Iron requirement for cellular DNA damage and growth inhibition by hydrogen peroxide and bleomycin

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Studies with *Euglena gracilis* and HL-60 cells have assessed the need for intracellular iron in the mechanisms of inhibition of cell growth and DNA damage by H_2O_2 and bleomycin. Cell culture media were directly depleted of iron in order to deprive cells of nutrient iron. Major pools of cellular iron were reduced in both cell types. Nevertheless, iron bound in e.s.r.-observable haem protein and ribonucleotide diphosphate reductase in HL-60 cells was not decreased. In both control cell populations, there was a concentration-dependent reduction in proliferation and cell survival caused by H_2O_2. In comparison, the proliferation rates of both iron-deficient cell types were significantly less sensitive to H_2O_2. H_2O_2 caused concentration-dependent single-strand breakage in DNA in control HL-60 and *Euglena gracilis* cells. Iron deficiency reduced the amount of strand breaks in HL-60 cells at each concentration of H_2O_2 used. Single-strand breakage caused by H_2O_2 in *Euglena gracilis* was a direct function of the concentration of iron in which the cells had been grown. Growth inhibition and both single- and double-strand DNA damage caused by bleomycin were substantially reduced or eliminated in iron-deficient cells. Copper bleomycin behaved like metal-free bleomycin when assayed for the capacity to cause DNA damage in iron-normal and iron-deficient HL-60 cells. In contrast, iron bleomycin was equally active under the two conditions in these cells.

INTRODUCTION

Activation of molecular oxygen to toxic species is thought to be a causative factor in a number of diseases and toxicological reactions and involved in the mechanisms of action of therapeutic drugs [1,2]. During the reduction of O_2, hydroxyl radicals may be generated, which are highly reactive species and can, for example, abstract hydrogen atoms indiscriminately from organic compounds in their vicinity [3–5]. Hydroxyl radicals can be produced by the Fenton reaction, in which a reduced transition metal ion or complex (M^{n+}) delivers an electron to H_2O_2:

$$H_2O_2 + M^{n+} \rightarrow {}^{\cdot}OH + OH^- + M^{(n+1)+} \quad (1)$$

In biological systems, M^{n+} is usually identified as Fe^{2+} or Cu^+. A number of investigations into the biochemical and biological features of oxidative stress have utilized H_2O_2 as a model oxidant [6–13]. H_2O_2 causes lipid peroxidation [7], DNA damage [6–7,10–12], and cell killing [6–10,12,13].

To probe the role of intracellular metal ions in the deleterious activities of H_2O_2, 1,10-phenanthroline or other metal-binding ligands have also been utilized to compete for cellular metals, particularly iron, thereby reducing their availability to participate in the Fenton reaction [14,15]. Although 1,10-phenanthroline has been effective in inhibiting the cytotoxicity of H_2O_2, its potential for multiple interactions with cells complicates its use [16–18].

Bleomycin (Blm), a widely used anti-tumour agent, is known to cause single- and double-strand breaks in cellular DNA [19,20]. On the basis of chemical studies, it is understood that DNA cleavage can be initiated by an activated form of iron-bleomycin, HO_2-Fe(III)Blm or a related species, which can abstract the C-4′ hydrogen atom from the deoxyribose moiety of the DNA backbone [21–24]. However, it is also known that cuprous ion and bleomycin can react with DNA in the presence of O_2 to cause strand breaks [25].

It has been difficult to extend these results to cells to determine whether metals are required for the cytotoxic activities of this drug. Iron-, copper- and metal-free bleomycin were each active against the Ehrlich ascites tumour in mice [26]; Blm remained active against this tumour in iron- and copper-deficient mice [16]; metal-restricted incubation conditions did not affect the cytotoxic activity of Blm or its Fe or Cu complexes *in vitro*, though short-term co-incubation of cells with a toxic level of 1,10-phenanthroline did decrease Blm-induced DNA damage [16,26,27].

Further investigation of the impact of nutrient iron deficiency on the cytotoxic properties of H_2O_2 and Blm has been undertaken with human leukaemia cells (HL-60) and with the micro-organism, *Euglena gracilis*. Each of these systems has the advantage that cells can be cultured in chemically defined media, in which the iron content may be readily controlled without recourse to the use of metal-chelating agents.

EXPERIMENTAL

Materials

1,10-Phenanthroline was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Pyridoxal hydrochloride, isonicotinic hydrazide, RPMI 1640 growth medium, gentamicin, bovine insulin, streptomycin, and penicillin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ultrapure H_2O_2 was acquired from J. T. Baker, Phillipsburg, NJ, U.S.A. [2-^{14}C]dT (specific radioactivity, 50 mCi/mmol) and [methyl-^{3}H]dT (specific radioactivity, 2 Ci/mmol) were obtained from RPI Products, Mt. Prospect, IL, U.S.A. Bleomycin was a gift from the

Abbreviations used: Blm, the clinical mixture of bleomycins, mostly A_2 and B_2; CoPBS, phosphate-buffered saline containing 0.5 mM $CoCl_2$; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; Fe–PIH, the 1:2 complex of Fe and pyridoxal isonicotinoyl hydrazone; HL-60 cells, human promyelocytic leukaemic cells; RDR, ribonucleotide diphosphate reductase; TPOH, tetrapropyl ammonium hydroxide.

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Bristol Myers Co. (Syracuse, NY, U.S.A.) and was used as the clinical mixture (Blm). Fe(III)- and Cu(II)Blm were made by direct addition of Fe^{3+} or Cu^{2+} to solutions of the drug. Christopher Chitambar (Medical College of Wisconsin, Milwaukee, WI, U.S.A.), provided the original culture of human promyelocytic leukaemia (HL-60) cells growing in defined medium. Cultures of the unicellular organism, *Euglena glacilis* strain Z no. 15-2802, were purchased from the Culture Collection of Algae (Carolina Biologicals, Burlington, NC, U.S.A.). All other chemicals were reagent grade or its equivalent. Solutions were prepared with distilled, deionized water and acid- or EDTA-washed glassware.

Fe–PIH was prepared from ferric citrate and pyridoxal isonicotinoyl hydrazone (PIH) as described [28]. H_2O_2 concentrations were determined using an absorption coefficient of 40 $M^{-1} \cdot cm^{-1}$ at 240 nm as described by Aebi [29]. Fresh solutions were made by dilution with water and stored at 4 °C.

Growth of cells in culture

HL-60 cells were grown in suspension culture in Falcon tissue-culture flasks at 37 °C with 6 % (v/v) CO_2 in serum-free medium comprised of RPMI 1640, pH 7.1, with the following additions: 2 g/l sodium bicarbonate, 30 mg/l bovine insulin, 0.25 ng/l sodium selenite, 100 units/l penicillin, 0.1 mg/l streptomycin and 50 μg/l gentamicin. The medium was replaced every 3–4 days.

E. gracilis was grown in the presence or absence of light (GE Gro and Sho Plant Light at 27 °C) in modified Hunter's medium in which glutamic acid and $MnCl_2$ were substituted for ammonium glutamate and $MnSO_4$ respectively [30]. Iron in the medium (36 μM) was supplied as $FeSO_4$. The final pH of the medium was 3.6.

Iron-deficient medium

Serum-free RPMI 1640 medium prepared as above without Fe–PIH contained 1.6 μM Fe. When incubated in this medium for 2 days, HL-60 cells had significantly less intracellular iron and a lower proliferation rate than control cells. Stringently iron-depleted medium (< 10 nM) for culture of *E. gracilis* was prepared by omitting $FeSO_4$ from the growth medium followed by its passage through a $Mg(OH)_2$ gel to exchange Mg for Fe, as previously described [16]. Iron concentrations were measured with an Instrumentation Laboratory-357 Atomic Absorption Spectrophotometer in the atomic emission mode.

Cell survival studies

HL-60 cells were initially grown with or without 20 μM Fe–PIH for 48 h to establish iron-normal and iron-deficient cultures. At this time each population of cells was in logarithmic growth. Then both cultures were transferred to iron-deficient medium to limit extracellular complexation of iron in subsequent incubations with drugs. Cells were routinely counted and the number of cells impermeable to Trypan Blue dye (Sigma Chemical Co.) determined.

Dark-adapted *E. gracilis* cells used in the Blm experiments were grown in an iron-deficient medium for at least 48 h. For experiments with H_2O_2 the iron content of dark-adapted cultures was adjusted by placing them in medium containing less than 10 nM Fe supplemented with specific levels of iron for 48 h. Then each population was taken up into iron-depleted, 0.4 % unbuffered NaCl and treated with various concentrations of H_2O_2 for 1 h in the dark. The cells were then washed and incubated for 11 h in medium containing 9 nM iron. The half-life of H_2O_2 in iron-deficient medium is about 2 h.

DNA damage in HL-60 cells

Single and double-strand breakage in HL-60 cells was determined by alkaline elution following the procedure of Kohn et al. modified as described [19,31]. HL-60 cells (7×10^5/ml) were grown in the presence or absence of Fe–PIH for 72 h. Cellular DNA was labelled by addition of 0.01 μCi/ml [2-^{14}C]dThd during the 24 h. Ehrlich ascites tumour cells, grown for 24 h in the presence of 0.5 μCi/ml [*methyl*-^{3}H]dThd and irradiated with 300 Rad of ^{137}Cs γ-radiation in PBS at 0 °C to introduce a uniform number of single-strand breaks, were used as internal standards. HL-60 cells grown with or without iron were washed and resuspended in fresh medium. Treatments with species of Blm and H_2O_2 were performed in 1 ml wells in tissue-culture plates (1.2×10^6 cells in 1.2 ml of medium) at 37 °C.

After treatment with forms of Blm, aliquots of medium containing 1×10^6 cells were added to ice-cold PBS containing 0.5 mM $CoCl_2$ (CoPBS) to stop strand scission and to inhibit post-lysis DNA degradation. Internal standard cells (5×10^5) were added, followed by addition of cells to 2 μm-pore-size polycarbonate filters (Nucleopore, Fullerton, CA, U.S.A.) and washed with additional CoPBS followed by PBS to remove cobalt [19]. Cells were lysed with 5 ml and 2 ml of 2 % (w/v) SDS plus 25 mM disodium EDTA, pH 10.0, (both from Sigma Chemical Co.) in the upper chambers of elution barrels. Proteinase K, 0.5 unit/ml (Sigma Chemical Co.) was present in the second lysis solution. After both additions, solutions were allowed to pass through the necks of elution barrels by gravity flow until the neck between lower and upper chambers was reached, at which times the filter outflow was stoppered. Proteinase digestion was conducted for at least 40 min. Then, tetrapropylammonium hydroxide (TPOH) (RSA Corp., Ardsley, NY, U.S.A.), containing 0.1 % SDS and 20 mM EDTA, pH 12.3, was added to the upper chambers. The denatured DNA on the filters was eluted with the aid of a peristaltic pump. Fractions (1.4 ml) were collected every 40 min.

The same procedure was followed when cells were treated with H_2O_2, except that to terminate exposures 1×10^6 HL-60 cells from each treatment were added to 15 ml of ice-cold PBS. This method was as effective, as cells were placed in cold PBS containing 10 μg/ml catalase to consume residual H_2O_2.

Ecolite liquid-scintillation cocktail (ICN Biomedical, Irvine, CA, U.S.A.) was added to each elution fraction, lysate portion, HCl/NaOH-treated filter, and NaOH-wash of barrels plus tubing. Radioactivity was determined using a Beckman Instruments Co. model LS-3801 liquid-scintillation counter.

Results for HL-60 cells are presented graphically (see Figure 6). The fraction of the total cell ^{14}C-labelled DNA contained in the two lysis fractions is indicated on the ordinate axis by the difference between 1 and the fraction of ^{14}C-labelled DNA remaining on the filter when the fraction of ^{3}H-labelled DNA is 1.0. This ^{14}C label represents DNA damaged by double-strand breaks [19,32,33]. After addition of TPOH, elution of ^{14}C-labelled DNA relative to internal standard ^{3}H-labelled DNA represents single-strand breakage. Control elutions of untreated, iron-deficient cells were identical with those of iron-normal cells.

DNA damage in *E. gracilis*

Alkaline elution was also used to assess damage to DNA in *E. gracilus*. Because this organism does not transport thymidine, a

fluorescence method of DNA quantification was modified to follow the elutions [34]. After assembly of the elution apparatus, 30 ml of cell lysis solution, consisting of 0.05% SDS plus 25 mM EDTA, pH 10.0, and then 30 ml of 20 mM phosphate buffer plus 0.9% NaCl, pH 7.4, were pumped through the filters to remove small particles over a period of 8 h. Then (2–5) $\times 10^6$ cells, treated with either H_2O_2 or Blm, were washed three times with ice-cold 0.4% NaCl (0.5 mM $CoCl_2$ was included in the second wash in the case of Blm) and layered on the filters in a 0.4% NaCl solution, which was rapidly pumped through the filter until 2–3 mm depth of liquid remained.

To quantify the DNA eluted before alkaline elution, the lysis solution (1 ml) and two successive 5 ml washes of 25 mM EDTA, pH 10.0, were pumped through the filter at a flow rate of 2.5 ml/h and collected. Then, 9 ml of 2% TPOH plus 25 mM EDTA, pH 12.3, were passed over the filter at 1.2 ml/h and collected in three 3 ml fractions. Finally, DNA remaining on each filter after alkaline elution was solubilized by shaking filters for 4 h in 1 ml of TPOH containing 0.1% SDS and 25 mM EDTA. These fractions were diluted into buffer used in the fluorescence measurements, which contained 15 mM sodium citrate, 154 mM NaCl, pH 7.0, 0.01% sodium azide and 0.01% SDS. The resultant solutions were stored at 4 °C. Measured DNA concentrations were stable for 1 week. There was no effect of cell number on the elution characteristics of control or damaged DNA between 2×10^6 and 1.6×10^7 cells per filter. The DNA content of these cells was 24 ± 1.5 pg/cell according to the diphenylamine test [35].

To determine DNA concentrations, 5 μl of combined lysis and EDTA washes, 15 or 25 μl of alkaline eluate, or 2.5 to 5 μl of solution of DNA solubilized from filters were mixed with 3.0 ml of the buffer described above, which also contained 0.15 μM bisbenzimide (Hoechst 33258 dye, Sigma Chemical Co.) [34]. Then after excitation of the dye at 350 nm, the enhancement of fluorescence emission at 440 nm due to DNA binding of the dye was determined with an SLM 9000 spectrofluorimeter. The fluorescence measurements obtained from control elutions without *E. gracilis* were subtracted and the resulting values referred to standard curves of fluorescence enhancement versus concentration of calf thymus DNA, determined by the diphenylamine test [35]. Generally, fluorescence of blanks accounted for 5–10% of the total fluorescence in samples containing DNA as described later in Tables 2 and 3. In addition, after readings were taken, internal standards of known quantities of calf thymus or *E. gracilis* DNA were added and the fluorescence of the samples remeasured to check the adequacy of the standard curve. These alkaline elution and DNA quantification methods gave results comparable with ones obtained with the standard procedure described above when tested with Ehrlich cells exposed to ionizing radiation or Blm.

To minimize extracellular reaction of iron with Blm or CuBlm during incubations, all cells, iron-normal and iron-deficient, were transferred to iron-deficient medium before they were treated with drugs.

Measurement of iron content and distribution in *E. gracilis* and HL-60 cells

Photosynthetic *E. gracilis* was grown in 1 litre growth tubes at ambient temperature under fluorescent lights. Sterile filtered water-saturated air was pumped through the system to maintain agitation and aeration. Cultures were initiated with 1×10^4 cells/ml in 1 litre of normal or iron-deficient medium. At the end of 7 days, 5×10^9 *E. gracilis* cells from each medium were isolated by centrifugation, washed with 10 ml of 0.15 M KCl/g wet wt. of pellet and recentrifuged. The cells were resuspended in 50 mM Tris buffer, pH 8.6, (5 ml/g wet wt of pellet) containing 3 μl of 2-mercaptoethanol/5 ml of suspension and sonicated with a Branson cell disruptor at 60% duty cycle and 75 W, for 2 min. The supernatant was separated by ultracentrifugation, applied to a 3 cm $\times$ 6 cm Sephadex G-75 column, and eluted at 4 °C using 50 mM Tris buffer, pH 8.6.

To determine the total iron content of dark-grown *E. gracilis*, cells grown to late logarithmic phase in the normal medium were washed twice with 50 ml portions of iron-free medium and then inoculated into a set of media containing different concentrations of iron (1.7×10^5 cells/ml). After incubation for 108 h with occasional shaking, all cultures contained about 2×10^8 cells. Cells were harvested, washed in iron-depleted buffer, and digested in 2–8 ml of Ultrex nitric acid. Iron contents were measured by flame emission spectroscopy.

Spin trapping

E.s.r. spin trapping was performed on normal *E. gracilis* growth medium in the presence and absence of added H_2O_2. Measurements of trapped radicals were performed with a Varian E-line Century series spectrometer as described previously [36].

E.s.r. studies of HL-60 cells

E.s.r. spectroscopy was used to assay the presence of different Fe(III) species in HL-60 cells. Cells were grown in tissue-culture flasks in the presence or absence of 20 μM Fe–PIH for 48 h. Then, 1×10^8 cells from each treatment were centrifuged into 1 ml of packed cells. After adding 0.2 ml of PBS, suspensions were transferred into a glass tube and frozen in liquid nitrogen. Scans to detect paramagnetic species of iron were run at an applied field of 0.205 T and a sample temperature of -196 °C. Spectra were also taken to demonstrate the presence of a tyrosyl radical associated with an oxo-bridged iron-dimer signal which is characteristic of ribonucleotide reductase [37]. A 0.02 T scan was used at an applied field of 0.325 T with the sample at -196 °C.

RESULTS

Effects of iron deficiency on *E. gracilis* iron content and distribution

Total iron content was examined in dark-grown *E. gracilis* as a function of iron status. As seen in Table 1, iron concentration changed from 6 to 150 μmol/g wet weight over a 1000-fold range

Table 1 Iron concentration in *E. gracilis* as a function of iron in the growth medium

Iron concn. in medium (nM)	Iron content of cells (μmol/g wet weight)
10	6.2
15	9.8
18	14
30	24
45	34
167	40
2000	140
10000	150

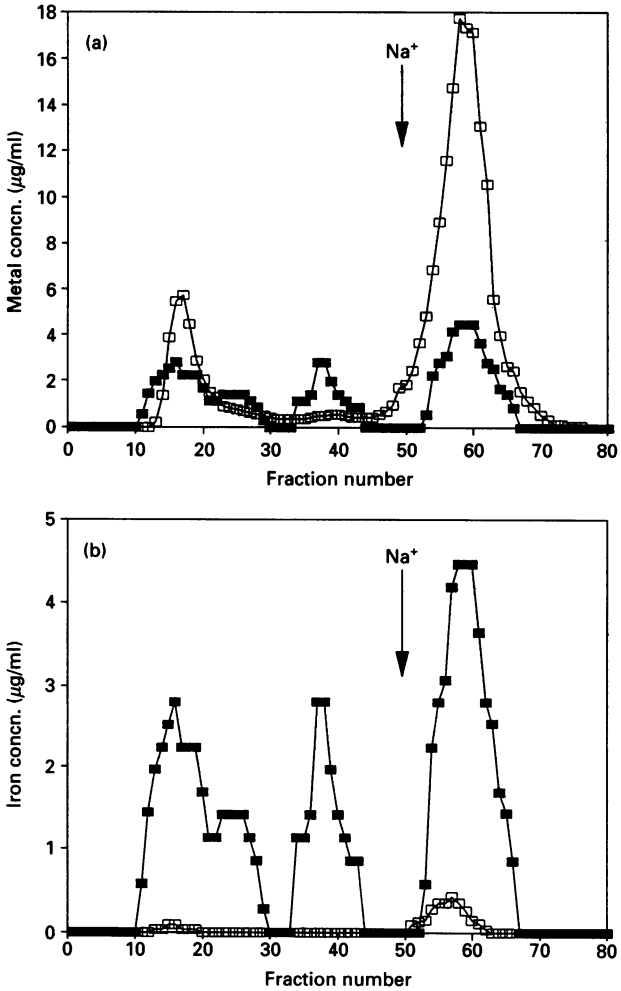

Figure 1 Sephadex G-75 chromatographic profile of iron in *E. gracilis* cytosol

Cytosols were extracted from 5×10^9 cells grown in (**a**) iron-sufficient medium (■, Fe; □, Zn); (**b**) iron-sufficient (■) and iron-deficient (□) medium. The profile of Zn in (**b**) was similar to that in (**a**). Cytosols were fractionated on a 3 cm × 60 cm Sephadex G-75 column. Total cellular iron was reduced by 97% in iron-deficient cells.

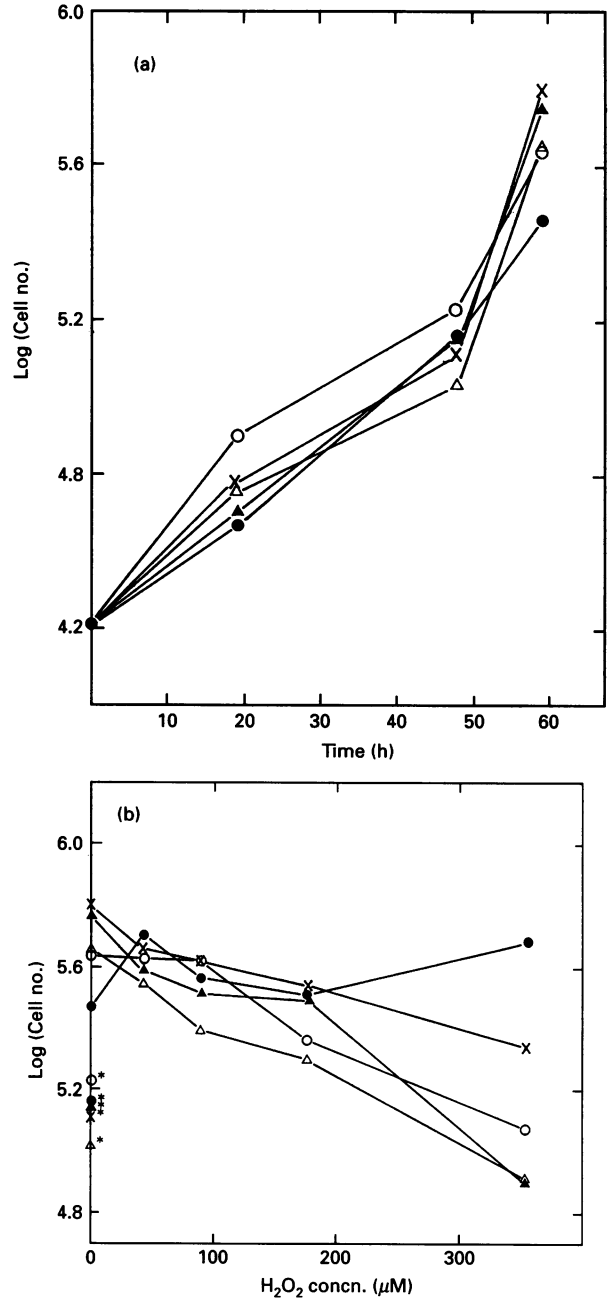

Figure 2 Effects of H_2O_2 on growth of *E. gracilis* as a function of iron status

Cell populations were grown in medium for 48 h containing less than 10 nM iron to which was added 0 (●), 9 (X), 18 (▲), 36 (○) and 180 (△) nM Fe (**a**). Then they were exposed to 0, 44, 88, 176, and 352 μM H_2O_2 for 1 h, washed free of H_2O_2 and incubated for 11 h (**b**). (**b**) * shows cell concentrations of each group at 48 h (**a**) just before H_2O_2 additions. The error in cell counts was ±5%.

of initial iron concentrations in the growth medium. It appeared to plateau between 2 and 10 μM extracellular iron. All of these concentrations sustained cell proliferation. Supernatant material was obtained from 5×10^9 cells grown in iron-normal (36 μM Fe) and iron-deficient (20 nM Fe) medium after lysis and ultracentrifugation. Each sample was then fractionated by Sephadex G-75 chromatography. Three distinct iron pools were observed in cells grown under iron-normal conditions (Figure 1). Spectrophotometric characterization of the middle band showed that it contained a reduced haem protein ($\lambda_{max.}$: Soret band, 416 nm; α and β band, 520 and 552 nm respectively), but this only accounted for about 10% of the iron in the peak. Growth in the iron-deficient medium nearly abolished the first two pools and reduced the third pool by a factor of 10.

Effects of H_2O_2 on the growth of *E. gracilis* cells

It was observed that iron-normal cells in control medium were less sensitive to H_2O_2 than similar cells in iron-deficient medium during a long-incubation experiment. For example, when 1×10^4 iron-normal cells were incubated with 100 μM H_2O_2 for 120 h, the percentage of untreated control growth was 96% for cells maintained in the iron-normal medium and 31% in the iron-deficient medium. It was suspected that H_2O_2 was being decreased by reaction with iron in the medium. This hypothesis was confirmed by means of an e.s.r. spin-trapping experiment. After addition of H_2O_2 to the cell-free iron-normal medium in the

presence of the spin trap, 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), a clear signal diagnostic of the presence of hydroxyl radicals was seen which was hardly evident in the iron-deficient medium (results not shown). It probably arose from reaction 1 and involved the participation of various ligated forms of Fe^{2+} in the medium. Therefore, subsequent studies on the effects of H_2O_2 on proliferation of *E. gracilis* cells were performed by placing cells into iron-deficient medium before reaction with H_2O_2.

Dark-cultured *E. gracilis* cells were grown in a set of media differing in iron concentration for 2 days. Then, after a 48 h period to establish proliferation kinetics (Figure 2a), cell populations were exposed to a range of H_2O_2 concentrations for 1 h in the medium containing no added iron, washed free of extracellular H_2O_2 and placed back in fresh, iron-free medium containing 9 nM added iron. The small amount of iron was included to support normal proliferation kinetics. Figure 2(b) shows the changes in cell number over the next 11 h as a function of H_2O_2 concentration. Concentration-dependent effects of H_2O_2 on cell viability were detected in cells grown in 18, 36 and 180 nM Fe. At 9 nM added iron, growth inhibition was observed only with 352 μM H_2O_2, whereas without added iron no concentration of H_2O_2 affected cell proliferation. These results clearly show that H_2O_2 toxicity is directly dependent on both cellular iron content and H_2O_2 concentration, with an apparent breakpoint in the iron effect between 9 and 18 nM added iron.

DNA breakage in *E. gracilis*

E. gracilis cells were grown in the dark in medium containing graded concentrations of iron for 3 days. Then each cell population was transferred to iron-depleted medium containing less than 10 nM iron (with no added iron) and exposed to 88 μM H_2O_2 for 1 h at 22°, after which time the extent of breakage of DNA was assessed. According to Table 2, at the lowest levels of added iron, 0 and 9 nM, there was no apparent double-strand and little or no single-strand cleavage of DNA. In cells grown in 18, 36, and 180 nM iron, H_2O_2 treatment elevated DNA in the lysis fraction by a factor of approx. two and increased the proportion of DNA eluted from the filter during the alkaline elution by a factor of four. An iron requirement for DNA breakage by H_2O_2 was evident in this experiment. Its sharp concentration dependence was similar to that described in Figure 2(b) for the growth effect of H_2O_2 in the presence of various levels of intracellular iron.

Effects of iron on HL-60 growth and intracellular iron content

Fe–PIH was used as a source of cellular iron in the defined growth medium for HL-60 cells [28]. The proliferation rate increased at low concentrations of added Fe–PIH, but then decreased above 20 μM Fe–PIH (Table 3). Apparently, at higher concentrations, the complex itself became moderately inhibitory of growth. Importantly, in the absence of added iron, cells grew at only a slightly lower rate for 3–4 days relative to control cultures containing 20 μM Fe–PIH. Thus all subsequent experiments were performed between 48 and 72 h after cells had been dispersed in either normal or iron-deficient media. Sephadex G-75 separation of cytosolic extracts from 2×10^8 cells after 72 h growth in the absence of Fe–PIH revealed a 5-fold decrease in high-molecular-mass iron levels taken to represent ferritin-bound iron.

Further analysis of cellular iron was performed by e.s.r. spectroscopy. The loss of a significant high-spin ferric iron signal

Table 2 DNA damage caused by H_2O_2 in *E. gracilis*

The DNA damage is shown in three fractions: Lysis DNA (double-strand damage), DNA eluted under alkaline conditions (single-strand damage), and DNA remaining on the filter after alkaline elution. Mean ± S.D. of total DNA in each set of cells is 26.0 ± 2.7 pg/cell. Control cells were without H_2O_2 treatment; the remaining members of the column were exposed to 88 μM H_2O_2.

[Fe]* added (nM)	DNA damage (% of total)		
	Lysis	Alkaline	Filter
Control	8.8	8.0	83
0	8.2	9.3	82
9	9.6	13	77
18	22	33	44
36	11	43	46
180	20	36	44

* Iron concentration added to medium containing less than 10 nM iron.

Table 3 Proliferation of HL-60 cells in medium containing different levels of Fe–PIH

HL-60 cells were grown for 72 h and then counted. Control cells in 20 μM Fe–PIH normally grew by a factor of three over this period.

[Fe–PIH] (μM)	Proliferation (% of control)
0	72
10	83
20	100
50	83
100	67

believed to be Fe–PIH ($g = 4.1$) may be seen in a comparison of the spectra of iron-normal and iron-deficient cells (Figures 3a and 3b). A probable haem iron signal was also present in the spectrum and remained with iron-deficient cells (Figures 3a and 3b). Its identification was based on a similarity with the high-spin haem signal of prostaglandin H synthase (g values = 6.6, 5.4 and 2.0) [38]. In addition, the tyrosyl radical associated with the oxy-bridged iron protein ribonucleotide reductase (RDR) was not lost in iron-deficient cells (Figures 3c and 3d). Therefore, although the amount of iron significantly declined during growth in the absence of Fe–PIH, some iron was still present in key sites to maintain cell function.

Response of HL-60 cells to H_2O_2

Normal cells grown in 20 μM Fe–PIH and cells depleted of iron were exposed to 0.5 and 5 μM H_2O_2 in iron-deficient medium. As with iron-deficient *E. gracilis*, iron-deficient HL-60 cells displayed reduced growth inhibition in comparison with iron-normal cells (Figure 4). At the lowest H_2O_2 concentration, iron deficiency almost abolished the effect of the oxidant on growth (Figures 4a and 4b). In accord with the results with *E. gracilis*, the effect of iron deficiency became less pronounced at higher H_2O_2 concentrations. This is clearly indicated in the set of values in parentheses in Figure 4(a), as defined in the legend.

The effect of iron deficiency on H_2O_2-induced DNA damage was also assessed. Cells were treated with 5, 10 or 25 μM H_2O_2 for 1 h before DNA alkaline elution. At each concentration, iron-deficient cells exhibited less single-strand and double-strand

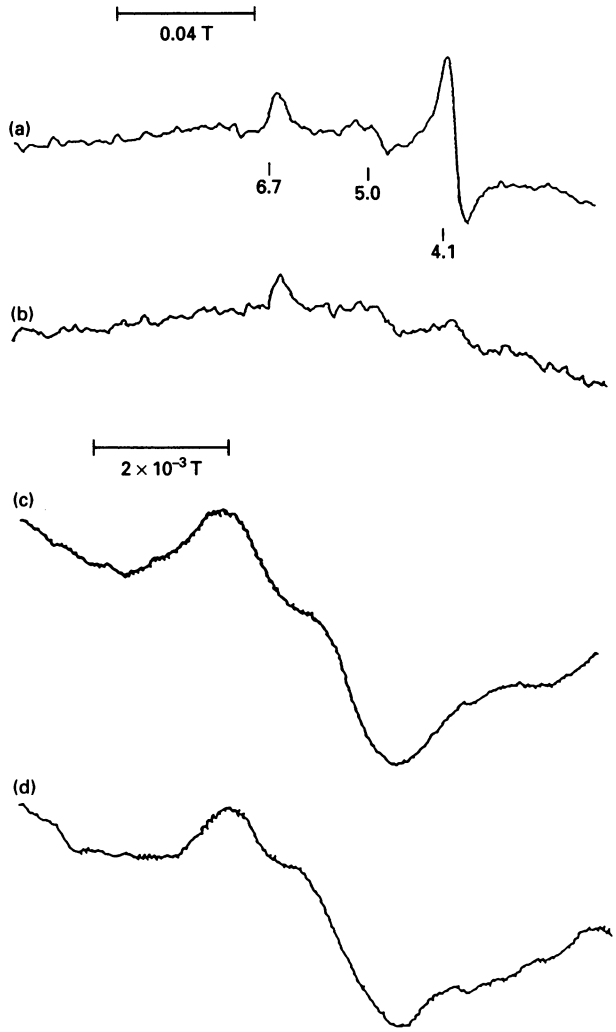

Figure 3 E.s.r. of HL-60 cells to determine the presence of iron(III)

E.s.r. spectroscopy was performed on 1 ml of packed HL-60 cells which had grown for 72 h in the presence (**a**, **c**), or absence (**b**, **d**) of Fe–PIH. Spectra (**a**) and (**b**) scan for cellular iron; (**c**) and (**d**) for the presence of the tyrosyl radical of RDR. Spectrometer conditions: temperature, 77 K; modulation frequency, 100 kHz; modulation amplitude, 1×10^{-3} T; time constant, 0.5 s; incident microwave power, 100 mW; microwave frequency, 9.1 gHz.

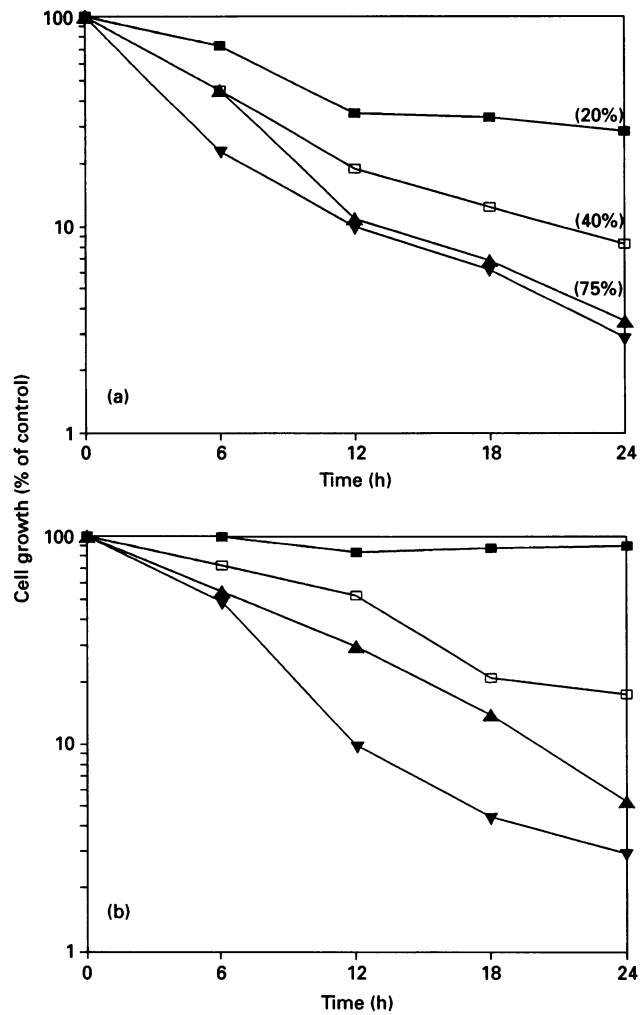

Figure 4 Comparison of growth of iron-sufficient or iron-deficient HL-60 cells during H_2O_2 exposure

Cells grown in the presence (**a**) or absence (**b**) of Fe–PIH were exposed to 0.5 μM (■), 1 μM (□), 3 μM (▲), or 5 (▼) μM H_2O_2. Counts were taken every 6 h and compared with control growth: % control cells = (concentration of control cells)/(concentration of cells in treated medium) × 100%. In parentheses in (**a**) are the ratios of ordinate values in panels (**a**) and (**b**) at 24 h × 100. The error in cell counts was ±5%. Iron-normal cells double every 24 h; the deficient cells double every 60 h.

breakage than iron-normal cells (Figure 5). Analysis of the initial slopes of the alkaline elution curves revealed an approximate 2-fold decrease in DNA damage in iron-deficient treated cells in comparison with iron-normal cells between 5 and 10 μM H_2O_2. Above this concentration of H_2O_2 both sets of cells approached similar indistinguishable levels of damage. It appears that reduction of iron in HL-60 cells lessens, but does not totally prevent, the single-strand cleavage of DNA by these concentrations of H_2O_2.

Growth of HL-60 cells as a function of iron and Blm

Cells that had previously been grown in normal or iron-depleted medium for 48 h were transferred to the iron-deficient medium and treated with Blm. According to Table 4, over the next 12 h neither concentration of drug had any effect on the extent of cell proliferation in iron-deficient cells relative to untreated iron-deficient controls, whereas under iron-normal conditions both levels of Blm almost completely inhibited growth of the cell populations. Thus iron deficiency counteracts the growth inhibitory action of Blm.

Blm-induced DNA damage in HL-60 cells as a function of iron concentration

The impact of iron deficiency on the DNA damage caused by species of Blm was also examined. Figure 6 shows a representative experiment from among five that were performed on the effects caused by a 1 h incubation of HL-60 cells with 10 μM Blm on the integrity of DNA in control and iron-deficient cells. In iron-normal HL-60 cells a large portion of the damage was due to double-strand cleavage [19,32,33]. It is evident in iron-deficient cells that double-strand cleavage was significantly reduced,

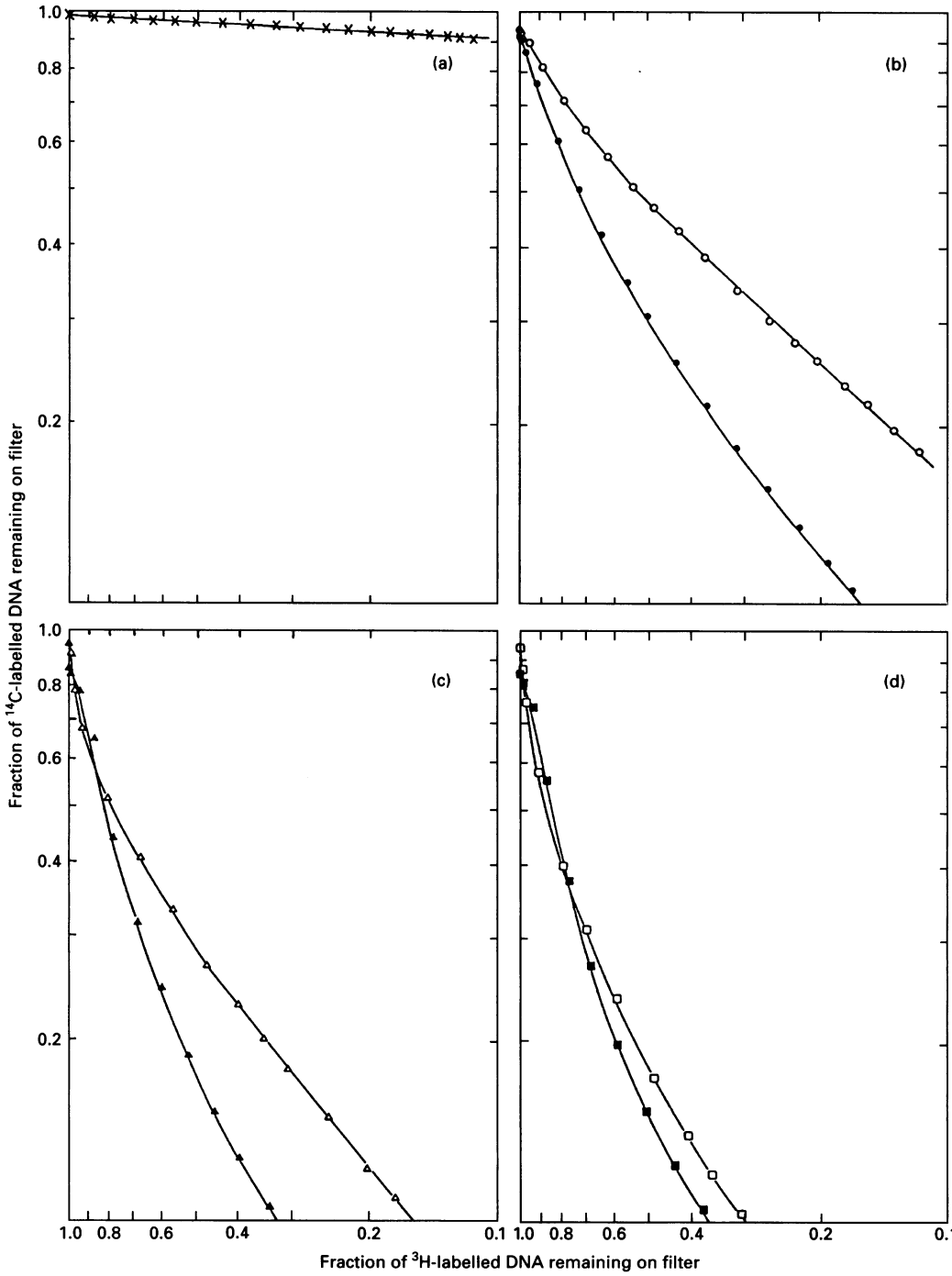

Figure 5 Alkaline elution of DNA from iron-normal or iron-deficient HL-60 cells exposed to various H_2O_2 concentrations

Control cells (**a**), treated for 1 h with 5 μM (**b**), 10 μM (**c**), or 25 μM (**d**) H_2O_2. Closed symbols denote cells grown previously for 48 h in the presence of Fe–PIH, whereas open symbols denote cells grown in the absence of Fe–PIH for 48 h.

almost to control levels. These findings are consistent with the need for iron in the mechanism of DNA strand cleavage by Blm.

This experiment was repeated three times using 25 μM Blm, FeBlm and CuBlm. According to Figure 7(a), iron deficiency also reduced the DNA damage caused by this concentration of Blm, inhibiting the double-strand component by 50%. In contrast, FeBlm was as effective in iron-deficient cells as in iron-normal HL-60 cells (Figure 7b). Indeed, FeBlm induced both more double- and single-strand breakage than Blm.

Finally, CuBlm was tested in this experiment; its activity was attenuated in iron-deficient cells to a similar extent to that of Blm (Figure 7c). Thus, unlike FeBlm, CuBlm was not independently functional in these cells. Under comparable conditions, Ehrlich cells take up similar amounts of tritiated forms of Blm, FeBlm

Table 4 Inhibition of HL-60 cell proliferation by Blm

The data shown are for one of four separate experiments. Each point has an error of less than ±5% of the number. Values in parentheses represent the fraction of the original cell count.

		Cell proliferation (% of untreated control)			
		Fe-normal cells		Fe-deficient cells	
[Blm] (μM)	Incubation time...	6 h	12 h	6 h	12 h
5		83 (0.98)	82 (0.87)	100	100
10		80 (1.16)	69 (0.97)	98	97

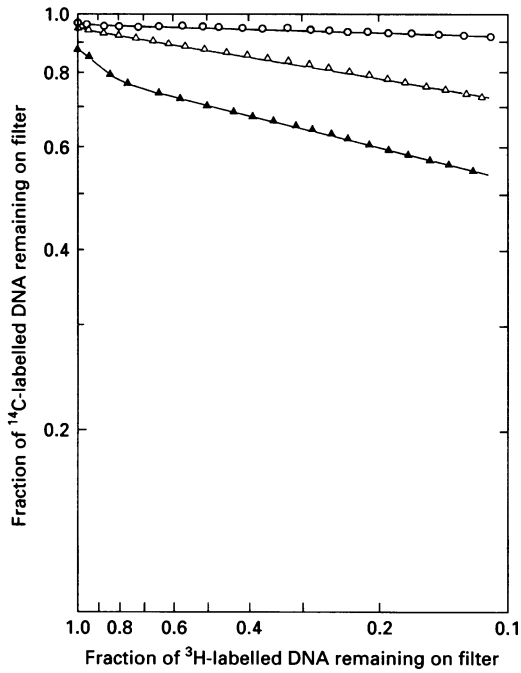

Figure 6 Alkaline elution of HL-60 cell DNA after exposure of cells to Blm

Cells (7×10^5/ml) were grown in iron-normal (20 μM Fe–PIH) or iron-deficient medium (0 μM Fe–PIH) for 48 h. Then, the iron-normal and iron-deficient cells were placed in fresh iron-deficient medium (7×100^5 cells/ml) before treatment with 10 μM Blm for 1 h. (○), Untreated cells representative of either iron-normal or iron-deficient cells; (△), iron-normal cells plus Blm; (▲), iron-deficient cells plus Blm.

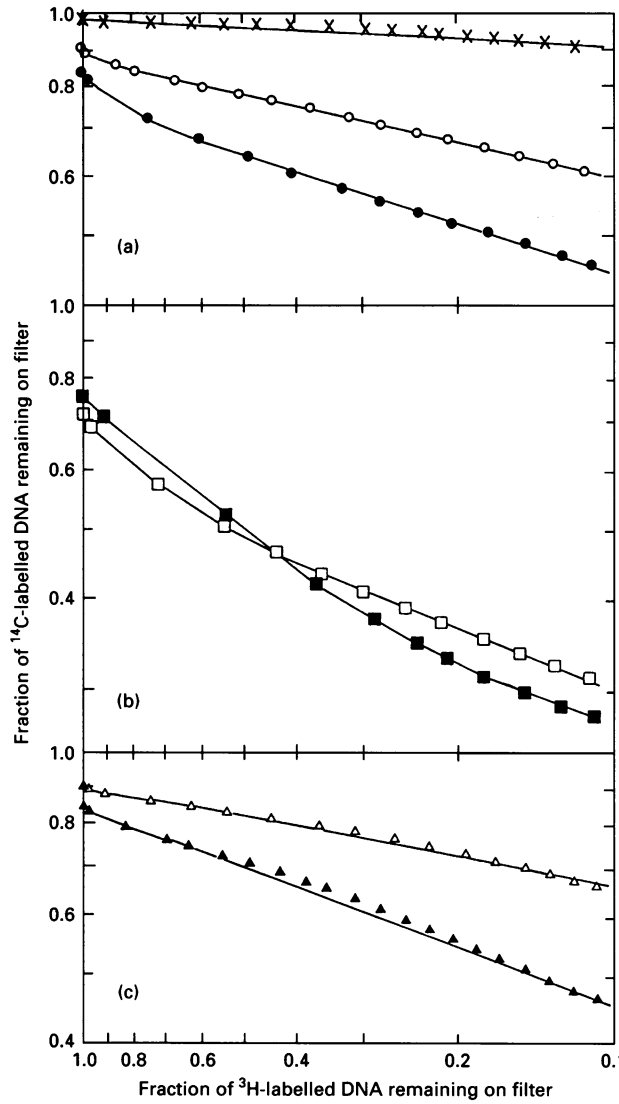

Figure 7 Inhibition of DNA damage caused by bleomycin and its Fe and Cu complexes in iron-deficient HL-60 cells

Cells were prepared as in Figure 2 and exposed to each form of the drug (25 μM) for 1 h prior to alkaline elution. (**a**), apoBlm; (b), FeBlm; (c), CuBlm. Key to symbols: (X), untreated control cells in (**a**); closed symbols, iron-normal cells plus drug; open symbols, iron-deficient cells plus drug.

and CuBlm, and accumulate similar amounts in nuclei [19]. So, the effects observed here probably did not result from differences in the internal concentration of these forms of Blm. Therefore these results support the direct role of FeBlm in the DNA cleavage reactions of the drug in cells.

Blm-dependent growth inhibition and DNA damage in iron-deficient *E. gracilis*

A second model system, the heterotrophically grown *E. gracilis*, was examined. In this system both extra- and intra-cellular iron content can be varied over a large range [16]. When 0.16 μM Blm was incubated with 6×10^5 cells/ml, the drug was without effect on cell proliferation in the iron-depleted medium over the first 24 h period. In contrast, FeBlm decreased the iron-depleted cell population to 4×10^5 cells/ml. Thereafter, FeBlm-treated cells began to divide again at the control rate.

To determine the effect of iron deficiency on the DNA damage caused by Blm, *E. gracilis* cells with varying levels of iron were incubated with Blm for 1 h and then analysed for DNA breakage. Table 5 summarizes elution results of a representative experiment. In treated cells, the percentage of double-strand-damaged DNA appearing in the lysis and EDTA washes was at control levels when added iron was 18 nM and below. Between 18 and 36 nM iron, it rose sharply. The percentage of total DNA eluted under alkaline conditions increased in a similar way except that with 180 nM iron there was a large increase in single-strand scission.

Table 5 Effect of iron on DNA damage from Blm treatment of *E. gracilis* alkaline elution analysis

Blm (160 nM) was incubated for 1 h with (2–5) $\times 10^6$ cells/ml in iron-deficient medium. Cells were previously grown for 48 h in medium (containing less than 10 nM iron) to which iron was added as shown. Average sum $\pm$ S.D. of lysis, alkaline-eluted, and filter DNA in the five sets of cells was 27.9 $\pm$ 3.6 pg/cell. The control percentage elution $\pm$ S.D. was 14 $\pm$ 6 ($n = 8$), in the absence of Blm.

	DNA distribution (%)		
Added iron concn. (nM)	Lysis*	Alkaline elution	Remaining†
0	8	5	87
9	8	10	82
18	13	10	77
36	31	28	39
180	38	72	0

* Percentage of total cell DNA damaged through probable double-strand breakage.
† Percentage of DNA remaining on filter after lysis and alkaline elution.

Table 6 Interaction of Blm and added iron in production of lysis DNA in *Euglena gracilis*

E. gracilis cells [(2–5) $\times 10^6$/ml] were incubated with Blm for 1 h in iron-deficient medium. Lysis DNA represents the percentage of total cell DNA damaged through probable double-strand breakage. The percentage lysis DNA for control treatments (0 nM Blm) were not subtracted. Control percentage elution $\pm$ S.D. was 13 $\pm$ 6 ($n = 4$).

	Lysis DNA	
Bleomycin (nM)	0 nM Fe*	1000 nM Fe
0	13	20
150	20	41
250	34	46
350	43	63

* Basal level of iron was less than 10 nM.

The results from another representative experiment shown in Table 6 verified that iron was required for double-strand DNA damage at all concentrations of Blm and indicated that above 150 nM Blm some damage occurred even in iron-deficient medium. Together, these results demonstrated that iron deficiency greatly suppressed the capacity of Blm to cause either double- or single-strand damage.

DISCUSSION

In order to assess the role of intracellular metals in the processes of oxidant damage, studies have typically resorted to the use of metal-chelating agents to bind iron which otherwise might participate in the Fenton reaction. Because they possess high affinity for Fe^{3+} or Fe^{2+} respectively, desferrioxamine and 1,10-phenanthroline have been commonly used for this purpose [9,19,20,29]. The former binds Cu^{2+} with a stability constant at pH 7.4 of about 10^{13} and so may not be specific for iron [39]. Indeed, it has also been demonstrated that the agent is a scavenger of hydroxyl radicals [40]. Thus when desferrioxamine inhibits oxidative stress, it is not unambiguously due to chelation of iron, but may result from the direct interception of radicals.

Similar problems of non-specificity exist for 1,10-phenanthroline. A previous study showed that this ligand depresses both iron and zinc content in Ehrlich tumour cells [22]. Results not described here reinforce these findings by showing that growth in the presence of 1,10-phenanthroline also lowers both cytosolic iron and zinc in *E. gracilis* (K. Radtke and D. H. Petering, unpublished work). In addition, this ligand might form adducts with co-ordinatively unsaturated zinc bound at active sites of various enzymes to inhibit their catalytic reactions [31]. There may be other cellular reactions of 1,10-phenanthroline which complicate any interpretation of its mode of action. Therefore, to avoid these complications two model systems have been developed in which iron concentrations are controlled by altering the level of nutrient iron in the growth medium.

The aquatic micro-organism *E. gracilis* remains viable indefinitely under conditions of iron deprivation when the external medium contains at least 10 nM Fe [22]. It maintains a doubling time of about 16 h during a 7–10-day growth period, even in the presence of iron-deficient growth medium. Because this micro-organism loses much iron during exposure to iron-depleted medium (Figure 1, Table 1), one can examine the role of intracellular iron status in the toxicity of oxidants.

Human promyelocytic leukaemia cells (HL-60) normally double in 24 h and withstand iron depletion for only 4–5 days, during which they continue to divide slowly. In these cells, cytosolic iron was reduced by a factor of five under iron-deficient conditions. However, an e.s.r. signal thought to arise from haem iron remained and the tyrosyl radical signal of RDR, indicative of the presence of an oxo-bridged iron, was still observed (Figure 3). Thus the level of iron deficiency in this study leaves intact at least some iron proteins, which, presumably, function to maintain cell visibility and proliferative capacity.

The present study revealed that systematic reduction of intracellular iron in *E. gracilis* lowered the toxicity of H_2O_2, as measured by inhibition of growth, at all concentrations of the agent and virtually abolished it at the lowest concentration employed (Figure 2). Thus it is clear in this system that iron supported the toxic action of H_2O_2, probably by providing the metal ion for the Fenton reaction. Nevertheless, one cannot exclude the possibility that other hypothetical iron-dependent processes involved in cellular protection against oxidants, such as DNA repair, have been compromised in iron deficiency.

Assessment of H_2O_2-induced DNA cleavage in *E. gracilis* was made by the alkaline elution method. As in the cell proliferation assay of cytotoxicity (Figure 2), it was evident that the DNA of cells grown in the lowest levels of iron in the extracellular medium was not measurably damaged, according to Table 2. A relatively small increase in added iron, from 9 to 18 nM, caused substantial single-strand breakage and a small increase in double-strand breaks. Further increases in iron concentration had no effect on damage. Apparently, the key source of iron needed for the effective conversion of H_2O_2 into its reactive form, presumably the hydroxyl radical, was not associated with the major pools of iron, which were depleted at much higher concentrations of nutrient iron [16,41].

A similar picture emerged from results of the reaction of H_2O_2 with HL-60 cells (Figure 4). Iron deficiency largely prevented the effects of the lowest concentration of H_2O_2 employed, i.e. 0.5 μM. However, it failed to completely protect cells which had been exposed to higher levels of the oxidant.

The effects of H_2O_2 on HL-60 cells were probed further by measuring DNA damage in these cells as a function of cellular iron status. Lesions induced in DNA by Fenton reactions involving iron have been suggested to be important in H_2O_2 toxicity [6,7,9,12,14,15,42]. As seen in Figure 5, H_2O_2 caused

concentration-dependent increases in DNA single-strand scission over the range 5–25 μM. At 1 μM H_2O_2 little DNA damage was observed (results not shown). Cells with lower intracellular iron displayed fewer strand breaks at each level of H_2O_2 used. Notably, however, iron depletion did not completely inhibit DNA damage at any concentration of oxidant.

The results presented here also unambiguously identify iron as a required cellular cofactor for the effects of Blm on the survival of two distinctly different cell types in culture and on the single- and apparent double-strand breakage that resulted when these cells were exposed to the drug (Figures 6 and 7 and Tables 5 and 6). Thus FeBlm retained and even showed enhanced DNA cleavage activity in iron-deficient HL-60 cells and showed growth inhibitory capacity in *E. gracilis* cells, whereas the actions of metal-free Blm and CuBlm were reduced or eliminated. It appears that CuBlm must be converted into FeBlm before DNA can be damaged (Figure 7). As such, these results agree with studies *in vitro* showing the lack of DNA strand scission activity of Cu(II)Blm [43,44]. These findings also weigh against copper as an important metal for the reactions of Blm leading to cellular DNA damage.

The fact that it has previously been difficult to demonstrate the iron requirement for Blm in cell or animal models attests both to the high affinity of Blm for residual cellular iron and to the difficulty of reducing the concentration of iron without affecting the viability of the test system [16,26]. For example, as seen in Table 6, there is a drug concentration-dependent increase in DNA damage in iron-deficient *E. gracilis* cells, which obscured the iron requirement of Blm in a previous study that had employed only a relatively large concentration of Blm, i.e. 260 nM [16].

The earlier findings that Blm and its Zn-, Cu-, and Fe-complexes are all cytotoxic can now be explained in terms of a set of reactions in which all species must be converted into FeBlm [15]. The fact that FeBlm is the active species against tumour cells and also appears to be significantly less toxic to the host than Blm or CuBlm suggests that consideration should be given to use of this form of the drug to treat cancers [26].

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REFERENCES

1 Aust, S. D., Morehouse, L. A. and Thomas, C. E. (1983) J. Free Radicals Biol. Med. **1**, 3–25
2 DiGuiseppi, J. and Fridovich, I. (1984) CRC Crit. Rev. Toxicol. **12**, 315–342
3 Ward, J. F. (1977) Adv. Radiat. Biol. **5**, 181–239
4 Girotti, A. W. (1985) J. Free Radical Biol. Med. **1**, 87–95
5 Davies, K. J. A. (1987) J. Biol. Chem. **262**, 9895–9901
6 Hoffman, M. E. and Meneghini, R. (1979) Photochem. Photobiol. **30**, 151–155
7 Bradley, M. O. and Erickson, L. C. (1981) Biochim. Biophys. Acta **654**, 135–141
8 Rubin, R. and Farber, J. L. (1984) Arch. Biochem. Biophys. **228**, 450–459
9 Imlay, J. A., Chin, S. M. and Linn, S. (1988) Science **240**, 640–642
10 Ananthaswamy, H. N. and Eisenstark, A. (1977) J. Bacteriol. **130**, 187–191
11 Lewis, J. G. and Adams, D. O. (1985) Cancer Res. **45**, 1270–1275
12 Prise, K. M., Davies, S. and Michael, B. D. (1989) Int. J. Radiat. Biol. **55**, 583–592
13 Repine, J. E., Fox, R. B. and Berger, E. M. (1981) J. Biol. Chem. **256**, 7094–7096
14 Mello Filho, A. C., Hofmann, M. E. and Meneghini, R. (1984) Biochem. J. **218**, 273–275
15 Birnboim, H. C. and Kanabus-Kaminska, M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6820–6824
16 Lyman, S., Taylor, P., Lornitzo, F., Weir, A., Stone, D., Antholine, W. E. and Petering, D. H. (1989) Biochem. Pharmacol. **38**, 4273–4282
17 Krishnamurti, C., Saryan, L. A. and Petering, D. H. (1980) Cancer Res. **40**, 4092–4099
18 Graham, D. R. and Sigman, D. S. (1984) Inorg. Chem. **23**, 4188–4191
19 Byrnes, R. W., Templin, J., Sem, D., Lyman, S. and Petering, D. H. (1990) Cancer Res. **50**, 5275–5286
20 Iqbal, Z. M., Kohn, K. W., Ewig, R. A. G. and Fornace, A. J. (1976) Cancer Res. **36**, 3834–3838
21 Sausville, E. A., Peisach, J. and Horwitz, S. B. (1978) Biochemistry **17**, 2740–2745
22 Sausville, E. A., Stein, R. M., Peisach, J. and Horwitz, X. B. (1978) Biochemistry **17**, 2746–2754
23 Burger, R. M., Peisach, J. and Horwitz, S. B. (1981) J. Biol. Chem. **256**, 11636–11644
24 Stubbe, J. and Kozarich, J. (1987) Chem. Rev. **87**, 1107–1135
25 Ehrenfeld, G. M., Shipley, J. B., Heimbrook, D. C., Sugiyama, H., Long, E. C., van Boom, J. H., van der Marel, G. A., Oppenheimer, N. J. and Hecht, S. M. (1987) Biochemistry **26**, 931–942
26 Rao, R. A., Saryan, L. A., Antholine, W. E. and Petering, D. H. (1980) J. Med. Chem. **23**, 1310–1318
27 Byrnes, R. W. and Petering, D. H. (1991) Biochem. Pharmacol. **41**, 1241–1248
28 Chitambar, C. R. and Zivkovic, Z. (1987) Blood **69**, 144–149
29 Aebi, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) pp. 673–684, Euglena media 684, Verlag Chemie, Weinheim
30 Price, C. A. and Vallee, B. L. (1962) Plant Physiol. **37**, 428–433
31 Kohn, K. W., Ewig, R. A. G., Erickson, L. C. and Zwelling, L. A. (1981) in DNA Repair: A Laboratory Manual of Research Procedures (Friedberg, E. C. and Hanwalt, P. C., eds.), vol. 1, Part B., pp. 379–401, Marcel Dekker, New York
32 Byrnes, R. W. and Petering, D. H. (1994) Radiat. Res. **137**, 162–170
33 Byrnes, R. W. and Petering, D. H. (1993) Radiat. Res. **134**, 343–348
34 Murray, D., Van Ankeren, C. S. and Meyn, R. E. (1987) Anal. Biochem. **160**, 149–159
35 Burton, K. (1969) Methods Enzymol. **12B**, 163–166
36 Byrnes, R. W., Mohan, M., Antholine, W. E., Xu, R. X. and Petering, D. H. (1990) Biochemistry **29**, 7046–7053
37 Sahlin, M., Petersson, L., Graslund, G., Ehrenberg, A., Sjoberg, B. M. and Thelander, L. (1987) Biochemistry **26**, 5541–5548
38 Kanthein, R., Nastainczyk, W. and Ruf, H. H. (1987) Eur. J. Biochem. **166**, 173–180
39 Sillén, L. G. and Martell, A. E. (1964) Stability Constants, The Chemical Society, Burlington House, London
40 Halliwell, B. (1985) Biochem. J. **34**, 229–233
41 Radtke, K., Byrnes, R. W., Kerrigan, P., Antholine, W. E. and Petering, D. H. (1992) Man. Environ. Res. **34**, 339–343
42 Schraufstätter, I. U., Hyslop, P. A., Hinshaw, D. B., Spragg, R. G., Sklar, L. A. and Cochrane, C. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4908–4912
43 Antholine, W. E., Solaiman, D., Saryan, L. A. and Petering, D. H. (1982) J. Inorg. Biochem. **17**, 75–94
44 Suzuki, T., Kuwahara, J. and Sugiura, Y. (1985) Biochemistry **24**, 4719–4721

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