Eur J Cancer Clin Oncol, Vol. 19, No. 4, pp. 547–551, 1983.
Printed in Great Britain.

0277-5379/83/040547-05$05.00/0
Pergamon Press Ltd.

Correlations between Cell-cycle Perturbations and Survival Levels after Exposure to Adriamycin for Two Chinese Hamster Cell Lines*

N. TOKITA,† J. H. JETT,† M. R. RAJU† and J. A. BELLI‡

†*Life Sciences Division, Los Alamos National Laboratory, University of California, Los Alamos, NM, U.S.A.*
and ‡*Joint Center for Radiation Therapy, Harvard Medical School, Boston, MA, U.S.A.*

Abstract—*The cell-cycle perturbation and cell inactivation after adriamycin (ADR) exposure were measured in two Chinese hamster cell lines differing in ADR sensitivity. Based on DNA distribution analysis, it was found that the rate of G_1 fraction depletion following low-dose ADR treatment was related to the level of cell inactivation. The results indicate that gross cellular sensitivity to ADR can be predicted rapidly by this approach.*

INTRODUCTION

CULTURE-GROWTH kinetic studies and cell-survival studies are, in principle, independent entities based on two different phenomena of cell proliferation. The former involve measurements of changes in properties such as cell-cycle distribution and proliferation rate. In contrast, the latter involve the reproductive integrity of the cells which can be measured only after longer time periods in which multiple cell divisions have taken place. If the magnitude of changes in cell-cycle distribution after drug treatment reflected the ultimate survival pattern of the treated cells, rapid flow cytometric studies of cell-cycle distribution could be used for predicting cellular responses to drugs in a short period of time. Some attempts to correlate cell-cycle changes with cell survival have been made recently [1–4].

In the present study we have extended these correlations by quantifying the relationship between cell-cycle perturbations and cell survival. This was accomplished by comparing the magnitude of cell-cycle distribution changes after adriamycin (anthracycline HCl, ADR) treatment to the cell survival. In addition, two cell lines differing in ADR sensitivity are compared to determine the relationship between the magnitude of cell-cycle perturbation and cellular sensitivity to the drug.

Accepted 24 September 1982.
*This work was supported by a Grant CA 22585 from the National Cancer Institute, DHEW and by the U.S. Department of Energy.

Our data suggest that a good correlation exists between the magnitude of cell-cycle perturbation and cell survival in a limited range (for the first log of cell killing) and rapid flow cytometric studies of cell-cycle distributions (DNA histograms) could be used to predict gross cellular responses to low-dose ADR treatment.

MATERIALS AND METHODS

Two Chinese hamster cell lines, V79 and V79b, were maintained as monolayer cultures in Eagle's basal medium (Grand Island Biological Company, Grand Island, NY) supplemented with 15% fetal calf serum and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. The line V79b, an adriamycin-resistant subline of V79 cells, was developed by exposing V79 cells continuously to low doses of ADR for prolonged periods [5]. The cell-cycle times for V79 and V79b cells were 12 and 13.5 hr respectively.

Experimentally, 4×10^5 cells were plated onto 25-cm^2 plastic tissue culture flasks (Becton-Dickinson Company, Oxnard, CA) in 12 ml of growth medium 18–20 hr prior to drug exposure. Adriamycin (ADR, Adria Laboratories, Columbus, OH) was dissolved in saline (100 μg/ml) just before use and diluted with medium to obtain concentrations ranging from 0 to 6 μg/ml. Exponentially growing monolayer cultures (10^6 cells/flask) were exposed to ADR in the medium at 37°C for 1 hr (in the dark). After exposure the cells were rinsed twice with phosphate-buffered salt solution (PBS, 128 mM NaCl, 1.5 mM

KH_2PO_4, 8.5 mM Na_2HPO_4), overlaid with 12 ml of drug-free medium and kept at 37°C until the time of cell harvest. The cells were harvested after one-quarter or one-half of the cell-cycle time had elapsed (3 and 6 hr after ADR exposure for V79 cells and 3.4 and 6.8 hr for V79b cells respectively). The cells were dissociated with 0.25% trypsin in PBS (5 min at 37°C), and after adding 5 ml of medium to inhibit the trypsin the cells were centrifuged at 200 *g* for 5 min, rinsed twice with PBS and fixed in 70% ethanol prior to storage at 4°C.

DNA content analysis of individual cells was made by staining cells with mithramycin [6] and then measuring the fluorescence of the DNA-bound stain using the Los Alamos Flow Cytometer FMF II [7]. In untreated cultures the cell-cycle distributions were analyzed using the method of Dean and Jett [8]. The DNA distributions derived from the drug-treated cultures could not be analyzed by the technique of Dean and Jett since the shapes of S-phase in drug-treated cultures did not conform to a second-degree polynominal required for that analysis procedure. Therefore estimates of the fraction of cells in G_1 and early S phase were obtained by a method similar to that previously used by Krishan and Frei [2]. This procedure calculates the fraction of cells below a predetermined channel number which was the same for all histograms in a series. That predetermined channel number corresponds to an inflection point (G_1–S boundary) on the DNA histogram of an untreated culture. No shift of the G_1 peak channel was observed within this series of experiments, although ADR-induced shift in the G_1 peak channel has been observed previously [9].

For cell-survival assays, monolayer cells were exposed to various concentrations of ADR for 1 hr in the same manner described above. The cells were then suspended by trypsinization and counted with a Coulter counter to determine the cell concentration. Appropriate numbers of cells were plated onto 60-mm Petri dishes in drug-free medium. The dishes were incubated 6 days at 37°C. The dishes were washed, fixed and stained and the resultant colonies (≥50 cells/colony) counted. Cell survival is expressed as a percentage of the colonies formed in parallel untreated cultures.

RESULTS

Figure 1 shows the cell-survival curves for exponentially growing V79 and V79b cell cultures exposed to various concentrations of ADR for 1 hr. The survival curve for V79 cells showed an exponential decrease in cell survival, whereas that for V79b cells displayed a shoulder and a resistant

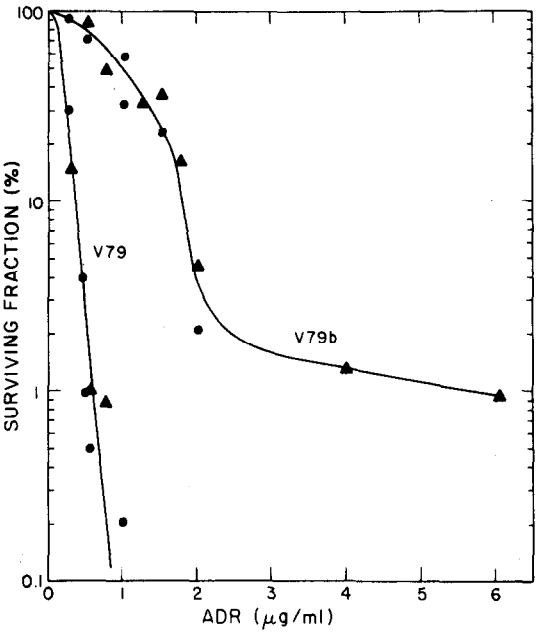

Fig. 1. Survival of exponentially-growing V79 and ADR-resistant V79b cells after exposure to ADR for 1 hr. *Circles and triangles represent duplicate experiments.*

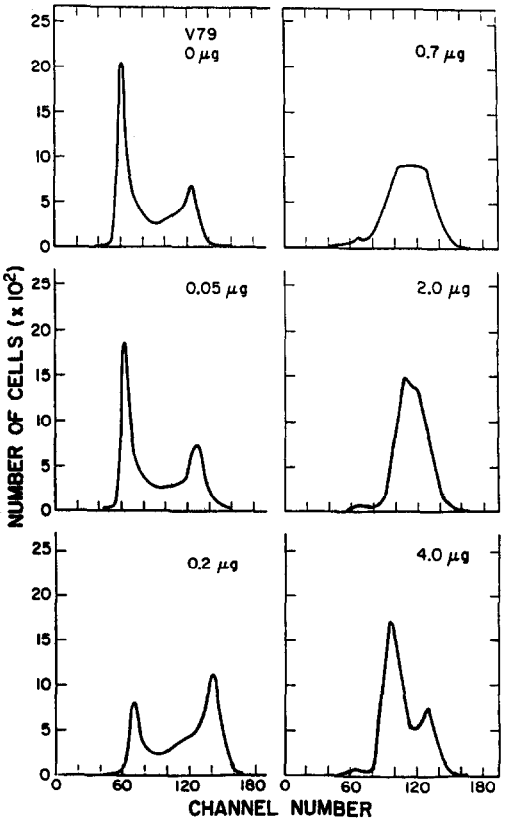

Fig. 2. Cell-cycle distributions for V79 cells sampled at 6 hr *following ADR exposure* (0–4 μg ml/*for* 1 hr). *Each DNA distribution represents* 50,000 *cells.*

subpopulation which comprised about 3% of the total population. The survival after exposure to 0.5 μg/ml was 4% for V79 cells and 80% for V79b cells.

Figure 2 compares the DNA distributions for

V79 cells exposed to ADR at concentrations from 0 to 4 μg/ml and sampled at 6 hr (one-half of the cell-cycle time). In these histograms, channel number is proportional to DNA content. The channel numbers 60 and 120 correspond approximately to the G_1 and G_2/M peaks respectively. The magnitude of the late-S block was more prominent at higher dose levels. Almost complete depletion of the G_1 peak was evident by 6 hr following exposure to 0.7 μg/ml ADR and less than 1% of the cells had survived (Fig. 1). Exposure to 0.05 μg/ml for 1 hr resulted in approximately 95% survival (Fig. 1) and a detectable increase in the fraction of late-S/G_2/M was seen when compared to the control (Fig. 2).

Figure 3 illustrates the DNA distributions for V79b cells 6.8 hr (one-half of the cell-cycle time) after a 1-hr exposure to various concentrations of ADR. There was an accumulation of V79b cells in mid-S phase and the cells in G_1 were not particularly arrested at lower ADR concentration ranges. No significant increase in the G2/M fraction was seen (Fig. 3). The DNA histogram of V79b cells after exposure to 0.25 μg/ml ADR is similar to that of V79 cells after exposure to 0.05 μg/ml ADR (Figs 2 and 3).

A consistent pattern exhibited in Figs 2 and 3 is the large depletion of the G_1/early-S region of the DNA distribution with increasing drug dose.

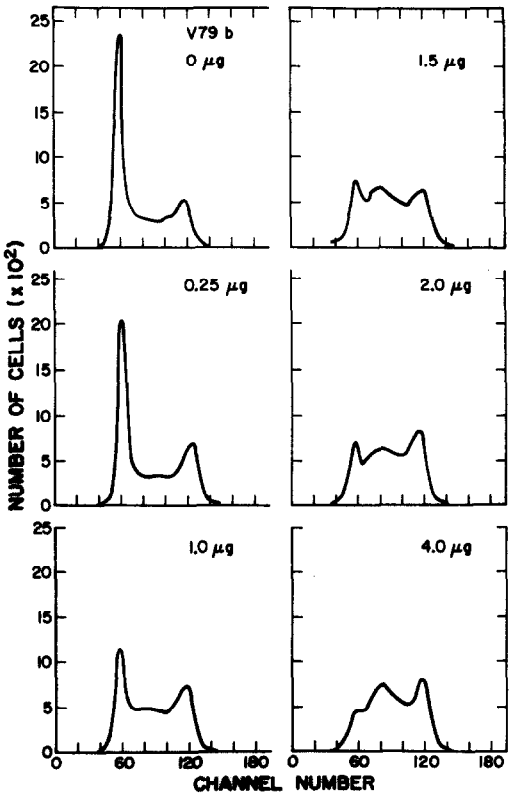

Fig. 3. Cell-cycle distributions for V79b cells sampled at 6.8 hr following a 1-hr exposure to ADR at concentrations of 0–4 μg/ml. Each DNA distribution represents 50,000 cells.

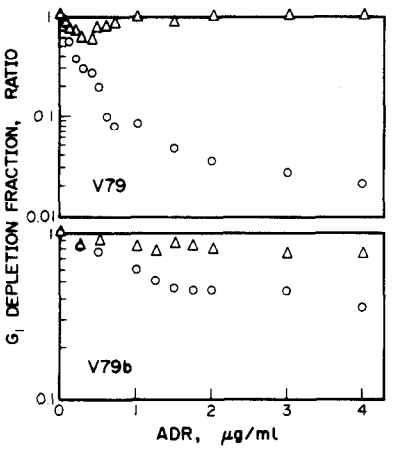

Fig. 4. The G_1/early-S fraction depletion is plotted as a function of ADR dose. The data represent cell populations sampled at 3 hr for V79 and 3.4 hr for V79b cells (-Δ-); and 6 hr for V79 and 6.8 hr for V79b cells (-O-) after a 1-hr treatment with ADR.

This is true for both the sensitive (V79) and resistant (V79b) cell lines. In order to determine if this G_1/early-S depletion quantitatively follows cell survival, the G_1 depletion fraction was calculated at each dose point. This fraction is the ratio of the fraction of G_1/early-S cells in treated cultures to the same quantity in untreated cultures.

The results of this calculation are shown in Fig. 4, where the G_1/early-S depletion ratios were plotted as a function of ADR dose one-quarter or one-half of a cell cycle after drug treatment. For both sensitive (V79) and resistant (V79b) cells the ratios calculated from the 3-hr distributions reached a constant value at relatively low doses. The 6-hr ratios descended to lower values but also displayed a plateau above the ADR concentration of 1.5–2 μg/ml. The period chosen for this study (6 hr for V79 and 6.8 hr for V79b cells) is longer than the calculated G_1 period for either cell line (Table 1); thus the G_1 cells at the time of drug application should have had time to enter S phase.

The G_1/early-S depletion ratios followed the survival data more closely if a correction was made for the depletion plateau in which the G_1/early-S fraction reached a constant value as the drug concentration increased. This correction was made to each fraction by subtracting the G_1/early-S fraction for the high (plateau) dose point from each of the other G_1/early-S fractions (Fig. 5).

The dose–response patterns for corrected G_1/early-S depletion fractions (Fig. 5) and cell survivals (Fig. 1) were compared for the two cell lines. The drug concentrations required for 50 and 10% survival levels were approximately 5

*Table 1. Cell-cycle distributions of exponentially growing cells**

	G_1 (hr)	S (hr)	G_2/M (hr)	Cell-cycle time (hr)
V79	2.2	7.4	2.4	12
V79b	4.3	7.3	1.9	13.5

*Cell-cycle distributions were obtained by the DNA-content distribution analysis using a method of Dean and Jett [8] and the cell-cycle time.

Table 2. Comparison of ADR doses required for survival and G_1 depletion

	(%)	V79 (μg/ml)	V79b (μg/ml)	ADR dose ratio
Survival	50	0.20	1.00	5.00
	10	0.36	1.75	4.90
G_1 depletion	50	0.16	0.70	4.40
	10	0.45	2.00	4.40

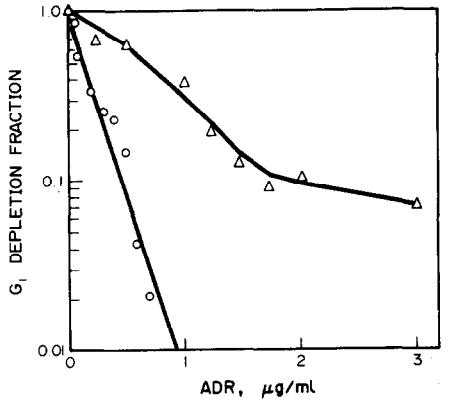

Fig. 5. The corrected G_1 depletion fraction for V79 (-O-) and V79b (-Δ-) cell samples taken 6 or 6.8 hr after a 1-hr ADR exposure shown as a function of ADR dose. The curves were drawn by eye.

times higher for V79b cells than for V79 cells. Similarly, the drug concentrations required for G_1 depletion fractions of 0.5 and 0.1 were approximately 4.4 times higher for V79b cells than for V79 cells (Table 2). These results suggest that the G_1/early-S depletion fraction correlated well with the survival pattern for the first log of cell killing.

DISCUSSION

The present study indicates that the magnitude of G_1/early-S depletion is related to the level of cell survival achieved at least for the first log of cell killing. Changes induced in cell-cycle distributions suggested that cells were accumulated in late-S at 6 hr for V79 cells and in mid-S at 6.8 hr for V79b cells. The results suggested, however, that the G_1 cells were not arrested, and a significant increase in the G_2/M fraction did not occur after very low-dose ADR treatment for either cell lines (Figs 2 and 3). Cell synchrony studies have indicated that the S-phase is the most sensitive cell phase to ADR-induced cell-traverse delay [1, 2, 10–13]. Cells in S are also most susceptible to ADR inactivation based on colony formation [14, 15]. Barlogie and his associates observed a reversible block in G_1 or at the G_1/S boundary when higher drug concentrations were maintained for a prolonged period [1]. Barranco found cell progression delay in all phases of the cell cycle except mitosis in CHO cells and that cells treated continuously in G_1 with 0.1 μg/ml ADR were delayed 1 hr but then proceeded normally into S-phase [16]. Göhde and his associates also reported that an ADR treatment with 1 μg/ml for 30 min reduced the rate of traversal from G_1 to S at 6 hr but that the G_1 fraction was almost depleted by 12 hr [3]. In the drug concentration ranges tested, our results seem to agree with the former observations that cell-cycle traverse delay is significant for cells in S but not for cells in G_1 or G_2/M. These published observations, as well as our data (Fig. 5), indicate a less significant correlation between the survival and G_1/early-S depletion when higher drug concentrations are used; therefore the gross estimate of cellular response to ADR by flow cytometry appears to be valid only at low drug concentrations.

Using time-course sampling, we determined that the magnitude of G_1/early-S depletion reached a plateau at higher ADR concentration ranges (<2 μg/ml) for V79b cells (Fig. 5). Tobey and his associates previously identified a small fraction of cells which entered a non-cycling state as a result of experimental manipulation [17]. It is yet to be determined whether this G_1/early-S depletion plateau resulted from the conversion of cells to a non-cycling state or simply an expression of ADR-induced G_1 block.

In conclusion, although the ultimate determination of cellular sensitivity to ADR requires a clonogenecity assay, the results of this study indicate that cell sensitivity differences to ADR to a limited extent can be demonstrated by flow cytometric studies.

Acknowledgements—We thank Elvira Bain for her technical assistance, and Lawrence Gurley, Tod Johnson, Robert Tobey and Mark Wilder for their comments and advice.

REFERENCES

1. BARLOGIE B, DREWINKO B, JOHNSON DA, FREIREICH E. The effect of adriamycin on the cell cycle traverse of a human lymphoid cell line. *Cancer Res* 1976, **36**, 1975–1979.
2. KRISHAN A, FREI E. Effect of adriamycin on the cell cycle traverse and kinetics of cultured human lymphoblasts. *Cancer Res* 1976, **36**, 143–150.
3. GÖHDE W, MEISTRICH M, MEYN R, SCHUMANN J, JOHNSTON D, BARLOGIE B. Cell-cycle phase-dependence of drug-induced cycle progression delay. *J Histochem Cytochem* 1979, **27**, 470–473.
4. TRAGANOS F, EVENSON DP, STAIANO-COICO L, DARZYNKIEWICZ D, MELAMED M. Action of dihydroxyanthraquinone cell cycle progression and survival of a variety of cultured mammalian cells. *Cancer Res* 1980, **40**, 671–681.
5. BELLI JA. Radiation response and adriamycin resistance in mammalian cells in culture. *Front Radiat Ther Oncol* 1979, **13**, 9–20.
6. CRISSMAN HA, TOBEY RA. Cell-cycle analysis in twenty minutes. *Science* 1974, **184**, 1297–1298.
7. HOLM DM, CRAM LS. An improved flow microfluorometer for rapid measurements of cell fluorescence. *Exp Cell Res* 1973, **80**, 105–110.
8. DEAN PN, JETT JH. Mathematical analysis of DNA distributions derived from flow microfluorometry. *J Cell Biol* 1974, **60**, 523–527.
9. ALABASTER O, TANNENBAUM E, HABBERSETT MC, MAGRATH I, HERMAN C. Drug-induced changes in DNA fluorescence intensity detected by flow microfluorometry and their implications for analysis of DNA content distributions. *Cancer Res* 1978, **38**, 1031–1035.
10. TOBEY RA. Effects of cytosine arabinoside, daunomycin, mithramycin, azacytidine, adriamycin, and camptothecin on mammalian cell cycle traverse. *Cancer Res* 1972, **32**, 2720–2725.
11. HITTLEMAN WN, RAO PN. The nature of adriamycin-induced cytotoxicity in Chinese hamster cells as revealed by premature chromosome condensation. *Cancer Res* 1975, **35**, 3027–3035.
12. TOBEY RA, CRISSMAN HA, OKA MS. Arrested and cycling CHO cells as a kinetic model: studies with adriamycin. *Cancer Treat Rep* 1976, **60**, 1829–1837.
13. CLARKSON JM, HUMPHREY RM. The effects of adriamycin on cell cycle progression and DNA replication in Chinese hamster ovary cells. *Cancer Res* 1977, **37**, 200–205.
14. KIM SH, KIM JH. Lethal effect of adriamycin on the division cycle of Hela cells. *Cancer Res* 1972, **32**, 323–325.
15. DREWINKO B, GOTTLIEB JA. Survival kinetics of cultured human lymphoma cells exposed to adriamycin. *Cancer Res* 1973, **33**, 1141–1145.
16. BARRANCO SC. Review of the survival and cell kinetics effects of adriamycin (NSC-123127) on mammalian cells. *Cancer Chemother Rep* 1975, **6**, 147–152.
17. TOBEY RA, CRISSMAN HA, KRAEMER PM. A method for comparing effects of different synchronizing protocols on mammalian cell cycle traverse. The traverse perturbation index. *J Cell Biol* 1972, **54**, 638–645.