Adriamycin-dependent damage to deoxyribose: a reaction involving iron, hydroxyl and semiquinone free radicals

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Adriamycin under partially anaerobic conditions degrades deoxyribose with the release of thiobarbituric acid-reactive products. This reaction is seen when electrons are transferred to adriamycin by xanthine oxidase or ferredoxin reductase to form the semiquinone free radical. Under the conditions described, damage to deoxyribose was inhibited by hydroxyl radical scavengers, catalase and iron chelators. When the ratio of iron chelator to iron salt is varied both EDTA and diethylenetriamino penta-acetic acid (DETAPAC) show stimulatory properties whereas desferrioxamine remains a potent inhibitor of all reaction.

Deoxyribose degradation *Thiobarbituric acid-reactivity* *Adriamycin semiquinone radical* *Hydroxyl radical* *Superoxide dismutase* *Desferrioxamine*

1. INTRODUCTION

Adriamycin, a member of the anthracycline antitumour antibiotics, is widely used in modern cancer therapy. Its mode of action is believed to relate to its ability to intercalate with cell DNA. In addition, it has been observed to cause strand scissons in closed covalent circular DNA [1,2]. Damage in this system is thought to result from the formation of hydroxyl radicals ($OH_2^{\cdot}$) by an 'iron-catalysed Haber-Weiss reaction' initiated by reduced adriamycin transferring electrons to dioxygen to form superoxide radicals ($O_2^{\cdot -}$) [3].

A novel reaction has been described in which the adriamycin semiquinone free radical ($Ad^{\cdot -}$) is thought to react directly with hydrogen peroxide to form the hydroxyl radical ($OH^{\cdot}$) [4]. Both xanthine oxidase and ferredoxin reductase provide simple systems for directly transferring electrons to a variety of quinone-containing agents such as adriamycin, to form their semiquinone radicals [5,6]. When dioxygen is present, however, superoxide radicals will form the major radical species:

$$Ad^{\cdot -} + O_2 \rightarrow O_2^{\cdot -} + Ad \qquad (1)$$

The sugar moiety of DNA, deoxyribose, is particularly susceptible to $OH^{\cdot}$ radical damage when these are generated in close proximity to it; a reaction which readily occurs when the antitumour antibiotic bleomycin binds to DNA in the presence of dioxygen and a reduced iron salt. This damage can be measured by the release of thiobarbituric acid-reactive (TBA) products [7,8]. In a similar way, deoxyribose has been shown to release TBA-reactive material following incubation with a reduced iron salt [9,10]. This latter technique provides a sensitive method for detecting the occurrence of $OH^{\cdot}$ radical damage [11] and has therefore been applied to our studies with adriamycin.

2. METHODS AND MATERIALS

Superoxide dismutase (bovine erythrocyte, 3000 units/ml), catalase (bovine liver, 27000 units/ml, xanthine oxidase (grade I,28.1 units/ml), albumin (human, fatty acid free), transferrin (human, iron free), ferredoxin-$NADP^+$ reductase (2.4 units/mg), 2-deoxy-D-ribose, diethylenetriaminopenta-acetic acid, NADPH, allopurinol, and hypoxanthine were obtained from Sigma Chemical Co. All

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units of enzyme activity were as defined in the Sigma catalogue. Desferrioxamine (Desferal) was from Ciba-Geigy and adriamycin (doxorubicin hydrochloride) from Farmitalia Carlo Erba. $E^{1\%}_{495\,nm}$ showed the sample to be ~70% pure. All other chemicals used were of the highest grades available and obtained from BDH. All solutions were prepared in Chelex resin-treated distilled water.

2.1. *Degradation of deoxyribose*

(A) *Xanthine oxidase system*: 0.4 ml deoxyribose (5 mM), 0.4 ml aqueous saturated hypoxanthine and 10 μl adriamycin (1 mg/ml) were placed in a new clean glass tube, 9 × 1.5 cm, with a tight-fitting screw cap. Where appropriate, inhibitors were added at the final reaction concentrations indicated in table 1. Tubes for incubation under nitrogen were gassed for 1 min and immediately capped; tubes for incubation in air were left open to the normal laboratory atmosphere; 0.07 units of xanthine oxidase (grade I) were added to each tube and the contents incubated at 37°C for 30 mn. The pH of the mixture was 6.9.

(B) *Ferredoxin reductase system*: 0.4 ml deoxyribose (5 mM), 0.2 ml phosphate–saline buffer (pH 7.4) (0.024 M phosphate, 0.15 M NaCl), 10 μl adriamycin and 0.2 ml NADPH 1.6 mM were treated as above with inhibitors and nitrogen gas. This was followed by the addition of 0.8 ml ferredoxin reductase (1 unit/ml) and incubated at 37°C for 1 h, both in air and under nitrogen.

(C) *Measurement of TBA-reactivity*: After incubation, 0.5 ml, 2.8% (w/v) trichloroacetic acid and 0.5 ml 1% thiobarbituric acid in 0.05 M NaOH was added to each tube before heating at 100°C for 15 min. The resulting pink chromogen was measured at 532 nm against appropriate blanks.

Table 1

Degradation of deoxyribose by adriamycin using the enzyme system xanthine oxidase and hypoxanthine

Inhibitors added to the reaction	Amount of TBA-reactive material formed from deoxyribose in 0.5 h (A_{532})			
	In air		Under nitrogen	
	A_{532}	Inhib. %	A_{532}	Inhib. %
Blank 1 (no XOD)	0.05		0.06	
Blank 2 (no adriamycin)	0.38		0.30	
Control (all reagents)	0.48		1.40	
+ EDTA 0.1 mM	0.31	40	0.38	76
+ DETAPAC 0.1 mM	0.12	84	0.21	89
+ Desferrioxamine 0.1 mM	0.16	74	0.13	95
+ Transferrin 0.1 mg/ml	0.26	51	0.25	86
+ Albumin 0.1 mg/ml	0.49	0	1.26	10
+ Superoxide dismutase (0.05 mg/ml)	0.20	65	1.28	9
+ Catalase 0.1 mg/ml	0.15	77	0.09	98
+ Catalase (boiled)	0.43	12	1.13	20
+ Mannitol 10 mM	0.24	56	0.35	78
+ Thiourea 1 mM	0.30	42	0.78	46
+ Allopurinol	0.10	88	0.32	81

The % inhibition is calculated after subtraction of A_{532} given by the chromogenic properties of adriamycin (i.e., blank no. 1). Concentrations of inhibitors are the final reaction concentrations. Allopurinol was prepared as a saturated aqueous solution. The results shown are a mean of two separate assays which could be reproduced within ± 10%

3. RESULTS

Enzymic reduction of adriamycin, under partially anaerobic conditions, brings about the formation of active radicals capable of degrading deoxyribose with the release of TBA-reactive products. However, when more strict anaerobic conditions were secured, using a Thunberg tube, little degradation was observed (not shown).

The effects of including superoxide dismutase, catalase or hydroxyl radical scavengers, such as mannitol and thiourea, show that the two active oxygen intermediates hydrogen peroxide and the $OH^{\cdot}$ radical (or species with similar reactivity) are essential for the observed degradation of deoxyribose under nitrogen (tables 1,2). Since hydrogen peroxide is not added to the reaction it must be formed by the reduction of the dioxygen remaining after gassing with nitrogen. This reaction would not be inhibited by superoxide dismutase.

Aerobic reduction of adriamycin does not bring about significant deoxyribose degradation within the two systems (tables 1, 2). Some 90% of the aerobic degradation seen with xanthine oxidase cannot be attributed to the presence of adriamycin (table 1). It is this adriamycin independent deoxyribose degradation that is inhibited by superoxide dismutase and which has been characterised in [11]. Both the aerobic and partially anaerobic reactions were inhibited by the specific xanthine oxidase inhibitor allopurinol.

The addition of iron-chelators or the iron-binding protein transferrin, to reactions carried out in air and under nitrogen demonstrated that deoxyribose degradation requires the presence of traces of iron salt (tables 1,2). Saturation of transferrin with iron abolished its inhibitory properties. When iron salts were added to the various reactions, only desferrioxamine inhibited deoxyribose degradation under all the conditions studied (fig. 1). DETAPAC stimulated deoxyribose degradation at low ratios of chelator to iron salt only when adriamycin was present (fig. 2). However, EDTA stimulated degradation, whether adriamycin was present or not only aerobic conditions and over the whole range of chelator to iron salt concentrations tested (fig. 3).

Table 2

Degradation of deoxyribose by adriamycin using the enzyme system ferredoxin reductase and NADPH

Inhibitors added to the reaction	Amount of TBA-reactive material found from deoxyribose in 1 h (A_{532})			
	In air		Under nitrogen	
	A_{532}	Inhib. %	A_{532}	Inhib. %
Blank 1 (no ferredoxin reductase)	0.08		0.08	
Blank 2 (no adriamycin)	0.05		0.10	
Control (all reagents)	0.15		1.77	
+ EDTA 0.1 mM	0.08	100	0.67	65
+ DETAPAC 0.1 mM	0.08	100	0.38	82
+ Desferrioxamine 0.1 mM	0.08	100	0.11	98
+ Transferrin 0.1 mg/ml	0.08	100	0.15	96
+ Albumin 0.1 mg/ml	0.20	0	1.68	5
+ Superoxide dismutase (0.05 mg/ml)	0.15	0	1.69	5
+ Catalase 0.1 mg/ml	0.09	86	0.17	95
+ Catalase (boiled)	0.24	0	1.70	4
+ Mannitol 10 mM	0.10	71	0.32	86
+ Thiourea 1 mM	0.14	14	1.22	33

The % inhibition is calculated after subtraction of the A_{532} given by the chromogenic properties of adriamycin, (i.e., blank no. 1). Concentrations of inhibitors are the final reaction concentrations. The results shown are a mean of two separate assays which could be reproduced within ± 10%

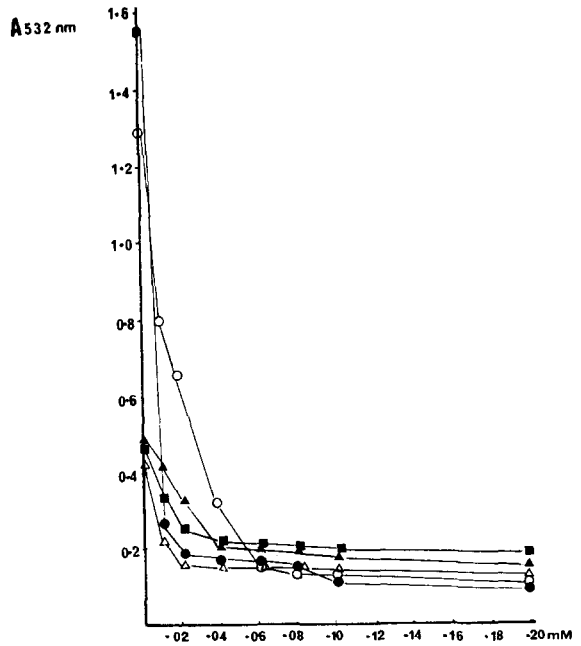

Fig. 1. Effect of different concentrations (shown as the final reaction concentration) of desferrioxamine under the conditions described in section 2.1. using xanthine oxidase and hypoxanthine. Where indicated, iron salts were added as $FeCl_3$ to 0.045 mM final reaction conc.: (△) incubated in air; (▲) Fe(III) added, adriamycin omitted and incubated in air; (■) Fe(III) added and incubated in air; (○) Fe(III) added and incubated under nitrogen; (●) incubated under nitrogen.

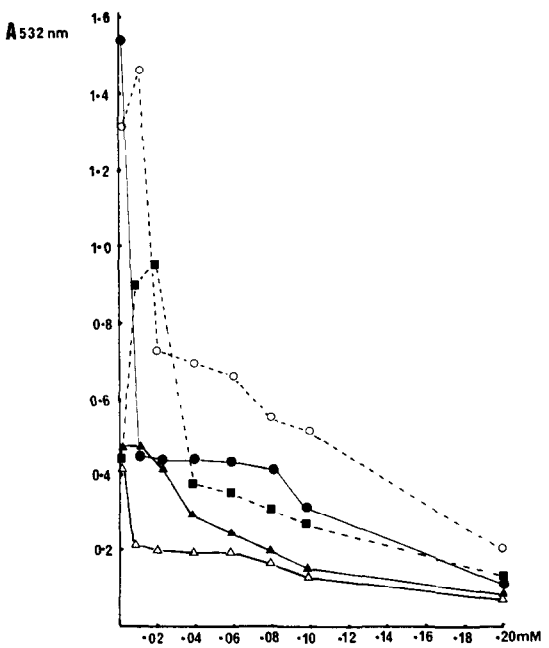

Fig. 2. Effect of different concentrations (final reaction concentrations) of DETAPAC under the conditions in section 2.1. using xanthine oxidase and hypoxanthine. Where indicated, iron salts were added as $FeCl_3$ to a 0.045 mM final reaction conc.: (△) incubated in air; (▲) Fe(III) added, adriamycin omitted and incubated in air; (■) Fe(III) added and incubated in air; (○) Fe(III) added and incubated under nitrogen; (●) incubated under nitrogen.

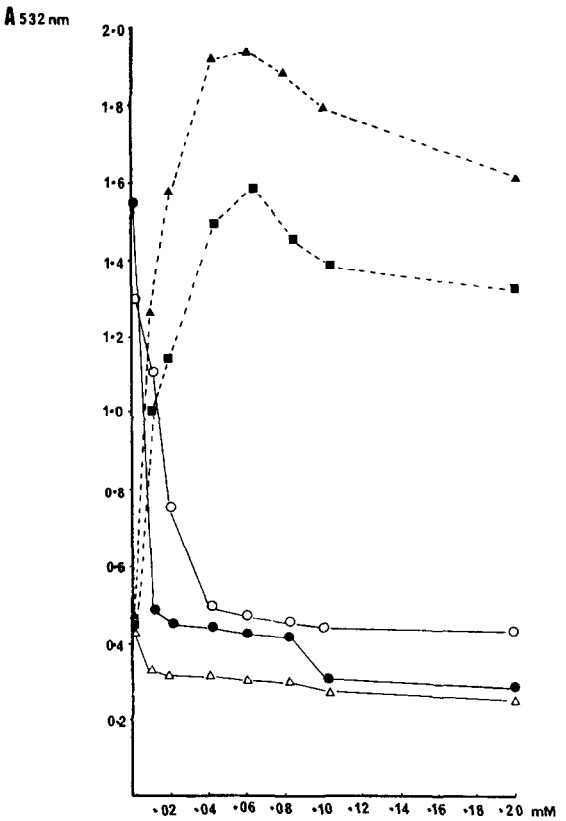

Fig. 3. Effect of different concentrations (final concentrations) of EDTA under the conditions described in section 2.1. using xanthine oxidase and hypoxanthine. Where indicated, iron salts were added as $FeCl_3$ to 0.045 mM final reaction conc.: (△) incubated in air; (▲) Fe(III) added, adriamycin omitted and incubated in air; (■) Fe(III) added and incubated in air; (○) Fe(III) added and incubated under nitrogen; (●) incubated under nitrogen.

4. DISCUSSION

TBA-reactivity following free radical damage to carbohydrates, amino acids, lipids and nucleic acids, has proved to be a sensitive and versatile indirect method for detecting the occurrence of both organic and inorganic oxygen radical reactions [10].

Xanthine oxidase transfers electrons directly to dioxygen to form superoxide radicals, whereas ferredoxin reductans does not. However, under the partially anaerobic conditions described, xanthine oxidase and ferredoxin reductase were both able to pass electrons directly to adriamycin to form the semiquinone radical ($Ad^{\cdot -}$). When diogen is present in abundance, electrons are transferred to the oxygen molecule to form superoxide radicals ($O_2^{\cdot -}$). This latter reaction must occur to a limited extent under the partially anaerobic conditions to account for the formation of the hydrogen peroxide:

$$2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

Superoxide dismutase will not inhibit this reaction, it will merely speed up formation of H_2O_2. The importance of hydrogen peroxide to the reaction is shown by the almost complete inhibition of deoxyribose degradation following addition of catalase, in agreement with studies on methional oxidation [3]. Boiled catalase and albumin served as controls for the specificity of this reaction.

In spite of using Chelex resin-treated distilled water for preparation of all reagents, iron salts were still present at significant levels to influence the reactions. Addition of iron salts to the reaction changed the behaviour of the iron chelators EDTA and DETAPAC but not that of desferrioxamine. DETAPAC stimulated deoxyribose damage in air and under nitrogen only when adriamycin was present with a low ratio of chelator to iron salt. However, EDTA stimulated over all ratios of iron salt to chelator only in the presence of air. Similar anomalies for these iron chelators have been observed during iron-catalysed lipid peroxidation [12].

The enhanced degradation seen under partially anaerobic conditions cannot be ascribed simply to an 'iron-catalysed Haber–Weiss reaction' since this should be more effective under aerobic conditions. Since adriamycin itself readily complexes iron [13, 14] $OH^{\cdot}$ formation may involve an interaction between hydrogen peroxide and a semiquinone–iron complex. The reaction described is clearly complex, and several anomalies have yet to be explained. For example, under our reaction conditions only hypoxanthine, not xanthine, as substrate led to significant deoxyribose degradation.

The observation that adriamycin in the presence of traces of iron salt (readily available in most biological systems) can be more damaging under reduced oxygen tensions may be particularly important in understanding how certain hypoxic tumours might be better treated with such drugs.

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REFERENCES

[1] Cone, R., Hasan, S.K., Lown, J.W. and Morgan, A.R. (1976) Can. J. Biochem. 54, 219–223.
[2] Lown, J.W., Sim, S.K., Majumdar, K.C. and Chang, R.Y. (1977) Biochem. Biophys. Res. Commun. 76, 705–710.
[3] Bachur, N.R., Gordon, S.L. and Gee, M.V. (1978) Cancer Res. 38, 1745–1750.
[4] Winterbourn, C.C. (1981) FEBS Lett. 136, 89–94.
[5] Bates, D.A. and Winterbourn, C.C. (1982) Biochem. J. 203, 155–160.
[6] Gregory, F.M. and Fridovich, I. (1973) J. Bacteriol. 114, 1193–1197.
[7] Gutteridge, J.M.C. (1979) FEBS Lett. 105, 278–282.
[8] Burger, R.M., Peisach, J. and Horwitz, S.B. (1981) Life Sci. 28, 715–727.
[9] Gutteridge, J.M.C. (1981) FEBS Lett. 128, 343–346.
[10] Gutteridge, J.M.C. (1982) Int. J. Biochem. 14, 649–654.
[11] Halliwell, B. and Gutteridge, J.M.C. (1981) FEBS Lett. 128, 347–352.
[12] Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1979) Biochem. J. 469–472.
[13] Gosálvez, M., Blanco, M.F., Vivero, C. and Vallés, F. (1978) Eur. J. Cancer 14, 1185–1190.
[14] Myers, C.F., Gianni, L., Simone, C.B., Klecker, R. and Greene, R. (1982) Biochemistry 21, 1707–1713.