Deoxyribose breakdown by the adriamycin semiquinone and H_2O_2: evidence for hydroxyl radical participation

Diana A. Bates and Christine C. Winterbourn

Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

Received 22 June 1982

We report our finding that the reaction between the adriamycin semiquinone (produced by reduction of the drug by xanthine oxidase) and H_2O_2 in N_2 causes deoxyribose degradation to a thiobarbituric acid-reactive chromogen. Deoxyribose breakdown was inhibited by scavengers of hydroxyl radicals, providing evidence for the participation of hydroxyl radicals. The reaction was detected in air, but was less efficient in air than in N_2. Deoxyribose degradation did not require a metal catalyst, and was inhibited by superoxide dismutase in air, but not N_2. A similar reaction with deoxyribose in DNA may be of major importance in the antitumour action of adriamycin.

Thiobarbituric acid *Superoxide dismutase*

1. INTRODUCTION

The antitumour effect of adriamycin (doxorubicin (HCl) is thought to involve intercalation of the drug with the DNA molecule and free radical-induced scission of the DNA strands [1–3]. One possible mechanism for this strand scission involves cleavage at deoxyribose residues. Breakdown of deoxyribose in DNA has been shown to occur with another antitumour agent, bleomycin, when complexed with iron [4,5], and Halliwell and Gutteridge [6] have found that other systems that generate the hydroxyl radical ($OH^{\cdot}$) also degrade deoxyribose to a thiobarbituric acid (TBA)-reactive substance (malondialdehyde). Adriamycin is known to undergo redox cycling to give the semiquinone ($Adr^{-\cdot}$), with subsequent formation of superoxide ($O_2^{-\cdot}$) and possibly $OH^{\cdot}$ [3,7–11]. $OH^{\cdot}$ can be formed from H_2O_2 and $O_2^{-\cdot}$ if an iron catalyst is present [12], but H_2O_2 has also been shown to react directly with $Adr^{-\cdot}$ to produce a species with the properties of $OH^{\cdot}$ which oxidizes methional or α-keto-γ-methiol-butyric acid to ethylene [13].

To further characterise this species, and to determine whether it could be involved in adriamycin-induced DNA strand scission, we have examined whether it is capable of degrading deoxyribose. We report our finding that the reaction between $Adr^{-\cdot}$ and H_2O_2 causes deoxyribose breakdown. The reaction does not require an iron catalyst, and in air is inhibited by superoxide dismutase. This provides additional evidence that the reaction produces $OH^{\cdot}$ and suggests that a similar reaction with deoxyribose in DNA may be of major importance in the antitumour action of adriamycin.

Abbreviations: $Adr^{-\cdot}$, adriamycin semiquinone radical; TBA, thiobarbituric acid; $OH^{\cdot}$, hydroxyl radical; $O_2^{-\cdot}$, superoxide; DTPA, diethylenetriamine penta-acetic acid.

2. MATERIALS AND METHODS

The adriamycin semiquinone ($Adr^{-\cdot}$) was produced by the xanthine oxidase-catalysed reaction of adriamycin with xanthine, in N_2-bubbled phosphate-buffered saline (0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4) as previously reported [14]. Reactions with deoxyribose were carried out for 1.5 h at 22°C, in 10 ml tubes sealed with rubber stoppers, previously bubbled with N_2 for 2 min. The reaction rate was found to be approximately linear over this time period. Unless stated other-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

wise, solutions contained 30 μM adriamycin, 180 μM H_2O_2, 0.38 mM xanthine, 75 μM diethylenetriamine penta-acetic acid (DTPA), 3.2 mM deoxyribose and 8.6×10^{-3} U xanthine oxidase/ml, in a total volume of 1.3 ml. The radical generation rate, determined by measuring the rate of reduction of 25 μM cytochrome *c* (ΔA_{550}, $\varepsilon = 2.1 \times 10^4$) in the presence of catalase (30 μg/ml), was 0.91 μmol . l^{-1} . min^{-1}. Similar reactions were carried out in air, or air/N_2 mixtures prepared by replacing the appropriate volume of N_2 in the tube with air, using a syringe. In the presence of O_2, the xanthine oxidase reaction produced a mixture of $O_2^{-\cdot}$ and $Adr^{-\cdot}$. All glassware was washed with 30% nitric acid and rinsed with deionised distilled water. Xanthine, deoxyribose and buffer solutions were treated with chelex resin to remove contaminating metal ions.

The method of Gutteridge [15] was used for the measurement of TBA reactivity. 1 ml of TBA solution (1% w/v in 0.05 M NaOH) and 1 ml glacial acetic acid were added to each tube, at the end of the reaction. The samples were heated for 30 min at 100°C, cooled, and A_{532} read against a reagent blank. The absorbance due to adriamycin was subtracted.

All biochemicals were obtained from the Sigma Chemical Corp., St. Louis, MO, USA except for adriamycin (Pharmitalia, Barnet, Herts), superoxide dismutase (Diagnostic Reagents Ltd., Thame, Oxon, UK), and Chelex-100 (Bio-Rad Laboratories, St. Albans, Herts, UK).

3. RESULTS

The reaction of xanthine oxidase with adriamycin under N_2, in the presence of H_2O_2 and DTPA, resulted in breakdown of deoxyribose to a TBA-reactive chromogen. The amount of TBA reactivity depended on the concentrations of adriamycin, deoxyribose and xanthine oxidase (fig.1). Essentially no reaction occurred if any one of these three reagents or H_2O_2 was omitted (figs. 1 and 2). The reaction was inhibited by the $OH^{\cdot}$ scavengers mannitol, ethanol, formate and benzoate (table 1). The amount of inhibition by these scavengers was comparable to their inhibition of deoxyribose breakdown caused by Fe^{2+}(ETDA), xanthine and xanthine oxidase in air, a known $OH^{\cdot}$-generating system [16].

Deoxyribose breakdown in the presence of adriamycin was less in air than in N_2, and decreased as the O_2 concentration increased (fig.2). In the absence of adriamycin, with DTPA present, virtually no reaction was detectable. When no H_2O_2 was added, deoxyribose breakdown in-

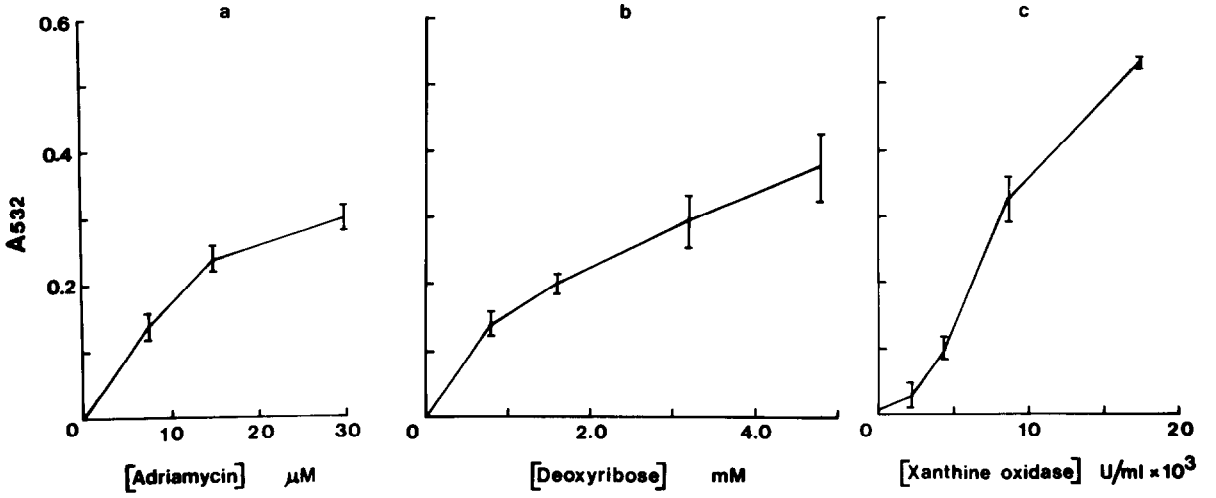

Fig.1. Adriamycin-dependent breakdown of deoxyribose. Dependence on the concentration of (a) adriamycin, (b) deoxyribose and (c) xanthine oxidase. Apart from the concentration of variable reactant, reaction conditions are given in the Methods section. Each point on the graphs represents the mean and standard deviation from 6–10 experiments. Absorbances were read after 1.5 h reaction.

Table 1

The effect of radical scavengers on deoxyribose breakdown to a TBA-reactive chromogen

Inhibitor added		Percent inhibition of deoxyribose breakdown by	
		[a]Adr$^{-\cdot}$ and H_2O_2	[b]$O_2^{-\cdot}$, H_2O_2 and Fe^{2+} (EDTA)
Mannitol (mM)	5	33 ± 10	32 ± 1
	10	30 ± 14	45 ± 6
	20	41 ± 13	48 ± 10
Ethanol (mM)	5	32 ± 12	25 ± 1
	10	40 ± 8	40 ± 6
	20	56 ± 10	48 ± 12
Formate (mM)	5	26 ± 6	24 ± 3
	10	35 ± 11	45 ± 10
	20	53 ± 12	58 ± 12
Benzoate (mM)	5	52 ± 5	40 ± 2
	10	67 ± 8	57 ± 7
	20	80 ± 7	53 ± 7
Superoxide dismutase (μg/ml)	15 (N_2)	12 ± 7	
	15 (10% air)	66 ± 21	
	15 (20% air)	72 ± 11	
	15 (air)	83 ± 12	
Catalase[c] (μg/ml)	17 (10% air)	82 ± 11	

[a] Reactions were carried out as described in the Methods section; in N_2, unless stated otherwise

[b] Reaction mixtures, in air, contained 15 μM $FeSO_4$, 100 μM EDTA, and $O_2^{-\cdot}$ generated using 0.38 mM xanthine and 8.6×10^{-3} U/ml xanthine oxidase

[c] In the absence of added H_2O_2

Means and standard deviations for 6–16 estimations are given

Table 2

The effect of Fe^{2+} and chelators on deoxyribose breakdown to a TBA-reactive chromogen

Additional reagent	TBA-Reactive product ($A_{532\ nm}$)			
	N_2		Air	
	30 μM adriamycin	No adriamycin	30 μM adriamycin	No adriamycin
No chelator	0.361 – 0.985	0.006	0.183 ± 0.030	0.128 ± 0.057
DTPA (75 μM)	0.328 ± 0.042	0.006	0.090 ± 0.048	0.021 ± 0.012
EDTA (100 μM)	0.575 ± 0.113	0.043	0.283 ± 0.028	0.226 ± 0.031
Fe^{2+} (15 μM), EDTA (100 μM)	1.000 ± 0.152	0.273 ± 0.042	1.348 ± 0.095	1.372 ± 0.083
Fe^{2+}, EDTA, No xanthine oxidase	0.191 ± 0.008	0.180 ± 0.006		

Reactions were carried out as described in the Methods section. TBA reactivity is expressed as means and standard deviations from 6–10 estimations

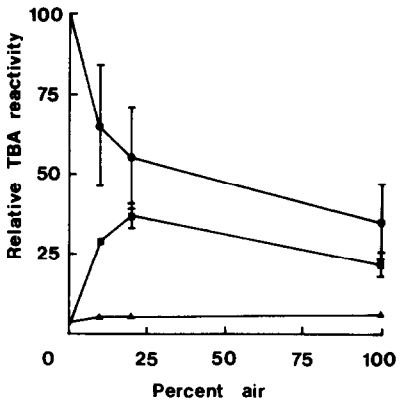

Fig.2. Effect of O_2 concentration on deoxyribose breakdown to TBA-reactive products. (•) adriamycin, H_2O_2, added. (▪) adriamycin, no H_2O_2 added. (▲) No adriamycin, with or without H_2O_2. Reaction conditions are given in the Methods section. Radical generation rates (from xanthine oxidase), in $\mu mol \cdot l^{-1} \cdot min^{-1}$, were: <0.2 (no adriamycin) and 0.91 (30 μM adriamycin) in N_2, and 2.14 (no adriamycin) and 2.43 (30 μM adriamycin) in air. Results are expressed as A_{532} relative to the value obtained with adriamycin and H_2O_2 in N_2, and represent means from 4–12 experiments.

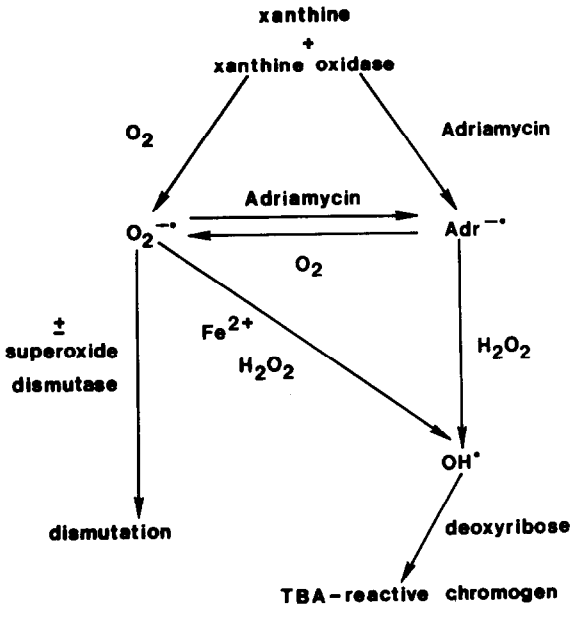

Fig.3. Sequence of reactions leading to deoxyribose breakdown.

creased to a maximum in 10–20% air as the O_2 concentration increased, and then decreased slightly (fig.2). This reaction was also H_2O_2-dependent, and was inhibited by catalase (table 1). Superoxide dismutase inhibited deoxyribose breakdown in air and air/N_2 mixtures (table 1). There was very little inhibition in N_2 and this was probably due to the presence of traces of O_2.

The reactions described above were carried out in the presence of DTPA, which complexes iron so that it cannot catalyse OH• formation from H_2O_2 and $O_2^{-\bullet}$ [17]. When DTPA was replaced by EDTA the amount of deoxyribose breakdown was about 80% higher (table 2). The increase caused by EDTA in the absence of adriamycin was insufficient to account for this difference. In the absence of a chelator deoxyribose breakdown was extremely variable, and was almost undetectable unless the reaction was carried out in tubes that had been washed in nitric acid. This was most likely to be due to the presence of an impurity that inhibits the reaction, but which has no effect in the presence of a chelating agent. The reason why EDTA and DTPA affected the reaction differently is unclear, but no requirement for an iron catalyst was evident. Although addition of 15 μM Fe^{2+} (EDTA) to the reaction mixture under N_2 increased the amount of deoxyribose breakdown, it was less than double that observed with EDTA alone (table 2). Most of this increase was accountable for by the reaction between Fe^{2+} and H_2O_2 (Fenton reaction), with a small additional contribution from xanthine and xanthine oxidase, probably via $O_2^{-\bullet}$ produced from any O_2 still present. In air, Fe^{2+}(EDTA) caused a much greater increase in the rate of deoxyribose breakdown, both in the presence and absence of adriamycin. Hence, under these conditions, OH• production via the iron-catalysed Haber–Weiss reaction predominated over the adriamycin-dependent reaction. EDTA alone also increased deoxyribose breakdown in air, presumably by complexing contaminant iron and catalysing the Haber–Weiss reaction.

4. DISCUSSION

Deoxyribose breakdown to TBA-reactive substances has been shown to occur when $Adr^{-\bullet}$ is generated from adriamycin in the presence H_2O_2.

The reaction was most efficient under N_2, where adriamycin can accept an electron from xanthine oxidase to generate $Adr^{-\bullet}$ [14]. In air or air/N_2 mixtures, where deoxyribose breakdown was less efficient, xanthine oxidase produces predominately $O_2^{-\bullet}$ [13], but because of the equilibrium between $Adr^{-\bullet}$ and $O_2^{-\bullet}$ (fig.3), subsequent reactions could be due to either radical. $OH^\bullet$ has been shown to cause degradation of deoxyribose to the TBA-reactive substance malondialdehyde [6]. Our present findings that the reaction between $Adr^{-\bullet}$ and H_2O_2 degrades deoxyribose, and that this reaction is inhibited by $OH^\bullet$ scavengers strongly support earlier evidence [13] that $OH^\bullet$ is produced. The alternative, that it produces a related species with very similar reactivity (e.g., crypto-$OH^\bullet$, as suggested by Youngman and Elstner [18]), cannot be entirely excluded. But biologically this is of little consequence, since the radical is energetic enough to undergo reactions typical of $OH^\bullet$, and is therefore capable of causing extensive damage to cell constituents.

The proposed mechanism of deoxyribose breakdown in the presence of adriamycin (shown in fig.3) is essentially the same as that proposed for methional oxidation by the same system [13]. The decrease in deoxyribose breakdown with increasing O_2 concentration is due to the fast reaction of $Adr^{-\bullet}$ with O_2 competing with the reaction of $Adr^{-\bullet}$ with H_2O_2. The inhibitory effect of superoxide dismutase is explained by its displacing the equilibrium between $Adr^{-\bullet}$ and $O_2^{-\bullet}$ to the left, and lowering the $Adr^{-\bullet}$ concentration. H_2O_2 was essential for the reaction, but only needed to be added in N_2. In the presence of O_2 the reaction could proceed, albeit more slowly, with H_2O_2 produced either through dismutation of $O_2^{-\bullet}$, or directly from the xanthine oxidase.

An alternative explanation, that $O_2^{-\bullet}$ was required for deoxyribose breakdown in the absence of an iron catalyst, must be excluded because the reaction was much more efficient in N_2 than air, and it did not occur when $O_2^{-\bullet}$ was generated in the absence of adriamycin. In the presence of iron complexes, $OH^\bullet$ can be produced from $O_2^{-\bullet}$ via the Haber–Weiss reaction. Little evidence for this or any other iron-catalysed reaction was seen in N_2, but in air with 15 μM Fe^{2+}(EDTA), the Haber–Weiss reaction predominated over adriamycin-dependent $OH^\bullet$ production. $OH^\bullet$ production by either mechanism could potentially occur in biological situations and both would be inhibited by superoxide dismutase. The Haber–Weiss reaction can take place only if a suitable catalyst is present. Whether metal catalysts are generally available has not yet been established, but if so, it is likely to be favoured at high O_2 concentrations [19,20]. The $Adr^{-\bullet}$-dependent reaction is more likely to be important if little or no metal catalyst is available. It will be more efficient at lower O_2 concentrations, although some O_2 is essential as a source of H_2O_2. These conditions could be met in most tissues.

If deoxyribose breakdown occurs within the DNA molecule, the likely outcome would be scission of the DNA strands. The cleavage of DNA by bleomycin has been shown to involve $OH^\bullet$-induced deoxyribose degradation [4,5]. The adriamycin mechanism must differ from that for bleomycin, which requires co-ordination of both Fe^{2+} and O_2, but the production of $OH^\bullet$ may be common to both mechanisms. The adriamycin-dependent deoxyribose breakdown we have observed requires the semiquinone and H_2O_2, but not a metal catalyst, and is inhibited by superoxide dismutase. If this reaction occurs with deoxyribose in DNA, it could be the cause of the DNA cleavage thought to be involved in the antitumour action of adriamycin.

ACKNOWLEDGEMENTS

The help and interest of Professor R.W. Carrell is gratefully acknowledged. This work was supported by a grant from the Medical Research Council of New Zealand.

REFERENCES

[1] Lown, J.W., Sim, S.K., Majumdar, K.C. and Chang, R.Y. (1977) Biochem. Biophys. Res. Commun. 76, 705–710.

[2] Lown, J.W. and Chen, H.H. (1981) Can J. Chem. 59, 390–395.

[3] Berlin, V. and Haseltine, W.A. (1981) J. Biol. Chem. 256, 4747–4756.

[4] Gutteridge, J.M.C. (1979) FEBS Lett. 105, 278–282.

[5] Giloni, L., Takeshita, M., Johnson, F., Iden, C. and Grollman, A.P. (1981) J. Biol. Chem. 256, 8608–8615.

[6] Halliwell, B. and Gutteridge, J.M.C. (1981) FEBS Lett. 128, 347–352.
[7] Handa, K. and Sato, S. (1975) Gann 66, 43–47.
[8] Thayer, W.S. (1977) Chem. Biol. Interac. 19, 265–278.
[9] Bachur, N.R., Gordon, S.L. and Gee, M.V. (1977) Mol. Pharmacol. 13, 901–910.
[10] Bachur, N.R., Gordon, S.L., Gee, M.W. and Kon, H. (1979) Proc. Natl. Acad. Sci. USA 76, 954–957.
[11] Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 77, 797–803.
[12] McCord, J.M. and Day, E.D. (1978) FEBS Lett. 86, 139–142.
[13] Winterbourn, C.C. (1981) FEBS Lett. 136, 89–94.
[14] Bates, D. and Winterbourn, C.C. (1982) Biochem. J. 203, 155–160.
[15] Gutteridge, J.M.C. (1981) FEBS Lett. 128, 343–346.
[16] Diguiseppi, J. and Fridovich, I. (1980) Arch. Biochem. Biophys. 205, 323–329.
[17] Halliwell, B. (1978) FEBS Lett. 92, 321–326.
[18] Youngman, R.J. and Elstner, E.F. (1981) FEBS Lett. 129, 265–268.
[19] Halliwell, B. (1981) Bull. Europ. Physiopath. Resp. 17 (suppl), 21–28.
[20] Winterbourn, C.C. (1981) Biochem. J. 198, 125–131.