SUMMARY

One-hr treatments of Chinese hamster ovary cells in plateau phase with the cell cycle-active drugs hydroxyurea or cytosine arabinoside produced no effects on survival. Plateau-phase cells treated with adriamycin (1 to 10 μg/ml for 1 hr) exhibited a biphasic survival curve similar to that for exponentially growing cells. The most sensitive population of exponentially growing cells had a $D_0 = 0.2 \mu g/ml$ for 1 hr, and a $D_0 = 9.4 \mu g/ml$ for 1 hr in the more resistant population of the biphasic survival curve. The survival curve for the plateau phase cells had $D_0 = 0.7 \mu g/ml$ for 1 hr and $6.4 \mu g/ml$ for 1 hr for the sensitive and resistant parts of the curve, respectively. Although the plateau cells were less sensitive than exponentially growing cells, the survival fraction was still reduced to 4% by 10 μg/ml for 1 hr. Therefore, these data suggest that adriamycin may be an effective agent for use on slowly growing solid tumors which contain large fractions of nondividing cells. In addition, the in vitro results of adriamycin on nondividing cells may be predictive of side effects on normal stem cell compartments in the bone marrow and small intestine.

INTRODUCTION

We have reported that nondividing CHO cells are more sensitive to bleomycin and 1-3-bis-(2-chloroethyl)-1-nitrosourea than cells in the exponential phase of growth (4). Evidence has been presented to indicate that cells in plateau phase are arrested in an “extended” G₁ period (4, 9, 18), and under the appropriate stimulus, these cells reenter the cell cycle synchronously. The G₂ compartments described by Patt and Quastler (15) for stem cells and by Mendelsohn (13, 14) for tumor cells in the viable but nongrowth fractions of some solid tumors thus resemble plateau phase cells. Since plateau or G₂ cells generally are not killed by the cell cycle-active drugs, these cells probably contribute to the repopulation or regrowth of the tumor. Therefore, the plateau phase cultures may serve as excellent model systems for studies of cancer drug effects on nondividing cells. This paper reports the survival responses of mammalian cells treated in plateau phase to ara-C, HU, or adriamycin.

MATERIALS AND METHODS

Cell and Culture Techniques. Culture techniques for maintaining stocks of CHO cells, for induction of plateau phase, and for kinetics studies of plateau cells reentering the cycle have been reported (4). Briefly, 2 to $3 \times 10^6$ exponentially growing CHO cells are placed in each 60-mm Petri dish containing 7 ml of Hsa's modified McCoy's 5A medium, supplemented with 10% fetal calf serum, and incubated. The cells are never fed again until the survival experiments are performed. Total cell counts are obtained at various intervals, and the cell number plateaus between 6 and $8 \times 10^6$ cells/plate after 48 hr. The survival experiments are performed only on cells that have been in plateau phase for at least 48 hr. The reentry of plateau phase cells into the cell cycle is followed on cells that have been resuspended (10⁶/dish) in fresh medium. At 3-hr intervals, cells are pulse labeled with 1 μCi thymidine-$^3$H per ml (specific activity, 1.9 Ci/m mole), and total cell counts are performed. The cells reenter the cell cycle synchronously, and progress from G₁ into S phase at 5 to 10 hr after subculturing. The cell number begins increasing at 18 to 22 hr and doubles by 30 hr after the cells are subcultured (4). In all experiments involving assays of radioisotope-labeled cells, Ilford K₁ liquid emulsion autoradiograph techniques were used as described previously (2).

Survival Determinations. The effects of drugs on survival were determined on cells that had been maintained in plateau phase for at least 48 hr. Concentrations of 1 to 10 μg adriamycin/ml, 1 to 100 μg ara-C per ml, or 1 to 7600 μg HU per ml were used in the 1-hr treatments. All plateau phase cultures were treated by adding the drug directly to plateau medium in which the cells had been growing, since the cells might have progressed out of plateau phase during treatment if fresh medium had been used. Exponentially growing cells were treated in fresh medium. Eight replicate plates were used for each survival study, and each survival
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RESULTS AND DISCUSSION

The effects of ara-C on survival of cells treated in plateau phase are shown in Chart 1. A survival curve of exponentially growing cells is included for comparison. The cells were treated in their respective modes of growth and plated immediately for colony formation. The results show that this cell cycle-active drug reduces the survival fraction in exponentially growing cells to 0.50 after a 1-hr treatment with 100 μg/ml; however, cells treated in plateau phase are unaffected by 75 μg/ml and show greater than 95% survival following a treatment with 100 μg/ml for 1 hr. The antitumor activity of ara-C is ascribed to its effect on inhibiting DNA synthesis after being phosphorylated by cytidine kinase to ara-CTP (5, 11). This inhibition may result by a direct effect on DNA polymerase (8) with ensuing cell lethality by unbalanced growth (1). In Don C cells, ara-C has been shown (10) to kill cells primarily in the S phase of the cell cycle. However, the decreased killing effect of ara-C on KB cells (10) was attributed to the fact that the drug was rapidly converted to an inactive product, arabinosyluracil, by cytidine deaminase. We have reported (3, 7) similar results on 4 human melanoma strains and a human lymphoma growing in vitro. Those cells with high cytidine kinase levels were sensitive to short (1-hr) treatments with ara-C. However, other strains exhibited differential sensitivities to the drug, due either to low kinase levels or high deaminase levels. Because the CHO cell populations in the present plateau model contained fewer than 1 to 4% S-phase cells, we find the lack of effect on survival to be consistent with the description of ara-C as a cell cycle-active drug.

The survival responses of dividing and nondividing cells following treatment with HU are shown in Chart 2. In exponential cells, survival decreases gradually at doses through 760 μg/ml for 1 hr. The survival fraction then plateaus at 0.40, with no increased killing at doses as high as 7600 μg/ml. Since HU has been shown to be an S-phase-
specific agent (17) and since approximately 60% of the exponentially growing CHO cells are in S phase (under the growth conditions of our laboratory), these data indicate that all S-phase cells were killed. No plateau phase cells were killed by any of the doses used. This is an expected response since essentially all of the plateau phase cells in our system are in a nondividing state (4).

Adriamycin interferes with DNA and RNA synthesis, while protein synthesis appears to be unaffected (12, 16). It has also been reported (2, 12) that adriamycin kills cells most effectively in G1 and S phase. We have presented evidence that adriamycin kills 99.9% of exponentially growing CHO cells after treatment with 2 µg/ml for 1 hr (2). At higher doses (up to 25 µg/ml), very little additional killing was observed. A portion of that survival curve is included in Chart 3 for comparison with the survival curve for CHO cells treated with adriamycin in plateau phase. Both curves are biphasic. The survival fraction decreases to 0.15 after exposure to 2 µg/ml for 1 hr. At 10 µg/ml for 1 hr, the survival fraction was reduced to 0.04.

The response of CHO cells in plateau phase to HU and ara-C further supports other findings (5, 8, 10, 11, 17) that these are truly cell cycle-active drugs. Therefore, these drugs have been effective only on tumors that had large growth fractions. The high killing efficiency of adriamycin on dividing and nondividing cells in vitro suggests that this agent may be an effective drug for use on slowly growing solid tumors containing large fractions of nondividing cells.

We are aware of the limitations inherent in comparing plateau phase cells in vitro with G0 cells in vivo. However, these results do suggest a possibility that considerable killing in the G0 compartment in vivo may be observed. A better understanding of the effects of anticancer drugs on these viable but nondividing cell populations has potential clinical significance. Since the plateau or G0 cells generally are not killed by the cell cycle-specific drugs, these cells probably contribute to the repopulation or regrowth of the tumor. Therefore, it is extremely important to identify and characterize drugs that will specifically influence the survival, repair, and repopulation kinetics of G0 and plateau phase cells. Such studies are continuing in our laboratory.

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Survival Responses of Dividing and Nondividing Mammalian Cells after Treatment with Hydroxyurea, Arabinosylcytosine, or Adriamycin

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