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# FERROUS IRON-MEDIATED ENHANCEMENT OF DNA DAMAGE AND RECOVERY POTENTIAL IN BLEOMYCIN-TREATED HUMAN CELLS

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**Abstract**—Damage to cellular DNA is generally considered to be responsible for the antitumour activity of bleomycin. In view of the ferrous oxidase properties of the drug, ferrous iron supply has been manipulated to enhance bleomycin-induced DNA cleavage so that the relationship between the initial yield of DNA damage and cell survival could be explored in a human transformed fibroblast cell line. Bleomycin-induced DNA strand breaks were quantitated by either alkaline denaturation or neutral nucleoid sedimentation techniques. Exogenously supplied ferrous iron greatly enhanced the initial frequency of frank DNA strand breaks and alkali-labile lesions without affecting gross cellular repair capacity. Despite the increased levels of DNA damage, the presence of ferrous iron (>3 $\mu$M) significantly increased (factor up to 2-fold; $P < 0.05$) the survival capacity of drug-treated cells. It is concluded that the induction of DNA damage which can promote cellular recovery may contribute to the variability of the *in vivo* efficacy of bleomycin.

The bleomycins are a group of glycopeptide antibiotics which have activity against a variety of human and animal tumours. The clinical preparation (a copper-free mixture of bleomycins with a predominant bleomycin $A_2$ component: BLM) is used most often in combination with other chemotherapeutic agents (review in ref. 1). It is well established that BLM can induce cleavage in DNA (review in ref. 2) and it is generally considered that this property is responsible for the cytotoxicity of the antibiotic.

A number of molecular mechanisms have been proposed [2] to explain the ability of BLM to induce the liberation of free bases and the formation of single- and double-strand breaks. A central feature of recent models is the capacity of the antibiotic to complex with $Fe^{2+}$ ions and reduce molecular oxygen to the superoxide radical, hydrogen peroxide and the potentially damaging hydroxyl radical [3]. The $Fe^{3+}$ formed during this ferrous oxidase cycle [4] of BLM can be reduced by cellular reducing agents or replaced by $Fe^{2+}$ to continue the cycle. The importance of metal cation availability in controlling bleomycin activity is demonstrated by the observations that copper-, zinc- and cobalt-chelated bleomycins are ineffective in causing DNA strand scission [5–7]. The clinical preparation contains contaminating levels of iron which, together with adventitious iron *in vitro* or *in vivo*, facilitates a basic level of DNA damaging activity. However, the complexity of the putative relationship between cytotoxicity and DNA damaging potential is exemplified by reports that the iron chelator deferoxamine can effectively inhibit DNA cleavage in cell-free systems [6, 7], but does significantly alter drug cytotoxicity towards Chinese hamster cells [8].

An interesting although variable feature of *in vitro* survival curves for bleomycin-treated cells is a biphasic response in which cell killing appears to be less efficient at high drug concentrations [9, 10]. This resistance of cells does not appear to relate consistently to cells of a given cell-cycle age or subpopulations with inherent resistance [11], but is of obvious importance in determining the effectiveness of bleomycin in the clinical situation. It has been suggested [9] that limitation of bleomycin activity may result from the depletion of cofactors such as ferrous ions.

In the present report, $Fe^{2+}$ availability has been manipulated to explore the relationship between the level of bleomycin-induced DNA damage, DNA repair capacity and cell killing in a human cell line. Unexpectedly the results indicate that exogenously supplied $Fe^{2+}$ promotes cellular recovery in BLM treated cells despite a background of increased DNA damage.

## MATERIALS AND METHODS

### *Cell lines and cell culture*

The fibroblast line (MRC5CVI) is an SV40 transformed derivative of the normal parental strain MRC5. The transformed fibroblast line was kindly supplied by Dr C. Arlett (MRC Cell Mutation Unit, Sussex). Cells were maintained in monolayer culture in Eagles MEM supplemented with 10% foetal bovine serum, 1 mM glutamine and incubated at 37° in an atmosphere of 5% $CO_2$ in air.

### *Bleomycin preparation and treatments*

Bleomycin sulphate (Lot No. U9U10AS8; Nippon Kayaku Co., Tokyo) was donated by Lundbeck Ltd. (Luton, U.K.) with a potency originally assayed as 1.7 mg (potency)/mg solid. The manufacturer's analyses indicated that 68.7% of the preparation was bleomycin $A_2$ and copper content was less than 0.008%. To facilitate the use of a single batch of bleomycin all experiments involved freshly thawed

BLM stock solutions (in PBS A stored at −70°). Exact concentrations of drug were determined spectrophotometrically (absorbance of a 1% solution at 294 nm, using a 1 cm path length taken as 121.5). All bleomycin concentrations refer to weight of solid drug in the standard treatment medium lacking serum and supplemented with 10 mM HEPES buffer (pH 7.5; maintained in an atmosphere of 2–3% $CO_2$ in air). Freshly prepared, filter-sterilized solutions of $FeSO_4.7H_2O$ were the sources of $Fe^{2+}$.

*BLM–$Fe^{2+}$ interaction detected by fluorescence quenching*

Fluorescence measurements were performed with a Perkin Elmer MPF-4 spectrofluorimeter in a 2 ml buffer solution (2.5 mM Tris–HCl, 1.2 mM NaCl, pH 8.4; 12) with a BLM concentration of 5 μg/ml (equivalent to 3.5 μM BLM $A_2$) and the sequential addition of fresh $Fe^{2+}$ to specified concentrations. BLM fluorescence was measured at 360 nm with an excitation wavelength at 300 nm as described previously by Huang *et al.* [12]. Preliminary studies (data not shown) indicated that a single freeze/thaw cycle for drug stock solutions did not alter the $Fe^{2+}$ chelating activity of BLM.

*Cell survival*

Cells were plated at $2.5 \times 10^2$–$2.5 \times 10^3$ cells/9 cm diameter plastic dish and allowed to attach for 16 hr at 37°. Dishes were then: drained and pre-warmed treatment medium added, incubated for 1 hr at 37°, drained, washed twice with PBS, and growth medium replaced for the estimation of viability by clonogenic potential.

*Assay for DNA strand breaks*

(i) *BLM treatments.* Exponential cultures of cells in 9-cm plastic dishes ($5–8 \times 10^6$ cells/plate) were exposed to BLM in the treatment medium described above) for 1-hr at 37°. Cultures were then handled as described below in Section (iii).

(ii) *Preparation of cells.* Assays were carried out on cultures which had been subjected to a standard protocol for the generation of freeze/thawed (permabilized) cells directly from monolayer, adapted [13] from the method described by Ganesan *et al.* [14]. Following the treatment of experimental or the sham-treatment of control cultures, monolayers were: washed twice with PBS a, drained well, overlaid with LS buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA; 1 mg/ml bovine serum albumin), the cells detached by one cycle of freezing and thawing, and cells resuspended by aspiration. The above procedure permitted the assay points in all experiments to be collected within 1 min of the cessation of treatment.

(iii) *X-irradiation.* Permeabilized cell preparations from exponential cultures (see below) were irradiated on ice as suspensions in LS buffer ($5 \times 10^5$ cells/ml; see below) at a dose rate of 0.676 Gy/min (using a 250 kV, 15 mA, Pantak X-irradiator, Windsor, U.K.; filtration of 2.32 mm copper half-value thickness).

(iv) *DNA unwinding assay.* DNA strand breaks (including alkali labile lesions) were measured by an adaptation [13] of the fluorometric method described by Kanter and Schwartz [15] involving the time-dependent partial unwinding of cellular DNA in alkaline solutions. Freeze-thawed cells were resuspended in LS buffer ($5 \times 10^5$ cells/ml) and distributed in 0.5 ml volumes into glass tubes for the determination of unwinding rates in quadruplicate as described previously [15] except that the denaturation period was 60 min at ice temperature and the DNA specific dye Hoechst 33342 was used at a final concentration of 0.25 μM. All tubes were homogenized by sonication prior to the measurement of the Hoechst 33342-DNA fluorescence (fluorescence enhancement greater for double- than for single-stranded DNA) using a Perkin Elmer MPF-4 spectrofluorimeter. Treatment-induced enhancement of DNA unwinding (F) was determined by the expression $F = -100 \log[Dx/Dc]$, where Dx and Dc represent the % double-stranded DNA in experimental or control samples respectively [16].

(v) *Nucleoid sedimentation.* Assays for DNA damage were carried out on freeze–thawed cultures. Nucleoid sedimentation detects changes, due to true DNA strand breakage, in the extent of DNA supercoiling in residual nuclear structures (i.e. nucleoids) obtained by exposure of cells to non-ionic detergent and high salt conditions. The current version of the technique is essentially that described by Farzaneh *et al.* [18], adapted as follows: permeabilized cells were filtered through a 35μ monofilament nylon mesh, resuspended in cold PBS A (50 μl containing $1 \times 10^5$ cells) and deposited into 150 μl of lysis buffer (giving a final concentration of 2 mM EDTA, 0.5% (v/v) Triton X-100, 100 mM Tris-(hydroxymethyl)-aminomethane pH 8.0, and 1 M NaCl), over 3.8 ml 15–30% linear sucrose gradients containing 1 mM EDTA and 10 mM Tris-(hydroxymethyl)-aminomethane pH 8.0 and 1 M NaCl. Cells were lysed on top of the gradients for 30 min at room temperature and then centrifuged for 40 min at 25,000 rpm in an MSE superspeed 65 ultracentrifuge using a $6 \times 4.2$ ml swing-out rotor. The sucrose gradients contained 1 μM Hoechst 33342 for the direct determination of the relative (versus control) distance sedimented by the nucleoids visualized using near-u.v. illumination.

## RESULTS

### A. *Induction and repair of bleomycin-induced DNA damage*

Previous studies [2, 19] have indicated that BLM induces a spectrum of DNA lesions, including double strand breaks, single strand breaks and alkali labile damage (representing sites of base loss). In the present study, drug-induced damage has been quantitated by two essentially different techniques, namely: (i) the alkaline denaturation assay (detecting all lesions; Fig. 1) and, (ii) the neutral nucleoid sedimentation assay (selectively monitoring true breaks including repair intermediates of alkali-labile lesions; Fig. 2). Both assays have been calibrated by the X-irradiation of permeabilized cells which preliminary studies have shown to be deficient in DNA strand break ligation (data not shown), thereby providing a true estimate of DNA strand breakage

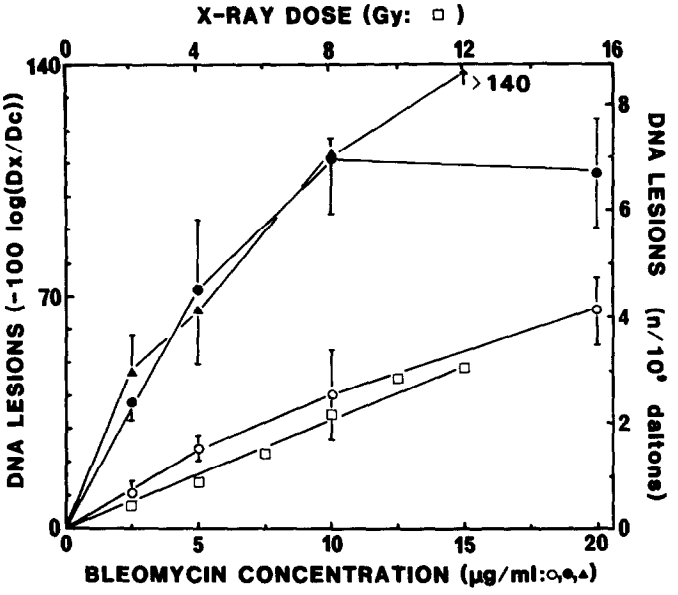


Fig. 1. Comparison of the dose dependent induction of DNA strand breaks (including alkali labile sites) by BLM, BLM + $Fe^{2+}$ and X-radiation, detected by alkaline denaturation. DNA lesion frequencies, measured by the enhancement of DNA unwinding, have been converted to absolute values by reference to the X-ray standards (see text for details). Symbols: ○, BLM alone (mean values from eight experiments, ± SE); ●, BLM + 3 μM $Fe^{2+}$ (mean values from five experiments, ± SE); ▲, BLM + 21.6 μM $Fe^{2+}$ (mean values from two experiments with range shown by error bars); □, X-radiation (representative experiment).

rates. Strand break frequencies, detected by alkaline denaturation can be calculated with reference to the X-ray standards assuming that X-rays induce 2.7 breaks/$10^{10}$ daltons mol. wt DNA/Gy [20]. True strand break frequencies, detected by nucleoid sedimentation, can be calculated by correction of the above induction rate for an assumed 28% of X-ray induced breaks arising from alkali-labile lesions [21].

The results for both assays (Figs. 1 and 2) reveal that BLM is more efficient at DNA lesion-induction at low drug concentrations (<5 μg/ml). Comparison of the results of the two assays at 5 μg BLM/ml yields 15.4 breaks/$10^{10}$ daltons for alkaline denaturation and 0.2 breaks/$10^{10}$ daltons for nucleoid sedimentation. Thus, in this human cell system and under the given drug exposure conditions and within the constraints of the assay methods used, only 1–2% of total lesions detected represent frank breaks.

Figures 1 and 2 also show the effects of the addition of $Fe^{2+}$ (at 3 or 21.6 μM) to drug treatment media on the patterns of DNA lesion-induction. Numerous preliminary experiments established that $Fe^{2+}$ (up to 21.6 μM) alone did not induce detectable levels of DNA strand breaks. However, the DNA damaging potential of BLM, detected by either assay, was enhanced by greater than twofold by the presence of $Fe^{2+}$ ($P < 0.05$ comparing the with and without $Fe^{2+}$ treatments at both 5 and 10 μg BLM/ml for either assay). The results also indicate that the initial $Fe^{2+}$-potentiation of BLM-induced damage at the low drug concentration (2.5 μg/ml) is only detected by the alkaline denaturation assay (Fig. 1) and not by nucleoid sedimentation (Fig. 2). The similarity in the potentiation effects of 3 or 21.6 μM $Fe^{2+}$ on a 5 μg BLM/ml exposure are expected, given the stoichiometry demonstrated for BLM–$Fe^{2+}$ interaction (see Results, Section B). In the presence of 21.6 μM $Fe^{2+}$, levels of drug-induced damage for BLM doses >10 μg/ml were essentially beyond the dosimetric limits of both assays. In the case of nucleoid sedimentation, this is presumably due to the loss of DNA from nucleoid bodies or the linearization of relaxed DNA loops containing double strand breaks.

We conclude that exogenous $Fe^{2+}$ significantly potentiates the intracellular DNA damaging capacity of BLM. Importantly, this effect does not appear to relate to a change in gross cellular repair capacity since total BLM-induced lesions disappear rapidly in cells following treatment, irrespective of the presence or absence of $Fe^{2+}$ during the treatment phase (Table 1).

### B. *Cell survival*

Initial studies on the responses of MRC5CVI to BLM indicated a $D_{37}$ value of 3 μg/ml, which is comparable with the general responses of transformed and non-transformed normal fibroblast strains ($D_{37}$ 1–2 μg/ml; PJS and M C Paterson, unpublished data; [22]). Figure 3 shows the effect of different concentrations of $Fe^{2+}$ on the toxicity of a fixed BLM dose (5 μg/ml; 25 ± 3% survival). Levels of $Fe^{2+}$ alone up to 21.6 μM (1 hr exposure) were not toxic. Unexpectedly, the supply of exogenous $Fe^{2+}$ resulted in a concentration-dependent enhancement of MRC5CVI cell survival. The effect of exogenous $Fe^{2+}$ reaches a plateau at 7.2 μM (51 ± 7% survival) yielding a significant ($P < 0.002$) survival enhancement of approximately twofold. The $Fe^{2+}$ depen-

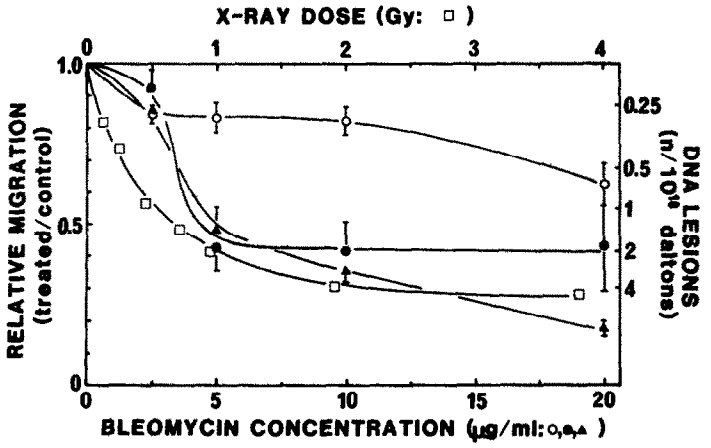


Fig. 2. Comparison of the dose-dependent induction of frank DNA strand breaks by BLM, BLM + $Fe^{2+}$ and X-radiation, detected by neutral nucleoid sedimentation. Absolute lesion frequencies were calculated by reference to the X-ray standard (see text for details). Symbols: ○, BLM alone (mean values from eight experiments ± SE). ●, 3 μM $Fe^{2+}$ (mean values from four experiments, ± SE); ▲, 21.6 μM $Fe^{2+}$ (mean values from three experiments, ± SE); □, X-radiation (representative experiment).

Table 1. Effect of the presence of ferrous iron during the bleomycin treatment of human cells on the induction and subsequent repair of DNA damage

| | DNA strand breakage assayed by alkaline denaturation at $t$ min after bleomycin exposure (5 μg/ml × 1 hr)* | | |
|---|---|---|---|
| $Fe^{2+}$ conc. (μM) | $t = 0$ $N/10^{10}$ daltons | $t = 30$ $N/10^{10}$ daltons | % Lesions unrepaired |
| 0 | 15.4 ± 2.4 | 1.6 ± 0.8 | ~10 |
| 3 | 45.1 ± 3.5 | 4.9 ± 1.9 | ~11 |

* See text for details of DNA strand break frequency calculations.
† Values represent arithmetic means (± SE) for at least four determinations.

dency of the survival enhancement was closely related to the interaction of free BLM with $Fe^{2+}$ assayed independently (Fig. 3).

The survival enhancement action of $Fe^{2+}$ was also confirmed by observing the effect of fixed concentrations of $Fe^{2+}$ (3 and 21.6 μM) on BLM dose–response curves (Fig. 4). There was a clear ability of $Fe^{2+}$ to reduce BLM toxicity resulting in more pronounced upward concave survival curves. The significance ($P < 0.01$) of this effect is demonstrable at 10 and 20 μg BLM/ml comparing $Fe^{2+}$ treated and untreated cultures.

## DISCUSSION

The role of the initial yield of DNA strand breakage in the cytotoxic action of the antitumour antibiotic BLM has been studied by observing the effects of $Fe^{2+}$-induced potentiation of DNA damage. The results are quite unexpected in view of a previous study indicating an overall correlation between the cytotoxicity and DNA damaging effects of various BLM analogues and derivatives [23] and the general consensus of opinion which links bleomycin cytotoxicity with DNA damage (review in ref. 2). It is reported here that despite the clear ability, as expected [6, 7], of exogeneously supplied $Fe^{2+}$ ions to increase greatly the level of BLM-induced DNA strand breakage, the survival capacity of transformed fibroblast cells (using a BLM concentration range which gave approximately 1–5 lethal hits) was significantly enhanced (>2-fold). The findings have implications for the action of the antibiotic *in vitro* and *in vivo* given the critical dependence of drug cytotoxicity on metal ion availability.

Various problems are encountered in interpreting the significance of BLM-induced damage, for example: the possible interactions of residual BLM with DNA during the preparation of cells for damage analysis [24]; the spectrum of lesions induced [2, 19, 25]; the rapid repair kinetics of the lesions together with the low absolute levels of residual damage; and the variation in apparent BLM activity between experiments [26]. The approach adopted in the present study was designed to permit the rapid collection of cells after treatment under conditions which would minimise the effects of ongoing repair, and reduce the DNA-degrading potential of residual drug and drug–metal cation combinations. This was achieved by the permeabilization of cells in the presence of a chelating agent ([6]; PJS, unpublished data). Although the possibility that internalized bleomycin can induce DNA damage during freeze–thaw procedures cannot be excluded entirely, it is reasonable to assume that the levels of drug-associated DNA

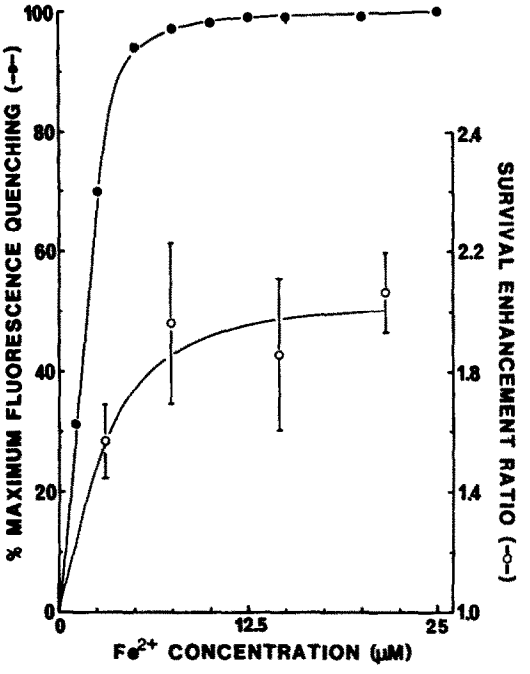


Fig. 3. Comparison of the interaction of $Fe^{2+}$ and BLM, monitored by fluorescence quenching, with the ability of $Fe^{2+}$ to reduce BLM (5 μg/ml for 1 hr) cytotoxicity when present during drug exposures. Data points represent arithmetic means (± SE) of values derived from nine independent experiments. Mean control plating efficiencies were 46 ± 6% (untreated control) and 25 ± 3% (BLM alone). Symbols: ○, survival enhancement ratio (% survival BLM + $Fe^{2+}$/% survival BLM alone); ●, quenching of fluorescence of BLM by $Fe^{2+}$ (assuming maximum effect at 25 μM $Fe^{2+}$).

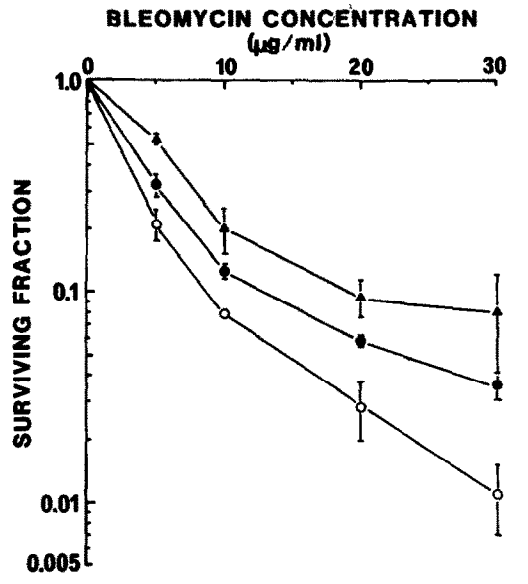


Fig. 4. Effect of ferrous iron on the BLM sensitivity of human cells. Data are arithmetic means (± SE) of collected values from independent experiments. Symbols: ○, BLM alone (five experiments); ●, +3 μM $Fe^{2+}$ (two experiments); ▲, +21.6 μM $Fe^{2+}$ (four experiments).

damage reported here reflect events occurring in intact cells. Furthermore, the current study principally addresses the effects of $Fe^{2+}$ on the initial yields of DNA damage rather than the residual levels of damage. The enhanced DNA degrading activity of BLM in the presence of ferrous iron is probably due to both the low level of $Fe^{2+}$ in the BLM preparation and the replacement of $Fe^{3+}$ ions by $Fe^{2+}$ ions permitting the ferrous oxidase activity of the drug to continue the generation of radicals [4, 6, 7, 27].

Previous cytogenetic studies suggest that the majority of rapidly-repairing single strand breaks are not genotoxic [26, 28]. However, critical targets such as active genes are preferentially digested by bleomycin but may not be distinguished in gross cellular DNA analyses since such active chromatin may represent only 5% of total cellular DNA [30]. Non-toxic damage could act to modify responses to the drug by inducing changes in cellular functions (e.g. modified repair enzyme activity, changes in chromatin organization, delay of cell-cycle traverse, or the extent of inhibition of *de novo* DNA synthesis) which are advantageous to the management of the genotoxic lesions. For example, given the extensive capacity of bleomycin-treated cells to exhibit potentially lethal damage (PLD) recovery (review in ref. 10), enhanced levels of non-toxic (but response-modifying) DNA damage may act to delay cell-cycle progression providing additional time for repair systems to act and effectively facilitating PLD recovery.

On the other hand the potentiation of DNA damage, but not cell killing, by the supply of $Fe^{2+}$, could reflect a change in the site and/or spectrum of lesions. D'Andrea and Haseltine [25], using restriction fragments of DNA, reported that in the presence of exogenously supplied $Fe^{2+}$, the sequence specificity for DNA cleavage was broadened to include TT, AT and TA sequences in addition to increased cleavage at the preferred GC and GT sites. However, other workers [31, 32] using similar cell-free systems reported that exogenous $Fe^{2+}$ reduced the requirement of the reducing agent necessary for the cleavage of DNA by bleomycin but did not alter the cleavage sequence specificity. Such effects are yet to be monitored in intact cells. A two-step mechanism for strand cleavage and the localized generation of radicals on DNA may also permit $Fe^{2+}$ to change the relative frequency of alkali-labile sites and frank DNA strand breaks. However, no evidence of significant changes in the relative frequencies of such lesions (except at low BLM concentrations; 2.5 μg/ml) could be found by comparing the results from the two assays for DNA damage used in the present study. Thus, on balance, there is no evidence that the initial yield of a given class of lesions is actually reduced by the supply of exogenous $Fe^{2+}$ to BLM treatments.

From the above considerations it is clear that several factors could control the sensitivity of a given cell type to BLM under conditions of varying $Fe^{2+}$ supply. A large capacity for PLD recovery or a restricted number of targets for genotoxic damage may explain the resistance of some cell types and it is suggested that facilitation of PLD type recovery should be explored as an explanation for the

reduction of BLM cytotoxicity by $Fe^{2+}$ in cells of moderate drug sensitivity (e.g. transformed fibroblasts).

The conclusions relate to two areas. Firstly, laboratory studies on the action of BLM should recognize the non-genotoxic, but potentially survival-modifying nature of the majority of lesions monitored by conventional assays for DNA damage and repair. The lowest levels of $Fe^{2+}$ which modified drug sensitivity are equivalent to those present in some fresh preparations of growth media (e.g. Hams F12 medium contains an equivalent of 3 $\mu$M $Fe^{2+}$) indicating the care with which treatment conditions should be selected for *in vitro* studies. The varying degrees of upward inflection shown by *in vitro* BLM survival curves [10] are not due to the depletion of $Fe^{2+}$ [9] but may reflect the development of rescue phenomena as the background level of DNA damage increases. Secondly, at the clinical level the cytotoxic action of BLM varies within normal tissues and tumour types [1]. The present findings identify one feature (namely the endogenous level of $Fe^{2+}$ or alternatively cellular oxidation-reduction potential) which can modify the sensitivity of a human cell to BLM in a manner not predicted from basic studies using cell-free systems.

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