Survival and Cell Kinetics Effects of Adriamycin on Mammalian Cells

S. C. Barranco, E. W. Germer, K. H. Burk, and R. M. Humphrey

Department of Human Biological Chemistry and Genetics, Division of Cell Biology, University of Texas Medical Branch, Galveston, Texas 77550 [S. C. B.,] and Departments of Physics, Section of Cellular Studies [E. W. G., R. M. H.] and Surgery (Gynecology) [K. H. B.], University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

SUMMARY

Adriamycin is extremely toxic to Chinese hamster ovary cells in relatively low drug concentrations. The survival response of asynchronous populations of cells is biphasic (n = 1, D0 = 0.2 µg/ml for 1 hr in the most sensitive population; D0 = 9.4 µg/ml for 1 hr in the more resistant population). Cells in mitosis and early S phase are most sensitive to the drug; G1 and late-S-phase cells exhibited the least sensitivity. Progression delay was observed in all phases of the cell cycle except mitosis. The length of delay appears to be dose dependent.

INTRODUCTION

Adriamycin, an anthracycline antibiotic isolated from Streptomyces peucetius var. caesius (1), is closely related to another antibiotic, daunomycin, differing only in replacement by a hydroxyl group of a hydrogen atom in the acetyl radical of the aglycone moiety (6). While adriamycin is not active as an antimicrobial agent (14), it exhibits antitumor activity with a high therapeutic toxicity ratio (6, 13). The drug interferes with DNA and RNA synthesis, while protein synthesis appears to be unaffected (10, 15). It has also been reported (10) that adriamycin kills cells most effectively in S phase.

The purpose of this paper is to describe the effects of adriamycin on cell survival, stage sensitivity, and cell progression throughout the cell cycle of CHO2 cells growing in vitro.

MATERIALS AND METHODS

Preparation of Adriamycin. Adriamycin manufactured for experimental use (Soc. Farmaceutici Italia An. p. Az., Milan, Italy) was used in these experiments. The adriamycin solutions were always made immediately prior to use by dissolving adriamycin in sterile 0.9% NaCl solution and diluting into the growth medium. The pH of the treatment medium was adjusted to 7.2 to 7.4.

Cell Culture Techniques. CHO cells were grown exponen-

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2 The abbreviations used are: CHO, Chinese hamster ovary; TdR, thymidine; MI, mitotic index; LI, labeling index.

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or collected for progression studies. G₁ synchrony and progression into S phase was monitored by labeling cells for 10 min at various times with TdR-³H, and the number of labeled cells was scored. In all cases, 500 cells were scored for each sample point.

For experiments involving progression of cells from G₂ phase to mitosis, asynchronous CHO cell populations were pulse labeled with TdR-³H, 1 µCi/ml (1.9 Ci/m mole), for 10 min, washed twice, and then treated continuously with medium containing adriamycin and Colcemid, 0.06 µg/ml (2, 3). Medium in the control plates contained Colcemid, but no adriamycin. At 20-min intervals thereafter for 3 to 4 hr, samples were taken. Cells were removed from the dishes with 0.025% trypsin, centrifuged, fixed in 50% acetic acid, and then stained with aceto-orcein. Slides for autoradiography were prepared and exposed for 2 weeks, and the percentage of unlabeled cells in metaphase (accumulated in Colcemid) was determined by the scoring of 500 cells.

RESULTS

Survival of Asynchronous CHO Cells. The survival of CHO cells following a 1-hr treatment with varying doses of adriamycin is shown in Chart 1. The survival response to this drug is biphasic (n = 1, D₀ = 0.2 µg/ml for 1 hr in about 99% of the population; D₀ = 9.4 µg/ml for 1 hr in the more resistant group of cells). Survival is reduced to less than 0.2% with a 2-µg/ml dose of adriamycin. No shoulder is observed in the response curve in the dosage range from 0 to 2 µg/ml. At doses greater than 5 µg/ml, the survival response of the remaining resistant 0.1% of the population assumes a shallow plateau shape through a dose of 25 µg/ml.

Survival Response of Synchronized Cells. For determination of relative sensitivities to adriamycin during each stage of the cell cycle, survival data were obtained on synchronous populations of cells. In these experiments, the location of the cells within the cell cycle at the time of treatment was determined with labeling TdR-³H and MI data. The sensitivity of mitotically selected cells is depicted in Chart 2. Aliquots of these cells were plated and treated with adriamycin for 1 hr at various times in M and G₁ phase. Drug concentrations of 0.1 (asynchronous survival, 67%) and 0.5 µg/ml (asynchronous survival, 8%) were used. Mitotic cells treated with adriamycin showed a reduction to 30% survival when treated with 0.1 µg/ml and reduction to a 2.3% survival following treatment with adriamycin, 0.5 µg/ml. In comparison to M cells, G₁ phase cells were less sensitive to both treatment doses (Chart 2).

Figures

Chart 1. The effect of adriamycin on survival in asynchronously growing CHO cells.

Chart 2. The effect of adriamycin on survival of synchronized CHO cells treated in mitosis or G₁ phase.
construed as an increase in toxic response of late-S or G2 cells. However, the possibility of a sensitive G2 population cannot be ruled out.

**Progression of Synchronized CHO Cells Treated with Adriamycin.** Mitotic cells treated with adriamycin, 0.5 μg/ml (continuously), progressed uninhibited from mitosis into G1 phase at the same rate as controls. The MI decreased from 97 to 0% within 1 hr after the mitotic cells were plated either in fresh medium or in medium containing adriamycin, 0.5 μg/ml. Synchronized populations of cells treated with adriamycin at the 1st hr into G1 phase were delayed in their progression into S phase. It can be seen in Chart 4 that the TdR-3H-labeled fraction in control G1 populations began increasing in a linear fashion 2.5 hr after the mitotic cells were plated, indicating progression of G1 cells into S phase. Exposure of the G1 cells to a continuous adriamycin treatment of 0.1 μg/ml, starting at the 1st hr of G1 phase, caused a 1-hr delay in G1 phase. However, the cells were able to overcome the progression block and subsequently moved into S phase at the same rate as control cells. Cells treated continuously with adriamycin, 0.5 μg/ml (Chart 4), were delayed for 3 hr in G1 phase, and when these cells recovered from the block they moved into S phase at a much slower rate than that of the controls or the 0.1-μg/ml-treated populations. The progression delay appears to be dose dependent.

Control populations synchronized in early S phase progressed normally through the DNA-synthesizing compartment of the cell cycle and into G2 phase, as seen from the LI data of Chart 5. The LI remains near 95% as the cells move through mid- and late S phase. As the cells progress into G2 phase, the LI decreases to a minimum at 8 hr followed by an increase in LI as the cells move again into S phase. The effects of a 1-hr and of a continuous drug treatment on progression of cells from early S phase into G2 and mitosis were studied. As seen in Chart 5, the LI of cells treated for 1 hr or continuously with adriamycin, 0.5 μg/ml, showed marked progression inhibition since the LI failed to decrease below 84% through the 12-hr sampling period. When the adriamycin concentration was reduced by a factor of 50, to 0.01 μg/ml (Chart 6), cells treated continuously were delayed for 2 hr but then progressed normally. In this population the LI remained above 90% through 6 hr and then decreased to a minimum at 9 hr as the cells moved into G2 (Chart 6, top). Further evidence for the progression delay of early-S-phase-treated cells is given in Chart 6 (top). As the control LI decreases and reaches a minimum at 8 hr (indicating progression from S phase into G2 and M), the MI reaches a maximum of 20% and then drops rapidly to 10%. In comparison, the MI of the treated population reached a peak at 9 hr, coincident with minimum LI in Chart 6 (bottom). The MI of the treated population also decreased at a slower rate than that of the control, indicating a difficulty in passing through mitosis. S-phase progression delay also appears to be dose dependent.

**Progression of Asynchronous Cells from G2 to Mitosis after Adriamycin Treatment.** For determination of the effects of adriamycin on cell progression from G2 phase into mitosis,
Cells treated continuously with adriamycin, 10 μg/ml, showed no increase in percentage of unlabeled cells. These results indicate that cells were being delayed in G₂ phase and were not progressing into mitosis in both treated populations. In addition, the progression delay from G₂ to M appears to be dose dependent.

**DISCUSSION**

Survival determinations made on synchronized cells (Charts 2 and 3) indicate that mitosis and early S phase are most sensitive to adriamycin, while G₁, late-S, and G₂ are less sensitive. This cell cycle age response was demonstrated with a range of adriamycin doses. When treating synchronized cells with drugs, we know for example that the cells initially treated in M phase may be in early G₁ phase by the end of treatment (Chart 2). The action of these drugs is usually so quick that one need not be concerned with cell progression effects; however, the survival points reported for M phase cells may reflect M-G₁ sensitivities. Nevertheless, since the 50% survival reported for cells treated at the 1st hr of G₁ is greater than the survival reported for cells treated in mitosis (3%), we believe the sensitivity reported for M cells in Chart 2 to be substantially correct. Synchronized populations of S-phase cells exhibited the greatest sensitivity in early S phase (Chart 3) when treated with adriamycin, 0.5 or 0.01 μg/ml. These data are in agreement with the work of Kim and Kim (10), who showed that adriamycin kills cells most efficiently in S phase. We feel, however, that adriamycin should not be labeled a cell cycle phase-specific drug. Although the drug kills mitotic and S-phase cells as well as a substantial fraction of late G₁ cells, the 4-decade decrease in survival, following a 1-hr treatment of asynchronous cells with doses of 3 to 5 μg/ml, indicates that adriamycin is extremely efficient at killing all cycling cells, no matter where they are in the cycle, at the time of treatment. The biphasic dose-response curve (Chart 1) suggests sensitive and less sensitive fractions of cells in asynchronously growing cell populations. The D₀ of the most sensitive fraction (0.2 μg/ml for 1 hr) agrees with the survival data for HeLa cells reported by Kim and Kim (10). Survival data reported in this paper for asynchronous cells indicates that, at doses greater than 3 to 5 μg/ml, the killing efficiency of adriamycin (killing efficiency defined as the number of cells killed per unit drug concentration) decreases dramatically. As seen in Chart 1, doses in the range of 3 to 5 μg/ml kill 99.9% of the original population, while doses 5 to 10 times larger produce only 0.1% greater killing effect.

Adriamycin caused progression delay in all phases of the cell cycle except mitosis. The length of progression delay is dose dependent (Charts 4 to 7). Cells treated continuously in G₁ with 0.1 μg/ml were delayed 1 hr but then proceeded normally into S phase (Chart 4). G₁ cells treated continuously with 0.5 μg/ml were delayed for 3 hr and then progressed erratically and at a much slower rate into S phase. The progression delay of G₁ cells may be due to dose-dependent interference of normal RNA activity or synthesis in this phase of the cell cycle. The drug rapidly inhibits RNA synthesis (10, 19). Furthermore, several investigators (4, 9, 12) have presented...
evidence that the inhibition of RNA synthesis in G1 phase delays the initiation of DNA synthesis. Why some of these G1 cells were able to overcome the progression block and move into S phase is unclear; however, it may be the result of drug inactivation or decay. This possibility was not tested.

CHO cells treated with adriamycin, 0.5 μg/ml, in early S phase (Chart 5) exhibited complete blockage in S phase regardless of whether the treatment lasted for 1 hr or continuously. However, cells treated continuously in early S phase with adriamycin, 0.01 μg/ml, were delayed in S phase for only 2 hr and then progressed into G2 (Chart 6). Our data at the cellular level do not indicate the cause of the S-phase progression delay; however, Wang et al. (19) have shown that adriamycin inhibits DNA polymerase activity, probably by binding to the template DNA. Since Wang et al. (19) also showed that the inhibition of DNA polymerase was dose dependent, it is possible that adriamycin may cause the cells to be irreversibly blocked in S phase after treatment with the high dose (0.5 μg/ml) by irreversibly inhibiting DNA polymerase, while treatment with a lower dose (0.01 μg/ml) would result in a short but reversible delay in S phase progression, due to a lesser effect on DNA polymerase activity. As shown in Chart 6 (bottom), S-phase cells treated with the smaller adriamycin dose (0.01 μg/ml) also exhibited some difficulty in progression through the subsequent mitosis. The control MI peaked at 8 hr (20% MI) and fell quickly to 3%, whereas the MI of the treated cells reached a maximum at 9 hr and decreased at a much slower rate. This phenomenon is currently being investigated in our laboratory.

Cells in G2 at the time of treatment with adriamycin are immediately prevented from progressing into mitosis (Chart 7). It has been shown (16–18) that interference with the translational and transcriptional events occurring in G2 affects the synthesis or function of a “division-specific protein.” The presence of this protein (or proteins) is necessary for the successful progression of cells from G2 into M. The onset of delay caused by the treatment of G2 cells with the protein synthesis inhibitors puromycin or cycloheximide (17, 18) produced a G2 block 10 to 25 min before mitosis. Although Kim and Kim (10) and Wang et al. (19) showed that adriamycin produced only very minimal effects on protein synthesis, our data suggest the possibility that adriamycin may affect the synthesis or structural configuration of the proteins necessary for division and progression since adriamycin-induced delay occurs at approximately the same point in G2 phase as that produced by puromycin (17, 18).

These data suggest several important implications relative to the chemotherapeutic use of adriamycin. First, although adriamycin shows some specificity for killing mitotic and S-phase cells, it cannot be considered as a true cell cycle phase-specific drug because doses as low as 2 μg/ml for 1 hr kill 99.8% of all of the cells in an asynchronously growing population. At doses greater than 5 μg/ml, the dose-response curve of the remaining 0.1% of the tested population assumes a shallow plateau shape, indicating a drastic reduction in cell-killing efficiency. Secondly, high doses of adriamycin pose a special problem in vivo. Adriamycin is cleared rapidly from the blood (5, 7), but it is retained for extremely long times in most tissues of the body. As a result, severe side effects can result from treatment with this drug, including alopecia, nausea, vomiting, myelosuppression, and heart irregularities (11). Therefore, because of the excellent cell-killing effect of adriamycin at relatively low doses, a reduction in the treatment doses used clinically may improve the therapeutic index of the drug by inducing fewer harmful side effects.

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