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DNA STRAND BREAKAGE IN ISOLATED NUCLEI SUBJECTED TO BLEOMYCIN OR HYDROGEN PEROXIDE

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Abstract—The sources of iron (Fe) and reductant required for DNA strand breakage by the antitumor drug bleomycin (Blm), H_2O_2 and ascorbate were investigated using nuclei instead of whole cells in order to study a simpler, related system that was subject to better control and easier chemical manipulation. Ehrlich ascites tumor cells were isolated and treated directly on filters, and analysed for DNA damage by alkaline and nondenaturing elution. Extraction and treatment buffers were depleted of trace Fe by passage through $Mg(OH)_2$ gel. Nuclei were treated for 1 hr at 37°. High levels of single- and double-strand breakage were obtained using Fe(III)Blm in the range 0.01 to 0.08 μM. In contrast, Blm was effective only at two orders of magnitude greater concentration. Cu(II)Blm was totally ineffective in causing damage. Depletion of nuclear protein thiols with *N*-ethylmaleimide reduced double-strand breakage at the upper end of the FeBlm concentration–response curve. A 1 mM concentration of NADPH or NADH greatly increased the extent of double-strand breakage by 0.01 μM FeBlm, suggesting roles for cytochrome P450 or cytochrome b_5 reductase in strand breakage. Fe(III)ATP (1:20 metal to ligand and 50 μM in Fe) and Fe(III)EDTA (1:2 metal to ligand and 50 μM in Fe) did not cause single-strand breaks. In the absence of added Fe, H_2O_2 or ascorbic acid (50 μM) caused less than one Gy-equivalent single-strand breakage. Addition of ascorbate plus Fe(III)ATP or Fe(III)EDTA produced breakage beyond the capacity of alkaline elution to analyse (5–6 Gy). Overall, the results indicate that Fe, which may contribute to DNA damage by Blm and forms of activated oxygen within cells, is not strongly bound in the nucleus and that nuclear thiols other than glutathione contribute reducing equivalents to Fe(III)Blm for the DNA damaging chemistry.

Key words: bleomycin; hydrogen peroxide; iron; nuclei; strand scission; thiols

Blm† is an antitumor agent that induces single- and double-strand DNA breaks [1]. Intracellular double-strand DNA breakage by the drug correlates with the extent of inhibition of growth of Ehrlich ascites tumor cells [1]. Both its cytotoxicity and capacity to cause DNA breakage require Fe [2].

The source of the Fe that activates Blm is uncertain. Short treatment of Ehrlich cells with Blm in medium containing little Fe causes growth inhibition, suggesting that intracellular Fe can provide this cofactor for the drug [3]. That intracellular Fe is utilized by the drug has been shown in human leukemic HL-60 cells made Fe deficient by direct depletion of their nutrient source of Fe [2]. Because Fe is strongly bound to many proteins distributed throughout cellular compartments, it has been difficult to define the reactive pools of metal. In addition, Fe is a frequent contaminant of laboratory reagents [4]. Thus, for example, the finding that CuBlm cleaves isolated DNA [5, 6] has been disputed by investigators who showed that sufficient Fe was probably present in the reaction mixture to support DNA damage by FeBlm [7]. Efforts to control the availability of intracellular Fe that can participate in redox chemistry by treating cells with chelating agents has not been satisfactory, because metal binding ligands are inherently nonspecific and might otherwise perturb the system [8]. As a consequence, virtually no information is available about the intercellular species of Fe that are accessible to form FeBlm. Similarly, relatively little is known about the molecular sources of Fe or copper that support oxidative damage to cells by other reagents such as H_2O_2.‡

We report here results of studies with Blm, H_2O_2 and ascorbic acid, which examine their ability to cause single- and double-strand breakage in nuclei. Nuclei were isolated *in situ* on filters used for elution of DNA. Subsequent treatments with potential strand-break reagents were initially carried out in the absence of external chelators and reductants. Rapid removal of cell debris, as well as reagent addition and removal, was achieved by allowing fluid to exit the volume above the filters. Our results suggest that sources of Fe required for DNA damage by Blm, as well as by oxidation–reduction processes driven by ascorbic acid, are not firmly bound to the nucleus. Unexpectedly, it was also found that

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† Abbreviations: Blm, bleomycin that contains predominately A_2 and B_2 congeners; BSO, DL-buthionine-[*S*,*R*]-sulfoximine; DTNB, 5,5′-dithio-bis(2-nitrobenzoate); FBS, fetal bovine serum; GSH, glutathione; MEM, Eagle's Minimal Essential Medium plus Earle's salts; NEB, nuclear extraction buffer; NEM, *N*-ethylmaleimide; NTB, nuclear treatment buffer; Pr_4NOH, tetrapropylammonium hydroxide.

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Fe(III)Blm caused DNA double-strand damage even in the absence of added reductants. A portion of the necessary reducing equivalents was apparently supplied by nuclear, probably protein thiols.

MATERIALS AND METHODS

Reagents and cells. MEM and FBS were from GIBCO, Grand Island, NY; DTNB and ascorbic acid (Gold Label) were from the Aldrich Chemical Co., Milwaukee, WI; EDTA and NaCl were from J. T. Baker, Phillipsburg, NJ; Triton X-100, glycine, Tris[hydroxymethyl]aminomethane, SDS, proteinase K, NEM, NADH, NADPH (tetrasodium salt), GSH, and BSO were from the Sigma Chemical Co., St. Louis, MO; $FeCl_3$ was from Fisher, Pittsburgh, PA; ATP was from P-L Biochemicals, Milwaukee, WI; phosphoric acid (meta form) was from Mallinkrodt, St. Louis, MO. Pr_4NOH (25% aqueous) was from the RSA Corp., Ardsley, NY.

All solutions were made up in doubly distilled water in glassware or plasticware that had been rinsed overnight in 10% nitric acid or 30 mM EDTA, respectively.

Ehrlich ascites tumor cells in suspension culture were grown in MEM plus 2% FBS. Cell DNA was labeled with [2-^{14}C]thymidine or [*methyl*-^{3}H]-thymidine according to established procedures [1, 9].

γ-Irradiations were performed in PBS at ice-cold temperature using a J. L. Shepherd Mark I ^{137}Cs irradiator at a dose rate of 4 Gy/min.

Blm in its clinical form, blenoxane, was a gift from Bristol Laboratories, Syracuse, NY. Blm concentrations were determined spectrophotometrically using an extinction coefficient, ε_{290}, of 14,100 $M^{-1}cm^{-1}$ [10]. Fe(III)Blm and Cu(II)Blm were prepared using $FeCl_3$ or $CuCl_2$ as previously described [1]. Concentrations of FeBlm are expressed in terms of Fe because Fe(III)Blm is only about 70% saturated with Fe at pH 7.4 [11]. Metal concentrations were determined by flame atomization atomic absorption spectrophotometry using an Instrumentation Laboratory Inc. (Lexington, MA) model 357 atomic absorption spectrophotometer. Analysis of the iron content of Blm used in this study showed that it contained 0.01 g-atoms of Fe/mol of drug.

Preparation of nuclear buffers. The compositions of NEB and NTB were adapted from those of Pommier and coworkers [12]. NEB was made up of 150 mM NaCl, 5 mM $MgCl_2$, 1–2 mM NaOH, and 0.3% Triton X-100; NTB was identical to NEB except that it lacked Triton X-100. The buffers were depleted of trace Fe by filtering through $Mg(OH)_2$ gel [13]. After passage through $Mg(OH)_2$, the pH was adjusted to 7.4 with distilled phosphoric acid. Mg content was checked by flame atomization atomic absorption spectrophotometry and corrected to 5 mM, if necessary, by addition of buffer of higher or lower Mg content, which had been similarly processed. Buffers prepared in this manner consistently yielded Fe contents of around 5 nM, as measured by atomic emission spectrophotometry. This is about 10-fold lower than can be achieved with Chelex-100 [14].

Isolation and treatment of nuclei. Nuclei were isolated directly on filters by a modification of a previously published procedure [12, 15]. [^{14}C]-Thymidine-labeled Ehrlich cells, 5×10^5 (for alkaline elution experiments) or 2×10^5 (for nondenaturing elution), were applied to each filter (polycarbonate, 2 μm pore size from the Nuclepore Corp., Fullerton, CA) in Swinnex units (Millipore) attached to syringe barrels according to procedure B of Kohn *et al.* [9]. The cells were washed with 5–10 mL PBS. Filter units were kept refrigerated until nuclei extraction. Nuclei were extracted by filling the lower chambers of the units with ice-cold NEB (about 1.5 mL). After 10–30 sec, the NEB was allowed to flow through the filter. The outflow was then stopped, and 5 mL of ice-cold NTB was added. After 10–30 sec, the NTB was allowed to flow through, and the units were again stoppered.

Treatment solutions were made up by mixing various forms of Blm and other components with NTB immediately prior to addition to the washed nuclei on filters. Sufficient treatment solutions (room temperature) were applied to completely fill the lower chambers, after which the units were transferred to a 37° water bath. The standard treatment time was 1 hr.

In some experiments, immediately after isolation, nuclei were depleted of reduced thiols by addition of a 0.2 mM concentration of freshly prepared NEM in NTB at room temperature for 15 min. The filters were then washed twice with NTB prior to addition of FeBlm.

After treatment, elution units were removed from the water bath, and 5 mL of ice-cold PBS containing 0.5 mM $CoCl_2$ (for Blm treatments) or non-$CoCl_2$-containing PBS (for other treatments) was applied to the upper chamber of each unit. Cobalt-containing PBS was used to inactivate traces of Blm bound to filters [1]. The solutions were then allowed to flow through by gravity until they reached the neck between the upper and lower chambers, at which time the units were stoppered. Cold PBS (2.5 mL) containing internal standard cells (5×10^5 for alkaline elution; 1.5×10^5 for nondenaturing elution) was added to the upper chambers. The solutions were allowed to flow down to the filters.

For alkaline elution, nuclei and internal standard cells were lysed with two aliquots of 5 and 2 mL of the following solution: 2% SDS in 25 mM EDTA, pH 10.0. Proteinase K (0.6 U/mL) was included in the second 2-mL step. Eluant (Pr_4NOH plus 0.1% SDS and 25 mM acid-form EDTA, pH 12.3) was added to upper chambers, and elution was initiated 40 min after beginning proteinase digestion [9]. Untreated control nuclei exhibited some damage, at levels above those normally observed in cells [1]. Apparently, it was due to random nucleolytic or physical cleavage during isolation of nuclei as this breakage was not reflected in internal standard DNA. Nevertheless, the breakage was observed to be highly reproducible within each experiment (as shown in Fig. 1, A and B). Thus, results were quantifiable in Gy-equivalent terms due to the additivity of single-strand break indices, which is permissible when linear or near-linear curves, indicative of random damage, are obtained. For these calculations, we determined the slope in

internal standard-corrected plots, which is equal to the first-order elution rate constant of DNA from treated nuclei divided by the first-order elution rate constant of internal standard cell DNA [1]. The corresponding ratio for untreated DNA in control nuclei was subtracted in order to obtain an index of single-strand breakage attributable to the treatment.

The nondenaturing elution procedure of Bradley and Kohn [16] was closely followed, except that 0.2% instead of 2% SDS was used. Eluting solution contained 0.2% SDS, 0.025 M disodium EDTA, 0.05 M tris[hydroxymethyl]aminomethane, and 0.05 M glycine, pH 9.6. The DNA was digested in 3 mL of eluting solution containing 0.9 U/mL proteinase K before elution was begun [16]. Relative elution was calculated as described [17], except that internal standard cells were irradiated with 50 Gy and elution data at 16.5 hr were used. Relative elution is the ratio of fraction [^{14}C]DNA eluted from a filter to that of the [^{3}H] internal standard DNA, after subtraction of the ratio for the filter containing untreated control [^{14}C]nuclei. High FeBlm concentrations (>0.1 μM) produced some [^{14}C]-DNA in the nuclear washes; however, over the concentration range used in this report, no DNA was observed in saline washes.

Fractions, filters, and filter support washes were counted, as described, using a Beckman LS3801 liquid scintillation counter [1].

Thiol assays. The total reduced thiol content of Ehrlich cells and nuclei was measured after sonication [18], using the colorimetric reaction with DTNB [19]. Nuclei were extracted by centrifugal removal of extranuclear debris using NEB and NTB [12]. Total GSH was measured by following the formation of 2-nitro-5-thiobenzoic acid at 412 nm in the presence of DTNB, glutathione reductase, and NADPH after acid extraction [20].

To determine the efficiency of thiol depletion by NEM, 2×10^7 nuclei were suspended in 30 mL NTB at room temperature and treated with 0.2 mM NEM for 15 min. NEM-treated or control nuclei were then washed by centrifugal pelleting, resuspension in PBS and recentrifuging, and finally resuspension in 2 mL of Ellman's reagent for thiol measurement [18, 19].

In some experiments, cells were treated with 0.2 mM BSO for 24 hr in order to inhibit GSH synthesis [21].

RESULTS

Nuclei from Ehrlich ascites tumor cells containing DNA labeled with [^{14}C]thymidine were prepared, as described in Materials and Methods, and used to determine whether isolated nuclei could furnish Fe for strand breakage by Blm or reduced forms of molecular oxygen. In the experiment of Fig. 1A, which shows results representative of three experiments, nuclei were treated with 0.1 μM Blm or CuBlm and two concentrations of Fe(III)Blm (6.5 and 65 nM). Neither Blm nor CuBlm (0.1 μM) showed any capacity for single-strand breakage. However, both concentrations of FeBlm produced significant single-strand breakage.

Figure 1B shows representative results of several experiments examining the effects of ascorbate and H_2O_2. Fifty micromolar ascorbic acid or H_2O_2 was only mildly active in producing strand breakage,

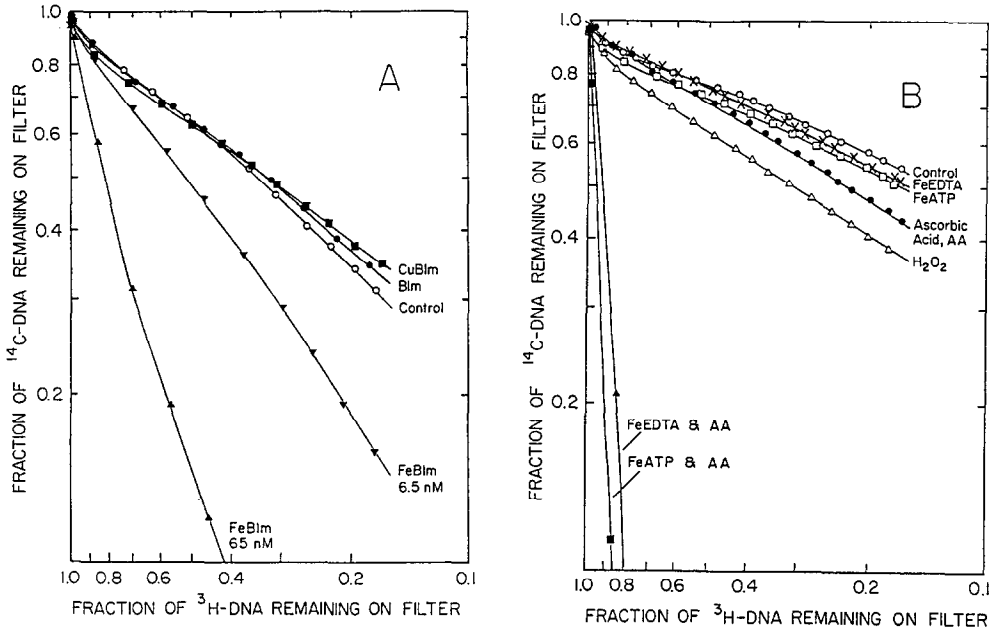

Fig. 1. (A) Alkaline elution of DNA from nuclei treated with forms of Blm, Cu(II)Blm (both at 0.1 μM), or indicated concentrations of FeBlm. (B) Alkaline elution curves of DNA from nuclei treated with Fe complexes, ascorbic acid, H_2O_2, or combined Fe complexes plus ascorbic acid (AA). All additions were 50 μM for each reagent.

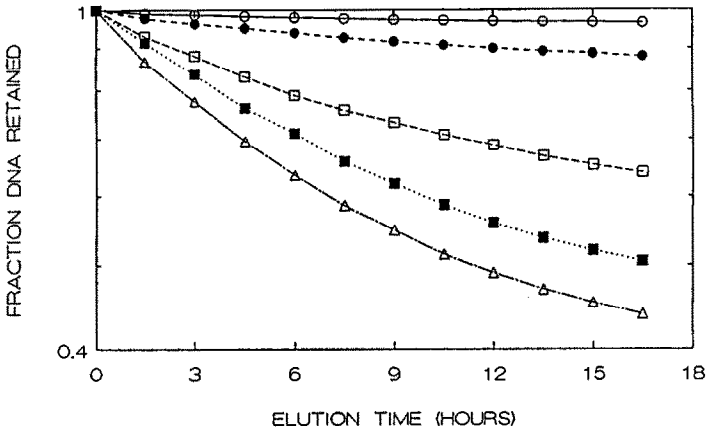

Fig. 2. Nondenaturing filter elution curves of DNA from nuclei treated with FeBlm or γ-radiation. Symbols: (○), control; (●), 0.01 μM FeBlm; (□), 0.04 μM FeBlm; (■), 0.08 μM FeBlm; and (△), 50 Gy γ-radiation.

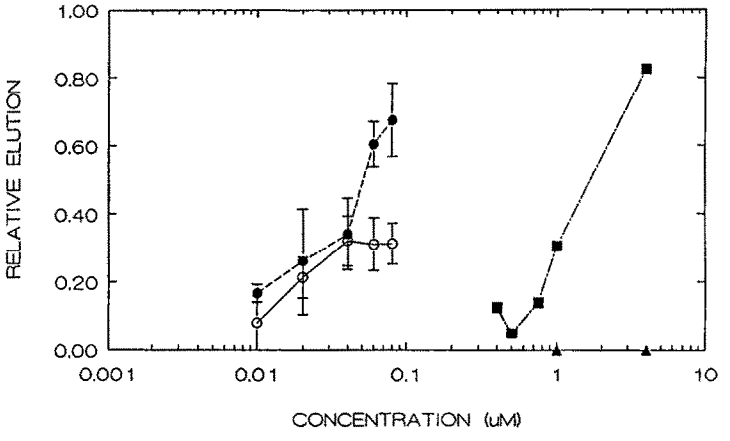

Fig. 3. Yield of relative elution from nuclei treated with various forms of Blm. Shown are the means ± 1 SD of 3–6 experiments for each FeBlm concentration and a single experiment for each Blm or CuBlm concentration. Symbols: (●), FeBlm; (○), FeBlm after pretreatment of nuclei with NEM; (■), Blm; and (▲), CuBlm.

with H_2O_2 producing breakage corresponding to only 0.65 Gy ionizing radiation. In a second experiment, 50 μM H_2O_2 generated 0.5 Gy-equivalent damage (not shown). Fe(III)ATP (1:20 Fe:ATP) and Fe(III)EDTA (1:2 Fe:EDTA) at concentrations of 50 μM Fe produced DNA elution that was nearly identical with that of untreated controls (Fig. 1B). However, 50 μM ascorbic acid plus either form of Fe produced rapid elution, indicating high levels of breakage, beyond the capability of this low-sensitivity alkaline elution method to measure [22].

Double-strand breakage by forms of Blm was characterized by nondenaturing filter elution. Representative elution curves obtained using FeBlm are shown in Fig. 2. The curves are similar to those of ionizing radiation, with a gradually decreasing slope as fractions are collected.

Figure 3 illustrates results of treatment of nuclei with Fe(III)Blm, Blm, and CuBlm. Concentration-dependent increases in relative elution were observed with FeBlm and Blm. However, approximately 100-fold greater concentrations of Blm than FeBlm were required to produce similar elution. This may be due to the presence of contaminating Fe in the drug, for the sample of Blm contained about 1% Fe (mol Fe/mol Blm as measured by atomic emission spectrophotometry) (unpublished results). No strand scission was observed with 1 or 4 μM CuBlm. Thus, there is little or no Fe in these isolated nuclei which can be scavenged by Blm to support DNA strand breakage reactions. Notably, DNA breakage occurred without addition of reductants to the reaction mixture.

In vitro Fe(III)Blm-dependent DNA strand scission requires a source of reducing equivalents [11]. It was surprising, therefore, that Fe(III)Blm efficiently caused single- and double-strand damage

in washed nuclei without the addition of an external reductant. Therefore, the availability of different types of reducing agents for double-strand breakage by FeBlm was investigated in additional experiments. First, the role of endogenous nuclear thiols as the source of reducing equivalents was investigated. The total reduced thiol content of Ehrlich cells and isolated nuclei was found to be 2.40 ± 0.12 and 0.239 ± 0.017 nmol/10^5 cells, respectively (N = 5 for both determinations). No detectable GSH was found in isolated nuclei. To check these findings, cells were depleted of GSH by growth in the presence of 0.2 mM BSO, followed by isolation of nuclei and assay of total thiol content [21]. The total thiol content of cells was reduced by about 35% (to 1.57 ± 0.19 nmol/10^5 cells, N = 3), whereas the thiol content of nuclei was unchanged (0.257 ± 0.039 nmol/10^5 cells, N = 3). This result supports the view that no detectable GSH remained in the isolated nuclei, which is consistent with the known porosity of the nuclear membrane [23]. Thus, it is hypothesized that the measured thiol content of nuclei was most likely protein bound.

Treatment of isolated nuclei with 0.2 mM NEM for 15 min at room temperature reduced the total thiol content by 90 and 100% in two experiments, respectively. Strand scission experiments were then conducted on nuclei isolated on filters and pretreated with 0.2 mM NEM. Exposure to NEM reduced the extent of nondenaturing elution at concentrations greater than 0.04 μM, but had no effect at lower concentrations of FeBlm (Fig. 3). The leveling off of breakage at about relative elution 0.3 suggested that two mechanisms of strand breakage were operative, with a thiol-dependent mechanism effective primarily at elevated FeBlm concentrations.

The time-dependent stability of the potential endogenous reductant(s) was investigated in two experiments in which nuclei were preincubated for 1 hr at 37° in nuclear treatment buffer followed by its replacement with buffer containing 0.02 or 0.04 μM FeBlm for a second hour. No decrease in relative elution was evident; respective results for preincubated vs non-preincubated nuclei for the two concentrations were 0.23 vs 0.17 and 0.46 vs 0.44. These are within the range of variability of the measurements (Fig. 3).

Finally, the capacity of cytochrome b_5 reductase and cytochrome P450 reductase to supply reducing equivalents to Fe(III)Blm was investigated by addition of NADPH or NADH, respectively, to the reaction mixture. Both agents greatly stimulated double-strand breakage by 0.01 μM FeBlm. Neither NADPH nor NADH appreciably affected elution in the absence of FeBlm (Fig. 4). This result extends the previous finding that these reducing agents enhance single-strand DNA damage by much larger concentrations of Blm in nuclei [24].

Because of the possibility that some residual cellular NADPH or NADH may have remained in extracted nuclei to provide a source of electrons, used later by added FeBlm, another experiment examined the ability of nuclei to retain these cofactors. One millimolar of each cofactor was added separately to isolated nuclei and incubated for 5 min at 37°. Each nuclear preparation was then washed once with NTB prior to addition of treatment with 0.01 mM FeBlm. The extent of relative elution for cofactor-pretreated nuclei was not increased (relative elution 0.04 for both cofactor-treated nuclei compared with 0.08 for FeBlm-treated only). As increased double-strand breakage attributable to added NADPH or NADH was not observed, it seems unlikely that these cofactors contribute significantly to breakage by FeBlm in otherwise untreated nuclei.

In further experiments (not shown), nuclei were either exposed to NEM or left untreated. Then they

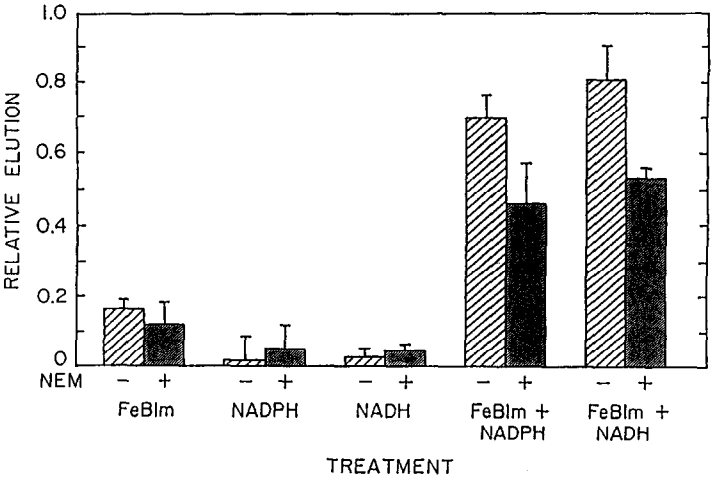

Fig. 4. Effects of NADPH and NADH on FeBlm-induced relative elution. Shown are the means ± 1 SD of 3–4 experiments for each treatment. Also indicated are results of pretreatment of nuclei with 0.2 mM NEM for 15 min (indicated by +) prior to addition of reactants. Concentrations were: NADPH or NADH, 1 mM; FeBlm, 0.01 μM. Treatment times were 1 hr.

were incubated with 0.04 μM FeBlm and analysed in the presence or absence of internal standards. Closely similar fractions of [^{14}C]DNA were eluted in the absence or presence of internal standards. Clearly, cells providing internal standard DNA did not supply reductants for strand breakage by possible filter- or nuclear-bound FeBlm [1]. Therefore, although concomitantly added cofactors such as NADPH or NADH, as well as endogenous protein thiols, were able to supply reducing equivalents for DNA strand breakage in nuclei by FeBlm, another reducing agent for DNA strand breakage was also associated with nuclei and remains to be identified.

DISCUSSION

The process by which Blm acquires cellular Fe, needed for DNA degradation and cytotoxicity by the drug, is a fundamental issue in mechanistic studies of this agent [11]. More generally, the nature of the Fe species which appear to be involved in the reduction of O_2 to mutagenic, toxic, and carcinogenic forms is also of longstanding interest [25]. We have addressed this question with a nuclear model system in which the production of DNA lesions in the form of single- and double-strand breaks could be monitored and manipulated. The combined results indicate that nuclei prepared in the manner described do not contain much Fe that is available for reactions involving Blm, ascorbate, or H_2O_2.

Some alteration of nuclear membranes prepared using Triton X-100 (2%) has been described [26]. However, at lower detergent concentrations and at reduced temperature such as were used here, most of the nuclear structure is maintained with little perturbation [12, 27]. Certainly, the DNA in such preparations is largely intact (Figs. 1–3 and Ref. 12). Additionally, the nuclear structure so obtained is regarded as highly porous, allowing ready diffusion of small molecules into and out of nuclei [23].

The results shown here reinforce the conclusion that Fe is the required metal ion cofactor for Blm; CuBlm was totally without activity (Figs. 1 and 3). Blm can chelate Fe(II) and Fe(III), the latter with a sufficiently high stability constant so that it may readily compete with kinetically reactive pools of intracellular or intranuclear Fe [11]. Yet, on the order of 1 μM Blm was needed to initiate a concentration-dependent increase in double-strand breakage by the metal-free form of the drug (Fig. 3). The relative concentrations of FeBlm and Blm required to produce similar extents of damage (*ca.* 1–100) correlated well with the amount of Fe present in both forms of the drug. So, although nuclear preparations have Cu and Fe tightly associated with them [28], these pools of metal ions apparently do not participate in the activation of dioxygen as examined here.

H_2O_2 causes single-strand DNA damage in whole cells, possibly through a Fenton reaction in which H_2O_2 is reduced to hydroxyl radical and hydroxide in a transition metal ion-catalysed process. Since the formation of $^{\cdot}OH$ must occur in close proximity to DNA in order for it to be the target for this highly reactive radical, it is hypothesized that in intact cells, nuclei contain a species of Fe that can react with H_2O_2. That Fe is the important metal ion in the reaction of H_2O_2 with cellular DNA has been shown by the finding that such damage can be reduced substantially in Fe-deficient cells [29, *]. Nevertheless, neither 50 μM ascorbic acid nor a similar concentration of H_2O_2 produced much single-strand damage without addition of an exogenous Fe complex such as Fe(III)ATP or Fe(III)EDTA (Fig. 1B). Thus, highly sensitive alkaline and neutral filter elution experiments did not detect a significant amount of chelatable Fe, or Fe (or other metal) that was redox-active with H_2O_2 or ascorbate in nuclei.

The results with H_2O_2 (Fig. 1B) are in contrast to those of Meneghini and Hoffmann [30], who showed random single-strand breakage in nuclei, obtained from human fibroblasts and treated with 100 μM H_2O_2 for 30 min, of about 1.1 ± 0.4 single-strand breaks/10^8 Da as measured by alkaline sucrose gradient centrifugation. If an efficiency for strand breakage by ionizing radiation of 2.7×10^{-12} cGy^{-1} Da^{-1} is assumed, then those levels of strand breakage should be equivalent to 41 ± 15 Gy ionizing radiation [31]. Such levels of breakage are enormous compared with the range of selectivity of high-sensitivity alkaline elution (up to 5–6 Gy [22]). In contrast, we observed less than one Gy-equivalent single-strand breakage (Fig. 1B). The reason for this may be that in our experiments, the Fe complex drained through the filter during extraction, whereas in previous work centrifugation of nuclei copurified the Fe. If this is so, then the present experiments have demonstrated that the species of Fe, which must be available for redox reaction with H_2O_2 in the nuclei of intact cells, is only weakly associated with cell nuclei.

The experiment of Fig. 1B was designed as an initial attempt to investigate the sensitivity of DNA strand breakage to reaction in the presence of ascorbic acid and model cellular Fe complexes. One estimate of the intracellular concentration of low molecular weight forms of Fe, which might be the Fe reacting with agents such as H_2O_2 to cause DNA damage, is 3–9 μg Fe/g non-blood tissues [32]. If a uniform distribution in cells is assumed, this works out to 50–140 μM. Fifty micromolar Fe in the form of ATP or EDTA complexes was highly effective in supporting single-strand DNA breakage by a similar concentration of ascorbic acid (Fig. 1B). Obviously, lower concentrations of Fe and/or reductant will need to be used in future experiments in order to achieve effective comparisons of the ability of various forms of Fe to cause DNA damage.

The concentration range of FeBlm used in these experiments, 0.01 to 0.1 μM, is considerably lower than those required to damage DNA in whole cells (1–5 μM) [1]. This is because very little Blm associates with cells and only a small fraction of this reaches the nucleus to react with DNA [1]. In cells, the ratio of DNA base pairs to Blm in the nucleus is about 10^5 to 1 [1]. In the present experiments, this ratio was 22–220 to 1 according to a previously measured concentration of DNA in Ehrlich cells (1×10^{10} base pair/cell) [1].

It is well established that activation of FeBlm in

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solution in the absence of DNA leads to a modified drug that has little or no capacity to cause *in vitro* DNA damage [7]. This occurs with a half-time of 2 min at 6° and by extrapolation with a half-time of about 15 sec at 37° [33]. Thus, as FeBlm first approaches DNA it might be reduced and activated before it binds. Then, it must interact rapidly with DNA and initiate DNA damage or else lose its activity. However, with the observation that double-strand scission continues for several hours in cells treated with Blm, it is recognized that most of the damage is probably caused by drug bound tightly to DNA [34, 35]. If the drug is distributed randomly along the DNA polymer, the two molecules are isolated and cannot interact [36]. Thus, the mechanism of DNA strand breakage by FeBlm, derived from studies with isolated DNA and high concentrations of Fe and Blm, requiring activation of O_2-Fe(II)Blm by Fe(II)Blm (reactions 2–3)

$$\text{Fe(III)Blm} + e^- \rightarrow \text{Fe(II)Blm} \quad (1)$$

$$\text{Fe(II)Blm} + O_2 \rightleftarrows O_2 - \text{Fe(II)Blm} \quad (2)$$

$$\text{Fe(II)Blm} + \text{Fe(II)Blm} + H^+ \rightarrow HO_2 - \text{Fe(III)Blm} + \text{Fe(III)Blm} \quad (3)$$

is unlikely, particularly at the larger ratios of DNA to FeBlm [11]. In this situation a single molecule of Fe(III)Blm must be reduced as in reactions 1 and 2, and then further reduced to form the activated species (reaction 4):

$$O_2 - \text{Fe(II)Blm} + e^- + H^+ \rightarrow HO_2 - \text{Fe(III)Blm} \quad (4)$$

The nature of the reducing agent represented by e^- has not been determined. One attractive possibility is superoxide anion, generated as byproducts during the normal catalytic cycle of cytochrome P450 reductase or cytochrome b_5 reductase [24, 37].

Ionizing radiation dose-dependent alkaline and nondenaturing filter elutions result from DNA fragmentation to single-strand lengths of 1.5×10^6–3×10^7 bases (alkaline elution) [31] or double-strand lengths of 2.3×10^5 base pairs (nondenaturing elution) [38]. Again, using the measured DNA content [1] and assuming the extreme case of an equal distribution of DNA throughout the supra-filter volume, one can calculate the presence of a minimum of 1.5×10^{-13} to 3.0×10^{-12} M cleavage sites for alkaline elution experiments and 9.6×10^{-12} M cleavage sites for nondenaturing elution experiments. These concentrations are much lower than the concentrations of FeBlm used (10^{-8}–10^{-7} M). Providing that the distribution of cleavage sites from FeBlm in nuclear DNA can be approximated by that of γ-radiation, in the present model, only a few of the FeBlm molecules needed to be activated during the course of the experiment to cause measurable, concentration-dependent DNA damage, suggesting that multiple cleavages by single FeBlm molecules did not occur. Presumably, in whole cells where nuclear Blm concentrations and drug/DNA ratios are lower, the presence of NADH and/or NADPH stimulates reductase-mediated mechanisms of DNA damage by the drug [24]. Similarly, in nuclei lower FeBlm concentrations probably could have been used in the presence of 1 mM nucleotide to achieve substantial DNA double-strand cleavage (Fig. 4).

One interesting problem that arises out of this work centers on the nature of the reducing equivalents for Fe(III)Blm (reaction 1). Under the conditions of the present experiments using Fe(III)Blm, nuclear reductants must ultimately provide all of the electrons to activate O_2. GSH was not involved, though it is likely that protein thiols played a role at higher FeBlm concentrations (Fig. 4). In the absence of added NAD(P)H, there was no evidence that cytochrome b_5 or cytochrome *c* reductase delivered reducing equivalents. Added NADH and NADPH did not associate with nuclei, and the reducing equivalents that presumably activated FeBlm remained stable for at least 1 hr in isolated nuclei. Furthermore, they were not apparently available to reduce Fe(III)ATP or Fe(III)EDTA complexes (Fig. 1B), which, in turn, can damage DNA *in vitro* through the reduction of dioxygen to hydroxyl radicals [39]. Thus, future studies will focus on the identification of the nuclear reducing agent or agents for FeBlm.

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