Free Rad. Res. Comms., Vol. 10, No. 3, pp. 159–165
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BLEOMYCIN-IRON DAMAGE TO DNA WITH FORMATION OF 8-HYDROXYDEOXYGUANOSINE AND BASE PROPENALS. INDICATIONS THAT XANTHINE OXIDASE GENERATES SUPEROXIDE FROM DNA DEGRADATION PRODUCTS

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(Received February 12, 1990)

Bleomycin, in the presence of ferric salts, oxygen and a suitable reductant, degrades DNA with the release of base propenals, detected as thiobarbituric acid (TBA) reactivity, and the formation of 8-hydroxydeoxyguanosine (8OHdG) detected by HPLC. When xanthine oxidase is added to the incubated mixture of DNA degradation products, TBA-reactivity is destroyed but 8OHdG formation is increased. EPR Spin trapping experiments show that hydroxyl radicals ($^{\cdot}OH$) are formed in the reaction mixture and can be inhibited by the inclusion of either superoxide dismutase or catalase. These findings suggest that the base propenals and possibly malondialdehyde, formed from them, are aldehydic substrates for xanthine oxidase and, the product of this reaction is superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Thus, TBA reactivity is destroyed in the formation of O_2^- and H_2O_2 which stimulate further oxidative damage to DNA resulting in increased 8OHdG formation.

KEY WORDS: Bleomycin-iron, DNA damage, 8-hydroxydeoxyguanosine, hydroxyl radicals, spin trapping, xanthine oxidase, superoxide dismutase.

INTRODUCTION

The bleomycins are glycopeptide antitumour antibiotics used in the treatment of several human malignancies. Bleomycin sulphate preparations are a mixture of bleomycins consisting mainly of bleomycin A_2 and bleomycin B_2. The bleomycin molecule has two important features that determine its ability to degrade DNA. The bithiazole and terminal amine residues effect binding to DNA, whereas the β-aminoalanine-purimidine-β-hydroxyhistidine portion is responsible for complexing metals (for a review see Gutteridge and Halliwell 1987).[1] Both iron and oxygen play a key role in the ability of the bleomycin to degrade DNA[2] and when iron is complexed to bleomycin it can bring about the specific cleavage of DNA at guanine residues.[3] The reactive species responsible for DNA degradation is, however, still a subject of some debate. Although hydroxyl radicals ($^{\cdot}OH$) have been spin-trapped in bleomycin-iron reactions,[4] DNA degradation, as might be expected from a site-specific reaction is poorly if at all influenced by scavengers of the $^{\cdot}OH$ radical.[2,5,6] The

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penultimate species detected before DNA damage occurs has been identified as a ferric peroxide of bleomycin ($BLM\text{-}Fe^{3+}\text{-}OOH^{-}$).

When DNA is damaged by the bleomycin-iron complex base propenals are released[6] which decompose under the conditions of the thiobarbituric acid reaction to release malondialdehyde[7] that reacts to give a characteristic pink chromogen. Recent studies have shown that hydroxyl radical damage to the DNA molecule results in the formation of a hydroxylated base, 8-hydroxydeoxyguanosine (8OHdG)[8] and that this product can be measured with high sensitivity using an electrochemical detector after separation by high performance liquid chromatography.[9]

In the present study we examine the reaction of bleomycin, with guanine residues, and the possible site specific formation of $^{\cdot}OH$ radicals.

Xanthine oxidase has previously been shown to destroy TBA-reactive material derived from base propenals[12] and we here compare the effect of xanthine oxidase on TBA-reactivity and 8OHdG formation.

MATERIALS AND METHODS

Bleomycin sulphate, calf thymus DNA (type I), superoxide dismutase (bovine erythrocyte), xanthine oxidase (Grade I units as described in the Sigma catalogue), DNAase I, alkaline phosphatase (calf intestine) and endonuclease were from Calbiochem and phosphodiesterase (snake venom) from Boehringer Mannheim. All other chemicals were of the highest purity available from Aldrich Chemicals, Milwaukee.

1. Bleomycin-iron dependent degradation of DNA

The following reagents were added to new clean plastic tubes and the contents incubated at 37°C for 1 hr: 0.1 ml DNA 1 mg/ml, 0.1 ml of $MgCl_2$ 50 mM, 20 μl of bleomycin 1 unit/ml, 1.7 ml of phosphate-saline buffer pH 7.4 (0.05 M Na phosphate in 0.075 M NaCl). The reaction was started by adding 50 μl of ascorbate 7.5 mM. Where appropriate 10 μl of xanthine oxidase (0.25 units) was added to the reaction mixture before addition of ascorbate. The DNA and $MgCl_2$ solutions were dialysed against conalbuin[10] then treated with Chelex resin. The phosphate buffer was not treated in any way and the adventitious iron in this buffer was the main source of iron present in the reaction.

2. Thiobarbituric acid reactivity

After incubation at 37°C for 1 hr, 0.2 ml of thiobarbituric acid (1% v/v in 0.05 M NaOH) and 0.2 ml of 25% v/v HCl were added and the tube contents heated for 10 mins at 100°C. The resulting pink chromogen was measured at 532 nm against appropriate blanks and controls.

3. 8-Hydroxydeoxyguanosine measurements

These were carried out as previously described in detail.[11] In brief, after reaction of bleomycin with DNA the DNA was precipitated from solution with NaCl and ethanol and the recovered pellets dissolved in 5 mM Bistris/0.1 mM EDTA buffer pH

7.1 and digested with DNAase I phosphodiesterase, alkaline phosphatase and endonuclease. The amount of 8OHdG in the DNA digest was measured using HPLC with electrochemical detection. The amount of deoxyguanosine (dG) in the DNA preparation was determined by HPLC with optical detection in line, but before, the eletrochemical detector.

4. Electron paramagnetic resonance measurements

Measurements were carried out in the capillary segment of a sealed pipette using an IBM-Bruker ER 300 electron spin resonance spectrometer at room temperature (25°C). Instrument settings were: modulation frequency 100 KHz, modulation amplitude 0.975 G. Time constants 81.92 ms, sweep width 100 G, microwave frequency 9.75 GHz, power 1.98 mw, gains are given in respective figures. DMPO (final reaction concentration 140 mM) was dissolved in Chelex-resin treated distilled water, with a pH value of 7.4 and added to the reaction mixtures described for bleomycin-iron dependent degradation of DNA. Samples were scanned in the EPR spectrometer after further incubating for 15 minutes at 37°.

RESULTS

As expected and in agreement with previous studies a bleomycin-iron complex in the presence of ascorbate and molecular oxygen released thiobarbituric acid-reactive (TBA) material from linear duplex DNA[2,3,7] characteristic of base propenals. Little or no TBA-reactive material, was formed from DNA in the absence of bleomycin.[12] 8-Hydroxydeoxyguanosine (8OHdG) was formed from DNA under similar conditions with ascorbate and its formation was greatly enhanced by the inclusion of bleomycin (Table I). DNA degradation both in the presence and absence of bleomycin was dependent on the presence of iron salts since, the metal chelator desferrioxamine inhibited damage (Table I). By carrying out the reaction in the presence of phosphate, DNA degradation by bleomycin is greatly increased.[13] Incorporation of Tris at a pH value of 7.4 into the phosphate buffer inhibited formation of base

TABLE I
Formation of base propenals and 8-hydroxydeoxyguanosine from DNA by bleomycin-iron and the effect of xanthine oxidase and inhibitors

		8OHdG: 10^5 dG	Base propenals as TBA-reactivity (A532 nm)
1.	DNA + BLM	0	0.002
2.	DNA + BLM + Ascorbate	140	0.758
3.	DNA + Ascorbate	11.0	0.002
	Reaction 2 + XOD	270.5	0.084
	Reaction 2 + SOD + XOD	157.8	0.082
	Reaction 2 + TRIS 48 mM	81.3	0.674
	Reaction 2 + TRIS 145 mM	7.9	0.520
	Reaction 2 + TRIS 290 mM	8.7	0.295
	Reaction 2 + TRIS 435 mM	4.0	0.180
	Reaction 2 + DEFOM	25.6	0.002

The reactions contained at final concentrations: DNA 0.05 mg/ml, bleomycin (BLM) 0.01 units, ascorbate 0.18 mM, Superoxide dismutase (SOD) 0.024 mg/ml, xanthine oxidase (XOD) 0.123 units. The results shown are the mean of 3 or more separate experiments. DEFOM = Desferrioxamine.

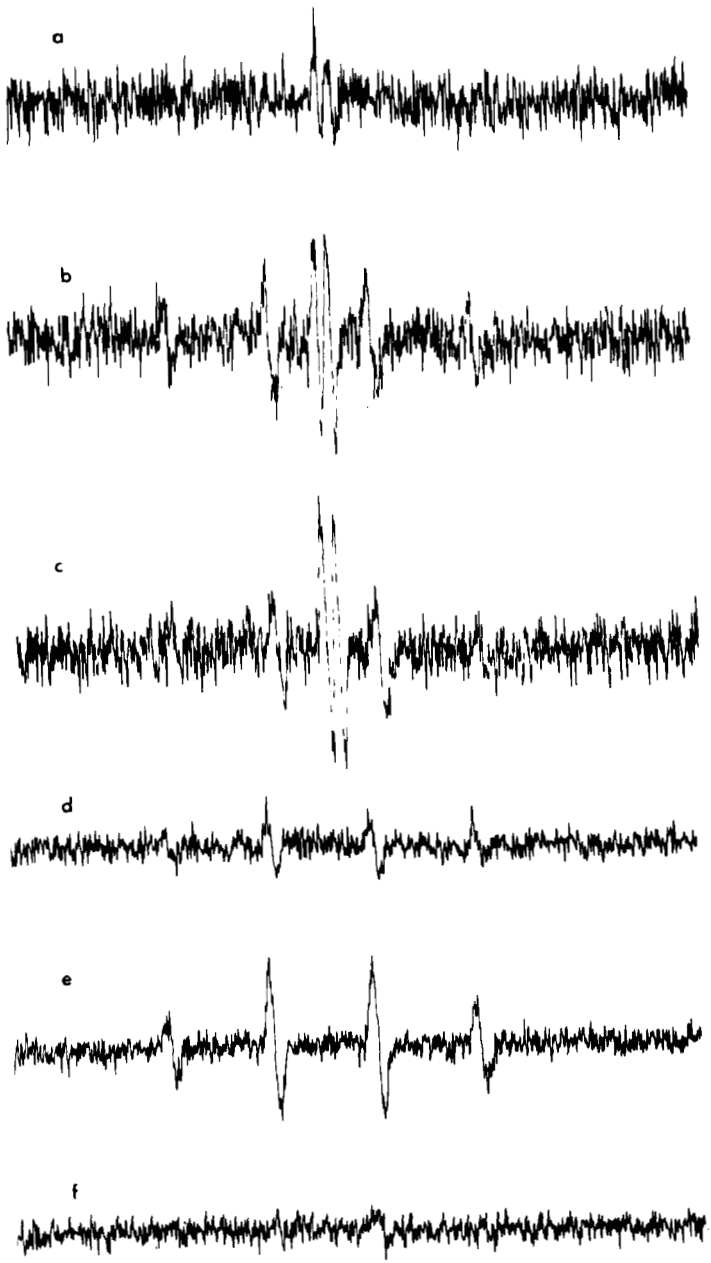

FIGURE 1 [1] DNA 0.35 mg/ml, bleomycin 0.07 units ml, magnesium chloride 8.8 mM, phosphate 8.8 mM, pH 7.4 and ascorbate 0.65 mM or [2] H_2O_2 0.8 mM (where appropriate) were incubated at 37°C for 2 hrs. To each reaction tube was added DMPO at a final concentration of 140 mM. The following reagents were added for further incubation at 37°C for 15 mins: (a) Control reaction mixture [1] with ascorbate as described above (no additions). (b) Control reaction [1] + xanthine oxidase (0.25 units). (c) Control reaction [1] + superoxide dismutase (0.087 mg/ml) + xanthine oxidase (0.25 units). (d) Control reaction [2] with H_2O_2 as described above (no additions). (e) Control reaction [2] + xanthine oxidase (0.25 units). (f) Control reaction [2] + superoxide dismutase (0.087 mg/ml) + xanthine oxidase (0.25 units). Scans a–f were with a receiver gain of 1.00×10^6, a–c were on scale 17, d–f scale 18 which is half the amplitude of 17.

propenals and 8OHdG from DNA (Table I). Mannitol at the same molar concentrations as Tris did not inhibit DNA damage and at high concentrations appeared to stimulate damage. In contrast formate was poorly inhibitory (data not shown).

When samples of DNA were degraded by bleomycin-iron-ascorbate mixtures and, the products incubated with xanthine oxidase, a complete loss of TBA-reactivity was seen. This occurred whether xanthine oxidase was added before the start of the bleomycin reaction or whether it was added after completion of the reaction, followed by a short 15 minute incubation period (Table I). When the same samples were examined for their 8OHdG content however an increase in 8OHdG content was usually observed after incubation with xanthine oxidase (Table I). The xanthine oxidase-dependent increase in 8OHdG formation was however, decreased by adding superoxide dismutase to the reaction mixture (Table I). This latter finding was further examined using epr spectroscopy and the spin trap DMPO. Incubation of DNA with bleomycin-iron and ascorbate produced a weak ascorbate radical signal (Figure 1a). Addition of xanthine oxidase appeared to increase the ascorbate radical signal and induce the presence of a weak DMPO trapped $^{\cdot}OH$ radical signal (Figure 1b). A weak $^{\cdot}OH$ signal is consistent with the known ability of ascorbate to destroy a DMPO-OH adduct.[17] Superoxide dismutase when added however, reduced the $^{\cdot}OH$ signal even further but the ascorbate radical remained and, possibly increased (Figure 1c). To avoid the complications of ascorbate radicals, and the destruction of the DMPO-OH signal the bleomycin reaction was driven by adding hydrogen peroxide in place of ascorbate. In Figure 1d it can be seen that DNA degradation by bleomycin-iron-H_2O_2 is accompanied by a small $^{\cdot}OH$ signal but, the signal is increased by xanthine oxidase (Figure 1e). Superoxide dismutase under these conditions removed the $^{\cdot}OH$ signal (Figure 1f). In both the ascorbate and hydrogen peroxide driven reactions catalase substantially inhibited formation of the DMPO-OH adduct (data not shown).

DISCUSSION

DNA is cleaved by a bleomycin-iron complex in the presence of molecular oxygen with the release of free bases and sugar fragments known to be base propenals with the general structure: Base-CH=CH-CHO.[3] The tight binding of the bleomycin iron complex to DNA ensures that a site-specific reaction takes place on the DNA molecule and, this is the most likely explanation of why most antioxidants are poorly effective in protecting the DNA molecule from such damage. Iron salts, in the absence of bleomycin, damage DNA but do not release substantial amounts of TBA-reactive material.[12] One of the products formed from DNA by simple iron salts that can be detected is the hydroxylated base 8-hydroxydeoxyguanosine (8OHdG).[8,11] Here we confirm that bleomycin greatly potentiates the iron-dependent formation of 8OHdG from DNA,[18] and, this may relate to its specific recognition of guanine residues in the DNA molecule.[3]

In previous studies using entirely different reaction conditions and in the absence of phosphate we have shown that some scavengers of the hydroxyl radical can weakly enhance DNA damage. This has been interpreted as the scavenger protecting the bleomycin molecule from self-destruction rather than protecting the DNA molecule.[5] Here, using a bleomycin reaction in which ascorbate reduces iron in the presence of phosphate we find that Tris is protective whereas mannitol stimulates damage and formate is weakly protective.

Xanthine oxidase has previously been shown to remove TBA-reactive material from the bleomycin reaction.[12] Here, we confirm that xanthine oxidase destroys the TBA-reactive product(s) when the bleomycin reaction is driven by either ascorbate or hydrogen peroxide (data not shown). Loss of TBA-reactivity is seen whether xanthine oxidase is included at the beginning of the incubation or after DNA damage has occurred when no further TBA-reactive material is being formed. In addition to substrates such as xanthine and hypoxanthine, xanthine oxidase is known to utilize aldehydes such as acetaldehyde.[14] Attempts to distinguish whether the base propenals or malondialdehyde are substrates for xanthine oxidase were unsuccessful. Acid or heat treatment to destroy base propenals formed in the reaction, followed by incubation with xanthine oxidase still resulted in a substantial loss of TBA-reactivity. This may suggest that propenals and malondialdehyde are substrates for xanthine oxidase although, in a previous study a sodium salt preparation of malondialdehyde did not appear to be a substrate for xanthine oxidase.[12] Xanthine oxidase has recently been ascribed an enzymic role as a ferroxidase[15] and this could conceivably explain its inhibitory role. However, evidence against a ferroxidase role for xanthine oxidase was the finding that it inhibited the peroxide driven reaction. It is known that hydrogen peroxide does not reduce ferric ions bound to bleomycin.[16]

Although xanthine oxidase caused the loss of TBA-reactivity in incubated mixtures it stimulated formation of 8OHdG and, this formation was inhibited by superoxide dismutase. Electron paramagnetic resonance spectra, using the spin trap DMPO, strongly suggested that hydroxyl radicals were formed in reaction mixture when DNA damage was initated by bleomycin and ascorbate or hydrogen peroxide. These signals did not arise from the decomposition of a DMPO-superoxide adduct (DMPO–OOH) since catalase was inhibitory in both reactions. With ascorbate and hydrogen peroxide the $^{\cdot}OH$ signal could be decreased by the addition of superoxide dismutase and, in the latter case completely removed suggesting superoxide radicals were formed when xanthine oxidase was added to the reaction mixture. Scavenger studies and spin trapping experiments, however, do not give a clear picture of what might be happening on the DNA molecule since the reactions described take place in a mixture of DNA degradation products. Scavengers do not influence $^{\cdot}OH$-dependent site-specific damage. Alternatively it could be that the species responsible for primary damage to DNA is not the $^{\cdot}OH$ radical. Once DNA damage has occurred however, and aldehydes are released as substrates for xanthine oxidase, it is likely that superoxide-dependent Fenton chemistry takes place in the complex mixture of fragments to produce $^{\cdot}OH$ radicals that can be trapped with DMPO.

The most likely explanation for our results is that aldehydes such as the base propenals, and possibly MDA, are substrates for xanthine oxidase and a product of this reaction is superoxide (O_2^-) which leads to hydroxyl radical formation. Oxidation of base propenals destroys the TBA-reactive material but produces O_2^- which stimulates 8OHdG formation by enhancing the reduction of ferric ions bound to bleomycin on the DNA molecule close to guanine residues.

Acknowledgements

JMCG was a 1988 Greenberg Scholar. We thank Lindsay Maidt and Donald Gibson for their valuable analytical help.

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Accepted by Prof. B. Halliwell