The Interaction between Radiation and Adriamycin Damage in Mammalian Cells

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SUMMARY

Interaction between radiation and Adriamycin damage reduced the width of the shoulder of the X-ray survival curve. This effect was found to persist for at least 24 hr after Adriamycin treatment. Adriamycin did not affect radiosensitivity. The drug did not suppress repair of radiation damage when cells were treated either before two-dose fractionation or with the drug present between X-ray exposures. Adriamycin is unique in that its major effect on radiation response is to reduce the survival curve shoulder without affecting two-dose recovery. It is suggested that Adriamycin and radiation produce independent classes of damage that are expressed mutually for killing, with regard to the registration of sublethal radiation damage, but that repair of the latter is unaffected by Adriamycin.

INTRODUCTION

ADRM is an anthracycline antibiotic with an aglycone sugar and a chromophore side chain (1). Used as a single agent or in combination with other chemotherapeutic agents, it has activity against a wide variety of human solid tumors (4) and has assumed a prominent place in the armamentarium of the medical oncologist.

The toxicity manifestations of ADRM are predominantly on cardiac (4, 16) and bone marrow (4) function. Apart from the toxicity to ADRM alone, a decreased tolerance of important normal tissue systems to radiation has been reported. Among these tissues are bone marrow (7, 15), heart (14, 19), lung (7), skin (7), and gastrointestinal tract (5, 20). It was observed that human skin exhibits "recall" effects similar to those reported for AMD (7, 8), another anthracycline antibiotic of proven antitumor effectiveness. Therefore, clarification of damage interactions between X-radiation and ADRM in mammalian cells is important because radiation remains one of the major treatment modalities for human neoplastic disease and because such information will allow the oncologist to design therapeutic strategies that do not compromise the efficacy of radiation on the one hand and do not result in unacceptable complications and toxicity on the other.

Like AMD, ADRM intercalates between base pairs of duplex DNA. Whereas the former preferentially binds to G-C regions, the latter appears to be more random in its binding properties (21). Because both agents are anthracycline antibiotics and both intercalate into duplex DNA, it is reasonable to expect them to exert similar effects on cellular radiation response. AMD modifies radiation response in a number of important respects. (a) Cells surviving exposure to this agent exhibit a decreased capacity to accumulate sublethal injury (10). (b) At moderately toxic doses cells have increased radiosensitivity; e.g., the terminal slope of the survival curve increases (9). (c) AMD decreases the radiation damage repair capacity of both exponential (9, 13) and plateau phase cells (23). (d) Delayed effects regarding damage interactions can be demonstrated for both exponential and plateau phase cells (22).

Contrary to expectation we show that ADRM differs from AMD regarding the radiation response of exponential cells. The major effect of ADRM is to reduce the capacity of cells to accumulate sublethal radiation injury with little or no effect on radiosensitivity; also, ADRM does not inhibit radiation damage repair.

MATERIALS AND METHODS

The cell line used was a clone (V79-182) derived from lung cells of a female Chinese hamster (22, 23). Surface-attached cells were grown in FST-15. Maintained in FST-15 at 37°C in a humidified atmosphere at 3% CO2, cells grew with a doubling time of 9 to 10 hr. Exponentially growing cell populations were dispersed with 0.05% trypsin in Puck's Saline A and diluted with FST-15; the cells were then counted in a hemacytometer. Appropriate numbers of cells were plated onto 9-cm plastic Petri dishes to yield about 200 colonies in control and experimental dishes. After overnight growth [\(N\) (average number of cells per colony) = 2.5 to 4.0], cells were exposed to ADRM dissolved in FST-15. Exposure times to the drug were 0.5 or 1.0 hr at 37°C. Following these exposure times drug-containing medium was removed, and cells were rinsed with 10 ml Puck's Saline F and overlaid with drug-free FST-15. At times after drug exposure, the medium was removed, and the dishes without their covers were placed in a holder that allowed for temperature control below and gas control above the dish. Attached cells were irradiated with a Machlett OEG-16 beryllium end-window X-ray tube operated at 50 kVp and 20 ma. At 23.2 cm from the end window, the dose rate at the cell-dish interface was 26.9 R/sec. The beam, filtered with 0.06 mm aluminum, had a half-value layer of 1.0 mm aluminum.

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2 The abbreviations used are: ADRM, Adriamycin (doxorubicin HCl); AMD, actinomycin D; FST-15, Eagle's minimal essential medium supplemented with Eagle's nonessential amino acids, glutamine, NCTC-109, (4%) penicillin, streptomycin, and fetal calf serum (15%).

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Cells were irradiated at room temperature and in room air. Two-dose recovery response was measured in treated and untreated cells by incubating cells in FST-15 at 37° for 0 to 8 hr between exposures. In some experiments this incubation time was in the presence of ADRM. Immediately after the final experimental manipulation, cells were overlaid with fresh FST-15 and incubated at 37° for 10 to 12 days. At this time, colonies were stained with 1% methylene blue solution and counted, corrections were made for plating efficiency, and survival fractions were determined. Departures from this experimental sequence are indicated in the appropriate chart legends, and unless specified the data were derived from the same starting cell suspension. Survival curve parameters (extrapolation number, $D_s$, and $D_0$) were calculated by least-squares regression analysis. These calculations were accomplished with corrections for plating efficiencies, and the standard error for each survival point was usually less than 10%. Single-cell extrapolation numbers were calculated by correcting for $N$ after correcting the latter for the plating efficiency. The regression analysis, therefore, was determined with observed survival data, which were subsequently corrected for colony multiplicity (corrected for plating efficiency) and initial ADRM killing (0.4 to 0.6). When $\sim$ is used, the value was obtained from eye-fitted curves and indicates estimated rather than calculated values.

RESULTS AND DISCUSSION

Dose Response to ADRM. The ADRM dose-response curves (Chart 1) are biphasic for the 2 exposure times studied and are similar to results reported by Barranco et al. (2). Survival responses characterized by inflection points are usually taken to mean the presence of sensitive and resistant cell subpopulations. A measure of the rate of response of each can be obtained from the drug dose ($D_0$) necessary to reduce survival by $e^{-1}$ on the exponential portions of the curves. Comparison of the $D_0$ values for sensitive and resistant responses revealed that the subpopulation responsible for the terminal portions of the curves in Chart 1 was 7- to 9-fold more resistant than were sensitive cells. The proportion of resistant cells, moreover, was dependent upon exposure time: $4.5 \times 10^{-4}$ for 1.0 hr and $3.8 \times 10^{-3}$ for 0.5 hr. The initial portion of both survival curves demonstrated a threshold but was larger for 0.5-hr exposure time ($\bar{N}$ (single-cell extrapolation number) $= 1.05$ versus $10.9$).

Taken together these results suggest a time-dependent drug action of ADRM on V79-182 cells in vitro that is characterized by the requirement for incorporation of this agent to a critical level before exponential survival is observed. It is possible that a number of intercalation sites must be saturated before a constant probability of killing results. Therefore, cells were exposed to ADRM (0.25 $\mu$g/ml) for various times at 37°. Survival response (Chart 2) was biphasic, and the survival curve shape was similar to that of the curves in Chart 1. Therefore, the survival response of exponentially growing cells to ADRM is both drug concentration and time dependent, and the resistant subpopulation is expressed whether or not cells are exposed to drug concentrations for a fixed time or to a single concentration for different times.

That this subpopulation represents a resistant compartment in the cell cycle is unlikely because of its very small size (approximately $4.0 \times 10^{-3}$ to $4.5 \times 10^{-4}$).

Terasima et al. (24) found similar responses to bleomycin in several mammalian cell lines in vitro and also found that the time inactivation curve demonstrated decreased rates of inactivation as the exposure time to a fixed drug concentration increased. It was suggested that resistance was induced as the time of exposure was prolonged. However, it was also found that this resistance did not persist when cells were removed from bleomycin-containing medium. The exposure times in these studies were somewhat shorter than those used in the studies reported here, and it is
difficult to postulate time-dependent induction of drug resistance over such short periods when the induced resistance reverts rapidly to predrug sensitivity after cells are removed from drug-free medium.

The suggestion of Urano et al. (25) concerning the response of C3H mouse mammary carcinoma to bleomycin is more attractive. They proposed a “binding-saturation model” to account for the dose-response relationship observed in which it is required that bleomycin be bound to a critical target for cell death and that saturation occur at higher drug concentrations. An equilibrium state eventually emerges. As noted above we have suggested a similar mechanism for the survival responses of V79 cells to ADRM.

Lastly, the possibility remains that a very small proportion of V79 cells have “inherent” resistance to ADRM and that these cells are selected for during exposure to drug. For isolation of this subpopulation, small numbers of cells were exposed to ADRM (3.0 μg/ml) for 1 hr, which produced, on the average, 1 surviving cell per dish. Resulting individual colonies were harvested and subcultured. When these populations were studied for ADRM response, the response curve was no different from those shown in Chart 1. Therefore, subpopulations inherently resistant to ADRM were not present among V79 cells, but “resistant” cells emerged in a random way during cell division regardless of prior ADRM experience.

**ADRM Effect on Radiation and Recovery Responses.**

The radiation survival properties of V79-182 cells after ADRM (Chart 3; Tables 1 and 2) were derived from several experiments. We exposed cells to a concentration of 0.4 μg/ml for 1 hr prior to irradiation (survival level to drug alone, 0.4 to 0.6). The radiation survival curve for V79-182 cells without drug exposure (Chart 3A) was typical for this cell line. The radiation response of V79 cells is characterized by a wide threshold manifested by extrapolation numbers of 8.0 to 20.0; D0 values are usually between 130 and 170 R. The data in Chart 3 and Tables 1 and 2 are representative of several experiments. Chart 3, B to D, traces the survival curves at 0, 2, and 24 hr after ADRM; Table 1 summarizes these data and data from other experiments for 4 and 6 hr after drug. The salient features of these radiation survival data are that the major effect of ADRM was to reduce the threshold of the survival curve, which suggests a loss in the capacity to accumulate sublethal injury, and that this effect persisted through 24 hr after drug treatment. This effect is manifested by decreased extrapolation numbers (n) and quasithreshold doses (D0). The latter is the radiation dose required to reach saturation of suble-
data presented above, the effect of ADAM was a reduction in the threshold of the survival curve; this reduction per-3660haps was not the consequence of studying microcolonies rather than single cells and was probably independent of cell-cell interactions within a colony at the time of irradiation. In addition, these data also suggest that small residual levels of ADAM were not responsible for the effects observed because cells were irradiated on dishes that were not used for drug treatment.

The finding that ADAM reduced the capacity for sublethal radiation damage accumulation in treated cells suggested that such cells would also be deficient with regard to radiation damage repair as expressed by survival fluctuations after split-dose irradiation. Chart 4 shows the 2-dose recovery responses for nontreated and ADAM-treated cells immediately and 2 hr after drug. Comparison of the 3 curves clearly shows that the initial degree of survival increase after ADRM is as great and as rapid in ADARM-treated as in nontreated cells. In these experiments the initial exposure dose was large enough to reduce survival to the exponential part of the single-dose survival curve with and without prior ADRM. Therefore, although ADRM suppressed the expression of sublethal radiation damage accumulation, it did not inhibit its repair. Increasing the time (up to 24 hr) after ADRM treatment did not change the extent of 2-dose recovery response.

When cells were tested for their 2-dose response in the presence of ADRM, the results in Chart 5 were obtained. For the drug concentration used (0.05 μg/ml), minimal toxicity was observed when irradiated cells were treated over a

<table>
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<th>Time (hr)</th>
<th>n</th>
<th>( D_0 ) (R)</th>
<th>( D_0^*(R) )</th>
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<tr>
<td>No drug</td>
<td>6.5</td>
<td>151 ± 4</td>
<td>282</td>
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<tr>
<td>0</td>
<td>2.5</td>
<td>160 ± 8</td>
<td>145</td>
</tr>
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<td>2</td>
<td>2.9</td>
<td>171 ± 10</td>
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</tr>
<tr>
<td>24</td>
<td>2.8</td>
<td>168 ± 5</td>
<td>172</td>
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* \( D_0 = D_0 \ln n \).
period of 8 hr. Following 700 R, however, the same concentration of ADRM produced additional killing that could not be accounted for by ADRM toxicity alone. That a biphasic survival response was not observed (see Chart 2) can be accounted for on the basis of a lower ADRM concentration (0.05 μg/ml versus 0.25 μg/ml). The possibility that survivors to 700 R constituted the ADRM-resistant population described above cannot be entirely ruled out by these data.

However, this is an unlikely possibility because radiation survival curve shape was not changed by ADRM (Chart 6) after X-irradiation.

In spite of ADRM toxicity in irradiated cells, the initial survival increase observed in a 2-dose exposure was not suppressed by ADRM present between irradiations (Chart 5). The substantial fall in survival after a fractionation interval of 2 hr can be accounted for by ADRM toxicity. Nevertheless, these data show that ADRM did not inhibit the initial repair of sublethal radiation injury, as measured by split-dose techniques, when the drug was present at low but toxic levels between exposures.

In the context of existing models for the registration and expression of radiation injury, these findings have the following possible explanations.

1. ADRM alters the distribution of cells in the cell cycle so that the radiation survival response reflects those cells that may have had a decreased capacity to accumulate injury to begin with. This is unlikely because the threshold of the survival curve was not restored after several cell divisions. If ADRM had resulted in substantial population shifts, these shifts should have been reversed after relative growth increases approaching 8.0.

2. ADRM results in dissociation of damage accumulation and its repair. The inconsistency between a decreased survival curve threshold on the one hand and repair capacity comparable to untreated cells on the other may reflect a damage-repair state unrelated to sublethal radiation injury and its repair. This possibility has been suggested by Hellman and Hannon (18) to explain similar findings relative to ADRM modification of mouse bone marrow stem cell radiation and recovery responses. They found that ADRM reduced the extrapolation number of the radiation survival curve.

Chart 6. Effect of ADRM after irradiation. Progressive loss of threshold was observed with time after X-ray. After each radiation exposure cells were exposed to ADRM at 0.4 μg/ml for 60 min. Dose points in B and C give survival to ADRM alone. XR, X-ray; PE, plating efficiency.
They postulated that ADAM-mediated dissociation of multiple intracellular repair processes, which allows expression of those that require shorter times, may have led to greater than expected survival when a radiation dose was split after ADRM (18).

3. Cells surviving ADRM may accumulate sublethal radiation damage to the same extent as do untreated cells, but they are unable to express such accumulation because both classes of damage may be additive to suppressing colony formation. However, those cells that survive both ADRM and X-ray exposure may repair the sublethal injury registered by the latter and, when irradiated with a 2nd dose, may express this repair by a survival increase comparable to that in untreated cells. ADRM damage and X-ray damage may be additive in production of killing but are expressed independently when repair of radiation damage is measured. This is a likely possibility because the level of ADRM studied (0.4 μg/ml for 1.0 hr) produced survival levels of about 0.4. In studies (not shown here) in which ADRM concentrations were used that resulted in survivals of 0.8 to 0.9, no effect on the X-ray survival curves was seen.

4. Since these studies were accomplished with exponentially growing cells, small subpopulations that were in radiation-resistant cell-age response states may have been present after ADRM and the 1st X-ray exposure and, thus, may have retained the capacity for sublethal damage accumulation. Such a subpopulation, if small enough, would not be reflected in a single-dose X-ray survival response but would contribute to substantial survival increases in a 2-dose study if the 1st dose were large enough to inactivate large proportions of cells in sensitive response states. Therefore, a single-dose X-ray survival response would show a small threshold, but survivors to a large single dose would express damage repair because these would predominate in the surviving population.

Therefore, ADRM most probably reduced the capacity of cells to express fully the level of sublethal radiation injury registered, but cellular repair capacity was unaffected in cells that were in radiation-resistant age-response states at the time of the 1st dose. Whether these interrelationships are mediated through ADRM-induced changes in 1 or more multiple repair systems, as suggested by Hellman and Hannon (18), or whether the damage induced by each is expressed mutually for survival but independently for repair remains to be determined. It is clear, however, that ADRM has similar effects on radiation survival and repair responses on cells in vivo and in vitro and that these effects have the unexpected but important consequence of possible dissociation between radiation damage accumulation and its repair.

If these suggestions were reasonable, it would be expected that the sequence of ADRM-X-ray would not influence the expression of sublethal radiation damage accumulation and that ADRM would interact with X-ray damage to produce effects similar to those described above. The data in Chart 6 bear on this point and show that irradiated cells treated with ADRM (0.04 μg/ml for 60 min) as a function of time after irradiation exhibit progressive loss of the threshold region of the survival curve (the extrapolation numbers fall from 12.3 for untreated cells to 1.7 two hr after irradiation). These findings are consistent with our suggestion that ADRM interferes with the expression of radiation damage accumulation. Sublethal injury (Chart 6) had already been registered before exposure to ADRM and, by 2 hr after irradiation, a major portion of that injury would have been repaired (see Chart 4) and not available for interaction with ADRM to produce enhanced killing. Contrary to expectation the degree of reduction in extrapolation number by ADRM increased rather than decreased with time after irradiation. Similar effects with postirradiation treatment with AMD were reported by Elkind et al. (8). They found that the shoulder width of the radiation survival curve for mid-S cells was reduced by about 30% when cells were treated with nontoxic levels of AMD immediately after X-irradiation. This observation was taken to mean that AMD interfered with the repair of “lethal” injury and that this process was similar to the process responsible for sublethal damage repair. It is possible that some potentially lethal damage repair occurs in the presence of full growth medium; however, this class of damage repair proceeds more efficiently when irradiated cells are maintained in an environment that is deficient for growth (3). Furthermore, the repair of potentially lethal injury results in dose-modifying effects rather than shoulder changes (17); AMD or ADRM after single doses of X-ray exert their major effect on the shoulder rather than on the slope. Therefore, our data are not consistent with the possibility that ADRM interfered with potentially lethal damage repair and that it differed from AMD in this regard.

Apart from the interest our data may elicit concerning the identification of radiation sensitive sites in mammalian cells, there are important clinical implications. Clearly, these data suggest that caution must be exercised to avoid undue toxicity when patients who have had ADRM are concurrently or sequentially treated with radiation. For example, the gastrointestinal tract, which is a normal tissue system with substantial capacity to accumulate radiation injury (23), may exhibit toxicity at radiation doses and/or dose rates that would not ordinarily cause symptoms. Indeed, the use of low dose rates for treating patients with whole-body irradiation in preparation for bone marrow transplantation to avoid gastrointestinal radiation effects (6) should be approached with caution when ADRM is used as part of induction and consolidation therapy for leukemic states. In such patients the advantage gained by the use of low-dose-rate irradiation to avoid gastrointestinal toxicity may be severely reduced or lost altogether. In addition, our observation that ADRM injury persists in surviving cells and thus is available to increase the probability of radiation damage expression may account for the increased acute radiation effects seen in those tissues that do not depend upon rapid cell turnover for functional and morphological integrity. Among these are the myocardium and lung parenchyma. The oncologist must be aware of the damage interactions between important chemotherapeutic agents (of which ADRM is one) and radiation so that effective therapeutic strategies be designed that ensure the effectiveness of each modality without producing unacceptable acute and/or late complications.

In summary, the data presented here suggest that ADRM suppresses the capacity for mammalian cells to express the accumulation of radiation injury but does not inhibit the

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repair of such injury. In addition, this effect appears to persist in the progeny of ADRM survivors and is independent of the sequence of drug and radiation.

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