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TWO MECHANISMS OF ADRIAMYCIN–DNA INTERACTION IN L1210 CELLS*

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Abstract—Among the effects exerted by adriamycin (ADR), interaction with DNA is closely related to cytotoxicity. The interaction results in the formation of protein-associated DNA single-strand breaks (PA-SSB) and, at drug levels $\geq 2.8 \times 10^{-6}$ M, also in "direct" (nonenzymatic) DNA single-strand breaks (D-SSB). To characterize the two types of DNA lesions, euoxic mouse leukemia L1210 cells were treated with various antioxidant agents in the presence of 2.8×10^{-6}, $\times 10^{-5}$, or $\times 10^{-4}$ M concentrations of ADR. The enzymes superoxide dismutase (200 μg/ml) or catalase (250 μg/ml), the $OH^{\cdot}$ scavengers dimethyl sulfoxide (70 mM) or ethanol (70 mM), and an inhibitor of superoxide production, 2-deoxyglucose (1 and 10 mM), reduced the frequency of D-SSB to 18.3 to 68.2% of its level in ADR-treated controls, while the frequency of PA-SSB remained unchanged. These observations seem to indicate that ADR-mediated free radicals cause discernible DNA damage in euoxic cells only at very high drug concentrations, greater than the peak plasma level achievable clinically following i.v. bolus. At lower ADR levels, relevant to clinical use, another type of interaction between the drug and DNA prevails, which apparently does not involve a free-radical mechanism.

While ADR¶ is an effective chemotherapeutic agent widely used in clinical medicine [1, 2], its efficacy is seriously limited by life-threatening myelosuppression or cumulative cardiotoxicity [1–5]. Understandably, efforts have been made to develop new improved anthracyclines. A better understanding of mechanisms involved in ADR cytotoxicity seems to be critical for this endeavor. Since its isolation and initial testing in 1968 [6, 7], ADR, as a prototype anthracycline, has been the most extensively studied compound of its generic group [8–11]. The drug interacts with various cell components and shows multiple biological and biochemical effects. Among various macromolecules, ADR also binds to nuclear DNA. The consequent inhibition of DNA replication and RNA transcription is considered to be one, if not the principal, mechanism of cytotoxicity. Yet, the exact nature of this process remains unclear, and several explanations of the sequence of events leading to DNA damage have been suggested. The damage caused by ADR may be related to the formation of ADR-based free radicals *in vitro* in a fashion similar to that demonstrated in subcellular assay systems [12–16]. As a part of "electron shuttle", electrons from NADPH are transferred to the quinone moiety of ADR, most likely due to the interaction between the anthracycline ring C of ADR molecules and the flavin component of NADPH:cytochrome P-450 reductase. The ADR semiquinone may donate unpaired electrons to O_2, and oxygen-based radicals such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are formed. These active oxygen species disrupt macromolecular DNA [17]. Another concept suggests that the distortion of the DNA helix caused by drug intercalation may activate the endonucleases, possibly repair enzymes of nick-closure type [18–20], and that their activation results in DNA strand scission. Since DNA breaks and DNA-protein complexes (called DP-CL) are formed in equivalent amounts, it is assumed that molecules of nick-closure enzymes or ligases are actually the proteins covalently bound to broken DNA [21]. Finally, it has been hypothesized that under hypoxic conditions ADR acts as a bioreductive alkylating agent [22]. This concept, however, has not been supported by a recently published study [23].

We have shown [24, 25] that in ADR-treated L1210 leukemia cells the frequency of PA-SSB and DP-CL, detected by the alkaline elution technique, increases with the dose range of 2.8×10^{-8} to 2.8×10^{-6} M. At these drug levels, no differences were noted between cells treated under hypoxic or euoxic conditions. Higher drug concentration, however, induced in euoxic cells still another type of

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¶ Abbreviations: ADR, adriamycin (doxorubicin); PA-SSB, protein-associated DNA single-strand breaks; D-SSB, direct ("nonenzymatic") DNA single-strand breaks; DP-CL, DNA–protein crosslinks; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; and kR, kiloroentgens.

DNA lesions, not associated with proteins, called D-SSB. To characterize the nature of D-SSB and their relationship to PA-SSB in cells treated with various concentrations of ADR, we performed the experiments reported here.

MATERIALS AND METHODS

Preparation and radioactive labeling of L1210 cells. The line of mouse leukemia cells was obtained from Dr. E. Cadman, Yale University School of Medicine, New Haven, CT, and was grown in suspension culture in Modified Fisher's Medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated (56° for 30 min) horse serum (Biofluids Inc., Rockville, MD) and 1% penicillin-streptomycin (penicillin, 10,000 units/ml; streptomycin, 10,000 μg/ml, Grand Island Biological Co.). Exponentially growing cells had a 14-hr doubling time and were used pre-labeled for 21–22 hr either with [2-^{14}C]thymidine (sp. act > 50 mCi/mmole; 0.025 μCi/ml) or with [*methyl*-^{3}H]thymidine (sp. act. 40–60 mCi; 0.1 μCi/ml). The isotopes were purchased from the Amersham-Searle Corp., Arlington Heights, IL.

Treatment with ADR, antioxidant agents and X-ray. ADR (NSC 123127, Farmitalia Carlo Erba, Milan, Italy) was diluted with sterile 0.15 M NaCl, protected from light, and stored at −85°. Superoxide dismutase (superoxide oxidoreductase, EC 1.15.1.1, type 1, approx. 3000 units/mg protein), catalase (EC 1.11.1.6, from bovine liver, 2000–5000 units/mg protein), and DMSO were obtained from the Fisher Scientific Co., Springfield, NJ; 2-deoxyglucose (2-deoxy-D-glucose, grade III) was received from the Sigma Chemical Co., St. Louis, MO, and ethanol (USI pure ethyl alcohol) from the U.S. Industrial Chemical Co., New York, NY.

Cells were incubated with the enzyme or scavenger at 37° in 95% air/5% CO_2 for 15 min, and ADR was added for another 60 min. Exposure of cells to various concentrations of the drug alone, or to antioxidant agents alone, was used as a control in each set of experiments. In separate experiments, cells were pretreated with some of the antioxidant agents for 15 min in air at 37°, and irradiated in air with 1.5 kR of X-ray (kV 50, mA 10, target distance 30 cm to the center of the vessel, HVL 2.4 mm Al, flow 119 R/min) in the presence of the agents at room temperature. Total exposure time to 37° and room temperature (24 ± 2°) was 30 min; otherwise cells were kept at 0°. Cells incubated at 37° without antioxidants and irradiated with 1.5 kR served as controls.

DNA alkaline elution. A standard elution procedure [25–27] has been used throughout our studies. "Experimental" cells were labeled with [^{14}C]thymidine, then incubated with ADR or irradiated in the presence or absence of the antioxidants, as described above. Untreated cells and cells exposed to 300 R of X-ray at 0° were also used as a control in each set of experiments. "Internal reference" [^{3}H]thymidine-labeled cells were irradiated with 150 R at 0°. The trypan blue exclusion of treated and untreated control cells was better than 97%. Unless otherwise stated, all cells were kept during the preparatory stage of elution at 2–4°. A mixture of experimental and internal reference cells (6.5×10^5 cells total) was deposited on a polyvinyl chloride filter (Metricel membrane filter DM-800, Gelman Sciences Inc., Ann Arbor, MI), washed with ice-cold PBS (0.15 M NaCl, 0.71 mM KH_2PO_4, 4.28 mM K_2HPO_4, pH 7.4) and lysed at room temperature with 0.2% sodium-*N*-lauroylsarcosinate (ICN Pharmaceuticals Inc., Plainview, NY):0.04 M trisodium EDTA solution, pH 10. Cell lysates retained on filters were incubated for 50 min at room temperature in the lysing solution with or without proteinase K (0.5 mg/ml; MCB Reagents, Norwood, OH), washed with 0.02 M trisodium EDTA, pH 10, and eluted in the dark at room temperature and flow rate of 0.035 ml/min. The eluting solution consisted of 0.02 M EDTA and tetrapropylammonium hydroxide (RSA Corp., Ardsley, NY), pH 12.6, and eluted fractions were collected at 90- or 120-min intervals over 15–16 hr. The radioactive residual on the filter and the radioactivity of individual fractions were measured in a liquid scintillation counter. Data were analyzed and expressed as frequencies of D-SSB or PA-SSB per 10^6 nucleotides [25, 27].

RESULTS

Influence of antioxidant agents on the frequency of X-ray-induced SSB. The ability of antioxidant agents to prevent SSB was first tested using cells irradiated with 1.5 kR of X-ray. Representative DNA elution curves, following exposure of cells to X-ray alone or in combination with antioxidants, are shown in Fig. 1. The curves are biphasic, and approximately 20% of [^{14}C]DNA was eluted as a rapid component

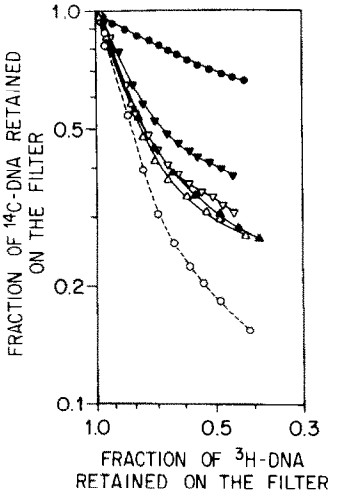

Fig. 1. Kinetics of DNA alkaline elution for cells labeled with [^{14}C]thymidine and irradiated with 1.5 kR of X-ray (0), and for cells irradiated with the same dose and treated with SOD (▲), catalase (△), DMSO (▼), or 2-deoxyglucose (▽). The elution slopes can be compared with the slope of untreated control cells (●). The horizontal scale shows the elution rate of "internal standard" cells, labeled with [^{3}H]thymidine and irradiated with 0.15 kR. More details are in Materials and Methods and in the text.

Table 1. Effects of antioxidant agents on the frequency of SSB induced by X-irradiation

Agent added with the exposure to 1.5 kR	SSB/10^6 nucleotides	Relative DNA damage (%)
None	7.74*	98.0
	8.06	102.0
Superoxide dismutase	4.18	52.9
(200 μg/ml)	6.08	76.9
Catalase	5.05	63.9
(250 μg/ml)	5.36	67.9
DMSO	3.26	41.2
(70 mM)	3.41	43.1
2-Deoxyglucose	4.13	52.3
(10 mM)	4.41	55.8

* Results of two independent experiments are listed. None of the antioxidant agents alone induced significantly different frequencies of SSB from the background values [27].

in the first three fractions collected from cells treated with antioxidants. When the rapidly eluted fractions were ignored, SSB frequencies still remained essentially unchanged as compared with calculations shown in Table 1, which include all eluted fractions. The table lists the four agents that significantly decreased SSB in cells irradiated with 1.5 kR, and this was demonstrated by the assay with proteinase K. The experiments have shown that the protection of macromolecular DNA by antioxidants is demonstrable in our system.

Influence of antioxidant agents on the frequency of D-SSB and PA-SSB induced by ADR. Three concentrations of ADR were tested (Table 2). The lowest dose, 2.8×10^{-7} M (0.162 μg/ml), of the drug, without any antioxidant agent, induced low levels of PA-SSB. A higher dose, 2.8×10^{-6} M (1.62 μg/ml), induced both D-SSB and PA-SSB, while a 2.8×10^{-4} M (162.0 μg/ml) concentration caused high incidence of D-SSB and substantially lower levels of PA-SSB. The results, obtained in thirty-four individual assays, confirmed the data reported earlier [25].

The protection provided by antioxidants against ADR-induced DNA damage was demonstrated by the decrease in frequencies of D-SSB, and this was shown in two different ways. First, the most pronounced decrease of the absolute number of D-SSB was noticed in cells treated with a 2.8×10^{-4} M concentration of the drug *plus* antioxidant. Treatments with this combination decreased the frequency of D-SSB from 1.97 (ADR only) to 0.36–1.07 breaks/10^6 nucleotides. The differences are statistically highly significant. The frequency of PA-SSB induced in cells by any of the three ADR concentrations was not changed significantly by antioxidant agents. Second, protective effects of antioxidants were also shown as changes in the frequency of D-SSB or PA-SSB expressed as the percentage of controls treated with ADR in the absence of antioxidants. Antioxidant enzymes and scavengers prevented 31.8 to 81.7% of D-SSB induced by 2.8×10^{-6} or 2.8×10^{-4} M concentrations of ADR. There were no noticeable differences in the extent of prevention between the two doses. Also, no difference in protection was noted between 1 and 10 mM concentrations of 2-deoxyglucose.

Figure 2 is a composite of representative elution curves obtained in several independent experiments. ADR at the highest concentration, as well as at lower levels (not shown), produced nearly first-order elution kinetics in the presence or absence of antioxidants. This is apparent at least in the region between 1.0 and 0.5 on the ordinate. When proteinase K was omitted from the assay (PK-), there was an overall increase in the elution rate above untreated controls, demonstrating the presence of D-SSB. However, the decrease in DNA elution rates caused by antioxidants with ADR over the rate of elution for ADR alone is evident. When proteinase K was used in the assay (PK+), the overall DNA elution rates increased even further and this indicates that, in addition to D-SSB, the PA-SSB, "unmasked" by the procedure, were present in cells treated with antioxidant agents and/or ADR.

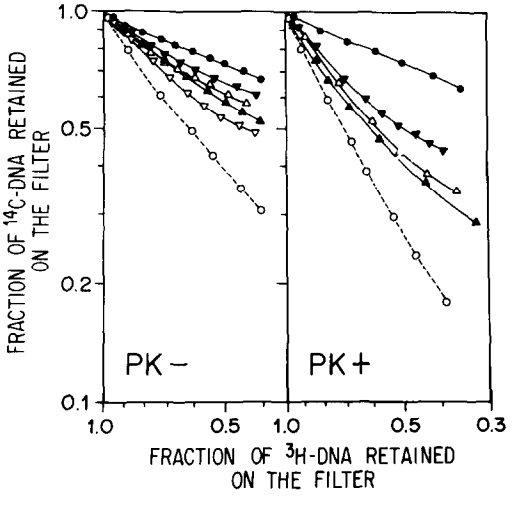

Fig. 2. DNA alkaline elution kinetics obtained for cells treated with a 2.8×10^{-4} M concentration of ADR alone (○) and in combination with SOD (▲), catalase (△), DMSO (▼) or 2-deoxyglucose (▽). Elution of DNA from untreated control cells provided a reference slope (●). Assays were performed without (PK-) or with proteinase K (PK+). For more details see the legend to Fig. 1.

DISCUSSION

An attempt has been made in the work reported to resolve the controversy regarding the origin of DNA SSB in mammalian cells treated with a clinically and experimentally widely used drug, ADR. Two separate mechanisms—one involving endonucleases of nick-closure type and the other the damaging effects of free radicals—have been proposed in the literature. The present work provides evidence that both mechanisms may occur, but that their detection depends on the concentration of the drug used in the treatment. Crucial to this contention seems to be the observation that antioxidant agents

Table 2. Effects of antioxidant agents on the frequencies of D-SSB and PA-SSB induced by various concentrations of ADR

ADR conc. (M)	Agent added with ADR	No. of experiments	SSB/10^6 nucleotides		Relative DNA damage (%)	
			D-SSB*	PA-SSB	D-SSB	PA-SSB
2.8×10^{-7}	None	8	0.03 ±0.08	0.47 ±0.14		100.0 ±29.7
	Superoxide dismutase (200 μg/ml)	2	0.05† 0.08†	0.55 0.55		117.1 117.1
	Catalase (250 μg/ml)	2	−0.08† 0.05†	0.53 0.41		112.2 86.8
	DMSO (70 mM)	2	−0.09† 0.0†	0.65 0.52		138.2 110.2
2.8×10^{-6}	None	14	0.85 ±0.27	1.04 ±0.22	100.0 ±31.5	100.0 ±21.1
	Superoxide dismutase (200 μg/ml)	4	0.43 ±0.02	0.97 ±0.10	50.9 ±2.7	93.3 ±9.8
	Catalase (250 μg/ml)	3	0.48 ±0.04	0.87 ±0.02	56.6 ±4.9	83.6 ±1.6
	DMSO (70 mM)	2	0.27 0.37	1.12 1.09	32.0 43.2	107.7 105.1
	2-Deoxyglucose (1 mM)	2	0.36 0.58	0.84 0.93	42.2 68.2	80.9 89.5
	2-Deoxyglucose (10 mM)	2	0.38 0.44	1.08 1.07	45.2 51.3	103.9 103.0
	Ethanol (70 mM)	2	0.28 0.34	1.13 1.13	32.6 40.3	108.9 109.0
2.8×10^{-4}	None	12	1.97 ±0.37	0.81 ±0.11	100.0 ±18.7	100.0 ±13.3
	Superoxide dismutase (200 μg/ml)	4	0.96 ±0.12	0.84 ±0.11	48.5 ±6.3	104.2 ±13.5
	Catalase (250 μg/ml)	5	0.91 ±0.31	0.72 ±0.10	46.2 ±15.9	88.4 ±12.8
	DMSO (70 mM)	2	0.36 0.46	0.95 1.06	18.3 23.1	116.6 131.0
	2-Deoxyglucose (10 mM)	2	1.07 1.22	0.77 0.98	54.5 61.8	95.2 121.5

* Background values for D-SSB and for PA-SSB ranged from 0.14 to −0.13. Their calculation has been described earlier [27]. Antioxidant agents alone, at the concentrations listed, did not induce D-SSB or PA-SSB that deviated significantly in their frequencies from the background values. The decrease of D-SSB in cells exposed to antioxidant agents with ADR, as compared with D-SSB induced by ADR alone, was highly significant (*t*-test of individual values: $P < 0.001$, d.f. 14–17). The frequency of PA-SSB in cells treated with an antioxidant and ADR did not differ significantly from cells treated with ADR only ($P > 0.1$, d.f. 14–17).

† The frequencies correspond to background values and were not recalculated as SSB/10^6 nucleotides.

partially prevent the formation of D-SSB which are presumably generated by the effects of oxygen-based radicals. The antioxidants used in the experiments included the enzymes SOD and catalase. SOD dismutases O_2^- and catalase removes H_2O_2. Both enzymes protect mammalian cells against ionizing radiation [28–30] and prevent biologically active DNA from radiation-induced degradation [31]. It was also shown that ADR forms H_2O_2 and O_2^-; formation of the latter can be prevented by SOD [32, 33], and the oxygen-dependent DNA degradation caused by ADR is strongly inhibited by SOD and catalase [34].

It is generally accepted that a reactive free radical $OH^\cdot$, an important effector of macromolecular damage implicated in cell injury as measured by DNA SSB or by cell killing [35], is a product generated by the reaction of the O_2^- anion with Fe_3. We have used two scavengers of $OH^\cdot$, ethanol and DMSO, a well-known radioprotector of aerobic mammalian cells [36]. Another compound used was 2-deoxyglucose. This analog of glucose lowers metabolic energy in cells by interference with one of the ATP-generating steps, consequently diminishing ATP cellular content [37, 38]. The agent protects cells against cytotoxic effects exerted by ADR [39], while its effect on survival of irradiated cells remains controversial [40, 41]. 2-Deoxyglucose has also been shown to lower O_2^- levels during respiratory burst in stimulated polymorphonuclear leukocytes, presumably by decreasing NADPH production [33]. Although various free-radical scavengers and, to some extent, the antioxidant enzymes used in this study are known to exert a multitude of effects on

the cell, the data show that their effects on ADR-induced D-SSB are comparable.

The selection of ADR concentrations used in current experiments is based on results of our recent study [25]. The dosages selected induce either PA-SSB (concentration of 2.8×10^{-7} M), or D-SSB and PA-SSB (2.8×10^{-6} M), or high levels of D-SSB (2.8×10^{-4} M) in treated cells. The frequency of SSB correlates well with increasing cytotoxicity of the drugs in the range of 1.4×10^{-7} to 5.1×10^{-6} M, with cell-surviving fractions of 0.31 ± 0.13 and 0.0075 ± 0.00037 at concentrations of 2.8×10^{-7} and 2.8×10^{-6} M respectively.* While protective effects by antioxidants are most apparent at 2.8×10^{-4} M ADR, when they prevented the occurrence of 27–58% of all SSB, this dose results in $\geqslant 5$ log cell kill, and the surviving fraction cannot be measured accurately by a clonogenic assay. It also should be mentioned that DNA elution rates of cells treated with the two higher concentrations are influenced by a complex situation. The speed of elution and the resulting slope depend on the frequency of SSB and DP-CL. While DNA SSB increase the speed of elution, DP-CL cause its retardation [26]. Consequently, the absolute frequency of D-SSB calculated from the slope is very likely an underestimate of their true frequency; this *caveat* should be considered for treatments with ADR, in the presence or absence of antioxidants. A biphasic shape of DNA elution curves was produced by irradiation (Fig. 1) and to a substantially lesser extent by ADR (Fig. 2). The elution curves of cells irradiated with a dose in excess of 1.0 kR have been shown by various investigators to be upwards concave in shape (see, for example, Refs. 18, 27 and 42). This may represent the elution of degraded DNA from dying cells [43], or a non-random distribution of DNA lesions in treated cells. Since all the curves have essentially identical shapes, the presence of proteinase K in the assay notwithstanding, DP-CL do not seem to be a major factor contributing to their concavity. While the results of our experiments indicate that the exposure of L1210 cells to high levels of ADR results in extensive D-SSB formation, most likely through anthracycline-activated O_2-based free radicals, it remains unclear why only a variable part, and not all, of D-SSB was prevented by antioxidant agents. Several factors should be considered, such as limited cell entry by larger molecules (SOD, catalase), catabolic changes of antioxidants during incubation resulting in products with diminished or lost antioxidant properties, or localization of some of DNA lesions in less accessible regions of chromatin. It is also possible that D-SSB do not represent a homogenous group of DNA lesions [25], and that some of them are generated by a mechanism different from free-radical induced damage.

In conclusion, results presented in this article, combined with results of a recent publication [25], seem to indicate that ADR-mediated free radicals induce DNA damage detectable in euoxic cells after their exposure to ADR in the range of 2.8×10^{-6}–2.8×10^{-4} M. Drug concentrations of this magnitude are used only exceptionally in clinical oncology, e.g. at initial levels of 2.2×10^{-6}–2.8×10^{-4} M as a 4-hr dwell in the peritoneal cavity in ovarian cancer patients [44]. High concentrations of ADR, in the range of 2.2×10^{-6} to 5×10^{-4} M, however, have been widely used in *in vitro* studies of drug–DNA interaction, including studies of anthracycline free radicals [15, 45–48]. Following an i.v. bolus delivery of 20–90 mg/M^2 of ADR to a patient, peak plasma levels of the drug are in the range of 5.2×10^{-7}–1.7×10^{-6} M (0.3 to 1.0 μg/ml), and a rapid decrease is noted within 1 hr [49–51]. Actual levels in tumor tissue are likely to be substantially lower, as can be extrapolated from studies of free plasma ADR and adriamycinol available in humans for distribution into tissues [52] and from direct measurements of ADR in animal tumors [53, 54]. At comparable concentrations of ADR used in our experiments, only one type of reaction between the drug and DNA takes place: formation of PA-SSB which is not oxygen-dependent and apparently does not involve free-radical mediation. Future work should clarify the mechanism of their formation as well as involvement in ADR–DNA interactions.

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