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 x 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 G_0-G_1, S, and G_2 + M phases of the cell cycle was 48.9 ± 0.6% (S.E.), 39.4 ± 0.6%, and 11.6 ± 0.3%, respectively. These parameters changed rapidly between Days 7 and 13 when plateau phase was reached. Between Days 13 and 18, 74.8 ± 0.7% of cells were in G_0-G_1, 15.3 ± 0.4% were in S, and 9.8 ± 0.8% were in G_2 + 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 G_0-G_1-phase cells, and a decline in percentage of S-phase cells. At doses >10 μM, 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 G_0-G_1 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 G_0-G_1 phase of the cell cycle was assessed by blocking their reentry into G_1 with ICRF 159. Following treatment of control cultures with ICRF 159, two populations of cells were distinguished by their rates of efflux from G_0-G_1 phase. The majority of cells left G_0-G_1 rapidly with a mean t_m of 2.3 hr ("rapidly cycling" cells). However, about 18% of cells had a much slower rate of exit with a mean t_m 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 G_1 transit times. Although this appeared to be the predominant effect, tamoxifen also decreased the rate at which the slowly cycling cells traversed G_1. Simultaneous treatment with estradiol returned these parameters to control values at doses of tamoxifen ≤5 μM, partially reversed the effect of 7.5 μM tamoxifen, but was without effect on the arrest of cell cycle progression induced by 10 μM tamoxifen. It is concluded that cells accumulate in G_0-G_1 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 antihormones 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 ³²P, and titrated thymidine into DNA, and inhibit DNA polymerase activity in ER-positive breast cancer cells (1-3, 10, 11, 14, 22, 24, 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-inhibited 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. Prentis 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 μg/ml), insulin (10 μg/ml), and 10% FCS], aliquots of 10^6 cells in 2 ml medium containing 200 μl dimethyl sulfoxide were frozen and stored in liquid nitrogen. Frozen cells were thawed, inoculated into two 150-sq cm flasks

1 Presented in part at a UCLA Symposium, "Rational Basis for Chemotherapy," Keystone, Colo., 1982 (36). 2 To whom requests for reprints should be addressed. Received December 28, 1982; accepted May 20, 1983.

3 The abbreviations used are: ER, estrogen receptor; FCS, fetal calf serum.
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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 10^5 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 10^6) 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 ml KH2PO4·8.1 ml NaHPO4·2.7 ml KCl·140 ml 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 10^5 cells/25-sq cm flask for regrowth and drug sensitivity experiments or plated into 5-cm-diameter plastic Petri dishes for the clongenic 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 10^5 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).

Clongenic Assay. Cell viability during various growth phases and following drug treatment was assessed in a clongenic 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-hydroxy-3-methoxyphenyl)-1,2-diphenylbut-1-ene] was supplied by I. C. I. Pharmaceuticals Division, Macclesfield, Cheshire, United Kingdom. 17β-estradiol was from Sigma. Stock solutions (10 to 50 μM) 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 the final ethanol concentration was 0.1%, a concentration without effect on cell growth. A stock solution of 10 mg/ml was prepared in 0.4 M NaOH and diluted 100-fold in culture medium to yield concentrations 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.

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 10^5/150-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 10^5 cells/flask was reached. This was maintained for a further 7 days with daily medium changes, but thereafter, cell numbers declined (data not shown). In flasks where the medium was not changed, a maximum cell density was reached by Day 8, and thereafter, the cell numbers declined. The rate of decline was more rapid in the medium supplemented with 5% FCS (Chart 1A).

Few cells were found free in the medium until Day 10. During the next 4 days, there was a rapid increase in free floating cells to a daily maximum of 2 to 3 x 10^5/flask, which represented <4% of the total cells in the flasks at this time.

The percentage of cells in the G0-G1, S, and G2 + M phases of the cell cycle was monitored under the same conditions, and the data are summarized in Chart 18. These cell cycle kinetic parameters remained constant during the first 7 days of growth with a mean (n = 33) distribution of cells between G0-G1, S, and G2 + M phases of 48.9 ± 0.6% (S.E.), 39.4 ± 0.6%, and 11.6 ± 0.3%, respectively. As the growth rate declined, the percentage in S phase decreased with a concurrent increase in the percentage of G0-G1 cells. The proportion of S-phase cells continued to decline until Day 13, despite static cell numbers being reached on Day 11. No further changes occurred between Days 13 and
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 kinetic parameters [percentage of $G_0/G_1$ (O), $S$ (C), and $G_2 + M$ (C) phase cells] were calculated from DNA histograms obtained by flow cytometry as described in "Materials and Methods." ---, data from unfed cultures supplemented with 5% (•, O) or 10% (O, C) 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 $G_0/G_1$ 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 $G_0/G_1$ 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).

Changes in plating efficiency with time in culture were also observed (Chart 1C). During the first 7 days in culture, when growth rates were maximal, cloning 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
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When experiments similar to those described in Chart 3 were performed with cells from cultures of Days 10 to 14 following replating at 2 x 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 ≥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 ≤5 μM were without effect on the growth of MCF-7 cells (Chart 6). When 10 μM...
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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 G₂ + 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  

Chart 4. Effect of exposure to tamoxifen for 6 days on cell number and percentage of S-phase cells of exponential and plateau-phase cells. Cells (2 × 10⁶) taken from exponentially growing (Days 3 to 6) or plateau-phase (Days 10 to 14) cultures were plated into 25-sq cm flasks in the presence of tamoxifen. After 6 days, cells were harvested and counted, and the percentage of S-phase cells was calculated from DNA histograms obtained by flow cytometry. I, cells from exponentially growing cultures; III, those from plateau-phase cultures. Points, mean of 2 to 6 replicate flasks; bars, S.E. or range.

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 cells than were 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 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 G₂ + 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  

Chart 5. Effect of exposure to tamoxifen for 24 hr on the clonogenic survival (A) and the colony size (B) of MCF-7 cells. Cultures that were plated at 3 × 10⁶/150-sq cm flask were then for 3 to 6 days (•) or 8 to 10 days (O) before receiving a 24-hr pulse of tamoxifen. Cells were then harvested and plated in the clonogenic assay, and after 14 days of incubation, the surviving fraction was calculated as described in "Materials and Methods." Points, mean of at least 8 plates; bars, S.E. B, size distribution of colonies formed when exponentially growing cells were treated with 0 (•), 10 μM (Δ), or 15 μM (◯) tamoxifen for 24 hr prior to plating in the clonogenic assay.

Chart 6. Effect of tamoxifen alone or in the presence of estradiol on the growth of MCF-7 cells. Cells (2 × 10⁶) in exponential growth phase were plated into 25-sq cm flasks in 5 ml of medium containing 5% FCS. Twenty-four hr later, tamoxifen ± estradiol was added. The drug treatments were: control (○); 5 (●), 10 (▲), and 15 (▽) μM tamoxifen; 1 μM tamoxifen + 100 μM estradiol (□); 5 μM tamoxifen + 500 μM estradiol (◇); 10 μM tamoxifen + 1 μM estradiol (△); and 15 μM tamoxifen + 1.5 μM estradiol (◯). Replicate flasks were harvested daily for 3 days, and viable cell counts were made. Points, mean of duplicate flasks or mean of 3 to 8 replicate flasks; bars, S.E.
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CONTROL

XGI - 47.1
XS - 37
XG2 - H - IS.7
C.V. = 2.3 X

4hr
XGI - 31.3
XS - 39.6
XG2 - H - 29
C.V. - 3.6 X

223 258 8hr
XGI
XSI
XG2 - H - 43.5
43.5

12hr
XGI - 28.7
XS - 24.4
XG2 - H - 82.1
XGI - 11
XS - 6.3
XG2 - H - 83.5
C.V. - 4.3 X

34hr
48hr

10^-3 X CELL COUNT

CHANNEL NUMBER

Chart 7. Effect of ICRF 159 on the DNA distribution histograms of MCF-7 cells. MCF-7 cells (2 x 10^6) 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.

Effect of Tamoxifen on the Rate of Efflux of Cells from Go-G1 Phase. The effect of pretreatment with tamoxifen on the rate of efflux of cells from Go-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 t, and the size of the slowly cycling pool were calculated, it became obvious that tamoxifen decreased (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 G0-G1 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 t, and the size of the slowly cycling pool were calculated, it became obvious that tamoxifen decreased

Chart 8. Rates of efflux of MCF-7 cells from the G0-G1 phase of the cell cycle following treatment with ICRF 159. The experimental design is described in the legend to Chart 7. Points, mean of 2 to 6 flasks; bars, S.E. or range. Where bars are not shown, the S.E. did not exceed the size of the symbol. In order to estimate the rate of efflux of the rapidly cycling component, data points between 0 and 12 hr were corrected for the effect of the slowly cycling component and replotted (×).

Chart 9. Effect of tamoxifen treatment on the rate of efflux of MCF-7 cells from the G0-G1 phase of the cell cycle. Cells (2 x 10^6) in exponential growth phase were plated into 25-sq cm flasks in 5 ml of medium containing 5% FCS and the following concentrations of tamoxifen: 0 (O), 100 (A), and 500 (×) nm; and 1 (□), 5 (•), 7.5 (△), and 10 (◆) μM. After 42-hr exposure to tamoxifen, ICRF 159 was added to a final concentration of 100 μg/ml. Replicate flasks were harvested at the times indicated, and the proportion of the cell population remaining in G0-G1 was calculated from DNA histograms obtained by flow cytometry. Points, mean of 2 to 6 observations; bars, S.E.
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-
cer agents by Bruce et al. (8) and more recently by Drewinko et al. (13) serves to underline this point (for review, see Ref. 18).

In this study, the pattern of growth of monolayer cultures of MCF-7 cells followed a typical sigmoidal curve with clearly defined lag, exponential, and plateau phases (Chart 1A). It is important to note that the duration of the lag phase was highly dependent on the stage of growth of the cells used to seed the cultures (Chart 2). This is of particular importance in drug studies, where it is usual to begin drug treatment soon after inoculation of cultures. We have therefore adopted the procedure of always setting up experimental cultures from cells in the fourth to sixth days of culture (Chart 1A), thereby minimizing and to some extent regulating the duration of the lag phase. The period of exponential growth which followed the lag phase is of particular interest because it was at this time that conditions approaching a steady state were attained. During this 5-day period, the growth rate remained constant, and no significant changes were observed in cell cycle phase distribution, proportion of rapidly cycling cells, or clonogenic fraction (Chart 1). This was in marked contrast to the changes seen during the transition from exponential to plateau phase of growth when, over a period of 7 days, the proportion of cells in G1 increased from 50 to 75%, with a corresponding decrease in the proportion of rapidly cycling cells, and the clonogenic fraction dropped to less than 1%. It was only after 14 days in culture that a true plateau phase with constant cell cycle parameters was achieved. As reported by others (40), we found that daily medium renewal from Day 7 was necessary to produce and maintain this plateau stage. Failure to renew the culture medium resulted in a rapid loss of cells from the monolayer from the eighth day of culture at a rate that was somewhat dependent on the serum concentration of the medium, suggesting that nutrient depletion was an important factor in the loss of monolayer integrity.

The mean population-doubling time of 24 hr measured in exponential growth phase is somewhat lower than the 36 hr reported for MCF-7 cells by others (4, 16, 41). This may be due in part to the almost continuous maintenance of cells in exponential growth phase. However, similar rapid doubling times have been observed when MCF-7 cells were plated at low density and given the opportunity to grow exponentially for 5 to 7 days (21).

If all cells in an exponentially growing population are progressing through the cell cycle in an asynchronous fashion, then the relative proportion of cells in any particular phase of the cell cycle reflects the duration of that phase. Thus, the mean phase durations for G0-G1, S, and G2+M in MCF-7 cells can be calculated as 11.8, 9.5, and 2.8 hr, respectively. However, stathmokinetic analyses using ICRF 159 suggest that, even in exponentially growing cultures, approximately 18% of the population are slowly cycling and that these are predominantly in G0-G1 (Charts 1D and 8). Adjustment of the G0-G1-phase time to account for this subpopulation of very slowly dividing cells gives a G0-G1 time of 9.6 hr and a cell cycle time of 21.9 hr.

The data illustrating the effects of tamoxifen on MCF-7 cells in exponential growth phase (Chart 3) 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

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**Table 1**

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<th>Tamoxifen</th>
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<td>171</td>
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* n, the number of observations used to calculate the regression equation; r^2, the coefficient of determination.
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 G1 and G0 phases of the cell cycle or to the subcompartments of G0 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-reversible components. The biochemical basis of these cell cycle effects is currently being investigated.

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