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The Effect of Adriamycin and Radiation on G_2 Progression[1]

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SUMMARY

The effect of the DNA-intercalating antibiotic adriamycin on the progression of Chinese hamster ovary cells into mitosis, and on the delay induced by ionizing radiation, was studied using the mitotic cell selection procedure to monitor the rate of cell division.

Following the addition of adriamycin, the mitotic rate remained unaltered for a refractory period and then decreased to zero. This effect was concentration dependent with transition points between the S-G_2 boundary for 0.1 μg/ml and late G_2 for 250 μg/ml.

Cells treated with either a 10- or 30-min pulse of 1.0 μg adriamycin per ml exhibited a refractory period identical to that observed for continuous treatment. However, after a delay of $\simeq$3.5 or $\simeq$5 hr, respectively, cell division resumed. The mitotic rate of cells that received 150 rads of X-ray at the onset of an adriamycin pulse declined coincident with that of radiation only, but resumed coincident with those receiving adriamycin only. This implies that radiation-induced division delay ($\simeq$3 hr) was repaired before cells recovered from adriamycin-induced division delay and that the two agents were not additive. This lack of synergism is in contrast to that observed for cell lethality.

INTRODUCTION

Adriamycin is an anthracycline antibiotic that in recent years has been widely used in cancer therapy. For an extensive review of the properties and uses of adriamycin, see Ref. 31. In addition to its use as a single agent (6, 13, 31), it has also been used in combination with other drugs (16) and with radiation (10, 12, 40). *In vitro* studies with adriamycin have shown it to be more lethal to cells in S than to cells in G_1 or G_2 (3, 4, 15, 19, 20) and to cause an accumulation of cells in G_2 (1-4, 18, 20, 34), in common with a number of other cycle-active drugs (1, 27, 34-37). Little has been done on the effect of adriamycin on cell kinetics other than with survival as an end point (3-5, 19) or with systems (2-4, 18, 20, 34, 35) that do not allow the precise resolution available with the present technique (see below). Experimental results on the interaction of adriamycin and radiation have been obtained for inhibition of DNA repair replication (21), for *in vitro* cell lethality (9), and for various *in vivo* end points (8, 38, 39). As part of the on-going line of experimentation into the effects of combined modalities on cell kinetics and ultimately on their utilization in cancer therapy, a series of experiments utilizing the mitotic cell selection procedure to analyze cell cycle kinetics was initiated to ascertain the relationship between damage induced by adriamycin and radiation.

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MATERIALS AND METHODS

Cell and Culture Conditions. Chinese hamster ovary cells were maintained in exponential growth as monolayer cultures at 37° in a humidified atmosphere of 6% CO_2 in modified McCoy's Medium 5A (Grand Island Biological Company, Grand Island, N. Y.) and supplemented with antibiotics and 10% calf plus 5% fetal calf sera (Flow Laboratories, Rockville, Md.). Under these conditions, the cells exhibited a generation time of approximately 12 hr with $G_1 \simeq 3$ hr, S $\simeq$ 7 hr, $G_2 \simeq 1.5$ hr, and M $\simeq$ 0.7 hr. The cultures were monitored for *Mycoplasma* contamination every 6 weeks by the method of Levine (25).

Collection Technique. The progression of cells through mitosis was analyzed by means of the mitotic cell selection procedure originally described by Terasima and Tolmach (33), as modified by Schneiderman *et al.* (28). The specific technique utilized has been published previously (22). Briefly, replicate 75-sq cm plastic Falcon flasks with approximately 10^7 cells in exponential growth for at least 18 hr were shaken for 20 sec every 10 min on a reciprocating horizontal shaker in a walk-in 37° incubator. The overlying medium was poured into test tubes immersed in ice and replaced with another 10 ml of medium that had been conditioned by log phase cells. The number of cells/collection (shake) was determined by Coulter counter. After several shakes at the beginning of an experiment, only the rounded-up and loosely attached mitotic cells were collected. By interfacing the Coulter counter with a cell-size analyzer, cell volume was monitored to verify that collected cells exhibited twice the volume of the smallest (G_1) cells. A precise determination of the mitotic index of the collected cells was obtained by fixing cells with aceto-orcein and scoring mitotic figures. The mitotic index, which was customarily greater than 98%, correlated with the cell volume analysis.

X-Irradiation. The flasks were irradiated with a 250 kVcp X-ray machine operated at 15 ma with an additional filtration of 2.0 mm Al giving a resultant dose rate of approximately 150 rads/min. The flasks were held in a 37° water bath during irradiation and were not out of the walk-in incubator for more than 3 min. The irradiation procedure

itself did not cause any perturbation of the mitotic rate.

Drug Treatment. Adriamycin (NSC 123127) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in Hanks' buffer and then sterilized by filtration. The drug was either diluted into complete medium and used immediately or was stored in the freezer until needed. No change in response was observed as a result of storage. Final adriamycin concentrations were confirmed spectrophotometrically. For drug treatment, normal medium was replaced with medium containing adriamycin for the desired number of shakes. For adriamycin-radiation combination treatments, the drug was added and then the flasks were irradiated within 1 min. A total of 4 experiments for the adriamycin-radiation studies and 8 experiments for the adriamycin studies were done to verify the results. The confidence intervals presented in the following section are the mean ± 1 S.E. Data from 2 representative experiments are shown (Charts 1, 3, 4, and 5) to aid in comparison of the results.

RESULTS

Adriamycin Treatment. Chart 1 shows the effect of continuous treatment with various concentrations of adriamycin upon the rate of mitotic cell collection (mitotic rate) with time. Compared to the control, there was an initial period during which the mitotic rate remained constant and then decreased. This refractory period was shorter for high concentrations of adriamycin. The mitotic rate declined in an exponential fashion until a background level of nonmitotic cells was obtained. The volume of these residual cells was routinely checked through the course of the experiment with the use of the cell-size analyzer to insure that no mitotic cells were present. As the concentration of adriamycin increased, the slope of the descending curve also increased until it reached a constant value at 1.0 μg adriamycin per ml (Chart 1). The mid-point is defined as the time from addition of drug (or radiation) to the time at which the mitotic rate decreased to the arithmetic mean between the upper and lower plateaus of the cell collection curve. By adding 16 min (the average time of collection prior to cell division) to this value, the average time prior to cell division that cells become refractory to the drug treatment (the transition point) can be obtained.

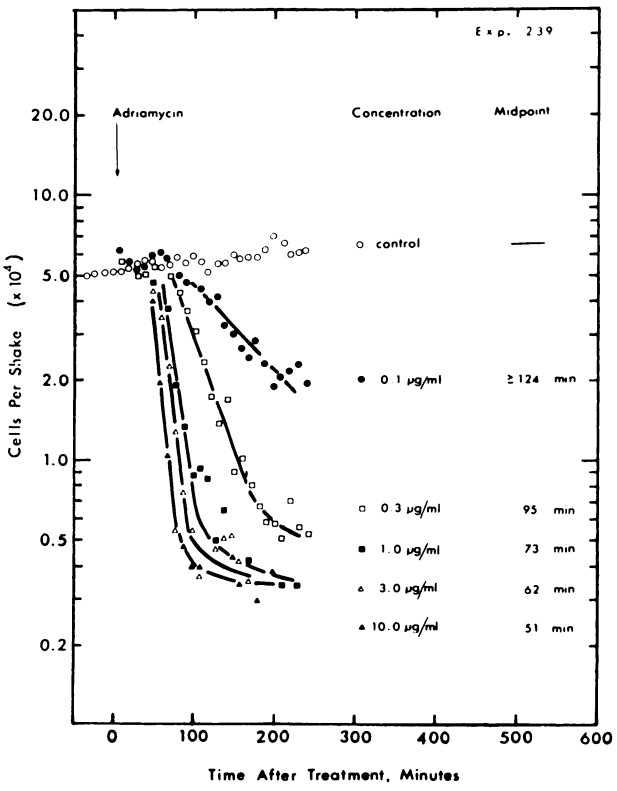

Chart 1. Effect of various concentrations of adriamycin on mitotic rate. At Time 0, the control medium was replaced with medium containing the indicated concentration of adriamycin for the duration of the experiment.

Chart 2 combines the results of 8 experiments and displays the transition points (prior to cell division) for various concentrations of adriamycin. The same transition points were observed whether the adriamycin was given as a pulse (see below) or continuously. There is an exponential relationship between transition point and concentration over a wide concentration range. At the higher concentrations, the curve becomes asymptotic to a value of 45 min ± 3 min (approximately the end of G_2). This is consistent with a large body of data that suggest that, for a given modality, there exists a minimum number of cells that are refractory to the treatment and will proceed through to collection. At the low end of the concentration range, the transition points deviate from linearity at the values that indicate the point of action is in late S or early G_2. This is in part due to incomplete suppression of the cell cycle progression by the low concentration of adriamycin (see the curves for 0.1 and 0.3 μg adriamycin per ml in Chart 1). Other experiments carried out to longer time intervals indicate that, for the low concentrations of adriamycin, a lower plateau of mitotic rate exists that is significantly above the background level. The exponential portion of this curve is approximately in the range of adriamycin concentration that is customarily used for therapy (6, 13, 16, 31). There may be some questions of whether the rate of diffusion of adriamycin into the cells contributes to the prolonged transition points at very low concentrations. Based on findings with actinomycin D (7) and daunomycin and adriamycin (26, 32), any effect would be on the order of a few min and not on the order of 40 to 100 min, as observed with refractory cells (Chart 1).

Adriamycin-Radiation Interaction. The effect of radiation on the progression of cells through to division is seen in Chart 3. If 150 rads X-ray were given at Time 0, there was a refractory period during which the mitotic rate remained constant and then an exponential decline to a background level. The transition point was 52 min prior to the completion of cell division. The mean value from all experiments done in this laboratory was 52 ± 3 min. After some time (which is a function of radiation dose), the mitotic rate began to increase in an exponential fashion until it reached and surpassed the control rate. The division delay of 156 min (169 ± 21 min) induced by radiation was determined as the time between the mid-points of the descending and ascending portions of the mitotic rate curve.

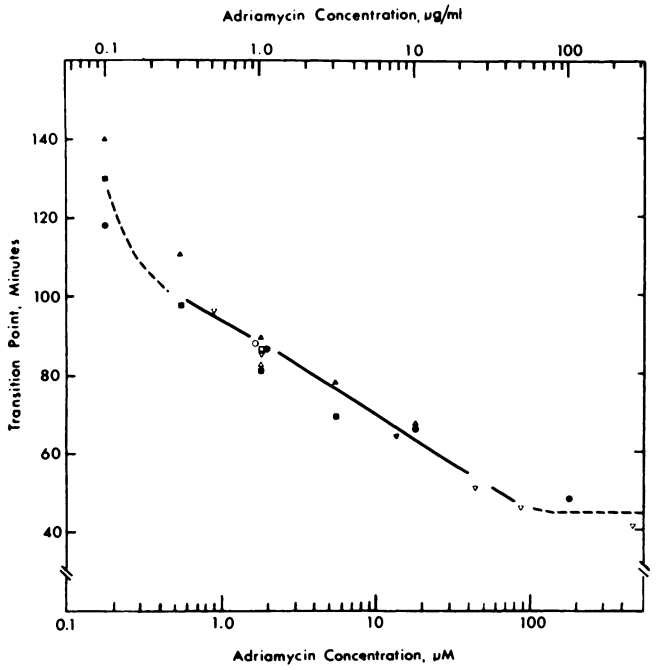

Chart 2. Effect of adriamycin concentration on the time prior to division after which cells are refractory to drug treatment (transition point). The different symbols refer to different experiments; *solid symbols*, continuous exposure to adriamycin; *open symbols*, a pulse treatment. ——, fitted by linear regression analysis; - - -, fitted by eye.

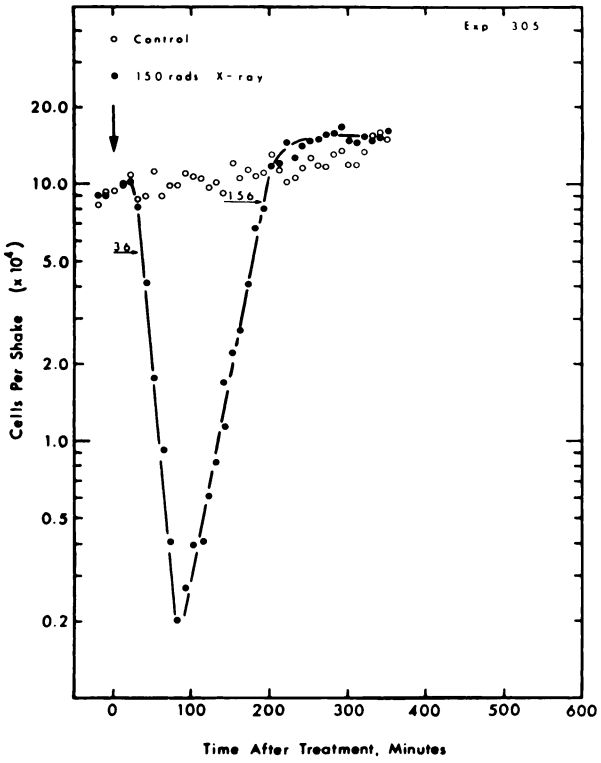

Chart 3. Effect of radiation on mitotic rate. Control flasks were untreated; irradiated flasks received 150 rads X-ray at Time 0. Transition point = 52 min (32 min + 16 min) from completion of cell division. Division delay = 156 min.

Chart 4 shows the effect of a 10-min pulse of 1.0 µg adriamycin per ml on the division delay induced by 150 rads X-ray. The transition point (85 min) for the adriamycin pulse was identical to the value (85 ± 3 min) observed for continuous treatment with adriamycin. For adriamycin plus radiation, the mitotic rate curve exhibited a transition point of 52 min (50 ± 4 min) that was identical to the radiation transition point (Chart 3). This result was also observed for radiation plus continuous treatment with 1.0 and 7.5 µg adriamycin per ml (data not shown). However, the adriamycin-plus-radiation-treated cells recovered at approximately the same time as those treated with adriamycin only, exhibiting division delays of 194 min (226 ± 41 min) and 170 min (209 ± 57 min), respectively. The plateau rate of cell division after radiation plus adriamycin was higher than after adriamycin alone, even though the adriamycin treated cells recovered to the pretreatment rate. This was similar to the recovery overshoot after radiation alone (Chart 3).

Chart 5 shows a treatment similar to that in Chart 4 but with a 30-min pulse of 1.0 µg adriamycin per ml, with and without radiation. The results are similar to those in Chart 4. The transition points for adriamycin alone or for adriamycin plus radiation are similar to those for continuous treatment with adriamycin or for radiation alone, respectively. As after a 10-min pulse, cells did not recover from radiation-induced damage at the normal time, but rather, were coincident with the adriamycin-treated cells. For a 30-min pulse of adriamycin, the division delay was 279 min (312 ± 74 min); for a 30-min pulse of adriamycin plus 150 rads, division delay was 298 min (335 ± 75 min). Obviously, although adriamycin-induced delay is reversible, the delay is much greater than merely the time of treatment. There was no recovery of cell

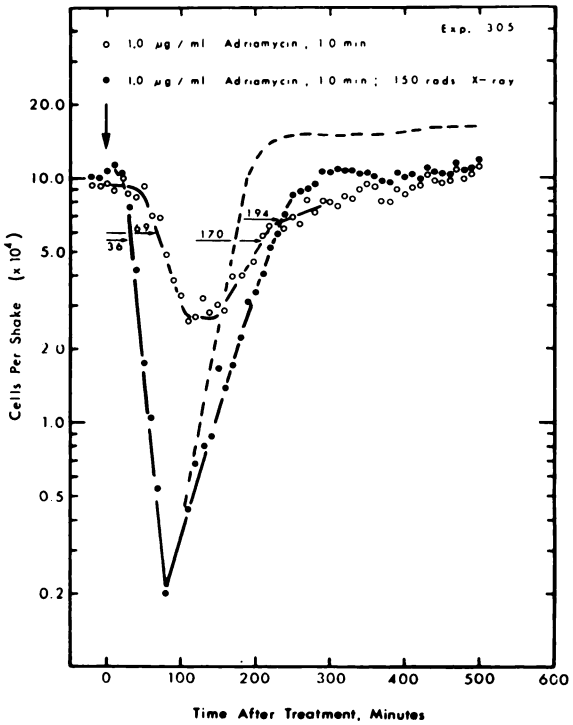

Chart 4. Effect of a 10-min pulse of adriamycin and radiation on mitotic rate. Conditions were the same as in Chart 3, except for the addition of 1.0 µg adriamycin per ml for the 1st 10 min of treatment. - - -, the response to 150 rads of X-ray only from Chart 3. Adriamycin only: transition point = 85 min (69 min + 16 min) from completion of cell division; division delay = 170 min. Adriamycin plus radiation: transition point = 52 min (36 min + 16 min) from completion of cell division; division delay = 194 min.

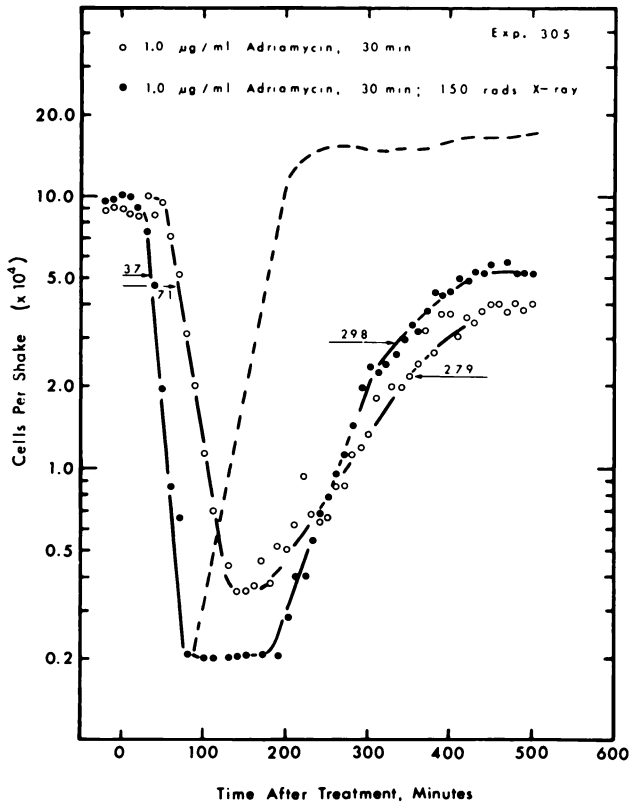

Chart 5. Effect of a 30-min pulse of adriamycin and radiation on mitotic rate. Conditions were the same as in Chart 3, except for the addition of 1.0 μg adriamycin per ml for the 1st 30 min of treatment. - - -, the response of 150 rads of X-ray only from Chart 3. Adriamycin only: transition point = 87 min (71 min + 16 min) from completion of cell division; division delay = 279 min. Adriamycin plus radiation: transition point = 53 min (37 min + 16 min) from completion of cell division; division delay = 298 min.

division if cells were irradiated and treated with adriamycin continuously (data not shown).

DISCUSSION

The G_2 block seen with adriamycin is likely to be caused by a mechanism similar to that observed with actinomycin D (14, 17, 35) and lucanthone (unpublished data): (*a*) at low concentrations of adriamycin, the inhibition of RNA synthesis (19, 26, 32) resulting in the lack of specific proteins necessary for the completion of cell division; and (*b*) at high concentrations, some immediate structural effect of adriamycin binding to DNA (29, 30) causing chromosome aberrations (18) and possibly interfering with spindle fiber formation or attachment to the centromere or centriole. The transition point results from the fact that cells refractory to treatment have already passed the time when the manufacture of specific substances or structures has been accomplished prior to the initiation of treatment (14, 17). There exists a minimum, concentration-independent transition point (45 min; see Chart 2) coincident with the onset of prophase (Refs. 14, 17, and 28; personal observations), indicating that once a cell has progressed to mitotis it will continue through division without perturbation. A similar hypothesis for a minimum refractory period (52 min) has been proposed for radiation-induced division delay (14, 17, 22-24, 28).

The radiation transition point is not altered by the presence of either 1.0 or 7.5 μg adriamycin per ml; those cells that are refractory to radiation are also refractory to adriamycin. At high concentrations (Chart 2), the adriamycin transition point achieves a minimum value (45 min) 7 min closer to the completion of cell division than the radiation transition point (52 min). The fact that we do not observe any change in the radiation transition point, even though the technique is sensitive enough to discern a difference within this 7 min, leads us to believe that there is no interaction of adriamycin and radiation on the refractory period.

As with the transition point, there does not appear to be any interaction of adriamycin and radiation on division delay, the delay being determined by the duration of adriamycin treatment. Rather than a synergistic action of the 2 agents, there appears to be less effect (division resumes earlier) for adriamycin plus radiation than for adriamycin alone because of the difference in transition points. If this were because cells that were refractory to adriamycin but not to radiation suffered radiation-induced delay (169 min) but not adriamycin-induced delay (209 or 312 min for 10- and 30-min pulses, respectively), then they would appear before cells that had been blocked by adriamycin alone. However, these cells do not appear coincident with cells recovering from radiation only, but rather with a delay similar to the adriamycin-treated cells (226 min and 335 min, respectively). Thus it would seem that cells treated with adriamycin and radiation are blocked at the radiation transition point but are delayed from the adriamycin alone. This could be caused either by the reversion of irradiated cells to an earlier stage in the cell cycle [as observed by Carlson (11)], or by the cells between the 2 transition points now suffering an adriamycin-induced delay. Whatever the mechanism, it produces a difference of only 30 min in the recovery of cells as compared to hours of division delay.

Although adriamycin and radiation are synergistic, when viewed from the end point of cell survival, these findings indicate that they do not interact either to alter the refractory period or the division delay. This difference between survival and cell cycle progression warrants further investigation as schedules of adriamycin and radiation in combination are more frequently used for cancer therapy.

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