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Effects of Actinomycin D on Cell Cycle Kinetics and the DNA of Chinese Hamster and Mouse Mammary Tumor Cells Cultivated *in Vitro*[1]

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SUMMARY

Actinomycin D produces changes in the cell cycle kinetics of Chinese hamster (HA1) and mouse mammary tumor cells (EMT6) cultivated *in vitro*. There was a reduced rate of progression of the cells through S phase and a G_2 arrest, the duration and degree of which were drug dose dependent. The lethal effects of the drug on the two cell lines were comparable.

At the molecular level, DNA single-strand breaks (true breaks and/or alkali-labile bonds) appear initially. After drug removal, the extent of disappearance of these DNA strand breaks was drug dose dependent. Similar results were obtained for the two cell lines. However, at the lower drug concentration (0.1 μg/ml), although the DNA strand breaks appeared to disappear after drug free incubation, about 60% of this cell population was not viable.

INTRODUCTION

Some properties and characteristic effects of the antibiotic actinomycin D on mammalian cells are well documented. These include intercalation into DNA (22, 23), interference with transcription of DNA (12, 14, 15), and DNA replication (2). Cycling mammalian cells have been reported to be most sensitive to the drug at or near early S phase (2, 3, 6, 9). Probably related to the observed drug-induced cell killing is the G_2 block caused by actinomycin D (*e.g.*, Refs. 4, 8, 18, and 24). Differential drug retention rather than differential drug uptake appears to be an important determining factor in specific tissue sensitivity to the drug (20, 21).

Attempts have been made to elucidate the drug effect at the molecular level. Elkind and Chang-Lui (5) found no evidence for the induction of DNA single-strand breaks in Chinese hamster cells after a 30-min treatment with actinomycin D at 2.5 μg/ml. After a 3-hr incubation with actinomycin D at 0.1 μg/ml, Pater and Mak (10) observed drug-induced DNA single-strand breaks in human KB carcinoma cells that persisted for as long as 10 hr after the drug treatment.

We have compared the relative sensitivity to graded con-

centrations of actinomycin D of Chinese hamster ovary cells (HA1) and mouse mammary tumor cells (EMT6) cultivated *in vitro*. We have studied the effects of this drug on viability, on cell cycle progression using cytofluorograms to measure the DNA content per cell, and on cellular DNA using hydroxylapatite chromatography to separate single- from double-stranded DNA.

MATERIALS AND METHODS

Cell Lines and Culture Methods. A description of the derivation of the Chinese hamster ovary HA1 subline (HA1) has been given (25). These cells were routinely cultured in Eagle's minimal essential medium supplemented with 15% fetal calf serum and antibiotics. A description of the routine maintenance involving periodic reinoculation into mice and the tissue culture conditions of the cultured mouse mammary tumor cells (EMT6) have been given by Rockwell *et al.* (16). These cells were cultured in Waymouth's medium supplemented with 15% fetal calf serum and antibiotics. The population-doubling time of both cell lines was about 15 hr.

Drug Incubations. Cells were plated 48 hr in advance of the experiment in 4.5 ml of culture medium in 15- x 60-mm Pyrex glass dishes. Cells were typically grown to a cell density of 2 to 4 $\times$ 10^4 cells/sq cm and then treated with the drug. A frozen stock solution of actinomycin D dissolved in water was used to prepare the desired concentration of drug dissolved in culture medium. A volume of 0.5 ml of culture medium or of concentrated drug solutions was added to the dishes to give the desired final drug level. Incubations were performed in a humidified air-CO_2 incubator.

Drug Toxicity Assays. After the desired length of drug incubation, the cells were washed 3 times with HBSS,[2] trypsinized with fresh 0.05% trypsin, diluted, and plated in 60-mm Falcon plastic dishes. Colonies were counted 8 to 10 days later, after staining with 0.25% crystal violet in methanol.

For determination of the effectiveness of the HBSS rinses, a few control experiments were performed in which drug attachment to plastic *versus* attachment to glass was investigated. For these experiments, 5 ml of the drug solutions of graded concentration were added to either 60-mm Falcon

[2] The abbreviations used are: HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate.

plastic or glass dishes. After 4 hr of drug exposure, the dishes were washed 3 times with HBSS, and about 200 HA1 cells in 5 ml of minimal essential medium were added to each dish.

Population Kinetics and Cell Cycle DNA Distribution. Population growth patterns of drug-treated and control cells were compared by cell counts using a Coulter counter and also by examining the DNA distribution pattern within the cell population by flow cytofluorimetry using the method described by Hahn (7). In these studies, cell cultures set up 48 hr in advance in 60-mm glass dishes were exposed to the drug as described and washed 3 times with HBSS. The cultures were then reincubated for the desired time period in 5 ml conditioned culture medium (*i.e.,* medium removed from an untreated mass culture grown in parallel) to minimize metabolic disturbances, before parallel cultures were harvested for cell counts and for cytofluorimetric analysis.

Molecular Changes in DNA. The technique of measuring DNA lesions, manifested as sites susceptible to DNA strand separation during mild alkaline denaturation, by separation of single- and double-stranded DNA from cell lysates on hydroxylapatite columns has recently been described by Ahnström and Edvardsson (1) and by Rydberg (19).

For these experiments, cell cultures were plated 48 hr in advance as described above. After 24 hr [2-^{14}C]thymidine (0.02 μCi/ml; 56.5 Ci/mole; New England Nuclear, Boston, Mass.) was added. Samples were prepared for hydroxylapatite chromatography immediately after drug exposure or after the desired reincubation time in drug-free medium by scraping the cells off the dish with a rubber policeman. In both cases a cell pellet was collected by centrifugation of the cells in the original culture medium to prevent loss of "swimming" cells. Samples reincubated after drug exposure were washed 3 times with HBSS prior to reincubation.

For the hydroxylapatite assays, we have modified the methodology of Rydberg (19). After centrifugation of the scraped cells, the supernatant was decanted, the cell pellet (10^5 > cells < 10^6) was lysed, and the DNA was denatured in 1 ml of 0.03 M NaOH:0.01 M Na$_2$HPO$_4$:0.9 M NaCl solution (pH 11.8) for 30 min at 20°. This was followed by a quick neutralization with 1 ml of about 0.03 M HCl. The neutralized lysate was then sonically disrupted for 15 sec at the No. 3 setting in a Branson Model W-185 microtip Sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) which was followed by addition of 0.28 ml of 10% SDS to give ~1.2% SDS. The product of sonic disruption was diluted 1:3, and 1 ml was added to a 250-mg hydroxylapatite (DNA grade Bio-Gel HTP; Bio-Rad Laboratories, Los Angeles, Calif.) column (3-ml plastic syringe tube) kept at 60–65° and prepared with the 0.0125 M phosphate buffer used for the 1st elution step. The column was then eluted at 60–65° with 2 volumes of 2.5 ml each of 0.0125 M, 0.125 M, and 0.25 M NaH$_2$PO$_4$:Na$_2$HPO$_4$ buffers, all at pH 6.9, giving 6 eluant fractions of 2.5 ml each. The last 2 buffers also contained 0.4% SDS. Elutions in the same manner with potassium phosphate buffers gave the same result; that is, the bulk of single-stranded DNA was almost entirely in the 3rd fraction and that of double-stranded DNA was in the 5th fraction. After acid hydrolysis of the eluant fractions (made to 5% trichloroacetic acid and heated at 85° for 20 min), ~0.6-ml aliquots were counted in

5 ml Insta-gel (Packard Instrument Co., Inc.) in a liquid scintillation counter. The channels ratio method was used to calculate dpm/eluant fraction in order to find the percentage of double-stranded DNA per sample.

RESULTS

Drug Cytotoxicity. The problem of drug attachment to glass or plastic surfaces was considered. In these trials, HA1 cells were added to dishes that had been exposed to actinomycin D for 4 hr. The results demonstrate that, even after three 4- to 5-min washings with HBSS, there is some drug-induced cell killing due to residual amounts of actinomycin D attached to the plastic or glass surfaces (Table 1).

In Chart 1 the cytotoxicity of actinomycin D is depicted for HA1 and EMT6 cells. Equivalent drug concentrations produced essentially equivalent cytocidal effects on the 2 cell lines.

Population Kinetics. The influence of actinomycin D on the growth pattern of an asynchronous population of HA1 cells is shown in Chart 2. Chart 2A shows the logarithmic growth of control cells until approximately 1.5 × 10^5 cells/sq cm was reached. Drug-exposed cells exhibited an initial growth delay, especially at the higher drug concentration of

Table 1

Actinomycin D-induced cell killing due to drug attachment to glass or plastic culture vessels

The survival values for HA1 cells are averages from 2 experiments; those for EMT6 cells are from 1 experiment.

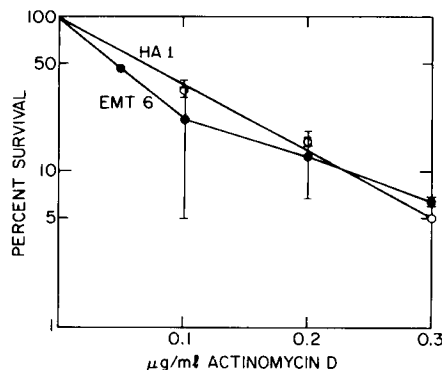| Cell line	Pretreatment of culture vessels with actinomycin D for 4 hr (μg/ml)	Relative % survival	
		Pyrex glass	Falcon plastic
HA1	0	100	100
	0.05	89	94
	0.1	85	100
	0.2	84	93
	0.3	89	76
EMT6	0	100	100
	0.05	84	73
	0.1	76	53
	0.2	69	47

Chart 1. Cytotoxicity of actinomycin D. Percentage of survival expressed in terms of colony-forming ability of HA1 (○) and EMT6 (●) cells exposed to various concentrations of actinomycin D for 4 hr. *Vertical bars,* range in survival found in 2 to 3 repeat experiments.
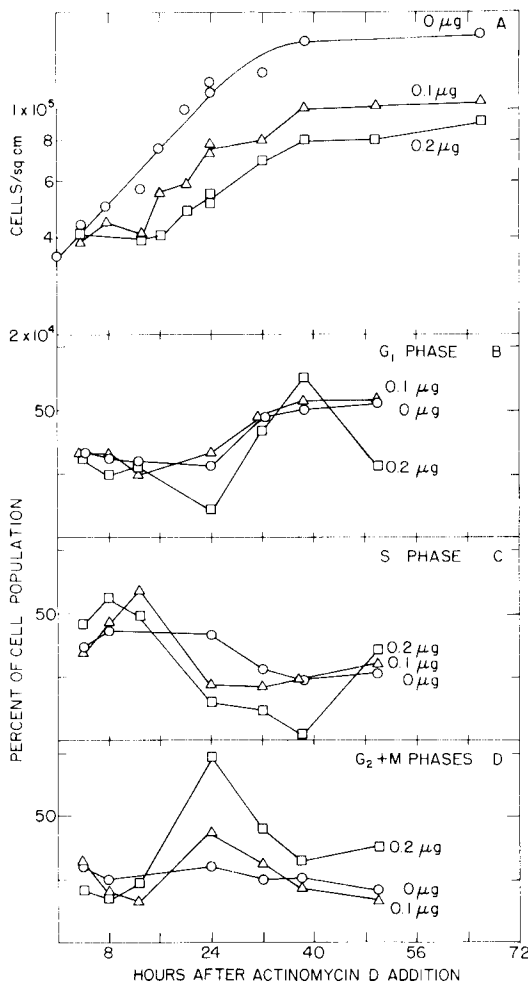
Chart 2. Actinomycin D-induced changes in the cell cycle kinetics of HA1 cells exposed to drug doses of 0 (O), 0.1 (△), or 0.2 (□) µg/ml for 4 hr. A, cell number as a function of time after drug addition, B, C, and D, fractions of cells with DNA contents of G_1, S, and G_2 + M phases, respectively, as determined from flow cytofluorograms analyzed at various times after drug addition that lasted for 4 hr. This experiment was performed twice yielding the same results.

0.2 µg/ml, a concentration that resulted in about 12% survival (Chart 1).

The rest of Chart 2 shows the distribution patterns of DNA content, obtained from the cytofluorograms, in the cell population at various times after drug exposure to either 0.1 or 0.2 µg/ml (4 hr), as compared to the DNA content of control cells. Chart 2, B, C, and D, represents the fraction of cells in the G_1, S, and G_2 + M phases, respectively. In control cells, the logarithmic growth evident up to at least 24 h (Chart 2A) is matched by a stable fraction of cells in G_1 (Chart 2B), S (Chart 2C), or G_2 + M (Chart 2D). At a cell density above 10^5 cells/sq cm, the fraction of control cells in G_1 increased and there was a concomitant decrease in the fraction of cells in the S and G_2 + M phases.

For the 1st 12 hr, drug-treated cells appeared to show a slower rate of progression through S phase with a corresponding decrease in the fraction of cells in G_2 + M. The actinomycin D-induced G_2 block is demonstrable by 24 hr at 0.2 µg/ml, although the same effect can be seen at 0.1 µg/ml.

In the case of the 0.2-µg/ml samples, at the 24-hr time point, i.e., 20 hr after drug removal, approximately 75% of the cell population was in the G_2 + M phases. The subsequent release of cells from the G_2 block is evidenced by the gradual decrease in the fraction of cells in the G_2 + M phases concomitant with the gradual increase in the cell fraction in G_1 and an absolute minimum of cells in S at the 38-hr point. Subsequently, there was some progression of cells into the S phase.

Drug-induced Changes in DNA. The results for the separation of single- and double-stranded DNA from Chinese hamster cells (HA1) and mouse mammary tumor cells (EMT6) by hydroxylapatite chromatography are shown in Table 2. The percentage of double-stranded DNA obtained from cells incubated for 4 hr in either 0.1, 1, or 5 µg/ml actinomycin D for times up to 20 hr after drug removal is compared to the percentage of double-stranded DNA normally found in an asynchronous control population. In some experiments with HA1 cells, the control and 0.1-µg/ml samples were followed for 68 hr after drug removal.

The initial low level of double-stranded DNA resulting from DNA lesions susceptible to mild alkali denaturation (4-hr points), which is evident for drug-treated cells and which correlates directly with drug concentration, appears to be a somewhat reversible process, at least at the lower drug concentrations.

DISCUSSION

Studies of drug-induced effects in a mammalian cell are made difficult by several factors. In the in vivo situation, drug uptake, metabolism, and tissue retention are of major concern. In the in vitro situation, residual drug attachment to the culture vessel may occur, even after several rinses, as is the case for actinomycin D (Table 1). Another complication is that after the desired duration of drug exposure, i.e., after washing the cells and replacing the medium with drug-free medium, the drug and/or metabolic products of the drug (e.g., Ref. 11) may be released into the medium from dying cells.

Although we had considered the possibility that asynchronous mouse tumor cells (EMT6) might show an actinomycin D sensitivity different from that for the Chinese hamster cells (HA1), this did not appear to be the case (Chart 1), although a greater variation between experiments was found to occur for the EMT6 cells. Differential tissue toxicity for actinomycin D has been discussed by Schwartz (20) who has considered the possibility that DNA may exist in different states in various tissues allowing, perhaps, a higher uptake of drug in the DNA of certain tissues. The correlation of tissue toxicity with drug retention rather than with drug uptake has been discussed by Schwartz et al. (21).

Actinomycin D has a profound effect on cell progression in the cell cycle (Chart 2). An actinomycin D-induced G_2 block has been noted by several authors (e.g., Refs. 4, 8, 18, and 24): Our data confirm the observation of a G_2 block (Chart 2D) and show that the duration of the G_2 block is drug concentration dependent (Chart 2A). Furthermore, it appears that there is also a reduction in the rate of progression of S-phase cells (Chart 2C). In cultures treated with

Table 2

Actinomycin D-induced molecular changes in DNA

In most cases, the mean values ± S.E. of data from 3 to 6 repeat experiments per point are shown. S.E. values for data consisting of less than 3 repeat experiments per point were not computed.

Cell type	μg/ml	% double-stranded DNA at following times after drug addition (4 hr drug exposure)							
		4 hr	6 hr	24 hr	28.5 hr	48 hr	52 hr	56 hr	72 hr
HA1	0	89.3 ± 1.9	86.0 ± 3.1	87.7 ± 2.5	91.0	94.0	92.0	90.0	90.0
	0.1	74.2 ± 3.7	79.3 ± 3.3	83.2 ± 5.1	92.0	86.0	83.0	90.0	86.0
	1	38.7 ± 8.9	74.5	77.3 ± 8.6					
	5	22.0 ± 4.9	74.5	63.0 ± 5.5					
EMT6	0	86.7 ± 4.8	97.3 ± 1.2	90.6 ± 1.2					
	0.1	80.0 ± 4.0	91.0	81.0 ± 2.1					
	1	55.5 ± 12.5	76.0	72.3 ± 7.5					
	5	46.0 ± 4.0	61.0	62.8 ± 7.1					

actinomycin D (0.1 or 0.2 μg/ml), very few floating cells were observed initially up to approximately 30 hr after drug removal. At longer times, however, floating cells became noticeable, especially in the 0.2-μg/ml samples.

Molecular changes in DNA have been studied for both the Chinese hamster and the mouse tumor cells as a function of time after drug addition (4 hr drug exposure) (Table 2). The assay technique measures DNA single-strand breaks that may either be true DNA single-strand breaks and/or breaks that may arise due to distortions of the DNA configuration by actinomycin D intercalation, which may make the DNA chain susceptible to enzyme attack or render it alkali labile. The initial degree of drug-induced lesions seems to disappear to some extent within 2 or 3 hr after drug removal. At high drug concentrations, e.g., 5 μg/ml, a significant percentage of abnormal DNA persists 20 hr after drug treatment. These data indicate that the drug-induced molecular changes in DNA are significant initially, even at lower drug concentrations, e.g., 0.1 μg/ml, and although these lesions tend to disappear with time significant lethality still persists. A 1% variation in the percentage of double-stranded DNA represents ~15 breaks/chromosome as determined from X-ray studies using the hydroxylapatite technique (unpublished observations) and a breakage efficiency of 20 breaks/cell/rad (17). Therefore, the persistence of a few, perhaps lethal, DNA breaks cannot be determined. Inaccurately repaired breaks may also contribute to drug killing.

Our data in Table 2 differ somewhat from the data presented by Pater and Mak (10), who measured actinomycin D-induced DNA single-strand breaks in human KB cells with the alkaline sucrose gradient sedimentation technique. In their case, after a 3-hr treatment with the drug at 1 μg/ml, there was a large increase in the initial DNA single-strand break yield after a further 10-hr drug-free incubation time (i.e., there was no apparent repair; in fact, there was extensive DNA fragmentation); however, only a small change in the sedimentation profile was observed for cells exposed to 0.1-μg/ml drug doses for 3 hr.

In our system, actinomycin D treatment caused the maximum number of HA1 cells to accumulate in the G_2 + M phases at about 24 hr after drug addition. Since the G_2 + M phases are very radiosensitive in many mammalian cell lines, including Chinese hamster ovary cells, irradiation at 24 hr after drug addition should be the optimum time to maximize the effect of the drug-radiation interaction in our cell system. However, such an interpretation is complicated by the fact that cytofluorograms give the DNA content of both viable and nonviable cells moving through the cell cycle for perhaps 2 generations before dying. Actually, for the higher drug concentration of 0.2 μg/ml, the duration of increased radiosensitivity of the cell population should last from 24 hr until at least 40 hr after drug addition, during which time a relatively low percentage of cells appear to be in the radioresistant S phase.

Apart from a possible radiation potentiation by actinomycin D due to partial cell synchrony, the drug apparently causes changes in DNA-lipid associations (5) and probably causes residual damage in the DNA molecule (Table 2). Such residual damage may well be one of the reasons for the apparently long-term drug-radiation interaction as pointed out by Piro et al. (13), based on data from clinical observations as well as from their in vitro experiments with actinomycin D.

ACKNOWLEDGMENTS

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