Differential Effect of Adriamycin on DNA Replicative and Repair Synthesis in Cultured Neonatal Rat Cardiac Cells

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ABSTRACT

The effect of the potent antitumor antibiotic Adriamycin (ADM) on DNA replication and unscheduled DNA synthesis in cultured rat cardiac cells was investigated. Autoradiography and [3H]thymidine incorporation studies were carried out on parallel cultures. DNA replication was depressed for up to 6 days following a 3-hr pulse of ADM administration. An ADM concentration of 1 μg/ml which was effective in reducing replicative DNA synthesis by as much as 75% did not reduce the ability of cardiac cells to repair UV-damaged DNA. However, cells exposed to higher ADM concentrations failed to undergo significant UV-induced repair. In the absence of UV treatment, ADM did not stimulate unscheduled DNA synthesis. To account for the differential response of the cardiac cell cultures to replicate and repair DNA, we propose that ADM exerts a localized effect on DNA synthesis covering a region proximal to its primary intercalation site.

INTRODUCTION

The anthracycline antibiotic ADM has been shown to be effective as a chemotherapeutic agent in the treatment of a variety of human tumors (2). Its success in retarding the growth of rapidly dividing malignant cells has been largely attributed to the ability of anthracycline drugs to intercalate between the parallel stacked bases of DNA (1, 8, 29). The resultant malformation in the double helix may be responsible for the inhibition of subsequent DNA and RNA synthesis (15, 21) and is implicated in the effectiveness of ADM in arresting tumor growth.

In spite of the antitumor value of ADM, its clinical administration has been restricted because of an associated cardiotoxicity (25, 27). Investigations on cardiotoxicity induced by ADM and related drugs have shown that gross morphological, physiological, and molecular changes occur in rat tissue culture (32) as well as in rats (9, 23) and other animal models (16–18, 20).

In the current study, we report that (a) neonatal rat myocardial cells in culture are capable of unscheduled DNA synthesis in response to UV irradiation, (b) ADM, at a concentration of 1 μg/ml, does not appear to affect the rate or extent of unscheduled DNA synthesis under conditions where replicative DNA synthesis is inhibited significantly, (c) repair synthesis occurs in virtually the entire cell population; and (d) at concentrations greater than 2 μg/ml, ADM-treated cells have lost the ability to repair DNA in response to UV-induced DNA damage.

MATERIALS AND METHODS

Neonatal (1 to 4 days old) Sprague-Dawley rats were supplied by Simonson Laboratories (Gilroy, Calif.); F-10 medium and fetal calf and horse sera were purchased from North American Biologicals, Inc. (Miami, Fla.); HU was purchased from Sigma Chemical Co. (St. Louis, Mo.); [3H]dThd was purchased from Schwarz/Mann (Orangeburg, N. Y.), had a specific activity of 62 Ci/mmol, and was used at a concentration of 10 μCi/ml unless otherwise noted. ADM, a gift of Dr. N. R. Bachur of NIH, was first dissolved in sterile distilled H2O and then diluted to the required concentration in F-10 medium. All photographic supplies such as the NTB autoradiographic emulsion, D-19 developer, fixer, and Ektachrome 160 film were purchased from Eastman Kodak Co. (Rochester, N. Y.).

Preparation of Cardiac Cultures. Heterogeneous cardiac cultures were prepared by a modified method of Harary and Farley (14), plated in F-10 medium supplemented with 10% fetal calf and 10% horse sera, and grown in a water-saturated 37°, 95% air-5% CO2 incubator. Such cultures contained spontaneously contracting cardiac muscle cells and quiescent epithelioid or nonmuscle cells. The 2 cell populations could be separated into greater than 80% pure myocardial cultures and greater than 95% nonmyocardial cultures. Advantage is taken of the faster rate of attachment of nonmyocardial cells to the dish. Following one hr of incubation, the medium containing the unattached myocardial cells is transferred to a new culture dish. The transfer procedure is then repeated after an additional 1 hr incubation. The cells attached to the first dish comprise the cardiac-derived, nonmuscle cell culture. The particular cell population type is characterized on the basis of cell density and beating and staining characteristics (32), thus indicating the physiological as well as the morphological integrity of the cells. During this study both "homogeneous" and heterogeneous cultures were investigated. However, since preliminary experiments showed no significant difference in the synthesis ability of the various cell types, the respective results have been pooled unless otherwise noted.

ADM Administration. At 18 hr in culture, one-half of the cell culture was placed in 4 ml of 1, 2, 5, or 10 μg/ml of ADM per ml of F-10 medium for 3 hr. After this exposure, HC and ADM-treated plates were rinsed once in F-10 medium, returned to 4 ml of supplemented F-10 medium, and rein-

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cubated for the desired periods.

DNA Replication Assay. At specific intervals post-ADM treatment, both HC and ADM plates were exposed in duplicate to [3H]dThd for 0, 90, and 180 min. At the end of the exposure time, the plates were rinsed 5 times in 0.9% NaCl solution, and 2 ml of 0.3 M perchloric acid were added to the plates. The cellular material was scraped from the plates and centrifuged at 14,000 rpm in a Sorvall type SS 34 rotor at 4° for 20 min. The supernatant was discarded, and the pellet was suspended in 0.5 ml of 0.5 M perchloric acid, heated for 15 min at 70° to extract the DNA, and centrifuged for 30 min. The supernatant was saved, and the DNA extraction was repeated. The supernatants were pooled and brought up to a final volume of 1.0 ml. A 0.5-ml sample was counted in 9 ml of a scintillation mixture containing 2% of toluene, 1% of Triton X-100, 16.5 g of PPO and 0.375 g of POPOP. Protein was determined on the pellet using the method of Lowry et al. (26).

DNA Repair Assay. At 68 hr in culture, HC and ADM plates were UV irradiated for 0 to 100 sec. It had been previously determined that at a distance of 4.5 cm above the exposed cells, a UVS-54 germicidal lamp delivered an incident dose of 15 ergs/sq mm/sec at 254 nm. Three ml of F-10 medium containing [3H]dThd (10 μg/ml) either with or without 0.1 M HU were added to each plate immediately after UV exposure. A 4-hr incubation was followed by five 0.9% NaCl solution rinses and the precipitation of proteins and nucleic acids by 2 ml of cold 10% trichloroacetic acid. The cellular material was scraped off and collected on Whatman GF/A glass filter discs mounted on a vacuum suction flask. The discs were dried for 20 min under an IR lamp and counted in 8 ml of toluene fluor, a scintillation mixture containing 3.79 g of toluene, 379 mg POPOP, and 18.95 g of PPO. Protein determinations were performed on parallel plates from the same culture. Irradiation did not measurably alter the protein levels.

Autoradiography. Trypsinized cardiac cells were seeded onto a glass slide placed in a 100 × 20-mm tissue culture dish. The media and growth conditions of the culture were as previously described. However, because cardiac cells do not attach as readily to glass as to plastic, an additional 48 hr were allotted prior to ADM treatment. Thus the slide-grown cardiac cultures were 66 hr old when exposed to a 3-hr pulse of ADM (0, 1, 2, 5, or 10 μg/ml). At 114 hr, the plates used for repair synthesis were treated with UV irradiation (675 ergs/sq mm) as described above for the DNA repair assay. Three ml of F-10 medium containing 0.1 M HU and 10 μCi [3H]dThd per ml of F-10 medium were added to the irradiated plates immediately following UV exposure. Control samples used for replication were unirradiated and treated with 3 ml F-10 medium containing 10 μCi [3H]dThd per ml. Following a 4-hr incubation, the slides were fixed in a freshly prepared methanol:acetic acid (3:1) mixture for three 5-min periods and then rinsed in 70% ethanol for an additional three 5-min periods and air-dried. Kodak NTB emulsion, which had been previously melted for 1 hr in a 45° water bath, was thoroughly mixed in the dark with an equal amount of deionized H2O. The fixed slides were dipped in the emulsion and dried for 1 hr in the dark. Then the slides were dipped for 10 sec in a freshly prepared scintillation mixture composed of 0.35 g PPO and 10 mg POPOP in 50 ml dioxane (11). Again, the slides were dried in the dark, sealed with a dessicant in a light tight box, and stored at −70° for 2 weeks. The sealed slides were brought to room temperature in a vacuum desiccator, developed with Kodak D-19 (diluted 1:2 with deionized H2O), and fixed with Kodak fixer. After a deionized H2O rinse, the slides were stained for 7 min in a buffered Giemsa stain (10 ml Giemsa stock, 6 ml 0.1 M citric acid, 6 ml 0.2 M disodium phosphate, 6 ml methanol, diluted to 200 ml with deionized H2O) and then dipped in a citrate buffer (1 ml 0.1 M citric acid, 1 ml 0.2 M disodium phosphate, and 48 ml deionized H2O). The slides were mounted with Permount (Fisher Scientific) and photographed under a Leitz Ortholux II microscope using Ektachrome 160 film.

RESULTS

To elucidate the effect of ADM on the synthesis of DNA by cardiac cells in culture, the incorporation of [3H]dThd into DNA both in the presence and absence of ADM was determined. Chart 1 shows that at every age tested, there is an essentially linear incorporation of [3H]dThd during the 3-hr exposure period both in the presence and absence of ADM. However, at each time point, dThd incorporation normalized to protein concentration is reduced significantly in ADM-treated cultures. Attempts to measure DNA content by the assay of Burton (4) were unsuccessful because an ADM-treated culture plate contained a reduced number of cells and, therefore, insufficient DNA for the assay. Thus, protein determinations were chosen as a reproducible parameter against which to standardize [3H]dThd incorporation into DNA. The possibility existed that a cell could increase in size without a concomitant increase in DNA. However, there proved to be a direct correlation between the average number of cells per field and the protein content per plate (data not shown). It is apparent from the cell number per plate that ADM has effectively caused a cessation of cell division, probably due to the inhibition of DNA synthesis. At all points studied, incorporation of [3H]dThd per μg protein was greater in the HC cells than in the ADM-treated ones. The latter reached and maintained a nearly steady low level of incorporation within a day after treatment. As the cultures aged, the dramatic differences between the 2 synthetic abilities lessened, possibly because the control cultures were now confluent and replicated infrequently. The ADM cells never reached confluency within the time course of this study. Therefore, only ADM and not contact inhibition was responsible for decreased synthesis.

Thymidine incorporation in Chart 1 is indicative of total DNA synthesis and does not distinguish between replicative and repair synthesis. To investigate the effect of ADM on DNA repair required the visualization of repair synthesis and the concurrent inhibition of replication. Increasing the dose of UV irradiation depresses replicative synthesis, but the level of DNA repair is so low as to still be masked. Therefore, irradiating the cells with UV in the presence of HU permits DNA repair to be more readily observed. HU is known to interfere with the reduction of ribonucleotides in the formation of DNA precursors (35), thus nearly eradicating replication while allowing repair synthesis to continue (10). A 0.1 M concentration of HU reduced replicative
Effect of ADM on DNA Replication and Repair

synthesis by 95% (see Chart 2, inset) yet allowed UV-stimulated synthesis to be observed over background replication levels which were designated as 100% activity (Chart 2). Although there was a great deal of experimental variation in the respective repair abilities of HC- and ADM-treated cultures, it is obvious that at all the UV doses investigated, stimulation over background levels was present. Enhanced repair synthesis is evident as increased \(^{3}H\)dThd incorporation due to the excision and subsequent repair of the pyrimidine dimers produced by UV irradiation. Such synthesis occurred up to exposures of 1500 ergs/sq mm. However, since this dose resulted in some morphological changes, 675 ergs/sq mm served as a maximum dose. The possibility of photoreactivation (33) in rat cardiac cells was unlikely since identical experiments run in the presence and absence of ADM, epithelial cells carried out replication better than did myocardial cells (data not shown). However, with respect to percentage of changes in activity, myocardial cells frequently did as well as or better than their epithelial counterparts. For example, the detrimental effect of ADM on \(^{3}H\)dThd incorporation, the decrease in replication by UV exposure, and the ability to repair damaged DNA are similar in the 2 subpopulations. With regard to total \(^{3}H\)dThd incorporation and percentage of activity, heterogeneous cultures generally reflected epithelial cell conditions, but this was not always the case. The great deal of overlap among synthesis abilities of the 3 culture types did not warrant separate figures for \(^{3}H\)dThd incorporation data. Therefore, all the results have been pooled for each parameter investigated (Charts 1, and 2; Table 1).

The above results did not eliminate the possibility that the low \(^{3}H\)dThd incorporation seen in HU-treated cultures may actually be due to a small amount of residual DNA replication and not to repair synthesis. To prove that substantial repair synthesis is occurring in the presence of HU, we studied the incorporation of \(^{3}H\)dThd in individual cells by means of autoradiography. Dark grains in the cell nucleus.
of unirradiated ADM-treated cells (Fig. 1, C, E, G, and I) show no indication of excision-repair induction. Thus, at the levels of ADM tested, there was no evidence of concomitant excision repair associated with possible ADM-induced strand breakage.

Since the inhibition of DNA synthesis by ADM is believed to occur during S phase (19, 22), the possibility existed that only those cells which were in the S phase would die. Unaffected cells which escaped the action of the drug could then proceed to synthesize DNA normally. This is not likely since we are able to observe fluorescence specific for ADM (12) in all myocardial and nonmyocardial cells irrespective of cell phase almost immediately after ADM exposure. Although neither the activity nor the intracellular concentration of ADM was determined, a drug-induced fluorescence related to either ADM or possibly one of its metabolites is present in the majority of the cardiac cells up to at least 48- hr post-ADM administration (data not shown). Thus, we conclude that UV-induced repair synthesis taking place in the presence of ADM cannot be attributed solely to cells which have managed to completely escape drug action.

**DISCUSSION**

The use of ADM as an antineoplastic agent has been limited by an associated cumulative, dose-dependent cardiomyopathy. Since ADM’s effectiveness is linked to its ability to destroy selectively the proliferating cells characteristic of tumor growth, it is not clear why cardiac cells, which replicate at a very slow rate if at all, are also damaged. In vivo studies using ADM have suggested several possibilities for its deleterious action in nondividing cells. For example, the drug may cause the formation of free radicals with resultant lipid peroxidation and cellular damage (28). Since it is well documented that ADM can both intercalate into DNA (29) and cause single-strand breaks (6, 31), numerous studies have been concerned with impairment by ADM of nucleic acid synthesis (15). We pursued this line of reasoning by investigating the effect of ADM on the 2 facets of DNA synthesis, replication and repair.

In the former case (Chart 1), ADM (1 μg/ml) reduces DNA replication in cardiac cells at every age tested with the reduction being most significant during the rapid growth phase (~30 to 120 hr). Yet, in spite of causing an approximately 75% reduction in replication, ADM (1 μg/ml) did not inhibit UV-stimulated repair synthesis (Chart 2; Table 1). However, at higher doses of ADM (2, 5, and 10 μg/ml), inhibition of UV-induced DNA repair was observed (Table 1). Supportive evidence of such inhibition is seen in recent studies on human leukocytes by Ringborg and Lambert (30). Comparable results have also been found by Byfield et al. (7) and Lee et al. (24) who showed that X-ray-induced excision repair is somewhat limited in mammalian tumor cells treated with and maintained in the presence of ADM.

To account for the differential action of ADM on DNA replication and UV-induced DNA repair in cardiac cell populations, we suggest that the focus of drug action may be limited to a site or sites proximal to where the drug intercalates into the double helix. Perhaps only one or a few ADM molecules positioned in the path of an oncoming...
Effect of ADM on DNA Replication and Repair

Table 1
Effect of UV irradiation on replicative and repair synthesis

<table>
<thead>
<tr>
<th>Conditions</th>
<th>N(^a)</th>
<th>[(^3)H]dTthd incorporation(^b) (cpm/ (\mu)g protein)</th>
<th>N</th>
<th>% stimulation(^b)</th>
<th>N</th>
<th>% stimulation(^b)</th>
<th>N</th>
<th>% stimulation(^b)</th>
</tr>
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<tbody>
<tr>
<td>HC</td>
<td>11</td>
<td>317 ± 42(^c)</td>
<td>9</td>
<td>75 ± 6</td>
<td>14</td>
<td>31 ± 1</td>
<td>14</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>HC + HU</td>
<td>11</td>
<td>11 ± 2</td>
<td>9</td>
<td>103 ± 5</td>
<td>16</td>
<td>142 ± 13</td>
<td>16</td>
<td>142 ± 12</td>
</tr>
<tr>
<td>ADM 1(^d)</td>
<td>11</td>
<td>75 ± 16</td>
<td>9</td>
<td>83 ± 5</td>
<td>11</td>
<td>39 ± 4</td>
<td>10</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>ADM 1 + HU</td>
<td>11</td>
<td>5 ± 1</td>
<td>9</td>
<td>137 ± 6</td>
<td>11</td>
<td>146 ± 20</td>
<td>11</td>
<td>178 ± 24</td>
</tr>
<tr>
<td>ADM 2</td>
<td>6</td>
<td>54 ± 11</td>
<td>5</td>
<td>94 ± 7</td>
<td>6</td>
<td>40 ± 5</td>
<td>6</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>ADM 2 + HU</td>
<td>7</td>
<td>4 ± 1</td>
<td>5</td>
<td>106 ± 15</td>
<td>7</td>
<td>137 ± 18</td>
<td>8</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>ADM 5</td>
<td>6</td>
<td>30 ± 7</td>
<td>5</td>
<td>94 ± 13</td>
<td>6</td>
<td>45 ± 8</td>
<td>5</td>
<td>44 ± 9</td>
</tr>
<tr>
<td>ADM 5 + HU</td>
<td>7</td>
<td>3 ± 0.8</td>
<td>5</td>
<td>150 ± 32</td>
<td>7</td>
<td>106 ± 22</td>
<td>7</td>
<td>114 ± 20</td>
</tr>
<tr>
<td>ADM 10</td>
<td>7</td>
<td>19 ± 3</td>
<td>5</td>
<td>85 ± 13</td>
<td>7</td>
<td>56 ± 16</td>
<td>7</td>
<td>72 ± 20</td>
</tr>
<tr>
<td>ADM 10 + HU</td>
<td>7</td>
<td>3 ± 0.5</td>
<td>5</td>
<td>89 ± 13</td>
<td>7</td>
<td>90 ± 15</td>
<td>7</td>
<td>76 ± 18</td>
</tr>
</tbody>
</table>

\(^a\) N, number of experiments.
\(^b\) Percentage of stimulation of the UV-irradiated samples (Columns 4, 6, and 8) is calculated by designating each corresponding unirradiated sample (Column 2) as 100\% [\(^3\)H]dTthd incorporation.
\(^c\) Mean ± S.E.
\(^d\) Numbers following ADM, concentration of the drug (\(\mu\)g/ml).

replication fork might be sufficient to impede further progress of the fork beyond the drug-binding site. Thus replication could be inhibited at relatively low ADM concentrations.

This, however, would not be the case for repair synthesis. The production of UV-induced pyrimidine dimers elicits an excision-repair response. If the ADM were administered at a high enough concentration for its molecules to be located within about 50 nucleotide pairs of a dimer (13), DNA polymerase could be physically inhibited from repairing the gapped region. It is not currently feasible to quantitate the number of intercalated ADM molecules per pyrimidine dimer in cardiac cultures. However, it can be reasonably hypothesized that (a) a drug:dimer ratio exists at which replication is decreased while UV-stimulated repair is unaffected, and (b) at some higher ratio, both forms of synthesis are reduced.

An alternate model might be proposed since a direct physical impediment by ADM may not necessarily be required to inhibit DNA synthesis. In fact, a precedent for indirect physical interference or "action at a distance" has been noted in biological systems in vitro by Burd et al. (3). They observed that a perturbation at a localized DNA region can affect the stability of a distant DNA region. Intercalating agents such as ADM which cause unwinding of DNA are excellent candidates for exhibiting biological effects over regions beyond their discrete binding sites. Our data are more compatible with the previous model in which ADM must intercalate near the dimer region to interfere with repair synthesis.

Since ADM has been observed in numerous instances to cause DNA breakdown in vivo (5, 7, 31), it was of interest to inquire whether ADM could itself stimulate DNA repair synthesis. This line of reasoning followed the supposition that ADM-induced DNA degradation could evolve from the abortive nicking action by endonucleases attempting to free the DNA of its tightly bound drug contaminant. Our results (Table 1) indicate that in the presence of HU, unirradiated ADM-treated cells do not stimulate [\(^3\)H]dTthd incorporation over unirradiated control (HC plus HU) levels. The inhibition of unscheduled DNA synthesis in cultured cardiac cells by ADM at 10 \(\mu\)g/ml but not at 1 \(\mu\)g/ml and an observed ADM-related fluorescence in the cells 2 days after treatment suggest a plausible mechanism for ADM cardiotoxicity. The toxicity of ADM when given in repeated doses in chemotherapy may result from an accumulation of the drug bound to DNA to the extent that repair of drug-induced damage can no longer occur.

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Fig. 1. Autoradiographs of cultured rat heterogeneous cardiac cells undergoing DNA-replicative or repair synthesis. ADM was added at a concentration of 1, 2, 5, or 10  
µg/ml at 18 to 21 hr of culture growth. At 68 hr, UV irradiation at 675 ergs/cm² was followed by a 4-hr pulse of [³H]TdR (10 µCi/ml) in the presence or absence of 0.1 M HU. Arrows: labeled nuclei. A, unirradiated HC; B, irradiated HC plus HU; C, unirradiated ADM (1  
µg/ml); D, irradiated ADM plus HU (1 µg/ml); E, unirradiated ADM (2 µg/ml); F, irradiated ADM plus HU (2 µg/ml); G, unirradiated ADM (5 µg/ml); H, irradiated ADM plus HU (5  
µg/ml); I, unirradiated ADM (10 µg/ml); J, irradiated ADM plus HU (10 µg/ml).
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