Influence of Adriamycin and Adriamycin-Radiation Combination on Jejunal Proliferation in the Mouse

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SUMMARY

The influence of adriamycin and adriamycin-radiation combinations on posttreatment proliferative activity of the mouse jejunum was examined by measuring [3H]thymidine incorporation. Single doses of 5 or 10 mg/kg produced a transient reduction in the proliferative activity, while 1 mg/kg had little effect. After 10 mg/kg, there was a rapid decrease in the number of mitotic figures, followed by a gradual decrease in the number of and rate of DNA synthesis in S-phase cells. A compensatory epithelial hyperplasia characterized by an enlarged crypt proliferative population and shortened mitotic cycle duration was observed beginning 8 hr after treatment. Multiple doses of adriamycin totaling 0 mg/kg inhibited cell production to a greater extent than the equivalent single dose. In combination with 1000 R, adriamycin (5 mg/kg) given from 96 hr before to 72 hr after irradiation reduced the amount of postirradiation proliferation.

INTRODUCTION

The anthracycline antibiotic adriamycin (NSC 123127) has shown recent promise as an antitumor agent. In many animal tumor systems, adriamycin is more effective than its parent compound daunomycin (11, 14, 26), and in clinical trials adriamycin has been active against a wide variety of neoplasms (4—7, 15, 23). The major mode of action of the drug appears to be intercalative binding to DNA with the subsequent inhibition of DNA polymerase activity and RNA transcriptions (12, 13, 22, 32). In vitro studies suggest that adriamycin is most effective in killing cells in the S phase of the mitotic cycle, although it does produce cell kill in all cycle stages (1, 2, 20). Adriamycin has also been reported to cause considerable chromosome fragmentation (31). Clinically, the major toxic manifestations are an acute dose-limiting myelosuppression (3) and a cumulative dose-limiting cardiomyopathy (21, 24). Gastrointestinal toxicity has been noted clinically but, in most schedules currently being used, is not dose limiting (3).

As more aggressive chemotherapy and/or combined modality therapies are used in the clinic, it becomes imperative to consider the time sequences of damage and recovery of rapidly proliferating normal tissues, such as the intestinal mucosa, to avoid excessive toxicity. Drugs which by themselves as single doses do not exhibit severe toxicity may, in combination with other treatment, produce further depression in cell production, leading to loss of tissue function. In this report, we examine the influence of adriamycin administered as a single dose up to 10 mg/kg, in several multiple dose schedules, and in combination with radiation on the posttreatment proliferative activity of the jejunal epithelium.

MATERIALS AND METHODS

Animals. Female Ha/ICR mice (Schmidt) 10 to 12 weeks old were used. The mice received Purina laboratory chow and water ad libitum and were housed in a temperature-controlled (23°C) animal room with a 12-hr photoperiod (lights on at 6:00 a.m.). Adriamycin. Adriamycin (NSC 123127) was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in U. S. P. Ringer’s solution immediately before use. Route of administration was i.p. at a constant volume of 0.01 ml/g body weight.

Irradiation. Physical factors of X-irradiation were: 270 kvpc 20 ma, half-value layer, 1.8 mm Cu, and a target to midline distance of 50 cm. The exposure rate measured in air was 124 R/min. Mice were restrained in individual Lucite containers with the portion of the mouse anterior to the zyphoid process, the femurs and tail shielded with 0.5 cm of lead.

Quantitation of Jejunal Proliferative Cell Population. The techniques for quantitation of the jejunal proliferative cell population have been previously described in detail (18, 19). Groups of animals were sacrificed 30 min after injection of 50 μCi of [3H]TdR2 (0.36 Ci/m mole; Schwarz/Mann, Orangeburg, N. Y.), and triplicate samples of the jejunum were rinsed in cold 0.9% NaCl solution, blotted on filter paper, weighed, and fixed in alcohol:acetic acid (3:1) to remove acid-soluble material. After 24 hr of fixation, the tissue was rinsed and solubilized; the incorporated 3H activity was counted in a Packard liquid scintillation spectrometer with an absolute activity analyzer for quench correction, with the result expressed as dpm/mg, wet weight, tissue.

For selected time points, pieces of jejunum were also obtained for crypt isolation. These were fixed and stained

1 Supported by Contract NO1-437-30 from the Division of Cancer Treatment and Grant CA 10438 from the National Cancer Institute.

Received August 12, 1974; accepted September 29, 1976.

2 The abbreviations used are: [3H]TdR, [3H]thymidine; [3H]UdR, [3H]iododeoxyuridine.
by the Feulgen reaction (hydrolysis for 12 min in 1 N HCl at 60°). The crypt dissection technique of Wimber et al. (33) was used to obtain isolated single crypts. Triplicate samples of 50 crypts were solubilized, the \[^3H\] activity was determined, and the results were expressed as dpm/crypt. From the same sample, other isolated crypts were squashed on slides, and radioautographs were prepared by coating the slides with Kodak NTB liquid emulsion diluted 1:1 with water. After 2 weeks of exposure time, the developed radioautographs were scored for the number of labeled nuclei and mitotic figures per crypt. If one divides obtained values of dpm per crypt by labeled nuclei per crypt, the result is dpm per labeled nucleus, which is an indicator of the rate of DNA synthesis of S-phase cells.

For each experimental point, jejunal samples from 5 mice were used. A mean value for each animal was determined from the replicate samples; these were used to calculate the mean ± S.E. for each sample time.

Mitotic Cycle and S-Phase Duration. For determination of the mitotic cycle and S-phase duration by the percentage labeled mitoses method, mice were given injections of 20 μCi [\(^3H\)]TdR and were sacrificed at various times after injection. Pieces of jejunum were fixed in alcohol:formalin:acetic acid (8:2:1); sections were cut at 3 μm and processed for radioautography. After 2 weeks of exposure, the developed radioautographs were scored for percentage labeled mitoses. At least 100 mitotic figures per animal were counted.

Fate of Damaged Cells. Proliferative epithelial cells which are damaged by a given treatment may be destroyed in the crypts or may remain intact and move out of the crypts onto the villi. In order to determine the fate of damaged cells following adriamycin treatment, animals were given injections of 10 μCi of [\(^3H\)]UdR (used in place of [\(^3H\)]TdR to minimize the reutilization of label lost from cells) 0.5 hr before drug administration. Mice were sacrificed at various times thereafter and the dpm/mg were determined.

DNA Determination. For DNA isolation and determination of dpm/μg DNA, tissue was homogenized in ice cold 0.9% NaCl solution, precipitated with 0.6 and 0.2 N HClO₄ and incubated at 37° in 0.3 N KOH for 1 hr. After the incubation, the solution was cooled in ice and precipitated with 1.2 N HClO₄. Finally, the material was incubated at 80° in 1 N HClO₄ for 10 min. After centrifugation, the supernatant containing the DNA was divided into 2 aliquots, one for the determination of \[^3H\] activity and the other for DNA content determination by the Burton diphenylamine reaction method (9).

RESULTS

Single-Dose Administration. Jejunal epithelial proliferative activity, as measured by [\(^3H\)]TdR incorporation per mg tissue, wet weight (dpm/mg), following single doses of either 1, 5, or 10 mg adriamycin per kg is presented in Chart 1. One mg/kg produced only a minor perturbation in [\(^3H\)]TdR incorporation, with a drop to 85% of the control value at 12 hr posttreatment and a subsequent return to a normal proliferative level. Following either 5 or 10 mg/kg, 3 temporally defined periods of [\(^3H\)]TdR incorporation were observed: (a) a period of declining activity lasting 12 hr, with a nadir of 45% for 5 mg/kg and 20% for 10 mg/kg; (b) a period of depressed DNA synthesis from 12 to 36 hr posttreatment with an eventual return to control levels by 48 hr; and (c) a period of increased [\(^3H\)]TdR incorporation, reaching a value between 72 and 120 hr of 150% of control after 10 mg/kg and 120% after 5 mg/kg. The cell population kinetic changes occurring during these 3 periods were studied in greater detail following a single dose of 10 mg/kg.

Within 2 hr after a dose of adriamycin (10 mg/kg), the number of mitotic figures per isolated crypt was reduced to 40% of the control value (Chart 2A). The number of mitotic figures per crypt dropped to 30% by 12 hr. Many of the mitotic figures observed during this period appeared aberrant. However, during the 1st 6 hr after treatment, the number of labeled nuclei per crypt increased (Chart 2A). It appears that cells continued to flow from G₁ into S, while the rate of passage through S, as measured by dpm/labeled nuclei (Chart 2B), was reduced. Between 6 and 12 hr...
posttreatment, the number of labeled nuclei per crypt declined, along with a further drop in the dpm per labeled nucleus.

The passage of cells through the S phase of the mitotic cycle following adriamycin (10 mg/kg) was further examined by administering \(^{[3}H\)TdR 0.5 hr before the drug treatment and then observing the percentage of labeled mitoses (Chart 3). The time period between the 50% levels of the ascending and descending portions of the curve, an indication of S-phase duration, is 13 hr. The S-phase duration for jejunal epithelial cells in untreated animals is 7 hr (see Chart 5). Although the dpm/labeled nucleus data summarized in Chart 2 suggest that rate of DNA synthesis per S-phase cell is reduced, one cannot conclude cellular traverse rates from such data alone. The data presented in Chart 3 suggest that those cells that successfully traversed S did so at a greatly reduced rate.

During the early times after treatment, cells that are reproducibly damaged may either disintegrate in the crypts or may remain intact and eventually move out onto the villi. By labeling before treatment and then following the loss of label from the tissue, relative amounts of cellular degradation \textit{in situ} may be ascertained. Chart 4 presents the results for the loss of \(^{[3}H\)UdR label administered 0.5 hr before adriamycin (10 mg/kg) and for untreated controls. Since the transit time from crypts to villi is 42 to 48 hr, there was little loss of label from the untreated animals during this time period. There was relatively little extra loss of label from the adriamycin-treated tissue, indicating that most of the cells remained intact. This observation was confirmed by the low frequency of pyknotic nuclei observed in the squash preparations of crypts at the early time periods.

At 18 hr posttreatment, both the number of labeled nuclei per crypt and the dpm per labeled nucleus reached a nadir (Chart 2). The number of mitotic figures per crypt remained below 50% through 24 hr (Chart 2). By 24 hr posttreatment, the rate of DNA synthesis of S-phase cells recovered to 80% of the control level and, by 48 hr, all 3 parameters in Chart 2 were back to or above control level.

The period between 48 and 120 hr was characterized by an increase above controls in both labeled nuclei and mitotic figures per crypt (Chart 2). The cellular rate of DNA synthesis was slightly above control value at 48 and 72 hr (Chart 2). The mitotic cycle duration of crypt epithelial cells during the recovery period was measured beginning 48 hr after the administration of 10 mg/kg (Chart 5). The cycle was estimated as 7.8 hr from the distance between the 50% levels of the 1st and 2nd ascending portions of the curve. The S-phase duration was 5.5 hr. Control cells have a cycle time of 13 to 14 hr, with an S-phase duration of 6.5 to 7.0 hr (Chart 5, \textit{dashed line}) (10).

**Multiple-Dose Administration.** Two series of experiments were undertaken to examine the effect of multiple doses of adriamycin on jejunal cell proliferation. In the first (Chart 6) 2 schedules adding up to 10 mg/kg over a 5-day period are compared with a single injection of 10 mg/kg. The area under the dpm/mg versus time curve is a relative measure of \(^{[3}H\)TdR incorporation during the observation period. The total amount of incorporation following a 10 mg/kg single-dose treatment was essentially the same as that of control animals. Schedules of 2 mg/kg every day for 5 days or 3.3 mg/kg every other day for 5 days reduced the area under the dpm/mg curve to 75 and 70% of the control value, respectively.

Another dose-scheduling experiment consisted of two 5-mg/kg doses of adriamycin separated by either 24, 48, or 72 hr (Chart 7). Two doses of 5 mg/kg separated by 24 hr prolonged the period of suppressed \(^{[3}H\)TdR incorporation, compared with a single dose of 10 mg/kg. A low level of increased incorporation occurred 96 hr after the initiation of treatment. If the 2nd dose was administered at, or after, a...
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• ADR 2.0mg/kg q 1dx5
• ADR 3.3mg/kg q 2dx3

TIME AFTER START OF TREATMENT (HOURS)

If adriamycin was given 24 hr after 1000 R, recovery was again delayed; a low level of incorporation was maintained for an extended time period (Chart 9B). If adriamycin was administered at a time when radiation recovery was complete, 72 hr after 1000 R, any further hyperproliferation was prevented for the remainder of the observation period (Chart 9C).

**DISCUSSION**

Single doses of adriamycin of 5 and 10 mg/kg produce a transient reduction in the proliferative activity of the mouse jejunum, while 1 mg/kg does not cause any appreciable decrease in proliferation. Gastrointestinal toxicity has not been a limiting factor clinically during adriamycin therapy when the drug was given in a single injection or on intermittent schedules with the drug doses spaced several days apart (3). Survival studies performed in this laboratory have not indicated gastrointestinal-mediated lethality with single sufficient time had elapsed for recovery from the 1st dose, there was a curtailment of further incorporation, with a secondary nadir in dpm/mg being reached 12 hr after the 2nd dose. Recovery from the 2nd dose occurred over the next 36 hr, with a low level of compensatory hyperproliferative activity following.

**Drug-Radiation Combination.** The influence of various temporally spaced doses of adriamycin (5 mg/kg) on the onset and extent of postirradiation proliferation following 1000 R was examined. The dashed line in Chart 8 represents the time course of [3H]TdR incorporation following 1000 R; beginning at 48 hr, there is a rapid increase in incorporation with a peak in dpm/mg of 240% being reached at 96 hr. If adriamycin (5 mg/kg) was administered immediately after irradiation, there was a prolongation of the depression of DNA synthesis, with a decrease in the amount of subsequent hyperproliferation (Chart 8, A). Adriamycin injected 24 hr before irradiation also reduced the amount of postirradiation [3H]TdR incorporation (Chart 8B). Administering the drug 48 and even 96 hr before irradiation reduced the level of postirradiation incorporation (Chart 8, C and D).

If adriamycin (5 mg/kg) was administered 4 hr after irradiation, control levels were not reached until 84 hr postirradiation (Chart 9A). Recovery to this level normally occurs by 48 hr following exposure to 1000 R. If adriamycin was given 24 hr after 1000 R, recovery was again delayed; a low level of incorporation was maintained for an extended time period (Chart 9B). If adriamycin was administered at a time when radiation recovery was complete, 72 hr after 1000 R, any further hyperproliferation was prevented for the remainder of the observation period (Chart 9C).
doses of adriamycin up through 10 mg/kg, although 10 mg/kg produced death in 20% of the animals within 30 days of administration (27). Although there is depressed jejunal proliferative activity for almost 48 hr following either 5 or 10 mg/kg, there is an adequate proliferative recovery, so that the total cellular output following drug exposure is near control levels. The ability of the intestinal epithelium to rapidly compensate for time periods of decreased cell production confers a considerable degree of tolerance to adriamycin-produced proliferative disturbances. It has been reported that 20 mg/kg doses of adriamycin in rats result in considerable gastrointestinal damage with attendant lethality (25).

The recovery of DNA synthesis following an i.p. injection of adriamycin (10 mg/kg) reported herein is more prompt than that previously reported by Tobias et al. (30), who found, in contrast to our results, that the DNA synthesis rate, as measured by dpm/µg DNA, dropped gradually over the 1st 48 hr after treatment to 30% of the control value and remained at that level through 72 hr, with no indication of recovery. The discrepancy between these 2 sets of data cannot be explained by the difference in experimental methods: the end points of dpm/mg tissue, wet weight, versus dpm/µg DNA. We have repeated 3 of the time points from our dpm/mg versus time curve for 10 mg/kg, with dpm/µg DNA as the measurement of DNA synthesis. The results of this comparison are shown in Table 1. There is no difference in the magnitude of change in dpm/mg, wet weight, compared with dpm/µg DNA. In this study, a method of assessing jejunal proliferative activity not based on thymidine incorporation (mitotic figures per crypt) also showed a recovery to control value by 48 hr and an overshoot at 72 hr posttreatment.

On the cellular level, adriamycin (10 mg/kg) promptly reduced the number of mitotic figures per crypt. The high incidence of aberrant mitotic figures suggests that the number of cells negotiating a successful division during the 1st 12 hr following treatment is not 40% of control, as shown in Chart 2, but, in reality, is between 10 and 20% of control. The number of labeled nuclei per crypt initially increased after treatment, with a concomitant decrease in the rate of thymidine incorporation per labeled nucleus, indicating that cells were still moving from G1 into S, while the transit time through S was increased. As fewer cells entered S from G1, a result of the decreased number of cells passing through mitosis, the number of S-phase cells dropped. This drop in the number of S-phase cells was gradual, since the dpm/labeled nucleus was also decreasing during this time period and those cells in S were remaining there longer.

In vitro studies have shown adriamycin to be most toxic for cells in S (1, 2, 20). All stages of the cycle except mitosis exhibit a dose-dependent progression delay (1). At drug concentrations which still allow the transition from G1 to S to take place in vitro, most cells in S are blocked from reaching mitosis (29). In an experimental tumor system, only moderate DNA precursor incorporation inhibition was detected 90 min after adriamycin administration, while mitotic activity was greatly curtailed (28). The highest single dose used in the present study, 10 mg/kg, did not cause immediate killing or cessation of DNA synthesis of S-phase cells; however the passage of cells through mitosis was interrupted within 2 hr after treatment. Most of the cells damaged by the adriamycin treatment remained intact and presumably moved out of the crypt onto the villi.

Recovery of the intestinal epithelium from cellular insult involves both a return to normal cell cycle progression, often accompanied by a decrease in mitotic cycle duration, and a repopulation of the functional compartment depleted during the period of decreased cell production (16, 17). In the case of adriamycin, a measure of S-phase progression, dpm/labeled nucleus, is back to 80% of control by 24 hr posttreatment. Although this is not a reflection of progression through the total cycle, it suggests that at that time cells are beginning to cycle at a near normal rate. By 48 hr after adriamycin (10 mg/kg), the mitotic cycle duration is reduced to 50% of the control time, with most of the shortening occurring in the G1 phase. In this configuration the S phase occupies 70% of the mitotic cycle. By 72 hr, the crypts are hyperplastic, with the number of mitotic figures and labeled nuclei per crypt above the control levels. At this time, the total cell number of isolated, squashed crypts is 135% of the control value. This increased cell production per crypt lasts through 120 hr posttreatment.

Subsequent treatments that interfere with the ability to compensate for time periods of little or no cell production may eventually result in a severe depletion of villous cellularity and subsequent loss of the functional integrity of the intestinal mucosa. Multiple doses of adriamycin, adding up to 10 mg/kg given over a 5-day period, eliminated the hyperproliferation seen after a single dose of 10 mg/kg and reduced the cell output during the experimental period, compared with that of the control, or after 10 mg/kg. It is interesting to note that mucositis is greatly reduced clinically in patients receiving adriamycin as a single dose instead of the same dose in a 3-day course (3).

At doses of 5 or 10 mg/kg given immediately after radiation exposure, adriamycin has been shown to modify the postirradiation response of the intestinal epithelium, not by causing additional cell killing, as measured by crypt survival, but by exerting an antiproliferative action on the surviving cells (8). The present results indicate that there is some reduction in the amount of postirradiation cell production when the drug is given prior to radiation, even if the drug is administered in a dose of 5 mg/kg 96 hr before irradiation. The long retention of adriamycin in tissues (34) may be a contributing factor to apparent long-term interactions between prior drug and subsequent radiation exposure.

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<th>No. of hr after treatment</th>
<th>dpm/µg DNA (% of control)</th>
<th>dpm/mg, wet wt (% of control)</th>
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ACKNOWLEDGMENTS

The excellent technical assistance of J. Kissel, M. Cost, and G. Thomas is gratefully acknowledged.

REFERENCES


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JANUARY 1977

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