

# Effects of Tamoxifen on Cell Cycle Progression of Synchronous MCF-7 Human Mammary Carcinoma Cells<sup>1</sup>

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## ABSTRACT

The effects of tamoxifen on cell cycle progression and clonogenic survival have been examined using synchronized cultures of MCF-7 human mammary carcinoma cells. Cell synchrony was induced by mitotic selection. Subsequent cell cycle analyses, using DNA flow cytometry, showed that 85% of synchronized cells had a mean cell cycle time of 21.3 hr with mean phase durations of 9 hr for G<sub>0</sub>-G<sub>1</sub>, 9.3 hr for S, and 3 hr for G<sub>2</sub> + M. A slowly cycling or noncycling subpopulation comprising 15% of the total population was also observed. Exposure to tamoxifen (5 to 12.5 μM) resulted in a dose-dependent reduction in the number of cells progressing through G<sub>0</sub>-G<sub>1</sub> and entering S phase. Those cells which were not retained in G<sub>0</sub>-G<sub>1</sub>, however, appeared to traverse G<sub>0</sub>-G<sub>1</sub> and the remainder of the cell cycle at a rate only slightly less than that of untreated controls. Further experiments demonstrated that the major sensitivity to tamoxifen in terms of both inhibition of cell cycle progression and drug cytotoxicity was restricted to a short interval in the middle of G<sub>0</sub>-G<sub>1</sub>. This 2- to 4-hr period of maximum drug sensitivity began approximately 4 hr after mitotic selection, with drug exposures outside this time frame having markedly fewer effects.

The significance of these observations in the light of previous studies with asynchronous populations of MCF-7 cells is discussed.

## INTRODUCTION

In previous studies from this laboratory, it has been demonstrated that tamoxifen treatment of asynchronous cultures of MCF-7 cells resulted in growth inhibition, which was associated with the accumulation of cells in the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle (3-5). Concomitant assessment of long-term cell survival by clonogenic assay indicated that tamoxifen also had a significant cytotoxic effect with drug concentrations of 10 μM or greater (4). However, although these studies suggested some correlation between the cytostatic and cytotoxic effects of tamoxifen, no positive identification of the temporal relationship between these events could be made.

In this study, we have used synchronous cultures of MCF-7 cells to examine further the mechanism of action of tamoxifen with particular reference to any possible relationship between the effects of the drug on cell cycle progression and subsequent clonogenic survival.

## MATERIALS AND METHODS

Stock cultures of MCF-7 cells were maintained in Roswell Park

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Memorial Institute Tissue Culture Medium 1640 supplemented with 5% fetal calf serum as described in a companion paper (4). The methods used for maintenance of stock cultures, clonogenic assays, drug additions, and flow cytometric analysis of cellular DNA content were as described previously (4). In all instances, tamoxifen was added directly to experimental cultures in a small volume of absolute ethanol (not greater than 50 μl). Control cultures received similar volumes of vehicle alone. For drug-pulse experiments, tamoxifen was added for the required period, the medium was removed, and the monolayer was gently rinsed with warm drug-free medium. Fresh warm drug-free medium was added, and the flask was returned quickly to the incubator. This procedure took no longer than 1 min and was not found to cause significant changes in control cultures.

**Cell Synchrony.** The observation that monolayer cells round up and adhere less firmly to a surface during mitosis was exploited by Terasima and Tolmach (6, 7) to selectively harvest mitotic cells from asynchronously growing cell populations. A modification of this technique was used to produce populations of synchronized MCF-7 cells. Plastic flasks (150 sq cm; Corning Glass Works, Corning, N. Y.) were set up with 10<sup>6</sup> cells from Day 5 stock cultures (4) and were used on Days 2 and 3 when cell numbers were 4 to 6 × 10<sup>6</sup>/flask. The numbers of MCF-7 cells and stage of growth at the time of selection were found to be critical determinants of the degree of synchrony obtained. Mitotic cells were harvested by gently shaking the flasks 25 times, allowing the medium to wash over the monolayer. The medium was removed and centrifuged at 200 × g for 5 min. The resulting cell pellets from a number of flasks were resuspended in fresh warm medium, pooled, and counted. About 3 to 5 × 10<sup>4</sup> mitotic cells could be obtained in this way from one 150-sq cm flask. Experimental flasks were then set up at 1 to 2 × 10<sup>5</sup> cells/25-sq cm flask. Replenishment of the 150-sq cm flasks with fresh warm medium allowed further harvests to be made at 3-hr intervals. In all experiments using synchronized cell cultures, the time required for completion of harvesting was about 30 min. Time zero under these circumstances was taken as 15 min after commencement of harvesting.

## RESULTS

The cell cycle distribution of MCF-7 cells 1 hr after mitotic selection is shown in Chart 1. Typically, 90 to 95% of the cells were in G<sub>0</sub>-G<sub>1</sub> phase at this time as all mitotic cells had divided after harvesting. No significant change in this distribution was seen with time of culture until 7 hr, when cells began to leave G<sub>0</sub>-G<sub>1</sub> and progress into S phase (Charts 1 and 2). The G<sub>0</sub>-G<sub>1</sub>-to-S-phase transition occurred over the following 3 hr when the proportion of G<sub>0</sub>-G<sub>1</sub> cells fell to 15% and when S phase increased to about 80%. By 16 to 20 hr after harvesting, the majority of cells had traversed S phase and, during the following 4 to 6 hr, undergone mitosis, once again returning to G<sub>0</sub>-G<sub>1</sub>. Estimates of cell cycle time and of mean phase durations for G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub> + M were calculated from the data shown in Chart 2 for untreated cells as 21.3 hr and 9, 9.3, and 3 hr, respectively. In addition, the presence of a more slowly cycling or noncycling subpopulation of MCF-7 cells representing approximately 15% of the cell population could be observed in G<sub>0</sub>-G<sub>1</sub> between 10 and 18 hr after mitotic selection (Charts 1 and 2). It should be

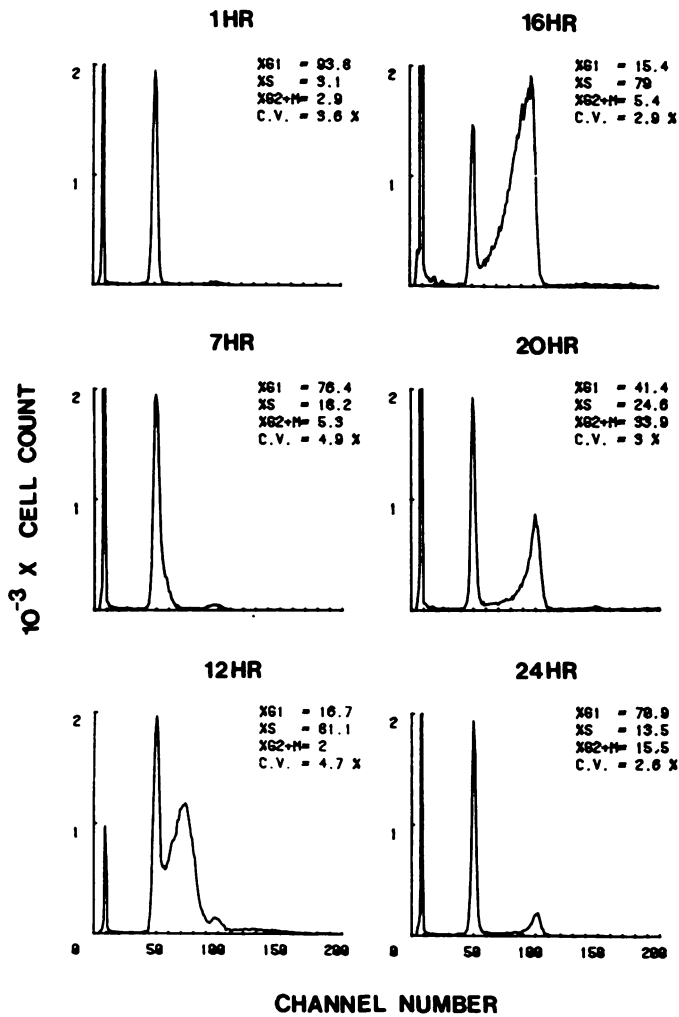


Chart 1. Changes in cellular DNA content distributions of MCF-7 cells with time in culture after mitotic selection. Channel number, relative fluorescence intensity (DNA content). The first peak in each histogram corresponds to chicken erythrocytes which act as an internal biological marker. The major peak in Channel 50 corresponds to MCF-7 cells in G<sub>0</sub>-G<sub>1</sub> phase. C.V., coefficient of variation of the G<sub>0</sub>-G<sub>1</sub> DNA peak.

noted that, while the degree of synchronization in these cultures was reasonably good as evidenced by the rapid transition from G<sub>0</sub>-G<sub>1</sub> to S, the cells rapidly lost synchrony as shown by the absence of a plateau G<sub>0</sub>-G<sub>1</sub> level after the first cycle.

The effects of tamoxifen (5 to 12.5 μM) on the progression of synchronized MCF-7 cells are shown in Chart 3. Tamoxifen was added immediately after harvesting, and the cultures were incubated for 9, 16, or 24 hr before analysis of cellular DNA content. These particular drug exposure times were chosen to allow assessment of the effects of tamoxifen on the progression of cells through G<sub>1</sub>, S, and G<sub>2</sub> + M, respectively. With drug exposure times of 9 and 16 hr, it was apparent that increasing the tamoxifen concentration resulted in an increased proportion of cells remaining in G<sub>0</sub>-G<sub>1</sub> and a corresponding reduction in the numbers entering S phase. This can be observed clearly from the DNA distributions of cultures treated for 16 hr with tamoxifen (Chart 4). At 24 hr, however, similar cell cycle distributions were obtained for all cultures irrespective of treatment. This suggests that the major effect of the drug is to reduce the proportion of cells which can exit the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle but with much less effect on the progression of cells through subsequent

phases of the cell cycle. This observation was supported by a detailed study of the effects of 7.5 μM tamoxifen (Chart 2) which showed that, apart from a slight delay in the 8- and 10-hr points, those cells capable of leaving the G<sub>0</sub>-G<sub>1</sub> phase progressed through the cell cycle at a rate similar to that for controls. It should be pointed out, however, that the plateauing of G<sub>0</sub>-G<sub>1</sub> cells after 24-hr exposure to tamoxifen was found to be un-

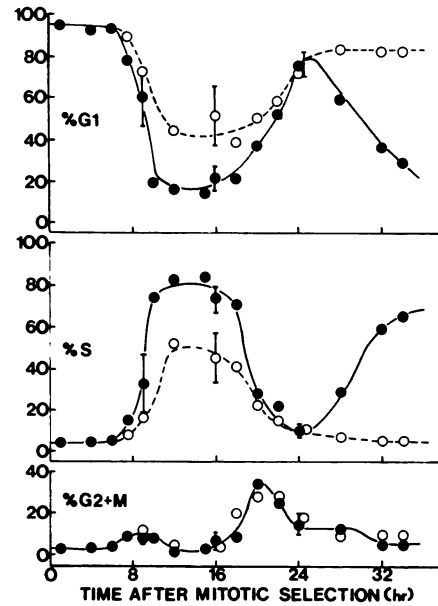


Chart 2. Changes in the cell cycle distribution of MCF-7 cells with time after mitotic selection. ●, non-drug-treated controls; ○, continuous exposure to 7.5 μM tamoxifen. Points, mean of at least 2 individual experiments; points with bars, mean of at least 4 individual experiments; bars, S.D.

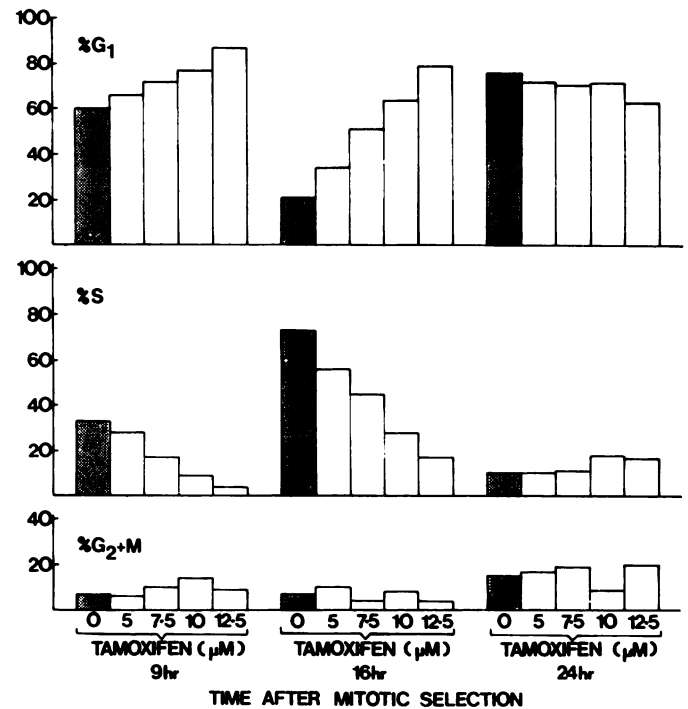


Chart 3. Changes in the cell cycle distribution of MCF-7 cells with time after mitotic selection in the presence or absence of tamoxifen (5 to 12.5 μM). The cell cycle distribution of non-drug-treated control cells 1 hr after mitotic selection is shown in Chart 2. All column histograms represent the mean of at least 3 individual experiments.

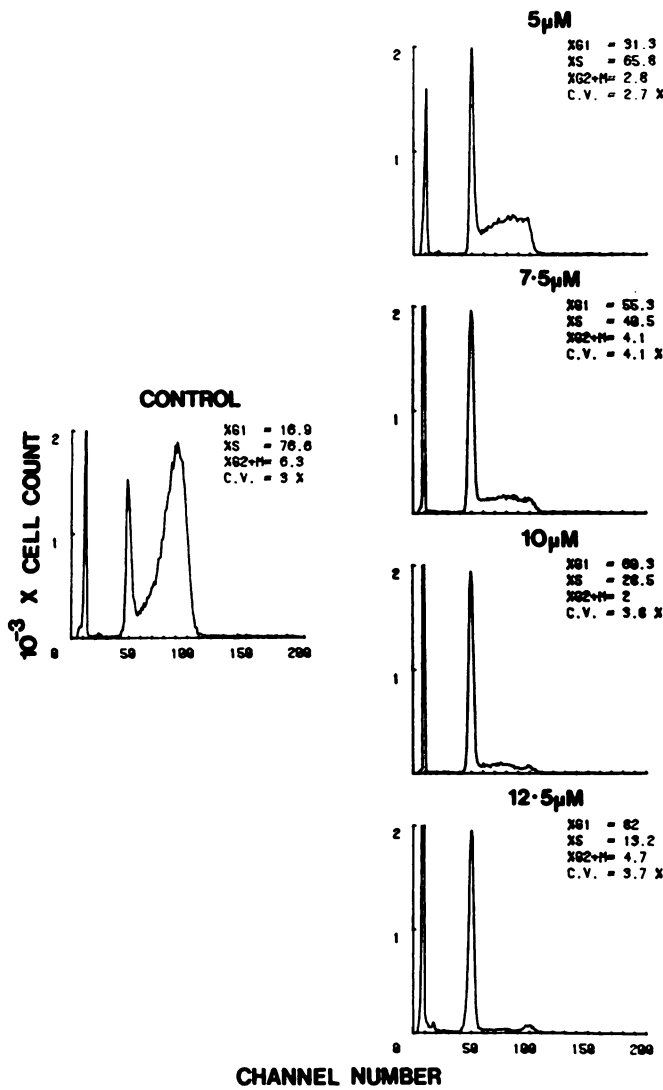


Chart 4. Effect of tamoxifen (5 to 12.5 μM) on the cell cycle progression of synchronized cultures of MCF-7 cells. Cellular DNA analyses carried out 16 hr after mitotic selection. Channel number, relative fluorescence intensity (DNA content). The first peak in each histogram corresponds to chicken erythrocytes which act as an internal biological marker. The major DNA peak in Channel 50 corresponds to G<sub>0</sub>-G<sub>1</sub> MCF-7 cells. C.V., coefficient of variation of the G<sub>0</sub>-G<sub>1</sub> DNA peak.

changed (G<sub>0</sub>-G<sub>1</sub> = 84%) when cultures were subsequently analyzed at 46 hr (data not shown). This suggests that the effects of tamoxifen are cumulative with increasing exposure, a finding which is perhaps more relevant to the prolonged drug exposures commonly examined in asynchronous cultures.

Because our previous studies (3-5) have shown that 7.5 μM tamoxifen exerts its effects primarily through a cytostatic rather than a cytotoxic action, further experiments were undertaken using a known cytotoxic concentration of tamoxifen (12.5 μM) to examine the cell cycle specificity of the cytostatic effects of the drug and corresponding cytotoxicity. Chart 5 shows the effects of 12.5 μM tamoxifen on the cell cycle distribution of MCF-7 cells measured 16 hr after harvesting mitotic cells. As before, 75% of untreated control cells progressed through G<sub>1</sub> and S phase during this period, while cells exposed continuously to 12.5 μM tamoxifen remained predominantly in G<sub>1</sub>. When tamoxifen was administered as a 2-hr pulse, however, only exposures between 4 and 6 hr after harvesting resulted in a significant accumulation

of cells in G<sub>1</sub> (Chart 5). The effects of 2-hr pulses of tamoxifen on cell cycle progression when administered outside of this time frame were much reduced, although a decrease in the apparent rate of progression through S phase compared to controls was observed between 0 and 6 hr. This reduced rate of S-phase progression was more marked than that seen with exposure to 7.5 μM tamoxifen. Concomitant analyses of cell survival by clonogenic assay (Chart 6) indicated that the cytotoxicity associated with 12.5 μM tamoxifen was expressed within the same time frame as were the major cytostatic effects of the drug. Previous studies with asynchronous cells gave a surviving fraction of 0.47 ± 0.09 for a 24-hr exposure to 12.5 μM tamoxifen (4), which is entirely consistent with the results shown here.

### DISCUSSION

Mammalian cells may be synchronized by a variety of chemical or physical techniques (for reviews, see Refs. 1 and 2). Chemical agents which cause reversible accumulation of cells in particular phases of the cell cycle can be conveniently used to produce large numbers of synchronized cells but only at the expense of significant drug-induced biochemical perturbations. Mitotic selection (6, 7), on the other hand, while restricted to monolayer

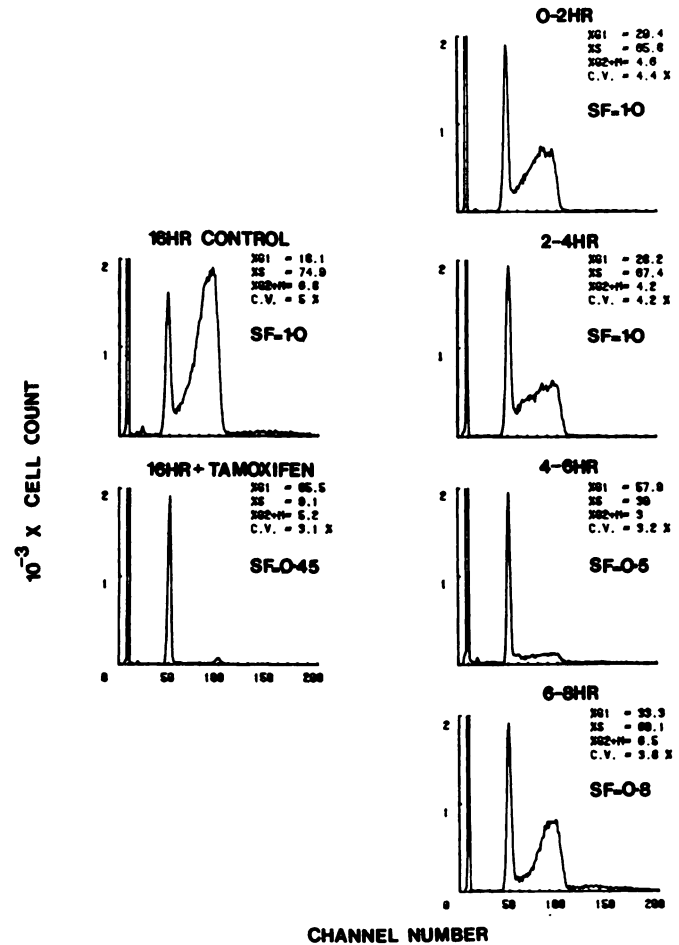


Chart 5. Changes in the cell cycle distribution of MCF-7 cells 16 hr after mitotic selection in the presence or absence of tamoxifen. Cells were exposed to tamoxifen (12.5 μM) either continuously or as a 2-hr pulse during the times indicated. Channel number, relative fluorescence intensity (DNA content). The first peak in each histogram corresponds to chicken erythrocytes which act as an internal standard. The DNA peak in Channel 50 corresponds to G<sub>0</sub>-G<sub>1</sub> MCF-7 cells. C.V., coefficient of variation of the G<sub>0</sub>-G<sub>1</sub> DNA peak; SF, surviving fraction obtained from a clonogenic assay (see Chart 6).

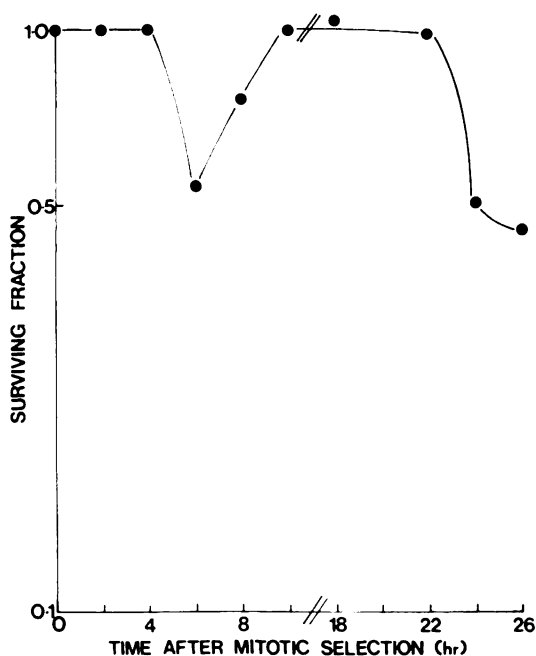


Chart 6. Changes in surviving fraction of MCF-7 cells with exposure to 2-hr pulses of 12.5  $\mu\text{M}$  tamoxifen commencing after mitotic selection. Points, mean of at least 2 individual experiments.

cultures, can produce synchronized cells where biochemical perturbations are minimized. This is particularly important in circumstances where the synchronized cell population is to be used to study cell cycle-related drug effects. It should be pointed out, however, that with MCF-7 cells the stage of growth, size of initial cell inoculum, and time of growth proved to be extremely critical factors in determining the subsequent purity of harvested cells. In this study, typically, 95% of the cells had a  $G_0$ - $G_1$  DNA content 1 hr after mitotic selection. In early experiments where flasks were harvested at a later stage of growth in an attempt to increase the yield of mitotic cells, the proportion of  $G_0$ - $G_1$  cells fell to 75% with significant numbers of cells in S phase and  $G_2 + M$ .

Subsequent analyses of non-drug-treated synchronized cultures of MCF-7 cells showed that 85% of the cells had a mean cell cycle time of 21.3 hr with mean phase durations of 9 hr for  $G_0$ - $G_1$ , 9.3 hr for S, and 3 hr for  $G_2 + M$ . The plateau in the proportion of  $G_0$ - $G_1$  cells seen in these cultures between 10 and 18 hr after mitotic selection suggests that the remaining 15% of cells represent a slowly cycling or noncycling subpopulation. These data compare favorably with those obtained from asynchronous cultures of MCF-7 cells in exponential growth, where 82% of cells formed a rapidly cycling subpopulation, which had a cell cycle time of 21.9 hr and similar phase durations as those reported here (4). The slowly cycling subpopulation was estimated at 18% in asynchronous cells by stathmokinetic analysis using ICRF 159.

In asynchronous cultures of ER<sup>3</sup>-positive MCF-7 cells, tamoxifen caused an inhibition of cell growth characterized by an increase in cells with a  $G_0$ - $G_1$  DNA content. A dose-dependent reduction in cell survival with exposure of exponentially growing cells to tamoxifen was also found, and this seemed to correlate to some extent with the growth-inhibitory effects. The use of

<sup>3</sup> The abbreviation used is: ER, estrogen receptor.

synchronized cell cultures in this study has allowed a more detailed study of the mechanism of action of tamoxifen. The data presented here suggest that the drug reduces the proportion of cells progressing from  $G_0$ - $G_1$  into S phase, thus effectively increasing the slowly cycling subpopulation as suggested previously (4). It was most interesting to note, however, that this effect on cells in  $G_1$  was predominant during a 2- to 4-hr period in the middle of this phase with drug exposures outside this time frame having markedly fewer effects. Moreover, the manifestation of cytotoxicity of tamoxifen was almost entirely confined to this same short period.

Our previous studies (4) did not allow estimation of the effect of tamoxifen on the rate of progression of rapidly cycling cells. However, in a number of instances in this study, it was noted that tamoxifen did cause some reduction in the rate of progression of this subpopulation. This was generally manifested by tamoxifen-treated cells being in an earlier part of S phase than might be expected from non-drug-treated controls (see Chart 5) and was observed to any degree only with relatively high tamoxifen concentrations (*i.e.*, 12.5  $\mu\text{M}$ ). In comparison to the effects of tamoxifen on the slowly cycling subpopulation, these changes in rapidly cycling cells were relatively trivial.

The data presented in this and the companion paper (4) have enabled precise definition of the proliferation kinetics of MCF-7 cells in monolayer culture. This includes the definition of 2 distinct subpopulations based on their rates of efflux from  $G_0$ - $G_1$  phase and cycle transit times. Together, this information provides a kinetic basis on which to define the cell cycle effects of a wide range of hormones and growth factors known to regulate the growth of human breast cancer cells.

In the present series of papers, the effects of the antiestrogen, tamoxifen, on these parameters have been investigated. Such studies have led to the conclusion that tamoxifen is a cell cycle phase-specific, growth-inhibitory, and cytotoxic agent acting at a very precise period in the middle to latter half of  $G_1$ . The apparent precision of the insult induced by tamoxifen coupled with the reduced effect of the drug on cells outside the critical 2- to 4-hr period makes it tempting to speculate that tamoxifen is in some way interacting with a fundamental biochemical process important in cell cycle regulation. Investigations are currently in progress to examine this phenomenon in more detail in both ER-negative and other ER-positive human cancer cell lines.

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