6 MCF-7 cells containing 10^6 chick erythrocytes as an internal standard were stained for DNA flow cytometry with ethidium bromide-mithramycin as described previously (37). RNase (type IA; Sigma Chemical Co., St Louis, Mo.) was added directly to the stained cell preparation 5 to 15 min before analysis to yield a final concentration of 1 mg/ml. Analysis was performed on a Model ICP22 pulse cytometer (Ortho Instruments, Westwood, Mass.) with excitation at 360 to 460 nm and fluorescence detection at greater than 550 nm. Estimates of the cell cycle kinetic parameters, i.e., the proportion of cells in the G0-G1, S, and G2 + M phases of the cell cycle, were calculated from the resulting DNA histograms using a planimetric method of analysis (28).

Clonogenic Assay. Cell viability during various growth phases and following drug treatment was assessed in a clonogenic assay. Following harvest from the monolayer, cells were counted, and the appropriate dilutions were made with medium containing the supplements listed above and 10% FCS. The desired numbers of cells (normally 100 to 1000) were plated into quadruplicate 5-cm-diameter plastic Petri dishes in 5 ml of medium. The dishes were placed in plastic boxes, gassed with 95% air-5% CO2, sealed, and incubated in high humidity at 37° for 14 days.

Following incubation, the medium was removed, the dish was rinsed with 0.9% NaCl solution, and the cells were fixed in 95% ethanol. After staining with 0.5% crystal violet, rinsing, and drying, the number of macroscopic colonies containing 50 or more cells was recorded.

In one experiment, colony size distribution was recorded using a microscope equipped with a micrometer eyepiece.

Drug Treatment. Tamoxifen [trans-1-(4-hydroxytamoxifen)-1,2-diphenylbut-1-ene] was supplied by I. C. I. Pharmaceuticals Division, Macclesfield, Cheshire, United Kingdom. 17ß-estradiol was from Sigma. Stock solutions (10 to 50 mM) were prepared in analytical grade ethanol and stored at -20°. Following the appropriate dilutions in ethanol, tamoxifen and/or estradiol was added to the culture medium such that a final concentration of 1 mg/ml. Analysis was performed on a Model ICP22 pulse cytometer (Ortho Instruments, Westwood, Mass.) with excitation at 360 to 460 nm and fluorescence detection at greater than 550 nm.

Drug treatment was assessed in a clonogenic assay. Following a lag period of 2 days, cell numbers increased exponentially for the next 5 days with a mean population doubling time of about 24 hr. After Day 7, the growth rate declined, but cell numbers continued to increase in fed cultures until Day 11, when a maximum cell density of 8 to 9 x 10^7 cells/flask was reached. This concentration of drug was sufficient to inhibit cell division, significant numbers of cells with DNA contents greater than G2 + M accumulate with increasing time of exposure to the drug (Chart 7). Since the computed cell cycle kinetic parameters apply to the diploid cell population only, the computed percentage of G2-G1-phase cells is an overestimate of the actual proportion of G2-G1 cells in the total population when cells with DNA contents greater than G2 + M accumulate. For this reason, the following correction was necessary to determine the actual proportion of cells remaining in G0-G1 phase at various times after ICRF 159 treatment.

Actual % of G0-G1 cells

= computed % of G0-G1 cells x analyzed cell no. total cell no.

where the analyzed cell number is the number of cells used in the computation of percentage of G0-G1, S, and G2 + M, and total cell number is the total number of tumor cells in the DNA histogram, i.e., total number of cells minus the chicken erythrocytes.

The rates of efflux of cells from the G0-G1 phase following ICRF 159 treatment were calculated from plots of log percentage of G0-G1-phase cells against time using least-squares linear regression analysis. The proportion of slowly cycling cells was determined by extrapolation of the terminal disappearance curve to the ordinate, and the half-times of disappearance of this and the rapidly cycling component were calculated from the rate constants assuming first-order reaction kinetics.

RESULTS

Cell Proliferation Kinetics. The growth of MCF-7 cells in monolayer culture, following inoculation of 3 x 10^6 cells into 150-sq cm flasks, is illustrated in Chart 1A. The inoculum was taken from Day 7 cultures when cell numbers were approximately 2 x 10^7/150-sq cm flask. Culture medium remained unchanged until Day 7, when some flasks received daily medium changes, and others remained unfed. Data for cells grown in medium supplemented with 5 and 10% FCS were pooled, since no differences between these growth media were observed except in unfed cultures after 7 days of growth (Chart 1A).

Following a lag period of 2 days, cell numbers increased exponentially for the next 5 days with a mean population doubling time of about 24 hr. After Day 7, the growth rate declined, but cell numbers continued to increase in fed cultures until Day 11, when a maximum cell density of 8 to 9 x 10^7 cells/flask was reached. This concentration of drug was sufficient to inhibit cell division, significant numbers of cells with DNA contents greater than G2 + M accumulate with increasing time of exposure to the drug (Chart 7). Since the computed cell cycle kinetic parameters apply to the diploid cell population only, the computed percentage of G2-G1-phase cells is an overestimate of the actual proportion of G2-G1 cells in the total population when cells with DNA contents greater than G2 + M accumulate. For this reason, the following correction was necessary to determine the actual proportion of cells remaining in G0-G1 phase at various times after ICRF 159 treatment.

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Chart 1. Changes in MCF-7 cell number (A), cell cycle phase distribution (B), clonogenic fraction (C), and percentage of slowly cycling cells (D) with time in culture. Cells were inoculated at $3 \times 10^4$/150-sq cm flask on Day 0. A, cell number. Some cultures had a daily medium change starting at Day 7 (O), and the number of cells lost into the medium per day was recorded thereafter (•). Data for cells grown in 5 and 10% FCS were pooled, except for the unfed cultures where cell numbers for 5% (O) and 10% (A) FCS are recorded separately. B, cell cycle phase distribution. The cell cycle parameters [percentage of G0-G1 (O), S- (G), and G2+M- (G) phase cells] were calculated from DNA histograms obtained by flow cytometry as described in “Materials and Methods.” … data from unfed cultures supplemented with 5% (x, A) or 10% (B, D) FCS. Points in both A and B, mean of 3 to 7 flasks; bars, S.E. Where bars are not shown, the S.E. did not exceed the size of the symbol. C, changes in the clonogenic fraction. Plating efficiency was assessed in a clonogenic assay as described in “Materials and Methods.” Points, mean of 8 to 28 plates from 2 to 7 separate experiments; bars, S.E. D, percentage of slowly cycling cells. Following treatment with ICRF 159 for 24 hr, cells were harvested and stained for flow cytometry, and the percentage of cells in G0-G1 phase was calculated from the resulting DNA histograms. Points, mean of 2 to 4 flasks; bars, S.E. or range.

18, when the mean ($n = 16$) percentage of S-phase cells was $15.3 \pm 0.4$ with an increase in G0-G1 cells to $74.8 \pm 0.7\%$. The rate of decrease in the proportion of S-phase cells was much more rapid in the unfed cultures (Chart 1C). During the first 7 days in culture, when growth rates were maximal, clonogenic efficiency was high with 50 to 60% of cells forming macroscopic colonies. As the growth rate declined, so did the clonogenic potential. By 12 days, <20% of cells formed colonies, while after Day 14, no colonies were observed (Chart 1C).
Tamoxifen and Growth Kinetics of MCF-7 Cells

Chart 2. Effect of time in culture on the growth of MCF-7 cells following replating of $2 \times 10^5$ cells into 25-sq cm flasks. The cells used to inoculate these flasks were from Days 3 to 6 (O), 10 (A), and 14 to 16 (•) of the growth curve illustrated in Chart 1A. Points, mean of 2 to 5 flasks; bars, S.E. or range. Where bars are not shown, the S.E. did not exceed the size of the symbol.

detected.

When the percentage of slowly cycling cells was monitored, it became apparent that this parameter was inversely correlated with the growth rate and the clonogenic potential of MCF-7 cells (Chart 1D). During exponential growth, the percentage of slowly cycling cells was <20%. As the growth rate declined, this percentage increased rapidly to reach a maximum of approximately 70% of cells by Day 13.

The effect of time in culture on the growth kinetics of cells following subculture at lower density was also monitored (Chart 2). Cells taken from exponentially growing cultures (Days 3 to 6) had a short lag period of about 6 hr before they grew exponentially with a mean doubling time of about 24 hr. As the time in culture was increased, so was the lag period following subculture, and with cells from cultures of Days 14 to 16, a significant decline in plating efficiency was also observed (Chart 2). However, after differing lag periods, cells were able to grow exponentially with a mean doubling time of about 24 hr. When the percentage of S-phase cells was monitored, it was observed that cultures of Days 3 to 6 maintained a constant S phase throughout, while cells from cultures of Days 10 and Days 14 to 16, that had markedly decreased proportions of S-phase cells at the start of subculture, showed rapid increases in the percentage of S-phase cells until exponential growth was attained with about 40% of the total cells in this phase of the cell cycle (data not shown).

Effect of Tamoxifen. Having determined the conditions necessary to generate populations of MCF-7 cells with markedly different growth kinetics, experiments were conducted to test if these populations displayed differential sensitivity to tamoxifen.

The effects of this drug on the growth of MCF-7 cells in exponential growth phase are shown in Chart 3A. Tamoxifen treatment resulted in a dose-dependent decrease in cell proliferation rate with doses as low as 100 nm producing a significant reduction in cell number, cf. control after 6 days of treatment. At doses of 10 µM and above, cell numbers declined from inocula levels, indicating that at these doses, tamoxifen was probably cytotoxic (Chart 3A). Cell cycle kinetic parameters measured in similar experiments showed that the decreased growth rate was accompanied by a dose-dependent decrease in the proportion of S-phase cells with a concomitant increase in the percentage of G0-G1-phase cells. At the highest dose tested, the percentage of S-phase cells was decreased to about 20% of that in untreated control cultures (Chart 3B).

When experiments similar to those described in Chart 3 were performed with cells from cultures of Days 10 to 14 following replating at $2 \times 10^5$ cells/25-sq cm flask, it was apparent that after 6 days of drug treatment, cells from plateau-phase cultures were less sensitive to tamoxifen than were exponentially growing cells. This is illustrated in Chart 4, where the percentage of reduction in cell number and the percentage of S-phase cells after 6 days of exposure to 4 doses of tamoxifen are recorded.

To further investigate the cytotoxic effects of tamoxifen, cells at different stages of their growth cycle were exposed to different doses of tamoxifen for 24 hr, and the surviving fraction was determined. Exposure of exponentially growing cells (Days 3 to 6) to doses $\geq 10$ µM resulted in a dose-dependent decrease in the surviving fraction with <20% of cells surviving 24 hr of exposure to 15 µM tamoxifen (Chart 5A). At doses of 10 µM and above, the colony size was also reduced significantly by drug treatment, suggesting that 24-hr exposure to high doses of tamoxifen also decreased the rate of proliferation of surviving cells (Chart 5B). When an identical series of experiments was performed with cells of Days 9 and 10, the surviving fraction was relatively unaffected over the same dose range, i.e., 5 to 15 µM. Exposure of plateau-phase cells to 15 µM tamoxifen for 24 hr reduced the surviving fraction by <15% (Chart 5A).

Effect of Estradiol on the Response to Tamoxifen. In the presence of estradiol, doses of tamoxifen $\leq 5$ µM were without effect on the growth of MCF-7 cells (Chart 6). When 10 µM...
G1 transit time relative to other phases of the cell cycle, it was necessary to monitor the rate of exit of cells from G0-G1 phase. This was done using the drug ICRF 159, an inhibitor of cytokinesis (38). Thus, ICRF 159 prevents nuclei from dividing and reentering the G0-G1 peak of the DNA histogram obtained by flow cytometry, enabling measurement of the proportion of cells in G0-G1 without contribution from cells reentering this phase from G1 + M (38).

Changes in the DNA histograms of MCF-7 cells following treatment with ICRF 159 (100 μg/ml) are presented in Chart 7. The DNA histogram of the control cells prior to treatment is typical of MCF-7 cells in exponential growth phase. After 4 hr of tamoxifen were administered together with 1 μM estradiol, cell numbers were not different from control at 24 hr, but thereafter, treated cells grew less rapidly. After 3 days of treatment, flasks receiving 10 μM tamoxifen and 1 μM estradiol contained only 40% of the cells in control flasks. This was significantly more than those present in cultures treated with 10 μM tamoxifen alone (Chart 6) and thus represents a partial reversal of the tamoxifen effect. Similarly, the cytotoxic effect of 15 μM tamoxifen was partially reversed by the addition of estradiol during the 3 days of study (Chart 6).

When the changes in cell cycle kinetic parameters were measured, it was apparent that, at doses of tamoxifen ≤5 μM, simultaneous treatment with estradiol resulted in cell cycle kinetic parameters that were indistinguishable from those of the control (data not shown). By contrast, the decrease in the percentage of S-phase cells and increase in the percentage of G0-G1-phase cells following 10 μM tamoxifen were only partially reversed 24 hr after simultaneous administration of tamoxifen and estradiol, and by 72 hr, estradiol had no effect on the tamoxifen-induced decrease of S-phase cells (data not shown). A similar result has been reported previously for MCF-7 cells grown in 5% charcoal-stripped FCS (34).

It is therefore concluded that tamoxifen has both estrogen-reversible and estrogen-irreversible components to its effects on cell growth and cell cycle kinetic parameters of MCF-7 cells in vitro.

Effect of ICRF 159. In order to test whether cells were irreversibly blocked in G0-G1 following tamoxifen treatment or accumulated there due to a tamoxifen-induced increase in the
**Tamoxifen and Growth Kinetics of MCF-7 Cells**

**CONTROL**

**XGI - 47.1**

**XS - 37**

**XG2 - H - 15.7**

C.V. = 2.3 X

**4hr**

**XGI - 31.3**

**XS - 39.6**

**XG2 - H - 29**

C.V. = 3.6 X

**223 258 288**

**8hr**

**XGI**

**XS**

**XG2 - H - 39.5**

C.V. = 4.1 X

**343 348 358**

**12hr**

**XGI - 28.7**

**XS - 24.4**

**XG2 - H - 34.8**

C.V. = 3.4 X

**248 258 268**

**24hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**348 358 368**

**36hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**48hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**58 108 158 298 258**

**4hr**

**XGI - 31.3**

**XS - 39.6**

**XG2 - H - 29**

C.V. = 3.6 X

**223 258 288**

**8hr**

**XGI**

**XS**

**XG2 - H - 39.5**

C.V. = 4.1 X

**343 348 358**

**12hr**

**XGI - 28.7**

**XS - 24.4**

**XG2 - H - 34.8**

C.V. = 3.4 X

**248 258 268**

**24hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**348 358 368**

**36hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**48hr**

**XGI**

**XS**

**XG2 - H - 28.7**

C.V. = 3.4 X

**58 108 158 298 258**

**58 IBi**

**24hr**

**XGI - 12.2**

**XS - 6.2**

**XG2 - H - 11**

C.V. = 4.3 X

**448 258 358 8 58 IBB 158 280 258**

**78 188 158 288 258**

**48hr**

**XS - 6.8**

**XG2 - H - 82.1**

**XGI - 11**

**XS - 5.3**

**XG2 - H - 83.5**

C.V. = 4.3 X

**348 358 368**

**Chart 7. Effect of ICRF 159 on the DNA distribution histograms of MCF-7 cells. MCF-7 cells (2 x 10⁶) in exponential growth phase were plated into 25-sq cm flasks in 5 ml of medium containing 5% FCS, and 42 hr later, ICRF 159 was added to a final concentration of 100 µg/ml. A flask was harvested at the times indicated, and the cells were stained for flow cytometry. C.V., coefficient of variation of the G0-G1 DNA peak.**

A detailed time course of changes in the percentage of G2-G1 cells, and cells were already beginning to accumulate in G2 + M. As the time of exposure increased, the percentage of G2-G1 phase cells continued to decline, and after 8 hr of treatment, the percentage of S-phase cells also declined, while the proportion of cells in G2 + M increased. By 24 hr, significant numbers of cells appeared with DNA contents greater than G2 + M, and by 48 hr, a distinct tetraploid peak was observed at Channel 200 (Chart 7).

A detailed time course of changes in the percentage of G2-G1 phase cells following treatment with ICRF 159 is illustrated in Chart 8. These data demonstrate that, after a short lag period of about 2 hr, cells began to leave G0-G1 with a single exponential rate of decay which was maintained for about 8 hr. After 12 hr, cells again left G2-G1 at a rate approximating a single exponential decay, but the t₁/2 was significantly increased to about 30 hr (Chart 8). These data are interpreted as indicating the presence of 2 populations of MCF-7 cells with markedly different G1 transit times. In this article, we refer to these 2 populations as rapidly cycling cells and slowly cycling cells. When the rate of efflux of the rapidly cycling component was corrected for influence from the slowly cycling component, the true half-time of efflux of the rapidly cycling cells was shown to be 2.3 hr (Chart 8).

**Effect of Tamoxifen on the Rate of Efflux of Cells from G1-G2 Phase.** The effect of pretreatment with tamoxifen on the rate of efflux of cells from G0-G1 phase is presented in Chart 9. It is apparent from these data that tamoxifen causes a dose-dependent decrease in the proportion of MCF-7 cells leaving G0-G1 phase. A significant decrease was apparent at 100 nM, and at 10 µM tamoxifen had almost completely arrested cell cycle progression. When the t₁/2 and the size of the slowly cycling pool were calculated, it became obvious that tamoxifen decreased...
the rate of disappearance of cells from this pool and increased the proportion of slowly cycling cells (Table 1).

The effect of estradiol on these parameters was also investigated. Estradiol alone (100 nM and 1 μM) had no significant effect on the rate of disappearance of MCF-7 cells from the G0-G1 phase (data not shown). When a 10-fold lower concentration of estradiol was administered together with 5 μM tamoxifen, the rate of disappearance was similar to control (Chart 10). In contrast, 1 μM estradiol had no effect on the arrest of cells by 10 μM tamoxifen. Pretreatment of cells with 750 nM estradiol and 7.5 μM tamoxifen resulted in partial reversal of the effects induced by this dose of tamoxifen alone (Chart 10).

DISCUSSION

_In vitro_ cultivation of tumor cells has been used widely as a means of studying modes of action of chemotherapeutic agents under controlled conditions. It has been recognized, however, that complex changes in a variety of cell cycle kinetic and biochemical parameters can occur during _in vitro_ growth which may influence profoundly subsequent responses to drug treatment (5, 40). The importance, therefore, of base-line measurements of cell cycle kinetic data for the understanding and interpretation of mechanisms of action of cytotoxic drugs should not be underestimated. The original kinetic classification of antican-

<table>
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<td>0</td>
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<td>41.9</td>
<td>39.0</td>
</tr>
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<td>50.5</td>
<td>75.0</td>
</tr>
<tr>
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<td>5</td>
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<tr>
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* n, the number of observations used to calculate the regression equation; r^2, the coefficient of determination.

The data illustrating the effects of tamoxifen on MCF-7 cells in exponential growth phase (Chart 10) confirm earlier reports that this drug is a potent inhibitor of growth in this ER-positive human mammary carcinoma cell line (10, 11, 20, 22, 24, 34–36). This response is time and dose dependent, with doses as low as 100 nM causing a significant reduction in cell number after 6 days of treatment. It is important to realize that the present experiments were conducted in the presence of FCS that had not been...
depleted of endogenous steroids, in order to maintain maximal growth rates. Since FCS contains estrogen, one would predict that, in the absence of estrogen, these cells would be inhibited by doses of tamoxifen below 100 nM. This is in general agreement with other published studies on the sensitivity of MCF-7 cells to tamoxifen (10, 11, 22, 24).

The predominant effect of tamoxifen in causing a decreased growth rate appears to be a decreased rate of cellular proliferation rather than an increase in the cell death rate. The dose-dependent accumulation of cells in the G0-G1 phase of the cell cycle and a concomitant decrease in the proportion of S-phase cells (Chart 3) are consistent with a decreased cell proliferation rate (35), while clonogenic survival data (Chart 5) demonstrate that increased cell death rate does not occur at doses of tamoxifen <10 μM. However, these latter experiments were based on 24-hr exposure times only, and it is not known whether exposure to doses ≤10 μM for longer periods causes significant reductions in clonogenic survival.

Tamoxifen effects on the changes in percentages of G0-G1- and S-phase cells appeared to increase with time except at doses >10 μM where the effects were maximal after 2 days (Chart 3B). A probable explanation for these data is that cells are susceptible to the effects of tamoxifen only at a specific stage in the cell cycle and that the proportion of cells affected is dose related. Thus, as cells pass through increasing numbers of cycles, the sensitive region of the cycle is reexposed to tamoxifen, and a greater proportion of the total population accumulates in G0-G1. In an accompanying paper, data are presented to support such a hypothesis (39). The reason that one does not see cumulative effects after 2 days of exposure to doses >10 μM tamoxifen can be explained by the observation that, after 42 hr of exposure to these doses, cell cycle progression is almost completely arrested (Chart 9). This would explain the decline in cell numbers seen after 3 days of treatment with >10 μM tamoxifen (Chart 3A), when cell proliferation is arrested and cell death rate is increased (Chart 5A).

These observations may also help explain the differential sensitivity of exponentially growing cells and plateau-phase cells to the effects of tamoxifen (Charts 4 and 5). Following replating at lower density in fresh medium containing tamoxifen, plateau-phase cells showed a markedly reduced response to the growth-inhibitory and cell cycle effects of tamoxifen after 6 days of treatment (Chart 4). This may be due to the fewer number of generation cycles completed by the plateau-phase cells, cf. exponentially growing cells, and/or the reduced proportion of plateau-phase cells in the tamoxifen-sensitive part of the cycle during lag phase (Chart 2). Likewise, the apparent resistance of plateau-phase cells to the cytotoxic effects of tamoxifen is probably related to the proportion of the total cell population in the tamoxifen-sensitive stage of the cycle during the 24 hr of exposure to the drug.

The observation, that not all the growth-inhibitory and cell cycle effects of tamoxifen could be reversed by estradiol, agrees with other published data, which illustrate that estrogen deprivation is less effective than is tamoxifen administration in inhibiting the growth of ZR 75-1 breast cancer cells in defined medium (1), and could be interpreted as evidence for effects of tamoxifen on cell proliferation in addition to those influenced by estrogen and mediated via ER.

The use of an inhibitor of cell division and flow cytometry to measure the rate of efflux of cells from the G1 compartment of the cell cycle is not novel. Böhmer (6) first described such a technique using Colcemid and mouse L-cells. Unlike the mouse L-cells, MCF-7 cells demonstrated 2 distinct rates of efflux from G1 phase, and both closely approximated a single exponential rate of decline (Chart 8).

When allowance was made for the contribution of the slowly cycling cells to the initial rate of efflux, a half-time of disappearance of 2.3 hr was calculated. Since 94% of cells would traverse G1 during 4 half-time periods, i.e., 9.2 hr, it is clear that this estimate is in good agreement with the mean G1 transit time of about 9 hr measured in synchronous populations of MCF-7 cells (39).

The physiological significance of these 2 subpopulations, i.e., the rapidly and slowly cycling populations, is difficult to interpret at this stage. Perhaps, they are related to G0 and G1 phases of the cell cycle or to the subcompartments of G1 described by Darzynkiewicz et al. (12), who have suggested that RNA concentration determines the rate of passage through G1, and have demonstrated subpopulations of cells with different RNA contents and different G1 transit times (12).

When the effects of tamoxifen on the rate of efflux of cells from G0-G1 were investigated, it became clear that the predominant effect was to increase the proportion of slowly cycling cells (Chart 9; Table 1). At the lowest concentrations tested, 100 and 500 nM, this was not accompanied by an increase in the half-time of disappearance, but at higher concentrations, both parameters increased in a dose-dependent manner. Due to the large contribution of slowly cycling cells to the total disappearance curve at doses >1 μM, we were unable to derive accurate estimates of the effects of tamoxifen on the rate of efflux of rapidly cycling cells from G0-G1. However, data with synchronous MCF-7 cells presented in a companion paper (39) indicate that this parameter was relatively less affected by tamoxifen treatment.

Simultaneous treatment with estradiol was without effect on the rate of disappearance of cells from G0-G1 phase induced by 10 μM tamoxifen (Chart 10). This is apparently anomalous with data in Chart 6 and Ref. 34, which illustrate partial reversal of the growth-inhibitory and cell cycle effects of 10 μM tamoxifen with a 10-fold lower dose of estradiol. However, the data presented in Chart 10 were obtained after 42 hr of pretreatment, and data presented elsewhere (34) show that the ability of estradiol to reverse the cell cycle effects of 10 μM tamoxifen decreases with time of exposure. Thus, the data presented in Chart 10 are compatible with other data presented on this system and add further support to the concept that tamoxifen has effects on cell proliferation in vitro that are independent of estrogen and perhaps the ER system.

In conclusion, this article has defined the cell kinetic basis of the G0-G1-phase accumulation of MCF-7 cells following tamoxifen treatment and demonstrated that it has both estrogen-reversible and estrogen-irreversible components. The biochemical basis of these cell cycle effects is currently being investigated.

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Cell Proliferation Kinetics of MCF-7 Human Mammary Carcinoma Cells in Culture and Effects of Tamoxifen on Exponentially Growing and Plateau-Phase Cells

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