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BLEOMYCIN-INDUCED UNSCHEDULED DNA SYNTHESIS IN NON-PERMEABILIZED HUMAN AND RAT HEPATOCYTES IS NOT PARALLELED BY 8-OXO-7,8-DIHYDRODEOXYGUANOSINE FORMATION

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Abstract—The genetic toxicity of the antitumour antibiotic bleomycin (BLM) is thought to involve the formation of a reactive oxygen intermediate. 8-Oxo-7,8-dihydrodeoxyguanosine (oxo^{8}dG), an oxidation product of deoxyguanosine, is one of the major products formed when isolated DNA is exposed to oxygen radical generating systems. Gamma-irradiation (10–500 Gy ^{60}Co; 10 Gy/min) or BLM and Fe^{2+} (37.5–150 U/L and 0.5 mM, respectively) treatment of isolated DNA (0.25 mg/mL) increased oxo^{8}dG above background. In the latter case, the effect was greater than that with Fe^{2+} (0.5 mM) alone and was dependent on the dose of BLM. When DNA was irradiated with 500 Gy ^{60}Co, deoxyguanosine oxidation was inhibited by antioxidants (ethanol: 37.5 and 98% inhibition at 2 and 20 mM, respectively; mannitol: 20.5, 60 and 92% inhibition at 0.1, 1.0 and 10 mM, respectively). Similarly the BLM-induced production of oxo^{8}dG was inhibited (64%) by mannitol (10 mM). BLM also caused production of base propenals on interaction with isolated DNA. In contrast, oxo^{8}dG was not induced above background concentration (27 mol oxo^{8}dG/10^6 mol dG) in permeabilized (37°) and non-permeabilized (4° and 37°) rat hepatocytes treated with BLM (260 U/L). Despite this, there was extensive BLM-induced unscheduled DNA synthesis (10 and 100 U/L) in non-permeabilized rat and human hepatocytes in the absence of hydroxyurea. These findings, in accord with other observations, draw into question the role of ·OH in BLM-induced DNA damage and the mimicry of ionizing radiation in cellular systems.

The glycopeptide antibiotic bleomycin (BLM§) possesses antitumor activity which is apparently due to its ability to degrade the DNA double helix [1]. BLM binds a variety of transition metal ions including Fe(II). The BLM–Fe(II) complex forms a redox active species with O_2, which degrades DNA [1–3]. The complex is activated through a one-electron reduction to form a high-valency iron-oxo species or, possibly, the hydroxyl radical (·OH) [4]. In the absence of reducing agents the electron is obtained from the disproportionation of two ferrous complexes producing an "activated BLM" and an inactive BLM–Fe(II) complex [5]. The reaction of the activated BLM complex with the deoxyribose portion of DNA [1], produces DNA base release [2], DNA strand breaks (single and double) and the inactive BLM–Fe(III) complex. The increase in efficiency of DNA strand breaks due to the addition of reducing agents is probably due to the ability to reduce ferric iron back to the ferrous state [6, 7]. Thus, BLM acts catalytically requiring two electrons and one molecule of dioxygen in each turnover.

Investigations of nucleotide sequence specificity have indicated that, unlike free Fe(II), BLM–Fe(II) shows a site-specific cleavage of nucleotides linked to the 3′ position of deoxyguanosine and favours adjacent dT and dC residues [8, 9]. Multiple single strand breaks in both strands of the DNA duplex give rise to double strand breaks [10].

BLM is said to be "radiomimetic" in that it produces biological effects similar to those of ionizing radiation [11]. Exposure of calf thymus DNA to ionizing radiation or other oxygen radical-generating systems results in the formation of several types of DNA lesion, including base adduct formation [12]. One of these adducts is 8-oxo-7,8-dihydrodeoxyguanosine (oxo^{8}dG) which is produced by reactive oxygen addition at the C-8 position of deoxyguanosine residues in DNA [13]. Oxo^{8}dG is a mutagenic lesion [14, 15] which can be sensitively measured by high performance liquid chromatography with electrochemical detection (HPLC-EC) [16]. DNA repair mechanisms exist for this lesion and these have been characterized in both bacteria [17] and mammalian cells [18, 19].

Increased levels of oxo^{8}dG were shown in the DNA of cells exposed to relatively high dose levels of ionizing radiation or hydrogen peroxide [20]. The BLM–Fe(II) complex has been shown to catalyse the *in vitro* oxidation of deoxyguanosine to oxo^{8}dG in isolated DNA [21–23]. However, the relative importance of this reaction in intact cells has not

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§ Abbreviations: BLM, bleomycin; oxo^{8}dG, 8-oxo-7,8-dihydrodeoxyguanosine; UDS, unscheduled DNA synthesis; HPLC-EC, high performance liquid chromatography with electrochemical detection; TBA, thiobarbituric acid, dG, deoxyguanosine; UHQ, "ultra high quality".

been defined and the nature and extent of DNA damage and repair in the non-permeabilized cell is uncertain.

We have compared DNA oxidation by BLM in isolated DNA and intact cells. In particular, we have investigated the extent of formation of oxo^{8}dG in intact cells compared to the induction of unscheduled DNA synthesis (UDS), a measure of DNA repair, in non-permeabilized hepatocytes to determine if the reactive oxygen species which induce the type of DNA damage leading to UDS are also responsible for the induction of oxo^{8}dG.

MATERIALS AND METHODS

Chemicals. The following reagents were obtained from Sigma Chemical Co. (Poole, U.K.): deoxyribonuclease I from bovine pancreas, *Neurospora crassa* endonuclease, phosphodiesterase I from *Crotalus atrox, Escherichia coli* alkaline phosphatase, calf thymus DNA and ribonuclease A. "Ultra high quality" (UHQ) water was obtained from a purification filtering unit purchased from Elga Ltd (High Wycombe, U.K.). The water was passed through a mixed bed ion exchanger, then through activated charcoal and this stage was followed by reverse osmosis membrane filtration.

Bleomycin sulphate was a kind gift from Lundbeck Ltd (Luton, U.K.) and was supplied as an ampule containing 15 U. The contents of each ampule were dissolved in distilled, deionized water and were standardized optically (based on the $\varepsilon_{292} = 1.45 \times 10^4\ M^{-1}cm^{-1}$ and $M_r = 1550$) to give a potency of 1.7 U/mg solid. All other chemicals were from BDH Ltd (Poole, U.K.).

The standard, oxo^{8}dG, was obtained both as a kind gift from Dr S. Nishimura (Biology Division, National Cancer Centre Research Institute, Tokyo 104, Japan) and by synthesis from deoxyguanosine (dG) using the Udenfriend system as described by Kasai and Nishimura [13]. The two preparations had identical UV spectra, retention times on HPLC and molecular ions by fast atom bombardment mass spectrometry (Dr P. Farmer, MRC Toxicology Unit, Carshalton, U.K.).

Animals. Adult male Wistar rats (approx. 250 *g*) were used throughout and were provided with 422-modified diet (Heygates and Sons Ltd, Northampton, U.K.) and water *ad lib.*

Preparation of hepatocytes. Rat hepatocytes were isolated by a collagenase perfusion method [24] with modifications [25]. Human liver samples (male, approximately 10 *g*, obtained from organ donors at Queen Elizabeth Hospital, Birmingham) were used to isolate hepatocytes by a similar perfusion protocol following the cannulation of blood vessels exposed on a single cut surface of the liver specimen [26], as described previously [27]. Yields of rat and human hepatocytes were determined using a Neubauer haemocytometer and their viabilities were assessed by the Trypan blue exclusion test [28]. Cell viabilities were 85–90% for rat liver and 60–80% for human samples. Cell yields were approximately 5×10^8 and 2×10^7 from entire rat liver and 10 g human liver, respectively.

Treatment of isolated DNA and rat hepatocytes. Calf thymus DNA (type I; 0.25 mg/mL in air saturated UHQ water) was treated in glass tubes at room temperature with 10, 100 and 500 Gy from a ^{60}Co source at a dose rate of 10 Gy/min in the presence and absence of the antioxidants ethanol (2 and 20 mM) and mannitol (0.1, 1 and 10 mM). Antioxidants were used with 500 Gy ^{60}Co. Under these conditions, superoxide radicals are produced in addition to hydroxyl radicals. Calf thymus DNA (0.24 mg/mL) was treated with BLM (0–600 U/L) in KH_2PO_4/K_2HPO_4 buffer (pH 7.0; 20 mM) and the reaction was started by addition of ferrous ammonium sulphate (0.5 mM), which was freshly prepared in N_2-purged distilled deionized water kept on ice. Reaction mixtures were in a final volume of 1.0 mL at the final concentrations stated. After incubation at 37° for 2 hr in a shaking water bath, 0.1 mL EDTA (0.1 mM) was added.

Freshly isolated rat hepatocytes (1×10^7 cells) suspended in 2 mL Hank's balanced salt solution (HBSS), were treated with a range of concentrations of BLM (0–260 U/L) for 1 hr at either 37° or on ice (4°). At the end of the incubation, the cells were centrifuged (500 *g*, 1 min) and the pellet was resuspended in 2 mL phosphate-buffered saline. In a separate experiment, the above procedure was repeated in permeabilized hepatocytes [29].

Isolation of rat hepatocyte DNA. Following exposure to BLM, hepatocytes were resuspended in 0.1 mL buffer (10 mM Tris–HCl containing 1 mM EDTA, pH 8.0) and 1 mL proteinase K (100 μg/mL in 5 mM EDTA and 0.5% sarkosyl, pH 8.0) and incubated at 50° for 2 hr. DNA was isolated by phenol extraction and enzymatically digested to the nucleoside level with deoxyribonuclease I, endonuclease, phosphodiesterase I and alkaline phosphatase [30]. DNA hydrolysates were centrifuged and supernatants were stored at 4° prior to analysis by HPLC-EC.

Determination of oxo^{8}dG by HPLC-electrochemical detection. Analyses of DNA hydrolysates were performed by reverse phase HPLC-EC [16] with modifications [31].

DNA-repair assay. The DNA-repair assay was based on that of Williams *et al.* [32] with modifications [27]. Both rat and human hepatocyte preparations (5×10^5 cells/plate, excluding non-viable cells which did not attach to plates during pre-incubation) were incubated with BLM (0, 10 and 100 U/L) for 20 hr in a 5% CO_2, humidified incubator (at 37°) or on ice (4°) in the presence of 5 μCi [^{3}H]thymidine (87 Ci/mmol; TRK 686, Amersham International plc, Amersham, UK). The medium (2 mL/plate) was Dulbecco's modified Eagles medium containing glutamine (2.0 mM), penicillin (50 IU/mL) and streptomycin (50 μg/mL). Visualization of unscheduled DNA synthesis in the cells was by an autoradiographic technique as described by Chipman and Davies [27]. Quantification of [^{3}H]thymidine incorporation was obtained by counting the number of silver grains seen in an arbitrary unit area of both cytoplasm and nucleus. This area was defined by the use of an eye-piece graticule with a ruled area of 0.49 mm^2 square which occupied at least 50% of the total nuclear area. Deduction of the cytoplasmic count from the nuclear grain count gave a net grain

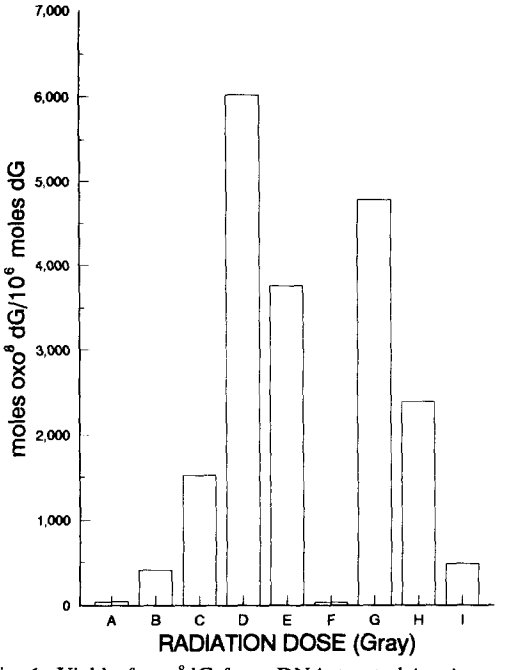

Fig. 1. Yield of oxo^{8}dG from DNA treated *in vitro* with irradiation. Calf thymus DNA (0.25 mg/mL UHQ water) was irradiated (^{60}Co; 10 Gy/min) at room temperature with the following doses (Gy) and additions: (A) 0; (B) 10; (C) 100; (D) 500; (E) 500 + ethanol (2 mM); (F) 500 + ethanol (20 mM); (G) 500 + mannitol (0.1 mM); (H) 500 + mannitol (1 mM); (I) 500 + mannitol (10 mM). The results are the mean of two separate experiments.

incorporation per unit area of nucleus. Typically, a total of 50 morphologically unaltered cells were counted per slide and duplicate slides were prepared for each treatment [27]. Heavily labelled nuclei (S-phase) were not looked at.

Assay of thiobarbituric acid (TBA) reactive substances. To assess the formation of TBA-reactivity in BLM-treated isolated DNA [33], samples (0.25 mL) were removed from the reaction mixtures and 0.5 mL 1 M HCl and 0.5 mL 1% TBA (in 50 mM NaOH) were added. The reaction mixtures were then heated at 100° for 15 min. After cooling, the absorption at 532 nm was read against water.

RESULTS

Production of oxo^{8}dG and TBA-reactive substances in isolated DNA

Calf thymus DNA was treated in solution with γ-rays from a ^{60}Co source (up to 500 Gy). The content of oxo^{8}dG (mol oxo^{8}dG/10^6 mol dG) was increased in a dose-dependent manner (Fig. 1). In the presence of 2 mM ethanol, the adduct levels produced by 500 Gy ^{60}Co were decreased 1.6-fold. When the concentration of ethanol was increased to 20 mM, the amount of adduct was reduced to background levels. Likewise, mannitol inhibited the induction of oxo^{8}dG by 500 Gy exposure to ^{60}Co in a dose-dependent manner (Fig. 1).

Incubation of DNA with Fe^{2+} and BLM produced a dose-dependent increase in levels of oxo^{8}dG in excess of that seen with Fe^{2+} alone (Table 1). Treatment of calf thymus DNA with BLM alone (600 U/L) did not increase the levels of oxo^{8}dG above background. Addition of iron (Fe^{2+}) to calf thymus DNA increased adduct levels by 15-fold above controls. As with radiation, the BLM-induced production of oxo^{8}dG in isolated DNA was inhibited (64%) by 10 mM mannitol (data not presented).

Formation of TBA-reactive substances, measured as A_{532}, was assessed in isolated DNA in the presence and absence of BLM and iron (Fe^{2+}). When isolated DNA was treated with increasing concentrations of BLM in the presence of a fixed concentration of added Fe^{2+} ions, there was a dose-dependent increase in TBA-reactivity (Table 1). In the absence of added Fe^{2+}, even a high concentration of BLM had only a small effect on TBA-reactivity and the addition of Fe^{2+} ions alone produced only a small effect. This absence of an effect of Fe^{2+} alone on TBA-reactivity is in contrast to the marked induction of oxo^{8}dG in isolated DNA by addition of Fe^{2+}. Furthermore, the dose–response to BLM–Fe^{2+} was linear in terms of TBA-reactivity, whilst the relationship between oxo^{8}dG induction and BLM concentration was non-linear over the range tested. In addition, the concentration of oxo^{8}dG produced by BLM/Fe^{2+} in isolated DNA was vastly lower than the amount of MDA (produced via base propenals) per nucleotide (Table 1).

Analysis of oxo^{8}dG in BLM-treated hepatocytes

Oxo^{8}dG was detected in DNA hydrolysates from control (untreated) hepatocytes incubated at either 37° or 4° (Table 2). The yield of oxo^{8}dG relative to dG was however not increased as a result of BLM treatment, the values for the maximum dose tested (260 U/L) being shown in Table 2. Even when the conditions of nuclear exposure to BLM were optimized by permeabilization of hepatocytes prior to treatment, the adduct was not increased above control levels.

Induction of unscheduled DNA synthesis in BLM-treated hepatocytes

In agreement with earlier studies [32], the control values for the net nuclear grain counts were negative (possibly a reflection of mitochondrial [^{3}H]thymidine incorporation). BLM at concentrations of 10 and 100 U/L, gave positive responses when unscheduled DNA synthesis was measured in cultured non-permeabilized rat and human hepatocytes (Table 3). This was shown to be dose-dependent using both rat and human cells (N = 3, with duplicate analyses of each experiment).

DISCUSSION

The present studies have shown that gamma-irradiation induces formation of oxo^{8}dG in calf thymus DNA. This result is expected from previous investigations which showed that ionizing radiation in the form of X-rays induced oxo^{8}dG in isolated DNA [34]. The radiomimetic action of BLM, regarding oxo^{8}dG formation, was demonstrated in

Table 1. Formation of TBA-reactive species and oxo^{8}dG on incubation of isolated DNA with ferrous iron/BLM

Reaction mixture	Concn (μM) MDA produced per incubation*	Mol MDA/10^6 mol nucleotides†	Mol oxo^{8}dG/ 10^6 mol dG
DNA	0.5	700	150
DNA/BLM (600 U/L)	1.16	1600	130
DNA/Fe^{2+}	1.60	2200	2210
DNA/Fe^{2+}/BLM (37.5 U/L)	8.64	11,800	2690
DNA/Fe^{2+}/BLM (75 U/L)	17.55	23,900	3310
DNA/Fe^{2+}/BLM (150 U/L)	32.01	43,700	3440

* $\varepsilon = 1.36 \times 10^5$ $M^{-1}cm^{-1}$ at 532 nm for MDA.
† Mean molecular mass of the four DNA nucleotides is 327.

Table 2. Formation of oxo^{8}dG in BLM-treated rat hepatocytes

Treatment	Temperature (°C)	Mol oxo^{8}dG/10^6 mol dG
Control	37	27 ± 16
BLM (260 U/L)	37	30 ± 13
Control	4	20 ± 12
BLM (260 U/L)	4	12 ± 3
BLM (260 U/L) (Permeabilized hepatocytes)	37	28 ± 7

The results represent the mean ± SD (N = 3).

Table 3. Induction of unscheduled DNA synthesis in BLM-treated rat and human hepatocytes

Treatment	Type	Net nuclear grain counts ([^{3}H]thymidine incorporation)
Control	Rat	−0.8 ± 0.2
	Human	−3.5 ± 4.7
BLM	Rat	3.8 ± 3.9
	Human	10.0 ± 6.9
BLM (100 U/L)	Rat	9.4 ± 8.6
	Human	24.5 ± 6.2
2-Acetylaminofluorene (positive control; 1 μM)	Rat	14.6 ± 3.2
	Human	7.9 ± 2.6

Cultures of hepatocytes (5×10^5 cells/plate containing 2 mL culture medium) were treated with test compounds. After incubation for 20 hr in the presence of 5 μCi [^{3}H]thymidine, the cells were processed for autoradiography and assessment of unscheduled DNA synthesis. The results represent the mean ± SD (N = 3).

isolated DNA and is in agreement with previous observations [21–23]. The formation of oxo^{8}dG induced by both radiation and BLM was inhibited by oxygen radical scavengers. The relative formation of base propenals and oxo^{8}dG did not correlate (Table 1) and since the formation of oxo^{8}dG but not TBA-reactive materials was inhibited by mannitol and dimethyl sulphoxide [23], the results suggest that different mechanisms for the induction of these effects may occur.

Although increased concentrations of oxo^{8}dG were shown in DNA isolated from cellular systems following high doses of X-irradiation [20] and H_2O_2 [35, 36], we have been unable to demonstrate this with BLM. In a single experiment, Kohda *et al.* [21] were unable to demonstrate oxo^{8}dG formation after treatment of Ehrlich ascites cells with BLM at doses of 0.35 and 0.7 mM (equivalent to 937.5 and 1875 U/L). In agreement with these authors we were unable to detect an increase of oxo^{8}dG above background cellular levels; control values were similar to those reported previously with rat liver [37, 38]. Thus, a

BLM-induced increase in oxo^{8}dG is detectable in isolated DNA but not in cells, despite the demonstration of UDS.

The reason for the absence of increased oxo^{8}dG concentrations in intact cells is not known. Nuclease [18] and glycosylase [19] repair systems for oxo^{8}dG have been identified, but repair is unlikely to explain the lack of adduct elevation since experiments conducted at 4° also showed no increase, despite the fact that Kohda *et al.* [21] showed formation of oxo^{8}dG in isolated DNA at 0° following BLM treatment. Lack of access of BLM into the cells also seems unlikely, since induction of UDS in hepatocytes was demonstrated. Furthermore, permeabilization of hepatocytes did not facilitate the formation of oxo^{8}dG.

The conformation of DNA in the nucleosome form may provide resistance to the agents, or cellular component(s) may inhibit the action of oxygen radicals. This explanation is consistent with the earlier observation that the formation of oxo^{8}dG in DNA is three orders of magnitude less on ionizing irradiation *in vivo* than on irradiation of isolated DNA [20, 34].

The results described above show for the first time that BLM induces UDS in non-permeabilized rodent and human hepatocytes in the absence of hydroxyurea. All previous studies on BLM-induced UDS have been performed on permeabilized cells [39, 40] or non-permeabilized cells in the presence of hydroxyurea [41]. Hydroxyurea decreases the stability of the non-haem iron centre in mammalian ribonucleotide reductase, an enzyme which catalyses a rate-limiting step in DNA synthesis [42]. The iron is rendered more readily available for chelation by BLM, increasing the potential for BLM to cause DNA damage [43].

Studies by Harbach *et al.* [44] showed that compounds which induce DNA damage resulting in "long-patch" repair [45] can be detected and quantified by UDS. However, UDS was not detected in hepatocytes exposed to X-rays (up to 100 Gy) [44], indicating that the UDS assay is insensitive to agents that result in "short-patch" repair of damage [44]. In contrast, BLM induced extensive UDS, suggesting a component that is characteristic of "long-patch" repair. At concentrations of 5 and 25 μg/mL, BLM produced repair patches in permeabilized human fibroblasts that were initially sensitive to staphylococcal nuclease as with agents repaired by "long patch" [46]. However, at a relatively low dose (1 μg/mL), the loss of nuclease sensitivity was slow in comparison to the rearrangement characteristic of "long patch" repair. These results suggest a dependency on dose for the type of lesions induced by bleomycin.

Since UDS was detected following treatment with a BLM concentration that did not produce an increase in oxo^{8}dG formation, it appears that the formation of the adduct is not a major factor in BLM-induced UDS. This is consistent with the recent finding that although oxo^{8}dG is induced by BLM treatment of isolated DNA, the production of $^{\cdot}$OH radicals is a minor side reaction resulting from the decomposition of a high valency iron-oxo species.

Gajewski *et al.* [23] compared the DNA base adduct formation induced by BLM with that produced by Cu^{2+}–phenanthroline complexes in the presence of reducing agents. Cu^{2+}–phenanthroline caused extensive damage to DNA and the pattern of products formed was characteristic of attack by $^{\cdot}$OH [47]. DNA strand scission can occur at sites slightly removed from the Cu^{2+}–phenanthroline binding site [48]. In contrast, DNA cleavage by BLM occurs locally at the binding site of the drug [48], and appears not to involve the hydroxyl radical [23]. Thus, it was concluded that the modification of bases by $^{\cdot}$OH formed in the BLM/iron system is a minor side reaction. In agreement with this conclusion, the results from our cell-based studies reported here draw into question the role of $^{\cdot}$OH in DNA damage by BLM, and the true radiomimicry of this agent.

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